Quantitative second harmonic generation microscopy for the structural characterization of capsular collagen in thyroid neoplasms

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Abstract: Quantitative second harmonic generation microscopy was used to investigate collagen organization in the fibrillar capsules of human benign and malignant thyroid nodules. We demonstrate that the combination of texture analysis and second harmonic generation images of collagen can be used to differentiate between capsules surrounding the thyroid follicular adenoma and papillary carcinoma nodules. Our findings indicate that second harmonic generation microscopy can provide quantitative information about the collagenous capsule surrounding both the thyroid and thyroid nodules, which may complement traditional histopathological examination.

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1. Introduction

Thyroid nodules commonly arise within a normal thyroid gland and most often they do not produce any symptoms. They cannot be regarded as the expression of a single disease but represent the clinical manifestation of a wide range of different pathologies [1]. They can be benign such as hyper-plastic, colloid nodules or follicular adenomas (FA). Nodular goiter is the most common thyroid lesion, with multiple, variably sized nodules clinically evident in most cases. However, up to 10% of the lesions that come to medical attention are malignant—papillary, follicular or medullary thyroid carcinomas. Although thyroid carcinomas have a low incidence rate, it is important to differentiate them from the much more frequent adenomas and nodular goiters [2]. Papillary thyroid carcinoma (PTC) is the most prevalent form of thyroid cancer, accounting for more than 80% of all thyroid cancer cases [2]. Most thyroid cancers can be treated successfully when diagnosed early. Surgical excision is the standard treatment of thyroid tumors by either a total thyroidectomy or partial removal of the thyroid gland (thyroid lobectomy). Diagnosis is confirmed after the surgical procedure by visualizing hematoxylin and eosin (H&E) stained thyroid sections in bright-field microscopy.

The thyroid gland is surrounded by a thin fibrous capsule made of connective tissue (collagen fibers) which adheres closely to the gland. The capsule extends into the thyroid forming septae which divide the gland into irregular and incomplete lobules [3]. The importance of the thyroid capsule is illustrated by studies showing that capsular invasion in PTC is associated with an aggressive form of carcinoma [4]. Capsular staining of follicular thyroid neoplasms with picrosirius orange-red was previously used to differentiate between widely invasive carcinomas, minimally invasive carcinomas and adenomas [5] using subjective parameters as intensity of the collagen color when viewed with polarized light.

To date, characteristic modifications in the fibrillar organization of collagen that could potentially serve as an early diagnostic marker have been identified for multiple pathologies. Such changes in collagen structure have been observed by analyzing the extracellular matrix (ECM) in cases of diseased human dermis [6], malignant colon biopsy samples [7], serous ovarian neoplasms [8], invasive breast carcinoma [9] and even two variants of papillary thyroid carcinoma [10]. Since collagen type I is the most abundant protein in the ECM and has a strong second harmonic generation (SHG), the ECM structure can be visualized by analyzing the collagen distribution by SHG microscopy [11–13]. This microscopy technique is based on a coherent second order nonlinear optical process known as second harmonic generation [14,15] in which photons interacting with optically nonlinear materials, are effectively combined to form new photons with exactly half of the excitation wavelength. SHG can only arise from materials lacking a center of symmetry, thus probing the absolute molecular structure and disorder in organized organic media like collagen fibrils [12], microtubules [16], and the myosin lattice of muscle cells [17].

Over the past decade important efforts have been placed for establishing SHG microscopy as a reliable diagnostic tool for cancers and other pathological conditions [18,19]. This is because SHG data sets that depict the collagen organization in tissues can be achieved in a non-invasive manner, in-vivo. The success of SHG microscopy in biomedical research relies on the quantification of collagen organization using different measures [11]. Although collagen SHG images have been previously used to describe empirical observations that were linked to a specific pathology (e.g. in the case of the thyroid gland [20]), recent studies highlight such correlation using quantifiable measures for objective comparisons. Some of the
methods used to quantify collagen organization from SHG images are anisotropy evaluation in polarization-resolved SHG microscopy [21], the 2D fast Fourier transform [22,23], wavelet transform [24], texture analysis of SHG images using first-order [25] and second-order statistics (e.g. gray-level co-occurrence matrix (GLCM) [6,25,26]), the structure tensor [27], fractal analysis [28] or Helmholtz analysis [29]. By combining such methods in an automated way, a quantification of liver fibrosis using liver capsule SHG images was proposed [30].

Although attempts of using the collagen capsule have been previously made [5], to our knowledge, no study has visualized the fine structure of the capsule surrounding the thyroid gland or thyroid nodules, either benign or malignant. In this paper we compare the collagen structure in the capsule surrounding FA and PTC nodules, since both cases can be easily distinguished by routine histopathology. Choosing this simple scenario allows us to test the idea that structural changes in the capsule surrounding thyroid nodules can be used for their differentiation. We have used SHG microscopy for imaging the collagen capsule and a set of quantification parameters which, in an objective manner, were able to detect differences in capsular structure in encapsulated nodules of PTC and FA nodules.

2. Materials and methods

2.1 The nonlinear optical microscope

SHG imaging was performed using a Leica TCS-SP confocal laser scanning microscope modified for nonlinear optical imaging [21]. As an excitation source we have used a mode-locked Ti:Sapphire laser (Tsunami, Spectra Physics) operated at 790 nm, with ~150 fs pulses and a repetition rate of 80 MHz. A 40X magnification and 0.75 numerical aperture (NA) objective was used for imaging. This objective (and consequently its corresponding 250 x 250 μm² field-of-view) was selected to be used in the conducted imaging sessions based on specific characteristics of the proposed experiment (e.g. collagen capsule thickness, collagen fiber and fibril sizes). The SHG signals were collected in the forward direction using a 0.9 NA condenser lens. A combination of a short-pass filter (FF01-750/SP-25, Semrock) to reject the fundamental beam and band-pass filter (FB390 -10, Thorlabs) to select the SHG radiation was used in the forward collection direction. Images were acquired with a circular laser beam polarization obtained by a combination between a Glan-Laser polarizer (GL10-B, Thorlabs) and an achromatic quarter-wave plate (AQWP05M-980, Thorlabs) placed before the microscope input port. This configuration does not yield a pure circular polarized beam after the objective due to ellipticity introduced by the various optical elements inside the microscope. Although circular polarization is typically preferred in SHG imaging experiments as it is considered that this illumination configuration does not discriminate between collagen fibrils aligned differently it was previously shown that an elliptically polarized laser beam can in some scenarios be better than a circularly polarized beam in terms of image quality [31], and therefore we consider that a circular polarization is not necessarily preferable to an elliptical one.

2.2 Image acquisition and data analysis

The 250 x 250 μm² (with 512 x 512 pixels) scanning range used in our experiment was selected as a result of our interest to quantify the collagen distribution within a collagen capsule, which requires the imaging of large tissue areas where relevant tissular structures can be observed. To reduce noise, each SHG image was acquired by averaging three unprocessed individual frames. The average power reaching the sample plane was kept less than 15 mW.

A z-stack of SHG images was taken at randomly selected sites of each thyroid sample with 6 optical sections with 1 μm depth between each section. For each image stack the maximum intensity projection was performed in ImageJ [32] to generate a 2D SHG image.

Collagen fiber alignment and distribution in the 2D SHG images were quantified by a set of parameters (Table 1) which were determined using the image histogram, the GLCM,
fractal and Helmholtz analysis. Their capability to characterize the collagen organization in SHG images and to differentiate between benign and malignant tumors was tested.

In the following section the parameters used for the quantitative analysis of collagen distribution are described.

A set of four parameters, directly related to the gray level distribution of pixel intensities extracted from the 2D SHG images (mean, standard deviation, skewness and kurtosis) were calculated using the histogram analysis toolbox from ImageJ. While mean and standard deviation are well known statistical moments, skewness and kurtosis describe the shape of a distribution. Skewness is a measure of the distribution symmetry relative to the mean value. A symmetrical data set will have a skewness equal to 0. Kurtosis describes the flatness of the distribution around the mean. We have calculated the excess kurtosis which is the kurtosis relative to the normal distribution which has a value of 3. The mean of the pixel intensities distribution was previously used [7] to provide an estimate of the collagen density. Because SHG intensity in each pixel depends on the density of emitters in the focal volume, but also on their organization and distribution [11], we have also considered two additional selective parameters that can be useful in differentiating between collagen structures exhibiting different distributions. The pixels in the SHG image having intensity above a threshold value are counted and the total collagen area ratio (TC-ratio) is determined as the ratio between this number and the total area of the image. The second parameter which we called significant mean (S-mean) is determined as the average value of the SHG signal in regions exhibiting significant SHG signals [33], using the same threshold as for the previous parameter.

### Table 1. Parameters used for the texture analysis of capsular collagen in SHG images.

| Parameter                              | Abbreviation |
|----------------------------------------|--------------|
| Histogram analysis Mean                | Mean         |
| Standard deviation                     | Hist-StDev   |
| Skewness                               | Hist-Skew    |
| Kurtosis                               | Hist-Kurt    |
| Alternative collagen estimation Ratio between the number of pixels with values above a threshold and the total number of pixels | TC-ratio     |
| Average value of SHG in significant areas | S-mean       |
| Gray Level Co-occurrence Matrix Contrast | Contrast    |
| Inverse Difference Moment Entropy      | IDM          |
| Angular Second Moment Correlation      | ASM          |
| Entropy                                | Entropy      |
| Correlation                            | Correlation  |
| Fractal analysis on binary images Fractal Dimension | FD-bin      |
| Lacunarity                             | Lac-bin      |
| Fractal analysis on grayscale images Fractal Dimension | FD-gray      |
| Lacunarity                             | Lac-gray     |
| Helmholtz analysis Standard deviation  | Helm-StDev   |
| Skewness                               | Helm-Skew    |
| Kurtosis                               | Helm-Kurt    |

A second set of parameters derived from the GLCM method which provides information on the spatial relationships between pixels intensities in a given image were calculated with ImageJ’s GLCM texture plugin. The GLCM is constructed by counting the number of occurrences of a gray level adjacent to another gray level, at a specified pixel distance. Each result is divided by the total number of elements to obtain a probability. The matrix elements are the probability of the gray level co-occurrence between pixels with the rows and columns of the matrix representing the gray levels in the image. The matrix can be computed for adjacent pixel either in horizontal (0°), vertical (90°) or diagonal (45°, 135°) direction. In our case, average values for the four directions were considered for the computed parameters. Information regarding the collagen organization in SHG images can be extracted from the
GLCM using different parameters which were previously classified as contrast, organization and statistical parameters [34]. Contrast parameters can be employed to give quantitative information on the intensity fluctuations in the SHG image. Among these parameters, contrast is a measure of the local variations present in the image, while homogeneity or inverse difference moment (IDM) gives information on the similarity of a pixel value in combination with its neighboring pixel to all the other pairs of neighboring pixels in the image. Organization parameters are particularly applicable to images containing fibrillar structures such as SHG images of collagen, and they can be employed in order to give a quantitative measurement on the mutual orientation of collagen fiber bundles [34]. For example, energy or angular second moment (ASM) is a measure of textural uniformity and reaches a maximum value equal to unity, corresponding to the highest structural uniformity, while entropy [35] is a measure of disorder in an image. ASM and entropy are inversely correlated. Statistical parameters are based on the statistical analysis of pixel value dependence, and they can be used to assess periodicity within an image. Among them, the correlation method represents a powerful approach applied to SHG images of collagen. We compared the GLCM correlation values at different pixel distances and fitted the correlation-distance dependence with an exponential curve to compute the correlation decay rate.

Another method for objectively quantifying organization in an image is fractal analysis. The SHG images were analyzed either as a grayscale image or as a binary image. Fractal analysis was performed using an ImageJ plugin (FracLac) that calculated the fractal dimension (FD) and lacunarity (Lac) using the “box counting” method. The image is covered in successively smaller boxes and at each level the boxes containing image details are counted. Such details are white pixels in the case of binary images and the difference in pixel intensity over the area covered by the considered box for grayscale images. The fractal dimension [36], which is a measure of complexity in the image is calculated as the slope of the log-log regression line for the number of boxes against the box size in the case of binary images and as the slope of the log-log regression line for the total sum of the difference in pixel intensity over the area covered by the box against the box size in the case of grayscale images. On the other hand, lacunarity is based on pixel distribution in an image which is calculated from scans at different box sizes and is a measure of gappiness in an image.

Helmholtz analysis [29] performed using an ImageJ plugin was used to determine the average collagen orientation distribution in SHG images. For the orientation distribution images obtained with Helmholtz analysis, the same set of statistical moments as for the 2D SHG images was computed: standard deviation, skewness and kurtosis. The mean values were not considered in this case because the average angle would depend on the orientation of the sample relative to the scanning plane.

To further assess whether the collagen distribution differences between malignant and benign capsules are due to small-scale or large-scale features, all the three image data sets were divided into 32x32 pixels images and all the texture analysis parameters were computed for these extended data sets as well. The obtained distributions were fitted with three Gaussian distributions for data interpretation. The quality of the fitting was quantified by the coefficient of determination [21] \( R^2 (0 < R^2 < 1) \), a value closer to unity indicating a better fitting.

The statistical analysis of the results was assessed by means of one-way ANOVA followed by Bonferroni’s post-hoc comparisons tests. For all statistical tests a confidence level of 95% (p-value of 0.05) was used to establish statistical significance. Different p-values thresholds were considered (p<0.005, p<0.0005) to assess different degrees of statistical difference whenever necessary. While from a clinical point of view, the problem of importance consists in distinguishing malignant vs. benign capsules, the third type, the thyroid capsule, was also included in our experiment in order to demonstrate a different collagen organization between natural thyroid capsules and pathological ones, either malignant or benign.
2.3 Sample preparation

The tissue samples were obtained according to an institutionally-approved protocol. For each considered case two consecutive thin tissue sections were cut from a formalin-fixed, paraffin-embedded tissue block. Before mounting these tissue sections on glass slides one of them was stained with H&E for traditional histopathological examination, while the other one was left unstained. Both samples were finally sealed with a cover-glass. A batch of 36 stained/unstained slide pairs composed of PTC and FA tissue was used for this study. The H&E stained sections were imaged with a bright-field microscope (Leica DM3000) for reference. Normal and malignant regions of interest were identified as well as capsules surrounding the thyroid gland and the nodules. Unstained pairs of the H&E stained tissue sections were used for SHG imaging. A total of 66 areas were imaged: 17 for thyroid capsules, 33 for PTC capsules and 16 for FA capsules. All the SHG images used in this study are available upon request to interested researchers.

![Thyroid capsule, Benign nodule capsule, Malignant nodule capsule](image)

Fig. 1. Traditional H&E histopathology (upper row) images acquired with a 20X objective and SHG microscopy images (lower row) for representative areas for the thyroid capsule, a thyroid follicular adenoma and a capsule surrounding a PTC nodule.

3. Results

We first present qualitative evidence that SHG imaging can reveal different collagen fibrillar organization in capsules for normal, benign and malignant ex vivo biopsies of thyroid tissue. Representative bright-field microscopy images of H&E stained tissue sections and SHG optical sections for the three types of investigated capsules are shown in Fig. 1.

Visual analysis of the SHG images for the normal thyroid capsule reveals wrapping collagen fibers. This wavy collagen structure is consistent with reports of crimped collagen fibers in mammary tissue that allow normal tissue deformation [37]. A similar behavior can also apply to the thyroid capsule, allowing for tissue deformation and normal growth without
over-stressing, yet providing adequate deformation resistance to the tissue. A similar collagen structure is identified for the benign nodule capsule as well. On the other hand, large differences in the collagen assembly are observed in the case of the collagen distribution for the capsule surrounding a PTC nodule. In this case a highly-directional and organized collagen structure, with straight fibers is observed in the SHG image (Fig. 1). This could be explained by the collagen fibers overstretching due to the rapid growth of the PTC nodule.

As the collagen features appeared distinct in the three investigated fibrous capsules, we determined whether quantitative parameters can distinguish between a normal thyroid gland capsule, a capsule surrounding a benign nodule and a capsule surrounding a malignant one. In order to accomplish quantitative image analysis we have used texture analysis methods which provide parameters of the collagen distribution and organization in the SHG images.

Figure 2 shows the values for the quantification parameters computed from SHG images. In the following we discuss the efficiency of these texture parameters in terms of quantifying the morphological changes in SHG images acquired on different collagen capsules.

The results in Fig. 2 indicate that for the case of one parameter (ASM) we obtained statistically significant differences between all the three considered capsules, while six of the 18 parameters detected statistically significant differences in the collagen distribution between benign and malignant nodule capsules: Hist-Skew, Hist-Kurt, IDM, ASM, FD-gray and Helm-StDev. While 16 parameters showed statistically significant differences between the thyroid capsule and the PTC capsule, only 10 of them were able to distinguish between the thyroid capsule and the benign nodule capsule. These findings indicate that a unique collagen topology can be detected in the PTC nodule capsule on the basis of SHG imaging and quantification compared to the benign nodule capsule and the thyroid capsule.
Fig. 3. Selected parameters distributions computed from the 32 x 32 pixels SHG image sets for the three investigated capsules. The black curves represent fitting Gaussians with their mean (m) and standard deviation (s) being given in the inset of each graph. A maximum of three Gaussians were fitted to each parameter distribution and the case with the maximum coefficient of determination (R² is also given in the inset) is shown.
Not all the parameters revealed statistically significant differences between the three capsules, although visual differences have been observed. One of the reasons might be the suitability of the texture analysis parameters to capsular collagen distribution quantification and the other is a higher dispersion of parameters caused by a heterogeneous collagen structure even for the same type of capsule. In other words, differences in collagen organization might be better identified at a smaller image scale. We have tested the latter hypothesis by dividing each image in 256 images with 32 x 32 pixels and calculated the parameters and their distributions for these new sets of images (Fig. 3).

Our results suggest that by combining small- and large-scale quantitative SHG image analysis, the capsular collagen assembly in the thyroid gland and thyroid nodules might be better characterized compared to the case when only large-scale images were used. On the other hand, an enhancement in method sensitivity might be achieved, resulting in an efficient differentiation between different thyroid pathologies, based on the texture analysis of the nodular collagen capsule.

4. Discussions

For the case of large-scale images, the quantitative results can partially be correlated with the qualitative observations on the SHG images (Fig. 1).

Lower mean pixel intensities obtained for both FA and PTC capsules with statistically significant differences with respect to the thyroid capsule are an indication of a lower SHG signal in the two considered pathological states, either due to a decrease in collagen density, or to a decrease in individual collagen fiber SH generation, or both. By calculating other two parameters, namely TC ratio and S-mean we have tried to answer this question. TC ratio estimates the total collagen area in an SHG image and only accounts for collagen density changes. For this parameter a statistically significant decrease was obtained between the thyroid capsule and both pathological nodule capsules. This result is consistent with the thinner capsule in the case of the benign nodule and the lower density of collagen fibers for the PTC capsule, without being able to distinguish between them. On the other hand, S-mean, which would show changes of pixel intensity only on collagen covered areas, didn’t provide any statistically significant differences between the three types of capsules. If we consider a subset of pairs for simultaneous comparison, relative to the normal thyroid capsule which in such a situation may be considered as the control, S-mean indicates a statistically significant decrease of the SHG signal in the case of the PTC nodule capsule. The lower value of S-mean might occur due to a reorganization of collagen at fibrillar level by either a loss of crystallinity or to a higher disorder in the focal volume [38].

Hist-StDev is used to quantify the amount of dispersion of the data set. Usually a lower standard deviation of a pixel intensity distribution is a sign of a sharper image, which in our case might indicate a more spread distribution of individual collagen fibers. This is the situation of the PTC capsule which has a statistically significant lower standard deviation than the thyroid capsule.

Regarding the Hist-Skew, all three distributions are highly positively skewed, with their right tails being longer. A higher value for skewness is an indication of a darker image [39], hence a lower intensity SHG from collagen or a lower collagen density in the imaged area. The higher value of Hist-Skew for the PTC capsule is consistent with the observation of thinner and more dispersed collagen fibers within the PTC capsule.

All three distributions have high positive Hist-Kurt. We have observed a statistically significant increase in Hist-Kurt from both the thyroid capsule and the FA capsule to the PTC capsule which indicates that pixel values are distributed closer to the mean than for a normal distribution, and little of the variance being caused by infrequent extreme deviations. A lower Hist-StDev, correlated with a higher Hist-Kurt indicates a narrower pixel value distribution around the mean and as for the case of Hist-StDev, indicates sharper features (collagen fibers) in the image.
The advantage of using distribution shape parameters such as skewness and kurtosis stands in the fact that they are intensity independent and are thus more robust to characterize capsular collagen structural features.

While first order statistic parameters computed by using the image histogram are directly related to the gray level distribution of pixels intensity, image second-order statistic parameters depend on the spatial arrangements and correlation of pixels intensities.

*Contrast* is associated with the average gray level difference between neighboring pixels and quantifies the heterogeneous distribution of collagen present in the image. A higher *contrast* indicates highly contrasted images, which can be associated with features that are distributed without a preferential alignment [25]. A statistically significantly higher *contrast* was obtained for the thyroid capsule than for the PTC capsule, which can be explained by the presence of aligned collagen fibers in the latter case.

*IDM* quantifies the local similarities in the SHG image and in our experiment, although *contrast* and *IDM* are inversely correlated, *IDM* is able to provide statistically significant differences between capsules of benign and malignant nodules, while *contrast* is not.

*ASM* detects disorders in textures, reaching a maximum value equal to one which occurs when the gray level distribution is homogeneous. Lower *ASM* values are due to a higher number of small entries in the GLCM and are an indication of heterogeneous images, in our case less homogeneous collagen distributions. *ASM* is the only parameter which reflects a statistically significant increase from the value corresponding to the thyroid capsule, then the FA capsule and finally the PTC capsule, indicating an increase in collagen homogeneity.

A larger *entropy* indicates more complex image texture. As in the case of *contrast* and *IDM*, although *ASM* and *entropy* are inversely correlated, *ASM* is able to provide statistically significant differences between the three considered classes, while *entropy* is not.

![Image](image_url)

**Fig. 4.** *Correlation* decay rate with the pixel distance for the three types of capsules. The inset displays the decay rate for the three *correlation* curves with the error bars representing the 95% confidence intervals.

The *correlation* is a measure of gray levels linear dependencies in the image. All the three considered capsules returned low *correlation* values. Because a low *correlation* generally means independent adjacent gray levels, no significant regular pattern can be identified in the image. All the GLCM parameters in Fig. 2 are computed considering a pixel separation distance of one for the calculation of the matrix elements. In order to use the ability of the *correlation* parameter to detect regular patterns in an image we have also compared the GLCM *correlation* values at different pixel distances (Fig. 4) and computed the *correlation* decay rate by fitting the correlation-distance dependence with an exponential. Quantitatively, the *correlation* for the PTC capsule fell off with a steeper slope suggesting distinct, linear fibers [26], while for the FA capsule the *correlation* remained elevated as the pixel distance increased, suggestive for a more disorganized collagen fibrous structure.

Fractal analysis was computed on both grayscale images (*FD-gray, Lac-gray*) and on binary images (*FD-bin, Lac-bin*) obtained after image thresholding. Although binary fractal
analysis is the most widely used, one of its drawbacks (e.g. the need of a threshold for image binarization) was also encountered in our case. Neither FD-bin, nor Lac-bin was able to detect significant differences between malignant and benign capsules. One possible explanation is that for the binary image, FD-bin offered information on the texture of the edges in the binary image, rather than the texture of the collagen distribution, which is the case in a previous study addressing the investigations on the membrane of tumor cells [40]. On the other hand, we consider that grayscale images are more suitable for fractal analysis when determining the texture of collagen distribution. FD-gray showed a significant decrease in complexity for the PTC capsule compared to both the thyroid and the FA capsule. Lac-gray increases for nodules, with a higher value for malignant nodules, a result which is consistent with the ones obtained for Mean and TC ratio discussed previously.

The last set of parameters is connected with the Helmholtz analysis. A higher Helm-StDev, associated with the moderately skewed distribution in the case of thyroid capsule (Helm-Skew = 1.12), the approximately symmetric distributions (Helm-Skew < 0.5) and the significantly lower Helm-Kurt for nodular capsules indicate a higher dispersion in the collagen distribution with a significantly higher value for the PTC capsule. This result is in accordance with the possible reorganization of collagen at fibrillar level by either a loss of crystallinity or to a higher disorder in the focal volume which might be indicated by S-mean.

A part of the considered quantification parameters that we evaluated on large scale images were not able to differentiate between FA and PTC capsules. One possible explanation is that structural changes in the collagen might reflect in a broad range of values occurring across the regions of a large-scale image (e.g. 250 x 250 μm²). Therefore, only judging by the mean and standard deviation calculated from these values no statistically significant differences could be obtained. Hence, we have further tested the considered set of parameters on small-scale SHG images (~16 x 16 μm²) and for part of them significant differences were obtained. In the following we only discuss the distributions of the quantification parameters for the benign and malignant capsule (Fig. 3).

Although when calculated for the entire SHG images the Mean decreased for the PTC nodule (Fig. 2), for small-scale images a shift of the Mean distribution towards higher values is evident. This result can be assigned to a local modification of collagen organization in the focal volume, contrary to the conclusion drawn from the Mean calculated on the entire images. No significant features can be observed in the case of Hist-StDev.

A common IDM component around a mean of 0.11 was obtained for both benign and malignant distributions. On the other hand, higher IDM values centered at 0.092 are obtained for the PTC nodule, with two regions centered at 0.049 and 0.078 resulting for the benign nodules. These results confirm the statistically significant increase in IDM for PTC nodules obtained on entire SHG images.

Similar results are obtained for ASM, with a high value component (~2·10⁻³) present for both FA and PTC capsules, while the other values are distributed around 0.63·10⁻³ and 1.02·10⁻³ for the FA capsule and 0.84·10⁻³ and 1.26·10⁻³ for the PTC capsule, respectively.

The small-scale analysis of ASM and IDM points towards a characteristic for the PTC nodules: local areas with a higher IDM assigned to a high similarity and areas with higher ASM, assigned to more homogeneous regions. Thus we assume that the collagen distribution suffers significant changes on a small-scale which might be missed by quantifying large field-of-view images.

This latter situation is evident in the case of Entropy. While the values obtained on the entire SHG images were not statistically different for the benign and malignant capsules, when computing this parameter on smaller images, we obtained a significant difference between these two capsules. While distributions centered at 6.44 and ~7 are present in both cases, higher Entropy (~7.45) is obtained only for the FA capsule indicating more complex image textures on a local scale, consistent with the wavy collagen structure observed previously (Fig. 1).
The *Correlation* values were fitted with two distributions, both centered on higher values for the malignant nodule than for the benign nodule. In the case of the *Correlation* computed on entire SHG images there was no significant difference between nodules. These higher *Correlation* values might indicate a regular pattern in the images, which might be the distinct, linear fibers present in the malignant capsule images (Fig. 1).

For small-scale images we have only tested the fractal analysis on grayscale images. *FD-gray* values were normally distributed for FA and PTC, with a broader distribution for the PTC nodule and no significant differences between the capsules. Hence, FD is more suited to detect changes on large-scale SHG images. On the other hand, two low *Lac-gray* components are present in both benign and malignant distributions, with a higher value only for the malignant nodules, consistent with the lower density of collagen fibers observed for this type of capsules. The case of *Lac* is another example of parameter computed on small-scale images which was able to provide a differentiation while on large-scale it did not.

Helmholtz analysis computed on small-scale images only provides a small increase in the mean values of the two fitted normal distributions, while the large-scale analysis returned a statistically significant increase in *Helm-StDev* for the malignant nodule.

By combining SHG microscopy with quantification parameters for image texture analysis provided by the histogram analysis, gray level co-occurrence matrix, fractal analysis and Helmholtz analysis applied on the collagenous capsule surrounding the thyroid gland, FA and PTC nodules, we have proven for the first time to our knowledge that the collagen distribution in the nodule capsules can be used to differentiate between benign and encapsulated malignant thyroid nodules. From the 18 tested parameters, 16 parameters returned statistically relevant differences between the thyroid capsule and malignant nodule capsule, while only 10 parameters offered statistically relevant differences between the thyroid capsule and benign nodule capsule indicating that common collagen organization characteristics are likely to exist between the latter two. The obtained results highlight a different collagen structure in the thyroid capsule compared to pathological nodule capsules. From the entire set of tested parameters, *Hist-Skew*, *Hist-Kurt*, *IDM*, *ASM*, *FD-gray* and *Helm-StDev* provided statistically significant differences between benign and malignant nodule capsules on large-scale images. On the other hand, after testing the entire set of parameters on small-scale images, we have identified additional parameters that provide statistical significant results. For example, *Entropy* does not provide statistically significant differences in the case of large-scale images, conversely to the case of small-scale images. The high *Entropy* values observed in the case of the evaluated small-scale images were present only in the case of benign nodules and thyroid capsule, which can be intuitively perceived by taking into account the wavy aspect of these two capsules. Hence, combining large-scale and small-scale analysis will be an asset that should be exploited. An important future research direction referring to the analysis of small-scale images consists in the evaluation of ultra-structural capsular collagen features, similar with the work done on the ECM [10]. Our quantitative results are consistent with the observed changes in collagen fibers organization and indicate randomly organized collagen fibers in benign nodule capsules and an organized PTC capsule with fibers aligned parallel with the malignant nodule. We hypothesize that this behavior might be interpreted as a defense mechanism against malignant tumors, since it was previously observed that aligned collagen fibers with the angle relative to the tumor boundary distributed around 90° promote invasion [37].

The present study deals with the comparison between FA vs. PTC. Our results point towards a modification/reorganization of the collagen fibers in the nodule capsule between the two pathologies and indicate that quantitative SHG microscopy is a reliable method to distinguish between benign and malignant thyroid nodules. This is important because it paves the way for quantitative SHG microscopy approaches that can be used in a clinical setting for the investigation of thyroid nodule capsules to provide important information that can be of help to a pathologist for consolidating his/her diagnostic decision. In the future, we plan to
extend our work in this direction by using the parameters which provided statistically significant differences between benign and malignant nodules in this current study, to automatically assign a malignant/benign capsule decision in a blind test conducted in clinical settings. In the same time, we consider that demonstrating the clinical utility of this tool in more challenging scenarios: e.g. noninvasive follicular thyroid neoplasm with papillary-like nuclear features vs. encapsulated papillary carcinoma or follicular adenoma vs. follicular carcinoma holds valuable potential for enhancing the diagnostic rate for thyroid related pathologies that are difficult to address with traditional histopathological approaches.

Another important advantage of a capsule-based differential diagnosis in the case of thyroid nodules is the possibility of in vivo examination and diagnosis of thyroid pathology. Nonlinear endomicroscopy using second harmonic generation has been demonstrated [41] and applying image quantification algorithms can enhance its potential and lead to minimally invasive thyroid nodules diagnosis.

In conclusion, our study shows that combining SHG microscopy with image processing may be used to provide complementary information to the current histopathology and cytopathology procedures for thyroid cancer diagnosis.

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**Disclosures**

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