A spectroscopy based procedure for in-vivo detection of liver metastasis in a rat model.

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Abstract

This report focuses on the diagnosis of metastases affecting the liver of an animal model through a fiber-optic probe delivering visible light and the analysis through the Diffuse Reflectance Spectroscopy. The metastases induction was limited to the right lobes of the liver, with the uncontaminated lobes of the same animal used as healthy control. The experiments were performed either on explanted organs or in vivo, on anaesthetised animals. The analysis of the reflectance intensities showed broad differences between the metastasis and the control. From both situations a panel of diagnostic wavelengths was argued, upon considering the specificity of the respective spectrum profile: 470 ÷ 480 nm, 485 ÷ 495 nm, 626 ÷ 632 nm and 636 ÷ 640 nm, plus smaller intervals centred at 500 nm and 535 nm. As consequence, we suggest that this probe configuration could be worthy of being further developed as a diagnostic tool.

INTRODUCTION

Biological tissue is assumed to behave as a turbid optical medium (Perelman LT et al, 1994, Yodh A el al, 1995). Several components are considered as responsible for both the absorption and the scattering of an incident light radiation (Perelman LT et al, 1998). The latter phenomenon provides significant attractiveness as far as the field of the optical biopsy is concerned, mainly due to the possibility of investigating tissue pathologies, such as cancerous or even pre-cancerous lesions, by means of a non-invasive approach. It is generally assumed that changes in the optical properties of biological tissues are the consequence of variations involving either the physiology or the morphology of the cells (Amelink A et al, 2004).

Several recent studies, aimed at the achievement of consistent solutions for the equations derived from the complex theories concerning the Light Scattering Spectroscopy (LSS), have been especially focussing on the measurements of the optical properties within the superficial layers of the biological tissue, for example at the level of mucosae (Perelman LT et al, 1998). As a matter of fact, many types of solid tumours arise within the epithelial layers and some common features have been reported, mostly concerning the morphology and the invasiveness of the malignant cells in the underlying tissues. The evidence of a significant enlargement of the nuclear dimensions in the tumour cells and cells crowding, as compared with those of the normal tissue, was obtained first through optical microscopy, the gold-standard technique, as reported for example in the case of breast, colon, bladder, prostate, cervix and liver cancers (Dukor R, 2002).

The equation proposed by Van de Hulst (vd Hulst HC, 1957) has been used in order to describe the optical scattering cross-section (\(\sigma\)) of the epithelial nuclei and the related reflectance (R), as measured by means of an optical probe and conformingly to the theory of Mie (Perelman LT et al, 1998). By means of such analysis it has been possible to differentiate effectively the nuclear size distribution of malignant cell lines from that of the normal counterpart.

Unfortunately, other sub-cellular structures of minor dimensions, ranging from the mitochondrion to the collagen based extracellular matrix, and including several chromophores such as the haemoglobin, contribute significantly to the scattering or absorption of the light (Perelman LT et al, 1998), so that the averaged scattering properties in a tissue depend ultimately on the individual scattering properties of such sub-cellular entities and their relative concentrations, yet the prediction of each sub-cellular particle’s size contribution to the transport scattering coefficient \(\left(\mu'_t\right)\) remains a controversial item (Mourant JR et al, 1998, Mourant JR et al, 2000). For example, in the study of Beauvoit and collaborators (Beauvoit B el al, 1995) a
correlation has been shown between $\mu'_s$ and the mitochondrial content of a panel of rat’s investigated tissues, the white adipose tissue representing an intriguing case, since the transport scattering coefficient was consistently high despite no correlation was observed with the content of the mitochondrial proteins. Such anomaly was explained by assuming that the high content of lipid particles in this anatomical district could be the main responsible for the scattering effect. Moreover, in a subsequent report (Beauvoit B and Chance B, 1998), where analogous experiments were specifically performed in the rat liver, it is claimed that the mitochondria rather than the nuclei are the main scattering centres. In spite of the discrepancies emerging from the conclusions regarding the identification of the scattering centres, the elastic light scattering spectroscopy (ESS) was proposed as a valid methodology for distinguishing the dysplasia and the cancer from the normal tissue or other benign conditions (Mourant JR et al, 1995, Mourant J el al, 1996, Ge Z el al, 1998), its use being claimed as little or non-invasive and suitable for providing real-time results. For example, ESS based techniques were applied at a clinical level for the breast cancer diagnosis (Bigio IJ el al, 2000). Beside such techniques, the differential path length spectroscopy (DPS) has been validated for the diagnosis of several tumour types (Amelink A et al, 2004). The basic instrumentation required for such analysis is analogous to that necessary in order to investigate the biological samples by means of LSS and proposed by Amelink and collaborators, although the probe’s configuration is different (Amelink et al, 2003). In particular, we have used the latter configuration in order to analyse the DRS signal acquired from the liver. The instrument’s capability of detecting the metastatic lesions was assessed in an animal model. The metastases were induced by an intra-splenic inoculation of a colon carcinoma derived cell line [DHD/K12/Trb (PROb)] (Caignard A et al, 1985) (Fig. 1); in anaesthetized animals a midline laparotomy was performed, the spleen was isolated and the hepatic portal branch to left lateral lobe (LLL) and left medial lobe (LML) were clamped to prevent the formation of metastases in the left side of the liver. Then $1 \div 2 \times 10^7$ neoplastic cells were injected into the spleen through a 25 G needle. The neoplastic cells invaded the right lobe, the right medial lobe and the caudate lobe only, while the remaining lobes (LLL and LML) were excluded from the blood inflow (due to the clamped portal branch, shown as a red bar in right panel of Figure 1).

**MATERIALS AND METHODS**

**INDUCTION OF LIVER METASTASES**

A total of 125 male BDIX rats, weighing 250-300 g, were used and treated according to the local authority’s guidelines. The surgical procedures were performed under general anaesthesia by intramuscular injection of Tiletamine and Zolazepam (8 mg/kg each). Cohorts of 25 animals were examined in parallel, during three independent ex vivo experimental sessions and two in vivo experimental sessions, respectively.

Liver metastases were induced by intra-splenic injection of neoplastic cells of a colon cancer cell line [DHD/K12/Trb (PROb)] (Caignard A et al, 1985) (Fig. 1); in anaesthetized animals a midline laparotomy was performed, the spleen was isolated and the hepatic portal branch to left lateral lobe (LLL) and left medial lobe (LML) were clamped to prevent the formation of metastases in the left side of the liver. Then $1 \div 2 \times 10^7$ neoplastic cells were injected into the spleen through a 25 G needle. The neoplastic cells invaded the right lobe, the right medial lobe and the caudate lobe only, while the remaining lobes (LLL and LML) were excluded from the blood inflow (due to the clamped portal branch, shown as a red bar in right panel of Figure 1).

**Figure 1**

Figure 1. Left panel: the injection of the neoplastic cells into the spleen; right panel: scheme of the invasion of the right lobe of the liver by the neoplastic cells (in purple). The red bar indicates the clamping of the portal branch

Thus, in the same organ both healthy and neoplastic areas were present (Fig. 2), so that the same animal could be used for recording the spectra of the metastases as well as the spectra of the healthy tissue. Upon infusion, the splenic vessels were ligated and the portal clamp removed. Splenectomy was performed and the abdominal wall sutured.
as already described (Chauffert B et al, 1988).

Ten days after the surgical intervention the animals were anaesthetised once again and the metastatic nodules were macroscopically evident in the right lobes of the liver as compared to healthy lobes (left). The animals were sacrificed and the liver explanted (a procedure referred to as ex-vivo approach) in order to be analysed through DRS. Alternatively, the DRS analysis was performed directly on the anaesthetized animals (referred to as in-vivo approach). In the former situation some areas were subjected to microscopic analysis upon staining with Hematoxylin and Eosin (Bancroft JD et al, 1996).

Figure 2
Figure 2. Upper panel: the liver is shown, with the metastases confined in the right lobes (visible on the left) while the remaining lobes are used as control. The panels in the middle (metast. and control) show specimens of the liver after fixation in a 4% solution of formaldehyde; dashed arrows put in evidence the metastatic nodules. The specimens were then subjected to the Hematoxylin and Eosin staining (bottom panels: magnification 10X, inset 40X).
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Figure 3
Figure 3. Upper panels: on the left the procedure used for recording the spectra upon explanting the organ is shown, while on the right the procedure used on the anaesthetised rats is shown. Bottom panel: The magnification of the acquisition of the data from the metastases.

DRS ANALYSIS
In the attempt of characterising the tissue by means of spectroscopy two approaches are common: the diffuse reflectance spectroscopy (DRS) (Amelink A et al, 2004) and the light scattering spectroscopy (LSS) (Perelman LT et al, 1998, Dukor R, 2002).

The DRS collects information from a multiple photon scattering inside the tissue, while, the LSS detects information from the single photon scattering after the light interaction with the most superficial cellular layer. In the schematic magnification of the tissue in fig. 4 the different outcome of DRS and LSS effects is evidenced.

As shown in the figure, the instrumentation used and the configuration of the probe are essentially those described by Amelink and co-workers (Amelink et al, 2003); briefly, an halogen source generated light, ranging from 360 to 2000 nm (Mikropack HL-2000-FHSA), that passed through a polarizer and a 50/50 beam splitter before entering the delivery/collection fiber (generally referred to as ‘d/c’ fiber), with a core of 400 μm. The light reflected from the sample passed through the beam splitter and was then directed toward the ‘master’ channel of a dual channel spectrophotometer (Ocean Optics SD 2000). The reflected light entering the probe’s collection fiber (also referred to as ‘c’ fiber), with a core of 400 μm, is directed toward the ‘slave’ channel of the spectrometer. The probe consists of the two fibers touching each other and placed within a metal tube. The relevant wavelength range for these studies is within 450-900 nm (including the visible range). The data acquisition process is controlled by a PC, as well as the positioning of the probe, obtained through a linear motor positioning stage.

Figure 4
Figure 4. The path of the light directed toward the sample (in yellow) and the reflected light directed toward the spectrophotometer (‘slave’ channel, in red) through the collection fiber ‘c’ is evidenced. Part of the signal was also directed to the spectrophotometer through the delivery-and-collection fiber, the beam splitter and the polarizer. This input to the ‘master’ channel did not provide additional information in terms of DRS/LSS (see legend below), but its possible relevance will be clarified in the Discussion.

In the schematic magnification of the tissue a representation of both the DRS and LSS effects is shown. The incident light is represented by an arrow pointing from the cyan area...
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toward the cells, depicted in red. The signal is acquired by collecting the light reflected from the cells (arrow pointing upward). The probe used in our experiments consists of two adjacent fibers. The former (fiber ‘c’) collects the DRS signal, the latter (‘d/c’) collects both the LSS and the DRS signal so that the LSS signal results from the difference between the “master” and “slave” channels, respectively.

Each acquired spectrum with DRS data corresponds to one file, coding for a (1418×2) matrix, that was analysed through a dedicated MatLab code. For statistical reasons, groups of 10 spectra were filed from each animal so that a total of 250 files were available per each experimental condition (i.e. concerning the healthy, the metastatic and the connective tissue, respectively), since cohorts of 25 animals were examined during each experimental session. Schematically:

**Figure 5**

Three independent experimental sessions concerned the ex-vivo analysis, while two independent sessions concerned the in-vivo approach, so that, globally, 3750 spectra were recorded. The 10 spectra included in each group (in total 375 groups of spectra, i.e. 25 animals × 3 experimental conditions × 5 experimental sessions) were named by means of progressive numbers so that a random number generator was then used for choosing one representative spectrum of a specific session per each animal and per each experimental condition.

Therefore the data presented are the average obtained from 25 independent measurements per each experimental condition.

The data providing the dark (d) and the white reference (ref) spectral intensities, respectively, were acquired according to the spectrophotometer manufacturer’s instructions (Ocean Optics) so that the reflectance (R) could be determined by the formula: \( R = \frac{S-D}{\text{ref} - d} \), being S the spectral intensity read by the spectrophotometer for a given acquisition. Each acquisition consisted of data that were subsequently corrected by considering the dark (d) and the white (ref) reference spectra, respectively: in the former case the signal resulted from the blocking of the path of the light in correspondence of the probe’s tip, while in the latter situation a spectrally flat reflectance was produced by means of a standard surface. For each spectrum, the values of S and ref had been preliminarily normalized by the respective underlying area and MatLab was used in order to create simple codes for the normalization, as well as for the statistical analysis of the data and for drawing the diagrams at a resolution of 0.36 nm. The approximation of the first derivative was obtained by means of the function ‘diff’ included in the software. Beyond the calculation of the standard deviation, the mean values were subsequently compared by means of the t-Student test (non paired data, two tailed) and the Mann-Whitney test (non-directional), respectively. Excellent and detailed explanations and examples about the purpose and the significance of the t-Student and the Mann-Whitney test can be retrieved from the work of Lowry available online and references therein (Lowry R, 2009). Briefly, both tests were used in our experimental situations, in order to establish whether the difference between the means of two sample populations (e.g., healthy vs. metastatic tissue, respectively, identifiable in terms of their mean and standard deviation, both arguable from the MatLab data of each specific spectral population) is, or not, statistically significant. The significance is conventionally assumed high enough as far as the probability that the observed difference of the means, as in case of experimental artefacts, is ≤ 5% (or, \( p \leq 0.05 \)). In other words, both tests should allow for rejecting the so-called null hypothesis (i.e., the difference observed is due to experimental artefact), that is assumed true when such probability should exceed 5%. While the t-Student test assumes, among a series of statistical conditions, that the distribution of the sample populations is normal, which thing in our case was not known a priori, the Mann-Whitney test assessed for the significance of the data, without the need of any statistical assumption about the sample distribution. The spectral data obtained from the MatLab matrix were then used as input to the calculators available online (Lowry R, 2009) for both tests, essentially performed through MS-Excel software. Finally, there are two choices for each test: a directional and a non-directional condition, respectively. The latter condition was chosen due to its higher stringency, given that, for our purpose, the observed difference between the means of two spectra populations could be either positive or negative.

MatLab scripts, as well as the list of specifications and technical detail for each component of the instrument, are
available upon request.

RESULTS

ANALYSIS OF THE RAT LIVERS

For the induction of the metastases in the rat liver, as described in Experimental details, a procedure was adopted in order to confine the metastasis within the right lobe of the organ (Nano R et al, 2004). Cohorts of 25 treated animals were analysed during each one of three independent experimental sessions and the same animal could therefore be used in order to acquire the data for the healthy and metastatic portion of the liver, respectively, so that the number of animals analysed could be limited to 75 rats. Upon explanting the liver, sets of ten spectra were collected from each animal for the healthy and the metastatic tissue, respectively, and 25 spectra, each representative for one animal (from a total of 250 spectra), were chosen randomly and mediated, as shown, for example, on top panels of Figure 5. Since each spectrum had been preliminarily normalised by the area included below the respective profile, the possibility that the observed differences between the metastasis and the healthy tissue could arise mainly from external physical factors, such as, for example, a variable distance between the sample and the probe’s tip during each measurement, or a variable intensity of the light directed toward each sample, rather than the molecular peculiarities and other characteristics specific for each anatomical district, resulted minimised. In the Figure 5 the ‘average’ spectrum for a given situation is presented alone and with the standard deviation, respectively.

Figure 6

Figure 5. Top panels: the ‘average’ spectra obtained after ex-vivo measurements of healthy (control) liver (in blue) and the metastatic counterpart (red). Bottom panels: on the left the average spectra shown on top are merged and the standard deviation shown (blue=control, red=metastasis). The enlargement of the horizontal scale in the 540 ÷ 600 nm interval is shown on the right in order to analyse the standard deviations at a higher resolution. Numbers on horizontal axis indicate wavelength values (in nm), while on the vertical axis the reflectance intensity is expressed in arbitrary units.

Upon the data acquisition and normalization, two analytical approaches were subjected to the statistical validation. The former was focussing on the differences between the reflectance intensities, as recorded at specific wavelength’s intervals for each kind of tissue, respectively. This approach revealed consistent differences (as indicated by the trend of the standard deviations) in broad intervals: 500 ÷ 590 nm and wavelengths above 685 nm. Another kind of information is provided by the approximation of the first derivative of the curves (Figure 6), obtained thanks to the differentiation procedure provided by the software, and highlighting the characteristic shape of each ‘average’ spectrum of Figure 5.
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**Figure 7**

Figure 6. Following the drawing of the spectra as in Figure 5 (top), the data were subsequently analysed by the differentiation procedure of MatLab. Control (blue) and metastasis (red) diagrams were merged. Numbers on horizontal axis indicate the wavelength (in nm), whereas values on the vertical axis (exponential factors arise from the scaling of the diagrams) indicate the ‘slope’ of the respective part of the diagram shown in Figure 5. Values above 0 correlate with ascending values of the reflectance, values below 0 correlate with descending values, while 0 indicates flat portions of the spectrum.

This approach resulted in the elucidation of several and relatively large wavelength intervals where the two tissues can be discriminated, with a confidence level ≥ 95% for all the points of each interval, according to the t-Student and Mann-Whitney tests. Consistent results were obtained from two independent experimental sessions and the definition of the critical wavelengths will follow the illustration of the results obtained in vivo (see below).

Provided that, on the basis of these data, the metastasis affecting the liver can be distinguished from the healthy hepatocytes, a key point to be taken into account concerns the fact that this discrimination could in part be favoured by the different colours of the two areas (see Figure 2) and determined by a differential absorbance of the incident light. In other words, it is plausible that a consistent fraction of the diagnostic capability could depend on such differences, rather than the morphological and/or molecular peculiarities, being the measured reflectance complementary to the absorbance (Jacob W et al, 2000). The impact of such potentially confounding factor was investigated in vivo, as described in the following section.

**THE HEALTHY LIVER AND THE METASTASIS CAN BE DISTINGUISHED UPON EXAMINATION.**

In order to test the diagnostic capability of the fiber-optic probe in a pre-clinical trial situation, the comparative analysis, performed as described above, was repeated on a total of 50 anaesthetised animals as exemplified in the right panels of Figure 3. Figure 7 shows the average spectra of the healthy liver and the metastatic counterpart (one representative experimental session).
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Figure 8
Figure 7. Top panels: the ‘average’ spectra obtained after ex-vivo measurements of healthy liver (control, in blue) and the metastatic counterpart (red). Bottom panel: the average spectra shown on top are merged and the standard deviation shown (blue=control, red= metastasis). Numbers on horizontal axis indicate wavelength values (in nm), while on the vertical axis the reflectance intensity is expressed in arbitrary units.

The profiles of these spectra, in particular, appear quite similar to those corresponding from the ex-vivo situation, as far as the respective reflectance intensity at a specific wavelength is concerned. The approach regarding the spectrum’s profile (Figure 8) reveals several intervals where the differences between the healthy and the metastatic tissue appear significant with a confidence level of 95% at least.

Figure 9
Figure 8. Following the drawing of the spectra as in Figure 7 (top), the data were subsequently analysed by the differentiation procedure of MatLab. Control (blue) and metastasis (red) diagrams were merged. The values are expressed as in Figure 6.

A set of diagnostic wavelengths was argued upon comparison of the significant differences shared by both the ex-vivo and the in-vivo specimens (c.l. ≥ 95%) and includes the following intervals: 470 ÷ 480 nm, 485 ÷ 495 nm, 626 ÷ 632 nm and 636 ÷ 640 nm, in addition to smaller intervals centred at about 500 nm and 535 nm, respectively.

Several differences, evident from the data of the spectra profiles rather than the reflectance intensities obtained from the ex-vivo approach versus that in vivo, suggested for such prudential restriction of the panel of the candidate diagnostic wavelengths. At the moment we cannot explain definitively such differential response, but assessed whether the cells’ transplantation procedure could be at least in part responsible for this effect. Repeated measurements from organ controls were performed, where sets of spectra were obtained from the liver of animals that did not undergo the intra-splenic injection of the cells, or received an injection of the sole medium (mock) where usually the cells are resuspended, in order to exclude the possibility of a cross
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Contamination by the tumour. A comparative analysis was done, either upon ex-vivo and in vivo measurements focussing on the healthy, red coloured areas of the liver in not treated and mock animals, respectively. The spectral features of the non contaminated liver confirmed analogous differences (not shown) in the profile, as those already observed upon comparison of the healthy side of the liver bearing the metastases between the ex-vivo and the in vivo context (as in Figures 5 and 7 top panels on the left). Given this result, significant changes in the optical properties may occur since the organ is explanted till the moment of the analysis, the metastatic portion appearing more susceptible, as arguable by the diagrams of Figures 5 and 7 (top right). In any case the metastasis resulted clearly differentiable from the healthy tissue.

As mentioned above, the healthy liver is characterised by a very homogeneous aspect, though a portion of connective tissue, approximately 10 mm in diameter, is localised internally and approximately in the middle of the organ and becomes accessible to the probe only after it is explanted (as in Figure 9 top panel, left). As can be seen on top of Figure 9, the discrimination between such tissue and the metastasis may be not easy without further investigation (for example by means of microscopy).

During the in vivo independent experimental sessions easily accessible areas of connective tissue, adjacent to the liver, were investigated and the respective spectra recorded in parallel to those from the metastases, then subjected to the usual mathematical approaches, and the results shown in Figures 9 and 10.

**Figure 9**

Top: on left panel the connective tissue of a healthy liver (explanted) is shown, on the right panel the metastases are shown for a visual comparison.

Middle panels: the ‘average’ spectra obtained after in vivo measurements of connective tissue adjacent to the liver (in light green) and the metastatic portion (red). Bottom panel: the average spectra shown on top are merged and the standard deviation shown (light green=control, red=metastasis). Numbers on horizontal axis indicate wavelength values (in nm), while on the vertical axis the reflectance intensity is expressed in arbitrary units.
DISCUSSION

The present study was aimed at determining whether an analytical approach complementary to ESS, namely based on the DRS, could be valid for distinguishing between the healthy and the metastatic portions of the rat liver in an in-vivo simulated surgical procedure. The experiments were based on the preliminary use of an animal model, where the metastases can be confined in an anatomical site that allows for the simultaneous and specific recording of the diffuse reflectance spectra from the healthy, the metastatic and the connective tissue, respectively. The significance of the analytical approach relies on a consistent number of recorded data. Indeed, the spectra were analysed upon being chosen through a random number generator and the tissues could be differentiated, following either ex-vivo or in-vivo independent experimental sessions. In particular, the validation of the in vivo data reveals that several wavelength intervals may be crucial for the diagnostic capability of the instrument’s configuration adopted for these experiments. As shown in the Results, the elucidation of several wavelength intervals, where the null hypothesis could be rejected by means of both the t-Student and Mann-Whitney tests (as explained in Materials And Methods), is consistent with a normal distribution of each spectral population, due to the agreement of the results obtained from both tests.

Although the identification of the intervals including the diagnostic wavelengths was limited on the basis of common features, shared by the ex-vivo and in vivo data, respectively, the results so far produced put in evidence a specific response to the pathological condition, so that the instrument configuration appears reliable from a diagnostic point of view. According to what has been already reported in the literature about an analogous approach for the diagnosis of the breast cancer (Bigio IJ el al, 2000), our future experiments should be conceived in terms of the most appropriate ex-ante analysis: data collected from multiple experimental sessions should contribute to a spectral classification based on the artificial intelligence pattern recognition, aimed at the assignment of parameters indicating the sensitivity and the specificity of our instrument.

The configuration of the probe used for the experiments described above is substantially equal to the instrumentation tested by Amelink and collaborators (see Figure 4) (Amelink et al, 2003).

These authors tested a tissue phantom consisting of
polystyrene spheres of different diameters by means of the visible light. Therefore, our configuration was expected to be suitable as well for discriminating the possible variations of the nuclear diameter, such as in the case of the neoplastic cells accumulation. According to the results from the tissue phantom (Amelink et al, 2003), the probability that the reflectance signal (the single scattering) is mainly generated by the Mie scattering centres closest to the probe’s tip is enhanced thanks to this specific configuration and the effect could in fact be reproduced also with our probe (Figure 11).

**Figure 12**
Figure 11. Example of spectra generated by the Mie scattering of a solution of microspheres of 4,5 μm in diameter. The profile in red is the recorded signal of the ‘slave’ channel of the spectrophotometer which was used for recording the DRS data from the liver, whereas the profile in blue, i.e. the signal of the ‘master’ channel, was not as informative in the case of organ examinations. Both signals are conveyed to the Fourier-transform procedure in order to measure the diameter of the spheres

As reported by Perelman and collaborators (Perelman LT et al, 1998), the data generated by means of the Mie scattering could in fact be used in order to detect cancerous or even precancerous lesions, on the basis that the epithelial nuclei determine a periodicity in the reflectance, its amplitude being a function of size and number of nuclei.

However, the results from the tissue specimens examined by means of this methodology failed to prove clearly the existence of the periodic oscillations typical of the single scattering effect (even upon analysing different organs, such as mammary tissue and lung). As far as the liver is concerned, the failure of the LSS (Light scattering spectroscopy) may be justified by the controversial identification of the nuclei as the main scattering centres (Beauvoit B and Chance B, 1998). Apart from our experiments, it is conceivable that the experimental condition based on the LSS could be less appropriate for the examination of the biological tissue in general; indeed, at the time of our experiments, three subsequent articles, published from the same team that had first reported about the experimentation of LSS on the tissue phantom, showed data obtained from biological models and based rather on the differential path length spectroscopy (DPS) (Amelink A et al, 2004, van Veen RL et al, 2002, Amelink A et al, 2007). In all these recent articles the attention is mainly concentrated on the optical properties of the biological tissue and do not focus on the dimensions of the scattering centres; a convincing validation of the DPS approach emerged from these reports, at least for the cases of malignant lesions of the bronchial and oral mucosae.

Our results, although obtained through an alternative technique (DRS) and from a different tissue, indicate as well that a metastatic lesion can be detected independently from the periodic oscillation of the reflectance profile. In principle it cannot be excluded that our reflectance signal may include a periodic fine structure component that could account for a very low percentage of the total signal, but the predominant phenomena could likely concern the absorption of the haemoglobin and the scattering effect produced by the collagen fibers, as already described in the literature (Perelman LT et al, 1998). These two chromophores generally determine a consistent signal, that can be neglected upon the application of the Monte Carlo simulations to specific models of light transport based on the Mie scattering (Perelman LT et al, 1994, Wu J et al, 1993). On the other hand, the haemoglobin concentration, its saturation degree, the blood vessels’ density, the lipid and the collagen content of the tissue have been proven to be as well informative parameters for a pathological situation (van Veen RL et al, 2005), like in the instance of cancerous lesions (Amelink A et al, 2004, van Veen RL et al, 2002, Amelink A et al, 2007).

In the present study the main purpose has been that of developing a relatively economic instrument, with the potentiality of being a real-time diagnostic tool to be used during surgical interventions, rather than being optimised a priori for the identification of the sub-cellular structures responsible for the spectral features at specific wavelengths; yet the data obtained so far show clearly that the optical properties related to the tissue composition play an important role, given the capability of the instrument to discriminate quite well the metastatic from the connective tissue, though both show an almost equal appearance. A
deeper analysis of the possible influence of the haemoglobin on the results could be a valid starting point, since several spectra are characterised by a profile that, at least for a restricted wavelength interval (typically within 500 ÷ 600 nm), is strongly resembling the trend documented in the work of Backman and collaborators (Backman V et al, 1999), that stressed about the presence of dips in such range, typically generated by the haemoglobin. Our speculation derives from the observation that the spectra of the metastasis and the connective tissue show similar dips in that interval. Then the possibility of a differential response in relationship with a different metabolism of the heme (Fujita H et al, 1997) in the healthy hepatocytes with respect to the connective or the metastatic tissue could be further investigated.

But before investigating on any molecular candidate, the results so far obtained in the animal model suggest that the same methodology should be worthy of being first exploited in humans affected by hepatic metastases, in order to confirm its reproducibility in a true clinical situation, expected on the basis of the analogous cytological characteristics.

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