Abstract. Background/Aim: The early diagnosis of breast cancer plays an important role in reducing mortality and optimizing the prognosis of the disease. The existing visual and histopathological methods do not give any information at a molecular level. Fourier transform infrared spectroscopy does not require any preparation, such as fixation and histological stains. The collected infrared spectral “biomarker bands” give information at a molecular level and could be used for biomarker screening, in order to minimize the false-positive or false-negative results.

Materials and Methods: For this prospective study, nine biopsies of lobular carcinoma (7 in situ and 2 invasive) and the adjacent healthy region of the biopsies were used. Each infrared spectrum consisted of 120 scans/spectrum (120 co-added spectra) at a spectral resolution of 4 cm⁻¹. Results: The infrared spectral analysis revealed three important “diagnostic spectral regions” between 3,300-2,850 cm⁻¹, 1,700-1,500 cm⁻¹, and 850-800 cm⁻¹, which are related to membrane, collagen, and DNA configuration damage, respectively. The shift of the absorption band at 1,161 cm⁻¹ at higher wave numbers up to 1,172 cm⁻¹ is assigned to vC-O-C bonds due to membrane, protein, and DNA glycosylation. Conclusion: The “biomarker bands” at 1,172 cm⁻¹ can be used as “diagnostic marker bands” for cancer progression. The shift of the absorbance band at 823 cm⁻¹ of the native configuration of B-DNA to lower wavenumbers at 810 cm⁻¹ Z-DNA in grade III, suggests the irreversible stage of the disease. The detection and possibility to differentiate the DNA structures may allow detection of carcinogenesis at the early stage of the disease, and development of new anticancer therapies.

Breast cancer is the most common neoplastic disease in the world in the female population, accounting for approximately 25% of all malignancies (1). The incidence of the disease varies across the world with low rates in undeveloped countries and higher rates in developed ones (2, 3). In contrast to the incidence of breast cancer, relative mortality is higher in undeveloped countries. Among the risk factors related to the disease manifestations, are the woman’s long fertility period (onset of menstruation at a very young age and menopause in old age), contraceptive use, obesity, postmenopausal estrogen use, lack of exercise, and alcohol use (4). On the contrary, childbearing and breastfeeding appear to be preventive factors (2, 5). With increasing age, the incidence of breast cancer increases and becomes significant close to 50 years. During pre-menopause, the rate increases 8-9% per year, while it drops to 2-3% per year after menopause, apparently due to hormonal changes (6-9). The most common breast neoplasia is lobular carcinoma (originated in the milk-producing glands, the lobules), which presents as in situ or invasive lesions, accounting for up to 15% of all breast cancers (3, 10). Studies have shown that early diagnosis of breast cancer plays an important role in reducing mortality and optimizing the prognosis of the
disease (11). Owing to early diagnosis and new therapeutic approaches, the mortality of the disease has decreased since the 1970s (12).

The most prevalent and well-documented method for disease screening, to date, is mammography. Ultrasound scanning, magnetic resonance imaging (MRI), positron emission tomography (PET), and other emerging and much more promising molecular imaging methods are used to elucidate and further evaluate mammography findings or screen high-risk groups for breast cancer. Although all the above methods are particularly important in the diagnosis of breast cancers, they do have some limitations related to factors, such as age, menopausal status, and the progression of the disease. Unfortunately, these methods cannot provide quantitative information regarding tissue composition. It is clear that for the early diagnosis and treatment of breast cancer, it is necessary to develop a bio-analytical method that will not be affected by factors such as age, breast density, size of the possible neoplastic lesions, method’s repeatability (i.e., without being harmful to the patient’s health) and others, which may simplify disease detection. Fourier transform infrared (FT-IR) spectroscopy is extremely rapid, non-destructive, and a very promising method in studying changes induced by diseases (13-21). Important is to interpret simultaneously all the spectral components of the tissues, without any previous special preparation of it, as in histopathology. Since the infrared spectrum is the “fingerprint” of a certain tissue, it is possible to detect and discriminate cancer cells from the surrounding microenvironment. The study and analysis of the final products of the disease are of particular importance for the understanding of the mechanism of carcinogenesis and therefore in generating the possibility of more advanced therapeutic schemes. In addition, the detection of infrared “biomarker bands” could be also used for biomarker screening, in order to minimize the negative false results.

Materials and Methods

Biopsies. For this prospective study, nine biopsies of lobular carcinoma (7 in situ and 2 invasive) were used. All biopsies immediately after surgical excision were stored in formalin solution. Then, the samples were washed with distilled water and dehydrated under vacuum at room temperature. Normal tissues of the adjacent healthy region of the biopsy were used for comparison. The hematoxylin and eosin (H&E) staining was used for the standard histological discrimination of grades I, II, and III. It is important to notice that the samples were not incubated in paraffin, since we have noticed that by removing the paraffin the hexane removes soluble products, such as aldehydes and glycation end-products, produced during cancer development, thus losing valuable information.

Statement of ethics. The samples were obtained according to the Helsinki declaration and the Greek law of ethics for ex vivo clinical research.

Fourier Transform Infrared spectroscopy (FT-IR). The success of the study depends on the collection of the spectra. The band intensities are the key of high-quality spectra for discrimination of the fingerprints of the breast tissue components in order to build a library for “spectral diagnostic bands”. In order to increase the signal-to-background noise ratio, each spectrum consisted of 120 scans/spectrum or 120 co-added spectra/spectrum. The specific spectral resolution of 4 cm–1 is sufficient for spectral collections. The attenuated total reflection (ATR) crystal, throughout the internal reflections into the samples increases the intensity of the bands. FT-IR spectra were recorded at room temperature with a Nicolet 6700 spectrometer (Thermo Scientific, Waltham, MA, USA). OMNIC 7.2a workstation software was used for data analysis (Thermo Scientific).

Mammography computation analysis. The open-source platform for biological-image analysis Imagej-Fiji was used to analyze the breast cancer images, which were received from mammography (23).

Results and Discussion

FT-IR spectroscopic analysis. The subtype lobular carcinoma in situ arises from the lobule at the terminal end of the duct. There is enlargement and expansion of the lobule with solid masses of tumor cells. The architecture of the lobes is not disturbed, while the malignant cells show great consistency; they are round-shaped, without coherence, with blurred borders, dense color nuclei, indistinguishable nuclei, and minimal mitoses. Representative FT-IR spectra of lobular in situ cancers of grade I, II, and III are illustrated in Figure 1. The spectra show vibration frequencies, band intensity, and shape changes.

In the spectral region of 3,500-3,000 cm–1 the stretching vibrations of vOH and NH of proteins and DNA are shown. It is observed that by increasing the grade of cancer from I to III the intensity of the NH band decreased and shifted from about 3,300 cm–1 of normal tissue to lower frequencies in the region 3,200-3,100 cm–1. These observations suggested that the secondary structure of proteins changed from the native Amide A (α-helix) to denatured Amide B form, due to the changes in hydrogen bonding, which stabilizes the collagen strands, upon the progression of cancer (24). This shift is a good criterion for the progression of the cancer stage. The band at 3,080 cm–1 is attributed to stretching vibration of the olefinic bond ν=CH 2 (16-19), indicating the involvement of oxidative stress and hydroxyl free radical (HO•) formation in the pathway of the disease (15-18). Interesting are also the changes observed in the spectral region 3000-2850 cm–1, where the asymmetric and symmetric stretching vibrations of methyl (νas,s CH 3) and methylene (νas,s CH 2) groups, mainly from membrane lipids and phospholipids, appear. With the increase in the grade of the tumor, the intensities of the methyl stretching absorption bands decreased, while the intensities of the stretching methylene bands increased. These changes indicate changes...
in the lipophilic environment of the cell membranes (13-20). Deconvolution of these bands showed the appearance of more bands resulting from branched polymers, indicating fibril formation. The formation of aldehydic functional groups, vCHO, is indicated by the absorption bands at 1,739-1,748 cm⁻¹, which confirmed the existing oxidative stress and inflammation during cancer development. It is known that aldehydes (malondialdehyde) are among the products of cancer detection (25). It is noticeable that the band of aldehydes at 1,739-1,748 cm⁻¹ is not observed in biopsies after paraffin fixation.

The high intensity band at 1,650 cm⁻¹ is attributed to amide I vibrational mode arising from the stretching vibration of C=O coupled to the bending vibration of the NH of peptide bonds of proteins (NHCO) with a possible contribution from C=O stretching modes of DNA nucleotides. The next intense band at 1,545 cm⁻¹ is attributed to amide II arising from the bending N-H vibration coupled to the stretching vibration of C-N. Comparison of deconvolution spectra between I-III grades in the region 1,750-1,500 cm⁻¹ (Figure 2) shows that with increasing tumor grade the secondary structure of proteins changes from α-helix to random coil and β-sheets, while in grade III cancers the protein structures are further damaged (15-18). The observed absorption bands of parallel and antiparallel β-sheets confirm the formation of beta-amyloid-like protein formation in agreement with the dominant lipophilic environment.

It is also important to notice that upon cancer formation the DNA changes its native conformation structure from B-DNA to cancerous Z-DNA. Analysis of the region 900-800 cm⁻¹, where the sugar-phosphate puckering of DNA absorption bands are located, showed that in grades I and II the double helix of C2’-endo/anti of native B-DNA is predominant. However, in grade III a shift from 825 cm⁻¹ to 810 cm⁻¹ was observed, indicating that the puckering is converted to C3’-endo/anti, which characterizes the structure of Z-DNA. This shift is proposed to be a “diagnostic shift” for the appearance of Z-DNA and cancer development (24, 26-27).

For a better understanding of the changes induced by cancer in proteins and DNA, the spectrum of grade I was abstracted from that of grade III lobular in situ cancer. As is illustrated in Figure 3 with the progression of cancer from grade I to grade III the appearance of the aldehyde absorption band at 1,746 cm⁻¹ is higher in grade III, while the protein secondary structure is transformed from α-helix to β-sheet.

The band at about 1,467 cm⁻¹ arises from bending δCH₂ and is overlapped with the stretching vibration of vCO₃²⁻ cations, due possibly to calcification of the breast tissues and

Figure 1. FT-IR spectra of representative normal tissue and lobular in situ cancers. The spectra 1, 2, 3, and 4 correspond to normal tissue, grade I, II, and III, respectively. Considerable differences in band intensity and shape are shown at the stretching vibration of vPO₂⁻ of DNA and phospholipids.
formation of calcium carbonate ($Ca^{2+}CO_3^{2-}$). The calcifications were confirmed from the mammography of the patient. The band at 1400 cm$^{-1}$ is attributed to vCOO- modes of proteins and peroxy-fatty acids. Another prominent spectral region is between 1,250-1,000 cm$^{-1}$. In this infrared region, the absorption vibrational modes C-O-P of membrane phospholipids and DNA appear, as well as the bands of the chemical groups of -C-O-C- and -O-C-C, where an oxygen atom is linked to two carbon atoms of the sugar moiety of glycosaminoglycan together with the exocyclic C-O-C inter-molecule groups. The bands between 1,150-1,165 cm$^{-1}$ are attributed to the –C-O-C bond of the sugar ring and the band at 1,080 cm$^{-1}$ to exocyclic –C-Ο-C- (oxygen bridge). The intensity of the band at 1,160 cm$^{-1}$ increases considerably and shifts to a higher wave number up to 1,172 cm$^{-1}$ in grade III spectra indicating the high rate of glycosylation, which takes place during the cancer development (15-17). This band can be used as a “biomarker band” to discriminate the grade of cancer. Our results are in agreement with literature data, which reports alterations in glycosylation during cancer progression and suggests these products as chemical labels for cancer (28-33). Enzymatic or chemical reactions may be the pathways of glycosylation. The observed conformational changes in DNA structure indicate that the nucleotides have lost their ribose sugars. The hydroxyl free radicals, that are produced during cancer progression, could abstract the hydrogen atoms at the C1’ site of nucleotides, leading to ribose and ribose-phosphate free radical formation (34). These reactions could lead finally to the observed single strand breaks (SSB) or double strand breaks (DSB) of DNA (35).

Since lobular in situ breast cancer is uncommon, while invasive lobular cancer is the second most common type, we compared the spectra of the in situ lobular and invasive lobular cancers. Figure 4 shows the FT-IR spectra of in situ (1) and invasive (2) lobular cancers, both grade III, in the sensitive spectral regions 1,760-1,540 cm$^{-1}$, where the amides I and II absorb, and in 1,000-760 cm$^{-1}$, where the conformational bands of ribose-phosphate groups of DNA appear. Substantial
differences are found between these two spectra. The intensity and shift changes of both amide I and amide II absorption bands suggest that in invasive lobular cancer the formation of amyloid-like proteins is more predominant.

Furthermore, the spectral region 1,000-760 cm⁻¹ shows that in situ lobular cancer is characterized by the absorption bands of all conformations of DNA, such as the left-hand native B-DNA, the right-hand A-DNA and the zig-zag Z-DNA (26, 27, 36, 37). On the contrary, in invasive lobular carcinoma the structures of A- and Z-DNA appear. The presence of the absorption band at about 785 cm⁻¹, which is attributed to cytidine, confirms conformational changes from the native B-DNA to Z-DNA. These bands confirm also that the ribose-phosphate groups of the DNA backbone have zig-zag conformation, which is characteristic of Z-DNA. The detection and the possibility to differentiate the DNA structures may give the possibility of understanding the mechanism of carcinogenesis (36, 37).

**ImageJ analysis of invasive lobular breast cancer mammography.** During breast cancer screening the histopathologists and radiologists encounter lesions that are difficult to identify with the existing methods and thus a combination with other techniques of molecular analysis is needed. Figure 5 (1) shows the left breast mammography of a 48-year-old patient. Features of in situ and invasive lobular cancer are present in the retro areolar (behind the nipple) area, as a rough group of calcifications.

Histopathological analysis showed an area of 2.5 mm with intense crushing effects in which foci of in situ and lobular invasive cancer existed. The in situ lobular cancer was grade II (moderate nuclear atypia) and had macrocalcification deposits. There was a coexisting in situ lobular cancer, comedo type, with epigenetic central macrocalcifications and a microscopic focus of invasive lobular cancer of a max diameter of 1.6 mm, that due to the intense crushing effects,
was not possible to evaluate its characteristics. Nevertheless, the cellular size in this microscopic focus and the mode of infiltration (presence of trabecular aggregates in the middle of the fibrous substrate) were in favor of invasive lobular cancer.

Figure 5 (2A) shows the surface plot (ImageJ) analysis of the squared region A. The arrows show the areas, which are characterized by a higher number of pixels, due to increasing conductivity induced by the calcium cations (Ca\(^{2+}\)) and appear as bumps (hill-like). Surface plot analysis of region B appears as a crater (arrow), indicating increase in lipophilic environment and amyloid protein formation (38).

These results are enhanced by FT-IR spectroscopic and mammographic data. Analysis of region C shows a channel formation. The channels demonstrate the breaking of the hydrogen bonds, which hold the breast connective tissue. The channels were also observed during \textit{ex vivo} cartilage irradiation with at a dose of 4 Gy (18, 39). The lesion was attributed to the hyaluronic acid hydrogen bonds break. It is known that hyaluronic acid binds to proteins (mainly collagen) with hydrogen bonds and forms the connective tissue. The negative ion positions of the protein amino acids (COO\(^{-}\)) are the binding sites of the positive calcium ions (Ca\(^{2+}\)), where the calcifications finally are formed.

Our analysis leads to the conclusion that in samples with grade III breast cancer features, where the lipophilic environment prevails, the formation of amyloid proteins has already begun. This consideration is reinforced by radiographic findings of a fibrous substrate. It is well known that fibrosis is strictly related to amyloid proteins.

These characteristic pictures received from surface plot ImageJ analysis show that with the development of appropriate software there will be a substantial contribution to the very early breast cancer detection.

Conclusions

The FT-IR spectral analysis demonstrated that the structural changes at a molecular level, induced by the disease, were related to the histological type of cancer and to the tumor grade and could give important results for the early diagnosis of breast cancer. The important “diagnostic” infrared spectral region at 3,300-2,800 cm\(^{-1}\) is related to the damage of the protein and DNA strands, as well as of the membrane lipids and phospholipids. The intensity decreases and shifts of the amide I (1,650 cm\(^{-1}\)) and amide II (1,544 cm\(^{-1}\)) bands indicate amyloid protein and fibril formation. The shift of the absorption band of 1,161 cm\(^{-1}\) to higher wave numbers up to 1,172 cm\(^{-1}\) is a result of glycosylation and cancer progression and could be used as a “diagnostic marker band”. It was also found that in grade III breast cancer the B-DNA changed its native configuration to Z-DNA, suggesting the irreversible stage of the disease. These findings would lead us to find new therapeutic methods for the very early stages of the disease. ImageJ analysis of
mammographs provides the lesions of hydrogen bonds by which hyaluronic acid binds to proteins (mainly collagen) and forms the connective tissues. Moreover, the analysis revealed the presence of calcifications in mammographs of the patients in the study and these findings were in full accordance with the findings of their histopathologic data.

Conflicts of Interest

No benefits have been or will be received from a commercial party related directly or indirectly to the subject matter of this article.

Authors’ Contributions

Athina Markouizou, MD, PhD, recorded the spectra and performed the clinical data of the patients. Pericles Tsekeris, MD, PhD, interpreted the clinical data of the results. Panayiota Kolovou, MD, PhD, received the breasts Echo Elastography and compared the illustrations with the corresponding illustrations of ImageJ. Theophile Theophanides, supervisor of the paper. Jane Anastassopoulou wrote the paper and analyzed the spectra.

References


