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## An FT-IR Spectroscopic Study of Metastatic Cancerous Bones

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### 1. Introduction

Breast cancer is the most frequently diagnosed cancer in women aged 40 to 65 years, with more than 464,000 new cases (13.5% of all cancer cases) per year in Europe [1,2]. Approximately 5% of breast cancer patients have advanced (metastatic) disease at diagnosis. Despite important progress in adjuvant and neoadjuvant therapies, up to 90% of cancer deaths are due to complications arising from metastatic dissemination of the disease [3,4]. For patients with established metastatic disease, therapy is usually for palliation.

Metastasis is a complex process, entailing tumor cells acquiring a set of features that allow them to develop new foci of the disease. Its exact mechanism remains unclear. Metastasis has been described as the migration of tumor cells from the primary tumor, followed by intravasation, survival, extravasation of the circulatory system, and progressive colonization of a distant site [5-7]. In a second definition, tumor cell genomic instability occurs that enables invasion and distant organ colonization [8]. In another definition, metastasis is described in terms of seed and soil [9]. Tumor cells (seeds) spread widely through the body, but grow only in supportive locations (congenial soil). Thus the various microenvironments (soils) of metastases contribute to the observed heterogeneity [9].

The most common sites of breast cancer metastases are the bones, brain, adrenal glands and other parts of the body [10,11]. Metastatic bone disease alters the mechanical properties of the involved bones, produces painful osteolysis, microfractures, and eventually complete fractures. The extent to which metastases are site specific and the transformation of healthy cells into cancer cells also remain poorly understood. A hallmark of breast cancer metastasis is the redundancy of pathways that mediate the process or its component steps, and the abundance of promoting genes [3]. Some pathways contribute to bone metastasis, while other pathways have

been reported to mediate lung and liver metastasis. Advanced next-generation sequencing techniques have also been used to interrogate whole cancer genomes at the single-nucleotide level and have distinguished between mutations in breast cancer metastases [12,13]. However, one metastasis may be distinct from another within the same patient accurate prediction of the molecular profile of metastatic disease by profiling the primary tumor is not feasible. In this setting, novel detection techniques are necessary [14].

Fourier transform infrared spectroscopy is a physicochemical, non-destructive, sensitive and reproducible method which provides important information about changes in the molecular structure of the bone due to the disease [15-29]. The advantage of the method is that it needs small amounts of the sample (only few micrograms) and we could study the sample without any preparation, such as coloring or demineralization as it is done in histopathology, since the spectra are based on individual chemical characteristics of the bone. Another important advantage of the infrared spectroscopy is that the method does not require any preparation of the sample, such as coloring or decalcification, as in methods like histopathology, where the samples are decalcified and labeled with color and are analyzed only the changes in the organic phase of the tissues. In infrared spectroscopy, the spectrum is the sum of all the frequencies of the components present and provides information on all components simultaneously [15-21].

Here within it is presented the influence of cancer on the molecular structure of the constituents of the bone (Hydroxyapatite, Collagen and Protein) and the characterization of the spectral differences between healthy and cancerous bones, in order to have a better insight of the process of the disease.

## 2. Materials and methods

### 2.1. Sample preparation

Although breast cancer metastases to skeleton bones are frequent sites of first distant relapse, however the bone samples are not easy to be obtained. Six bone sections from breast metastatic cancer patients (39 and 65 years), who suffered from breast cancer and underwent a reconstruction with an osteoarticular allograft, were used for the present study. Small amount of fresh bones (cancellous or/compact), were immersed successively in hydrogen peroxide solution ( $H_2O_2$ ) and in acetone, according to a modification method [15-17, 20,30]. Hydrogen peroxide and acetone processing is known to reduce the fat tissue and blood chromophores of fresh bone, but it does not remove the organic components completely.

### 2.2. Sample analysis

#### 2.2.1. Infrared spectroscopy

The conformational and molecular changes of healthy and cancerous bones were recorded using a Nicolet 6700 thermoscientific Transform Infrared (FT-IR) spectrometer, which was connected to an attenuated total reflection (ATR) accessory. This technique is convenient in

this case for the cancerous bones since it is almost impossible to powderize these tissues. Moreover, impregnated with PMMA bone appears less suited, because when remove the plastic there is dangerous to loose important components.

Each absorption spectrum of the samples was consisted of 120 co-added spectra at a resolution of  $4\text{ cm}^{-1}$  and all spectra were obtained in the same way in absorption mode in the spectral region  $4000\text{--}400\text{ cm}^{-1}$ . The interpretation of the spectra was done by analyzing the spectra and comparing the spectral data between cancerous and healthy tissues in order to follow and show the characteristic pattern of the disease. Data analysis was performed with the OMNIC 7.3 software.

#### *2.2.2. Scanning electron microscope*

Scanning electron microscopy (SEM) is also a non-destructive method, which allows the investigation of the surfaces of cancerous bone tissues, without any decalcification, coloring or coating. Under these conditions, there is not any change in chemical bonds between mineral and organic phase of the sample.

The distribution of the morphology of samples and bone mineral content were obtained using the (SEM) equipped with a microanalyzer probe EDX (Electron Dispersive X-rays analysis) from Fei Co, Eindhoven, The Netherlands

#### *2.2.3. X-Ray diffraction*

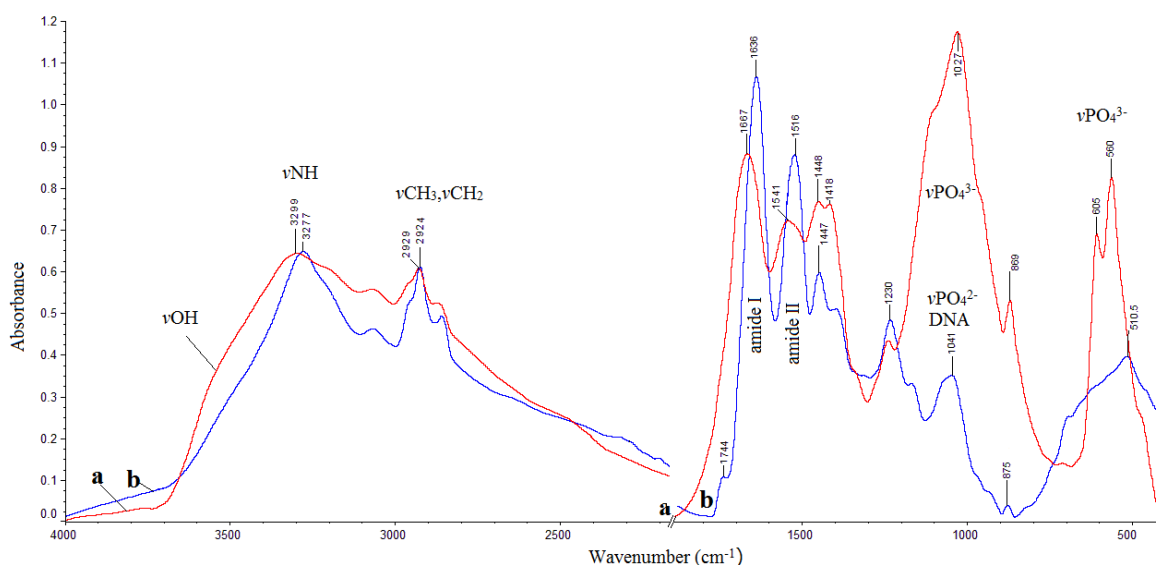
X-Ray diffraction, XRD, was used to identify the crystal structure of hydroxyl apatite and the presence of other calcium phosphate salts, which were produced with the progress of the cancer metastasis and development.

Powdered bone diffraction patterns were recorded using a Siemens D-500 X-Ray diffractometer based on an automatic adjustment and analysis system. The diffraction interval was of  $2\theta$   $5\text{--}80^\circ$  and scan rate of  $0.030^\circ/\text{s}$ .

### **3. Results and discussion**

#### **3.1. Infrared spectroscopic analysis**

In the treatment of breast cancer, the occurrence and growth of distant metastases is the major cause of morbidity and mortality. Long distant metastasis of breast cancer to bones induces micro-fractures changing the patients' quality of life. To understand the mechanism of the cancer cell dispersive and damaging effects which are induced to bone structure is of high interest in order to study the disease. Figure 1 shows the FT-IR spectra of a) healthy radius bones and b) of cancerous bones from a woman (59 years old) with a primary breast cancer.



**Figure 1.** FT-IR spectra of a) healthy bone and b) cancerous bone from a 59 years woman with metastatic bone cancer from breast cancer the original site

Comparison of the two spectra showed considerable changes in the whole spectral range of 3600–400  $\text{cm}^{-1}$ . The intensity of the shoulder band near 3550  $\text{cm}^{-1}$  for healthy bones was reduced or disappeared in all cancerous bones. Deconvolution of this band showed that it was double with maxima at 3515  $\text{cm}^{-1}$  and 3400  $\text{cm}^{-1}$ , which are dominated by the absorption of stretching vibration of  $\nu\text{OH}$  functional groups of water and hydroxyapatite, respectively [15-17, 19,20]. The intensities of these bands are sensitive at the demineralization of the bones due to disease cancer and they can show the progress of osteolysis. The broad band in the region 3300–3000  $\text{cm}^{-1}$  is made up from the two bands at 3209  $\text{cm}^{-1}$  and 3187  $\text{cm}^{-1}$ , assigned to NH vibrations [15-30]. In cancerous bone spectra the band at 3209  $\text{cm}^{-1}$  shifted to higher wavenumbers at 3277  $\text{cm}^{-1}$  and the 3187  $\text{cm}^{-1}$  was reduced in intensity, but it was not shifted. It seems that in cancerous bones the binding of proteins changed in structure and they did not give neither inter-nor intra-molecular hydrogen bonding of the NH hydrogen bonding leading to the result that decalcification takes place and finally the disease changes the secondary molecular structure of the proteins.

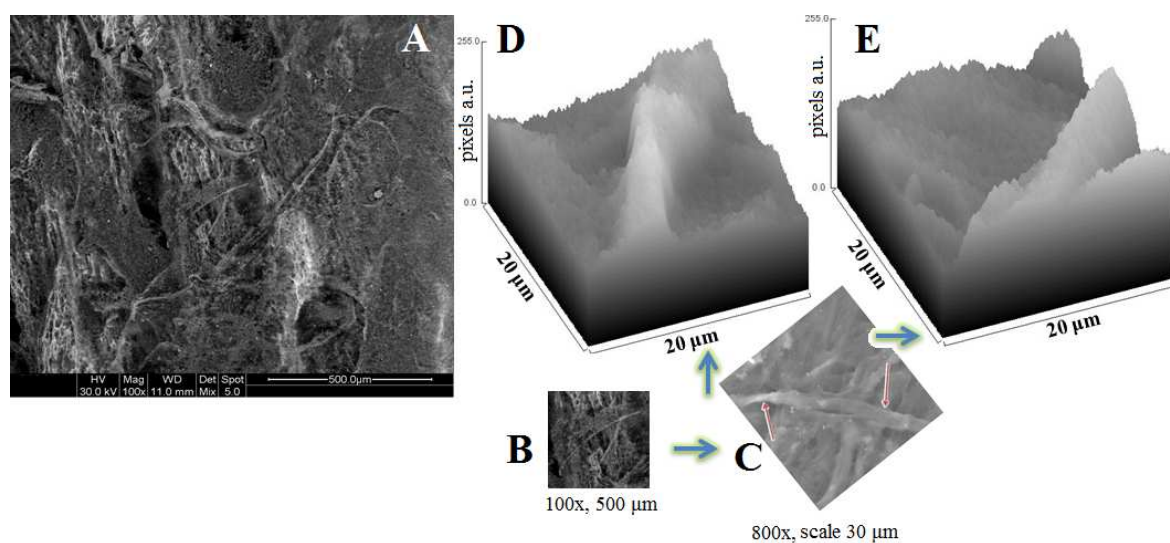
The absorption bands of the symmetric and asymmetric stretching vibrations of methyl ( $\nu_{\text{as}}\text{CH}_3$  and  $\nu_{\text{s}}\text{CH}_3$ ) groups were found at 2965  $\text{cm}^{-1}$  and 2880  $\text{cm}^{-1}$  and of methylene ( $\nu_{\text{as}}\text{CH}_2$  and  $\nu_{\text{s}}\text{CH}_2$ ) were near 2926  $\text{cm}^{-1}$  and 2854  $\text{cm}^{-1}$ , respectively [15-31]. These bands did not shift but their intensities increased for cancerous bones. These changes indicated that the environment of lipids, phospholipids and proteins has changed and that the permeability and fluidity of the membranes have increased, due to the damage induced by the disease and demineralization [15-30].

Interesting was the appearance of a new band in the spectra of cancerous bones at about 1745  $\text{cm}^{-1}$ , which was assigned to  $\nu\text{C}=\text{O}$  carbonyl stretching vibration of ester carbonyl groups ( $\text{RO}-\text{C}=\text{O}$ ) and was attributed to formation of aldehydes [31,32]. It is known that aldehydes are recognized as native “cancer markers” [33]. This carbonyl band was also proposed to charac-

terize the apoptotic cells [34]. This particular band indicates that lipid peroxidation was one of the pathways during the process of metastasis of bone cancer and could be used as “marker band” for the progression of the disease. This fact leads also to the hypothesis that the immune-like system is a contribution to the development of metastasis, in agreement with literature data [34].

Considerable changes were also observed in the region  $1700\text{--}1500\text{ cm}^{-1}$ . This region is known to be characteristic of proteins and is sensitive in order to evaluate the secondary structure of proteins and to distinguish that collagen exists as  $\alpha$ -helix [15-17,30-32]. A shift to lower frequencies of the absorption band of Amide I of proteins from about  $1650\text{ cm}^{-1}$  in healthy bones to  $1630\text{ cm}^{-1}$  in cancerous bones was observed. This shift to lower frequencies suggests that the proteins changed their secondary structure from  $\alpha$ -helix to random coil due to cancer processing. These results were confirmed also from SEM analysis.

Figure 2 shows the morphology of the cancerous bone. One can see that the bone is rich in fibrils, concerning the damage of collagenous and non-collagenous proteins, as well as the demineralization of the bone. The proteins changed their native structure as shown from the arrows on the points of damaged proteins (Fig. 2C). ImageJ analysis gives the relative intensity of the pixels which correspond to electron density of the proteins (Fig. D & E). The curves show the analyzed regions at the misfolding points.



**Figure 2.** SEM morphology of metastatic breast cancerous bone (scale 500 µm, Mag. 100x). B Selected region rich in proteins, C: Higher magnitude 800x (scale 50 µm), which shows the misfolding of proteins. D & E ImageJ analysis of the folding regions

The Amide II band at  $1555\text{ cm}^{-1}$ , which is consisted from  $\delta\text{NH}$  in-plane and  $\nu\text{CN}$  stretching vibrations, is very sensitive to environmental changes. It was found that the intensity of this band decreases and in almost all patients and shifts to lower wavenumbers. The ratio of the bands of  $[\text{Amide I}_{1650}]/[\text{Amide II}_{1550}]$  decreases with increasing the grade of cancer and it was found in the patients healthy bones to be equal to 1.11, whereas in the cancer it was 1.3. Although the differences are very small they are significant and it was found also in breast



cancer and colon cancer [18;21]. These changes represent the “structural change and abnormality” of proteins, induced from cancer disease, which decrease the probability of re-mineralization of the bones and thus increases the fragility of the bones.

Figure 1 clearly shows the absorption band at  $1418\text{ cm}^{-1}$ , which is assigned to stretching vibration of carbonate anions ( $\nu\text{CO}_3^{2-}$ ) of hydroxyapatite containing carbonate ions. This band is also reduced in intensity confirming the substitution of phosphate anions with carbonates in hydroxyapatite following the total demineralization of cancerous bones [17,20].

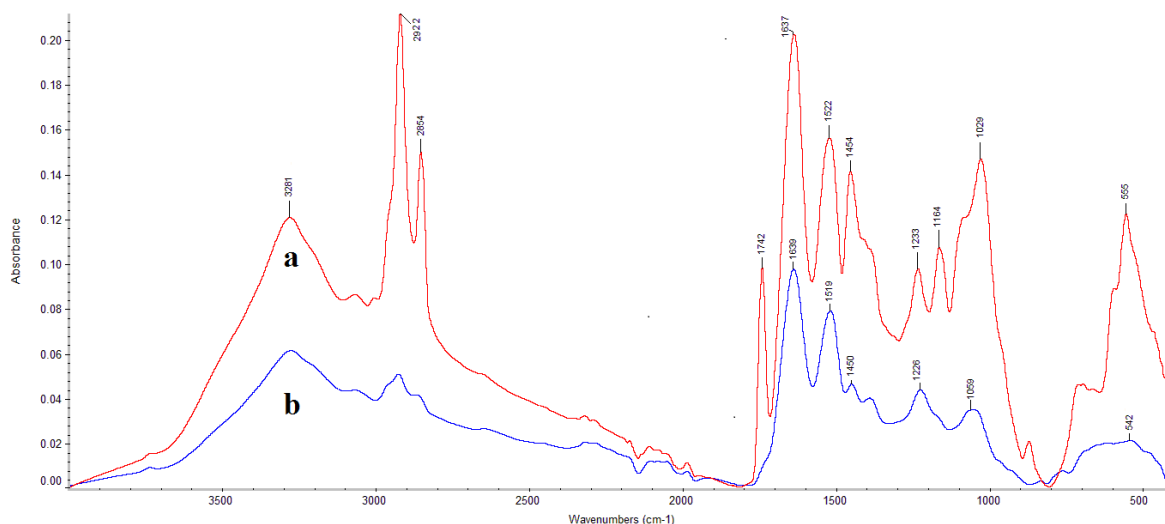
The band at about  $1040\text{ cm}^{-1}$  refers to stretching vibrations of  $\nu\text{C-O}$  coupled with the bending vibration of  $\delta\text{C-OH}$  groups of glycogenic bonds [36,37]. We found that the intensity of this band increases in the spectra of cancerous bones. The  $\nu\text{OH}$  absorption bands are sensitive upon isotopic substitution (deuteration), concerning the substitution of hydrogen atoms with deuterium in the C-OH groups of glucose (spectra not shown here). The increasing amount of glycogenic materials inhibits the mineralization of the bones [38]. From the conformational protein folding changes in combination with the increase glycogenic bonds (starch) it is suggested the production of amyloid-like proteins [39]. The formation of fibrils is also well shown in SEM morphology (Figure 2).

Furthermore, the spectra show characteristic differences between healthy and cancerous bone in the regions  $1200\text{-}900\text{ cm}^{-1}$  and  $600\text{-}500\text{ cm}^{-1}$ , where the phosphate groups ( $\nu_3\text{PO}_4^{3-}$ ) of hydroxyapatite absorb. The characteristic bands of  $\nu_3\text{PO}_4^{3-}$  and  $\nu_4\text{PO}_4^{3-}$  in cancerous bones have been dramatically decreased due to cancer, proving that osteolysis of the bones is taking place. These findings show that in cancerous bone a progressive mineral deficiency occurs in agreement with clinical and histological analyses indicating that the bone destruction is mediated by the osteoclasts [40]. Moreover, in the fingerprint region of the cancerous spectra between  $1200\text{-}900\text{ cm}^{-1}$  are coupled also the bands which are assigned to phosphodiester C-O- $\text{PO}_2$  of DNA [23-25,41-43]. These bands were not observed in normal bone tissue, maybe they were masked because of high concentration of hydroxyapatite.

### 3.2. FT-IR spectra after removal of organic components

To understand further the mechanisms of osteolysis of bone from metastatic cancer the bones were washed using organic solvents to eliminate the organic mass of the bones. Figure 3 shows the spectra of the bone before (spectrum a) and after elimination of dissolved organic components (spectrum b).

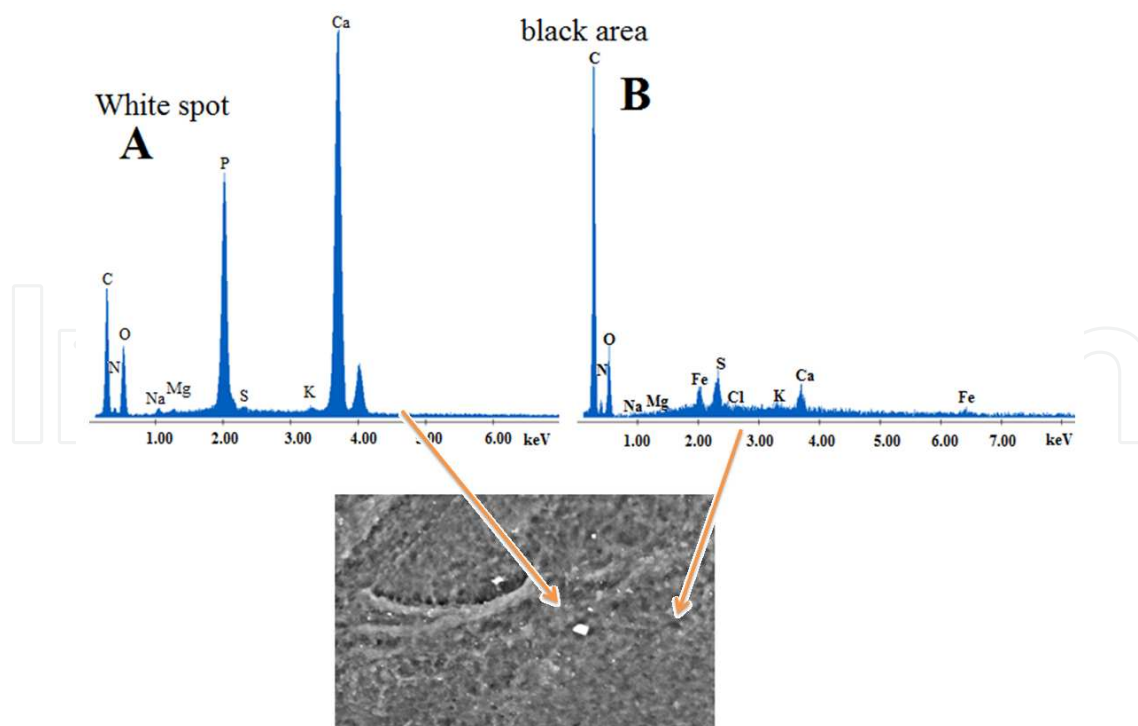
Comparison of the two spectra shows that after treatment with hexane all the organic components with low molecular weight have been dissolved and disappeared from the sample and are not shown in their spectra. It is interesting to note that the bands in the region  $1100\text{-}900\text{ cm}^{-1}$  and  $700\text{-}500\text{ cm}^{-1}$ , where the bands of hydroxyapatite absorb, the bands of  $\nu\text{PO}_4^{3-}$  have almost disappeared after treatment with hexane. From these findings it was suggested that the bands have resulted from other phosphate proteins, which also have phosphate groups with hydroxyapatite or fragments of phospholipids. However, these bands do not show that there is present biological hydroxyapatite. In addition, XRD analysis revealed that cancerous bones were not consisted from biological hydroxyapatite, but there was apatite rich in organic phase.



**Figure 3.** FT-IR spectra of cancerous bone tissues in the region 4000-400  $\text{cm}^{-1}$ ; a: without any preparation, b: after elimination of the organic components of the bone. The arrow shows free calcium phosphate.

The size of the crystals at  $2\theta=25,85^\circ$  was found to be 15 nm much smaller than the normal bone's hydroxyapatite which is 20-25 nm [44].

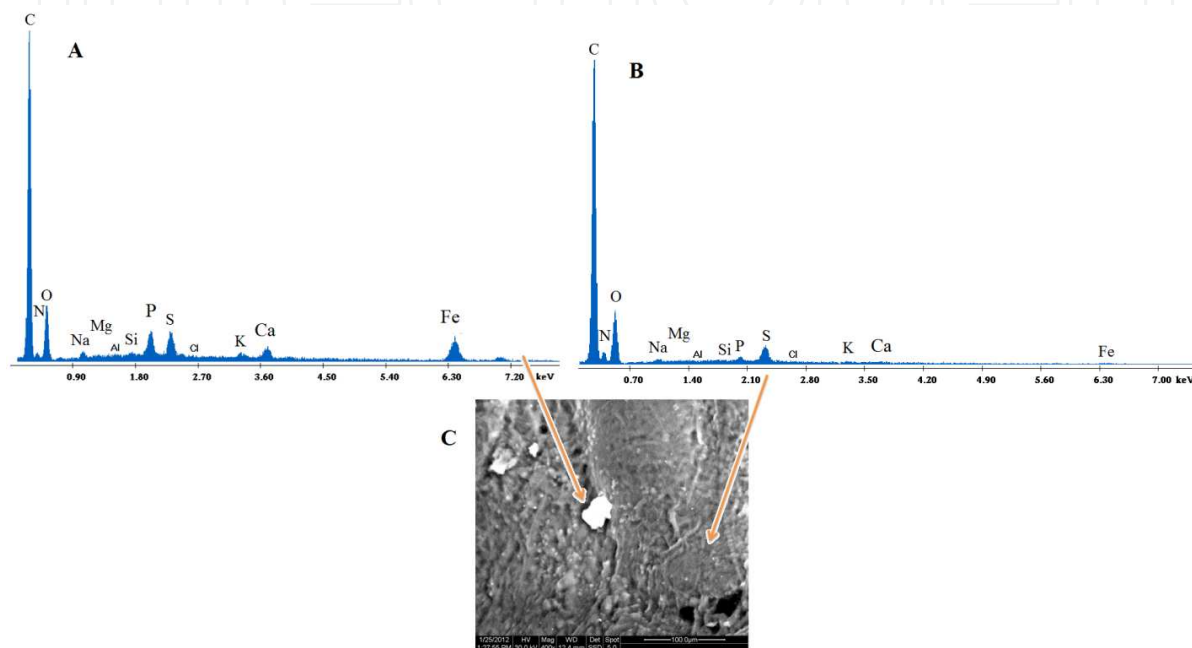
The SEM analysis of cancerous bones before treatment with hexane is given in Figure 4A. The relative element concentration of the white spot and of the dark region, are shown in the Figure.



**Figure 4.** SEM-EDX morphology and composition of metastatic breast cancerous bone before treatment: A; on white spot and B; on black area. Scale 100  $\mu\text{m}$ , Mag 400x



From EDX chemical analysis it was found that the extent area of the bone was poor in calcium and only the spread white spots were rich in minerals. By washing the bone with hexane the morphology of the surface changed (Figure 5). Figure 5 gives the SEM morphology of the bone after washing it with hexane. Figure 5A gives the relative concentration of the chemical elements of the white spots. It is obvious that the concentration of calcium of white spot is significantly reduced. The same picture was found for the extent area of the sample, where the calcium has almost disappeared (Figure 5B).



**Figure 5.** SEM-EDX results, composition of metastatic breast cancerous bone after treatment A; on white spot and B; on bulk area and C SEM morphology of the tissue. Scale 100  $\mu\text{m}$ , Mag 400x

These SEM results are in agreement with infrared spectroscopic results, which suggest that the mineral phase maybe a mixture of calcium salts of phosphate together with calcium carbonate, but not a biological hydroxyapatite. It was found from XRD analysis at  $2\theta=25,85^\circ$  that the crystal of hydroxyapatite showed also that the size of the hydroxyapatite crystals is 15 nm. This size is smaller than in healthy bones, which in adults was found 20-25 nm [44, 45].

## 4. Conclusions

The results obtained here using FT-IR spectroscopy and SEM analysis have shown that cancerous bone from metastatic breast cancer leads to considerable changes in bone density, especially in the structure of biological apatite that decreases dramatically or even it is destroyed due to osteolysis. Proteins change their secondary structure from  $\alpha$ -helix to random coil and aldehydes are produced during cancer metabolism. The intensity of the aldehyde band indicates the magnitude of the damage caused by the cancer. Furthermore, It is also noticed

that the proportion of inorganic mass is reduced in comparison to the organic matrix upon cancer invasion of the bones.

Furthermore, FT-IR spectroscopy can provide more information than histopathology, since it does not need any decalcification for analysis. From a clinical point of view, major disadvantages of bone decalcification are the laborious procedure that proceeds only slowly with incubation times up to several weeks depending on the extent of mineralization, the frequent loss of immune-reactivity, and the acid hydrolysis of DNA. In addition, excessive decalcification may lead to negative or non-diagnostic biopsy of the bone specimen (Rey, 1991). In this case, FT-IR spectroscopy is a rapid technique and it provides much more information compared to histology for a fast diagnosis of bone tumors.

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