Micro-ATR FTIR, SEM-EDS, and X-ray Micro-CT: An Innovative Multitechnique Approach to Investigate Bone Affected by Peri-implantitis

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Purpose: The aim of this work was to prove the synergic complementarity of attenuated total reflection Fourier transform infrared microspectroscopy (micro-ATR FTIR), scanning electron microscopy (SEM) coupled with energy-dispersive x-ray spectroscopy (EDS), and x-ray microcomputed tomography (micro-CT) by studying implant samples with bone affected by peri-implantitis. Materials and Methods: Six samples of implanted bone affected by peri-implantitis and one control healthy bone were analyzed. Thick bone sections included in epoxy-resin and removed implants were analyzed by micro-ATR FTIR, SEM-EDS, and micro-CT. Results: Micro-ATR FTIR revealed the complex nature of the bone composition. Vibrational bands characteristic of both mineral bone phase (acidic phosphates, CO$_3$$^2$– groups) and organic bone phase (mostly collagen) could be recognized, and their proportion could be seen to change accordingly with the bone degradation. Similarly, SEM-EDS clearly revealed the cortical nature of the control mandible and its homogenous mineral composition. On the contrary, EDS analyses performed over relevant portions of pathologic samples revealed that defective areas were almost Ca and P free. Micro-CT data showed that the morphology of the interface was smooth and linear in the physiologic peri-implant bone, while in the pathologic samples, an altered morphology was evident. Conclusion: This study demonstrated that morphologic, elemental, and biochemical modifications of peri-implant bone can be studied using micro-ATR FTIR, SEM-EDS, and micro-CT. The complement of these techniques can be considered a new multipurpose approach to investigate bone affected by peri-implantitis. INT J ORAL MAXILLOFAC IMPLANTS 2019;34:631–641. doi: 10.11607/jomi.7026

Keywords: bone-implant interactions, CT imaging, surface chemistry

Despite the impressive steps forward in implant dentistry, peri-implantitis still remains one of the major reasons for failure of implants. Peri-implantitis is a destructive infectious inflammatory disease, which occurs with a prevalence from 7% to 20% and affects soft and hard tissue around osseointegrated dental implants under functional loading. To date, the following parameters are considered the only available benchmarks in the differential diagnosis of peri-implant disease: implant mobility (lack of osseointegration), bleeding on probing (BOP), probing depth (the distance between the soft tissue margin and the bottom of the peri-implant pocket), and suppuration. Radiography is widely employed to detect bone loss around dental implants, while visible-near infrared spectroscopy has been recently proposed as a methodology for assessing multiple inflammation parameters.

This study proposed the integrated use of three analytical tools for the analysis of peri-implant bones affected by peri-implantitis: x-ray microcomputed tomography (micro-CT), scanning electron microscopy (SEM) coupled with energy-dispersive x-ray spectroscopy (EDS), and attenuated total reflection Fourier transform infrared microspectroscopy (micro-ATR FTIR). In the field of implant dentistry, the exploitation of x-ray micro-CT and SEM-EDS is not a novelty, and these two techniques have been applied in combination with...
optical and electron microscopy, cytologic, and histochemical analysis for evaluating the osseointegration capacity of the different metallic implants. Microstructure and microarchitecture are relevant aspects of bone strength. The study of the microarchitecture is based on the measure of parameters such as number, thickness, and separation of trabeculae as well as on their spatial distribution. Several methods are available to assess bone microarchitecture, particularly at the trabecular level. Histomorphometry, based on the use of optical microscopy and on the principles of quantitative histology and stereology, evaluates microarchitecture two-dimensionally (2D). Imaging techniques based on hard x-rays are also of particular interest, and microradiography has been proven to be useful for clinical diagnostics. Optical and SEM techniques are also widely adopted tools for the investigation of the texture and morphology of a large range of materials and biomaterials, as well as for medical and biologic applications. For its characteristics, SEM coupled with EDS was exploited in this work for chemical-compositional analysis. Measures of the microarchitecture appear well correlated to the three-dimensional (3D) structure and properties of the bone. In the last 20 years, great interest has been shown in x-ray micro-CT techniques, both employing microfocus and synchrotron radiation sources. These techniques produce three-dimensional (3D) images of the internal structure of the materials with a spatial resolution at the micron and submicron scale. Investigations performed directly in the 3D domain overcome the limitations of stereologic methods usually applied to microscopy-based analyses. In addition, a nondestructive approach is more suitable for further complementary analyses and for precious or unique samples (fossils and archeologic finds, in vivo imaging, etc). The potentialities of x-ray micro-CT for analyzing bone density and the characteristics of trabeculae have already been proven. This technique can potentially help in understanding how and to what extent chronic inflammation may affect the spatial arrangement and the characteristics of bone trabeculae as well as the implant-bone interface with respect to the peri-implant bone. In addition, microarchitecture seems to be a determinant factor of bone fragility, independent from bone density, and it is therefore important for understanding the mechanisms of bone fragility as well as the action of the drugs used to prevent pathologies.

Fourier transform infrared spectroscopy (FTIR) is a technique that has been extensively employed in many fields of science for the chemical characterization of materials. Until the 1990s, only average information on the sample composition could be achieved by conventional FT interferometers, but nowadays, the coupling of FT interferometers with specially designed infrared microscopes allows the providing of spatially resolved chemical information on a sample with a lateral resolution of few microns (FTIR microscopy). FTIR analysis provides details on the covalently bonded chemical species that constitute a sample, through the analysis of the vibrational modes of molecules and molecular functional groups. Specifically, position, intensity, and bandwidth of the spectral bands of an infrared spectrum allow retrieval of sample composition and relative concentration of the constituents. FTIR characterization, which is sensitive, fast, safe, and label-free, is nowadays recognized as an excellent tool for biologic and medical studies. Specifically, several studies have been published that exploit FTIR techniques for the analysis of bone samples (both human and animal) during the progression of diseases such as osteoporosis, osteogenesis imperfecta, arthritis, etc. However, to the best of the authors’ knowledge, there are no scientific reports on the use of FTIR microscopy in dentistry studies, and specifically, there are no analyses of bone affected by peri-implantitis done with FTIR. For the purpose of this study, FTIR microscopy technique with ATR sampling geometry (micro-ATR FTIR) was used to investigate bone sections. It was possible to get access to information on both organic (mainly collagen) and inorganic (bone minerals) bone constituents, and on the modifications that take place inside the tissues during the disease progression, without altering its composition. One of the main advantages of micro-ATR FTIR is that the analyzed sample remains intact and does not undergo any physical or chemical alteration. It was therefore possible to perform an in-depth characterization of the same 2D sections investigated by micro-ATR FTIR with SEM-EDS and micro-CT, gaining fine information on their morphology and elemental distribution. The study was finally completed with the 3D analysis of the sample microstructure by x-ray micro-CT, with the aim of linking the microstructural features of the bone affected by peri-implantitis to its organic and mineral pattern. Thanks to the complementary use of the three techniques, it was possible to have a more comprehensive and multiscale view of the sample features, both at the structural and chemical levels, enabling characterization of its properties.

**MATERIALS AND METHODS**

**Implant Removal and Sample Preparation**

For the micro-ATR FTIR and SEM-EDS analyses, six samples of peri-implant bone affected by peri-implantitis were used. Implants were selected among patients seeking care at the Dental Clinic of University Hospital “ASUI” of Trieste. Patients eligible for the
study were healthy (no systemic pathology) without risk factors for peri-implantitis. The following were selected: two men, aged 50 and 80 years, respectively; four implants from the mandible (localized 3.4, 3.6, 4.4, and 4.6) and two from the maxilla (localized 1.4 and 1.6). The implants had a diameter of 3.75 mm and cylindrical screw shape. The sample of healthy mandibular bone was collected from a patient 65 years of age who suffered a fracture, and the osseous fragments could not be recomposed through fracture treatment.

Before surgery, informed consent was signed by all the patients. A local anesthesia with mepivacaine hydrochloride plus vasoconstrictor (adrenaline) was then used. Once a trapezoidal flap was incised, the implant was removed by using a trephine cutter with diameter of 5 mm mounted on a handpiece 800 rpm with saline irrigation.

The bone removal should be as precise as possible, since the large majority of the pathologic bone tissue should remain adherent to the implant surface, given that the difference between implant diameter (3.75 mm) and cutter trephines (5 mm) is 1.25 mm. Sutures of the flap were performed.

The extracted implants were plunged into a solution of 10% neutral buffered formalin (formaldehyde 4% m/v 0.05 M phosphate buffer). The samples were then dehydrated through immersion into solutions of water and ethanol, with a gradually increasing alcohol concentration (25%, 50%, 75%, and finally, 100%). Solutions were replaced daily.

This step is the most critical of the preparation phase. The bone affected by peri-implantitis is more fragile than healthy bone, and during dehydration, it can detach from the surface of the implant. This is why only 6 samples could be analyzed among 19 removed implants, namely, C6, C7, C9, C10, C11, and C12.

Dehydrated samples were then included in epoxy resin, prepared by mixing 14 mL of hardener (triethylentetramine, polyoxypropylendiamine, Nonylphenol, m-phenylenebis, 4-tert-butylphenol) and 35 mL of resin (bisphenol-A-(epichlorohydrin) epoxide resin, propyldienetriethyltrimethacrylate Butyl 2,3,5-epoxypropoxy, 1,3-bis(2,3-epoxypropoxy)-2, 2-dimethyl(propene), to obtain a mixture as homogeneous as possible. Resin-included samples were then cut with a microtome (Micromet, Remet) into slices 0.6-mm thick, employing a water-cooled saw blade rotating at 3,000 revolutions/minute. Samples were later dried and stored in vials inside a desiccator prior to analyses. These types of samples have been used for micro-ATR FTIR and SEM-EDS analysis. The authors also attempted to cut the sample into thinner sections, a few tens of microns, in order to perform FTIR measurements in transmission mode, but the trials were unsuccessful because of the fragility of the bone and the hardness of the implant.

For the purpose of x-ray micro-CT analyses, the implants were removed from the maxillary bone. The first implant, used as control for healthy bone, is a titanium implant with a smooth surface, 10 mm in length and 4 mm in diameter; it was extracted for placement of a prosthesis, as requested by the patient. The second implant was extracted as a result of diagnosis of second-degree peri-implantitis; the titanium implant has a rough surface, and it is 10 mm long with a diameter of 3.5 mm. The third implant, having a smooth surface, 10 mm long and 4 mm in diameter, was also extracted after a diagnosis of second-degree peri-implantitis. The procedure for the removal of the implants from the patient and the preparation of samples up to dehydration was the same as followed for micro-ATR FTIR and SEM-EDS dedicated samples, but bulk samples characterized by x-ray micro-CT were not included in resin or cut.

### Micro-ATR FTIR
Micro-ATR FTIR analysis of the bone samples has been carried out at the Chemical and Life Sciences branch of the SISSI beamline at Elettra Sincrotrone Trieste. The end-station is equipped with a Vertex 70 interferometer coupled with the Visible-Infrared Hyperion 3000 microscope (Bruker Optics). For the purposes of the experiment, micro-ATR sampling was done by using the 20× micro-ATR objective (Bruker Optics) mounting a germanium internal reflection element (Ge-IRE) tip, 100 microns in diameter. The measurements were carried out on the bone sections embedded in epoxy resin as previously described, setting the pressure of the Ge-IRE tip onto the sample surface at approximately 16 N/m². Different colors of the regions should reflect different extents of bone damage. By identifying these regions using the optical images of the samples, 10 to 30 points were chosen, depending on the amount of residual bone. In Fig 1a, the optical micrograph of the control sample can be seen on the left, while on the right, a zoom of the measured areas and sampled points is shown. Figure 1b shows the optical micrograph of the peri-implant bone C9 on the left, while a zoom of one of the sampled areas with the measured points is shown on the right. A background spectrum was taken before each sample point acquisition pressing the Ge-IRE tip onto the surface of a clean BaF₂ window.

Each micro-ATR spectrum was acquired averaging 128 scans with a spectral resolution on 4 cm⁻¹, using a Mercury-Cadmium-Telluride detector. Acquired spectra were pre-processed for compensating spectral features related to atmospheric water vapor and CO₂ by running the atmospheric compensation routine of OPUS software (Bruker Optics).
SEM and EDS
Morphologic analysis was carried out using a Zeiss Supra 40 field-emission gun (FEG) SEM equipped with a Gemini column and an Inlens detector yielding increased signal-to-noise ratio. The SEM was operated at 10 kV, and 50× to 1,000× micrographs were acquired by a secondary electron detector on representative areas at the bone-implant interfaces of all the selected samples. For this kind of analysis, three samples among the ones previously investigated by micro ATR-FTIR were chosen: the control sample and two samples of patients affected by peri-implantitis, C6 and C9.

The chemical assessment of the samples was investigated by EDS using an EDAX spectrometer connected to the microscope. In particular, initial EDS spectra analyses were performed to ascertain the overall element composition of the investigated samples. Compositional maps were then carried out to determine the chemical distribution of the peri-implants and to follow the evolution of the Ca concentration across the bone-implant interface.

X-ray micro-CT
Bone samples were imaged by using the x-ray micro-CT technique at the laboratory station TomoLab of Elettra. The device is equipped with a Hamamatsu sealed microfocus x-ray tube that delivers polychromatic x-rays in cone beam geometry. The source operates in a voltage range of 40 to 130 kV, at a maximum current of 300 µA and with a minimum focal spot size of 5 µm. A water-cooled, 12-bit charge coupled device (CCD) camera was used as a detector. The camera (Photic Science VHR) consists of a full-frame CCD imager coupled to a gadolinium oxysulphide scintillator by a fiber-optic taper. The following source parameters were used in the experiments: voltage = 130 kV, current = 61 µA. A 1.5-mm-thick aluminum filter was inserted between the source and the sample to reduce the beam hardening effects. Samples were mounted onto a high-resolution rotation stage. For sliced samples, the source-to-sample and source-to-detector distances were set to 80 mm and 220 mm, respectively. This, combined with a 2 × 2 binning applied to the pixels, yields an effective pixel size of 9.1 × 9.1 µm². For bulk samples, source-to-sample and source-to-detector distances were set to 80 mm and 240 mm, respectively, giving an equivalent pixel size of 8.3 × 8.3 µm². The tomographic scans were performed rotating the sample over a total scan angle of 360 degrees. For the bulk samples, 1,800 projections were acquired with an exposure time/projection of 3.2 seconds; 2,400 projections with an exposure time/projection of 2.8 seconds were acquired for the sliced samples.

From the acquired tomographic projections, a set of 2D slices was reconstructed with the commercial software COBRA (Exxim Computing). The same software was used to reduce beam hardening artifacts, while the ring artifacts present in the slices were reduced with an algorithm custom-implemented in the Pore3D software library (http://www.elettra.eu/pore3d).

The reconstructed 2D slices were visualized by using the freeware software Fiji while the 3D visualizations (volume rendering) were obtained with the commercial software VGStudio MAX 2.0 (Volume Graphics). From these measurements, it was possible to characterize the internal microstructure of the cortical and trabecular bone in section and bulk samples. In particular, the interface between the implanted titanium screw and the bone could be visualized in 3D, and these results were related to the morphologic and chemical information extracted by SEM-EDS and micro-ATR FTIR techniques.
RESULTS

Clinical Examination
The choice of implants for analysis and their respective peri-implant bone was based on the fact that the implants were affected by peri-implantitis. Therefore, the considered implants had almost completely lost osseointegration and could no longer fulfill their functions. More specifically, according to a recent classification proposed by Froum and Rosen in 2012, the degree of peri-implantitis presented by the extracted implants could be graded at an advanced stage since they were characterized by a probing depth ≥ 8 mm, a bone loss greater than 50% of the length of the system, and by a bleeding and/or purulent exudate in the survey (Table 1).

Micro-ATR FTIR Analysis
A total of seven samples were analyzed, one sample of a healthy mandible and six samples of implants affected by peri-implantitis from the mandible and maxilla. In order to exclude the resin penetration into the analyzed bone sections, and eventually to discriminate its contribution to the bone spectra, the characterization of the included resin was done first. The resin absorbance spectrum and its second derivative are shown in Fig 2a. The most characteristic peaks of the resin are related to the C-O-C stretching of epoxy rings as well as to the linear ether C-O-C stretching of repetitive polymer units, which are in the spectral region below ∼1,300 cm⁻¹. At ∼1,730 cm⁻¹, the carbonyl ester band is also detectable. A very sharp peak centered at approximately ∼1,509 cm⁻¹, prominent in the second derivative spectrum, is related to C = C stretching and reveals the aromatic nature of the used epoxy resin. This peak is very characteristic for nonbiologic matter, and it has been selected as diagnostic for detecting the resin penetration into the sample and its spectral contribution.

Several points of the control sample were measured. They all showed vibrational features totally comparable to both spectral band position and intensity, and the absence of the second derivative peak centered at ∼1,509 cm⁻¹, which would reveal the included resin. Figure 2b shows the absorbance spectrum and second derivative of one representative sampled control. The FTIR spectrum reveals the complex nature of the bone composition, and spectral components of both inorganic and organic matrices can be recognized. The most intense band of the spectrum is originated by the mineral bone phase: it is centered at 1,014 cm⁻¹, with a shoulder at 959 cm⁻¹, respectively assigned to the asymmetric (ν₂) and symmetric (ν₁) stretching of PO₄³⁻ groups of hydroxyapatite. The shoulder at ∼1,100 cm⁻¹ can be assigned to acidic phosphate (HPO₄²⁻), a frequent anionic substituent in the crystal lattice of hydroxyapatite. In addition, the bands centered at 872 and 1,450 cm⁻¹ can be attributed to bending (ν₃) and asymmetric stretching (ν₂) modes of CO₃²⁻ groups, present as ionic substitutes in the apatite crystals.

The organic matrix of the bone consists of proteins and lipids. Among proteins, collagen type I represents almost 85% to 90% of the bone protein content. Peptide linkage of proteins gives rise to several bands diagnostic for proteins, called Amide bands. Among them, Amide I is the most sensitive to the protein secondary structure. In the spectrum of Fig 2b, it extends from 1,720 to 1,600 cm⁻¹, and it is made by three major contributions, centered at 1,630, 1,660, and 1,690 cm⁻¹, as can be better identified by second derivative spectral analysis. Several reports highlight that the components centered at 1,660 cm⁻¹ dominate pyridinoline (Pyr) cross-linked peptides, while the band components centered at 1,690 cm⁻¹ become stronger in dihydroxylysinonorleucine (DHLNL) cross-linked peptides. Therefore, the integrated area ratio of these two band components is indicative of collagen maturity. Amide A, Amide B, and Amide II band positions are also highlighted in Fig 2b, where the position of stretching (ν) and bending (δ) modes of CH₂ and CH₃ moieties and stretching mode of OH groups are shown as well.

Pathologic samples are characterized by a pronounced intersample and intrasample variability, in visual appearance, morphology (see later), and in spectral features. Some of the investigated samples, namely, C7 and C10, have a visual appearance comparable to the control one, being characterized by a yellow color. One of the investigated samples (C11) was almost completely black, while samples C6, C9, and C12 have both yellow and black regions. The selective measurements of both black and yellow regions of the later samples pointed out that the yellow regions have a spectral profile very similar to the one of the control samples, in both band position and relative band intensity, as can

Table 1  Classification of Peri-implantitis Proposed by Froum and Rosen

<table>
<thead>
<tr>
<th>Variables</th>
<th>Classification of peri-implantitis</th>
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<tbody>
<tr>
<td>Early</td>
<td>≥ 4 mm (bleeding and/or suppuration on probing)</td>
</tr>
<tr>
<td></td>
<td>Bone loss &lt; 25% of the implant length</td>
</tr>
<tr>
<td>Moderate</td>
<td>≥ 6 mm (bleeding and/or suppuration on probing)</td>
</tr>
<tr>
<td></td>
<td>Bone loss 25% to 50% of the implant length</td>
</tr>
<tr>
<td>Advanced</td>
<td>≥ 8 mm (bleeding and/or suppuration on probing)</td>
</tr>
<tr>
<td></td>
<td>Bone loss &gt; 50% of the implant length</td>
</tr>
</tbody>
</table>

*Noted on two or more aspects of the implant.
*Measured on radiographs from time of definitive prosthesis loading to current radiograph. If not available, the earliest available radiograph following loading should be used.
be observed in Fig 3a. Conversely, black regions have a spectral profile that greatly differs from the one of the control, as can be seen in Fig 3b. First of all, the spectral contribution of the epoxy resin becomes evident in black regions (Fig 3b), where the contribution of the diagnostic resin-band centered at ~1,509 cm$^{-1}$ emerges. This spectroscopic observation let the authors suppose a less compact and more porous bone structure in these bone areas, a hypothesis that has been verified by both morphologic investigations with SEM on the same thin sections and 2D and 3D characterization with micro-CT (see later). From a spectroscopic point of view, a relevant suppression of the intensity of the spectral band associated with the asymmetric ($\nu_3$) and symmetric ($\nu_1$) stretching of PO$_4^{3-}$ groups of hydroxyapatite and with acidic phosphate (HPO$_4^{2-}$) substituents can be seen. The spectral band assigned to the bending ($\nu_2$) of CO$_3^{2-}$ groups is barely detectable, while nothing can be said on the spectral contribution of the asymmetric stretching ($\nu_3$) of the same group due to its overimposition with resin spectral bands. With respect to the Amide I band, the components centered at 1,690 cm$^{-1}$ cannot be clearly distinguished in this sample, while the spectral component centered at 1,630 cm$^{-1}$ dominates
the spectral profile of proteins in black regions. The same considerations can be drawn out for both yellow and black areas of C6 and C12 samples. Consistently, for yellow C7 and C10 samples, all the sampled regions have spectral features comparable to the control, while for black C11 samples, all the sampled regions have spectral features comparable to the ones of the black regions of C9, C6, and C12 samples.

**SEM and EDS Analysis**

The SEM morphologic analysis of the control mandibular bone clearly reveals its cortical nature (Fig 4a): bone lamellae are compactly and concentrically disposed around blood vessels to form osteons. Elemental analysis has been performed at several points, revealing a homogenous composition of the control sample, with a Ca/P ratio almost equal to 1 for all the sampled points.

Morphologic SEM analysis of the pre-implant bones, and specifically, of sample C6 and C9, reveals a less compact and more heterogenous structure. In Fig 4b, a SEM image of a representative section of sample C9 is shown. In this sample, a consistent number of voids and defects intercalating within the overall

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**Fig 3**  (a) Absorbance spectrum (*upper panel*) and second derivative spectrum (*lower panel*) of a representative yellow point of the peri-implant bone from sample C9.  (b) Absorbance spectrum (*upper panel*) and second derivative spectrum (*lower panel*) of a representative black point of the peri-implant bone from sample C9.
bone matrix can be observed, and the extension of the defective regions increases in the darker regions of the peri-implant bone. This aspect is indicative of high bone turnover due to an inflammatory process. A darker contrast can be observed over the defective regions with respect to the surrounding matrix indicating local chemistry differences within the sample. EDS analyses performed over relevant portions of the sample, namely, over the healthy bone area and within the voids (solid and dotted boxes in Fig 4b, respectively), revealed that defective areas are almost Ca and P free (data not shown). This evidence is in agreement with micro-ATR FTIR, which also pointed out the severe demineralization process affecting the black regions of the peri-implant bone affected by peri-implantitis.

In order to better ascertain the signs of dental implant rejection, elemental maps have been acquired. Figure 5a is a SEM image of a portion of the equatorial peri-implant bone section of sample C9 obtained with secondary electrons. In Figs 5b to 5f, EDS maps measured over the corresponding imaged area are shown. EDS compositional maps provide evidence of C and O enrichment in the proximity of the bone-implant interface, namely, A and B regions in Fig 5a. In particular, more intense C and O signals are detected in region A, where the presence of Ca and P is also detected. Indeed, acquired EDS spectra showed a Ca/P stoichiometric ratio of approximately 1, as in the case of healthy bone. Conversely, in the B region, Ca and P signals are almost negligible. This evidence highlights the quite heterogenous morpho-chemistry of the bones affected by peri-implantitis, as also observed with the other analyses.

**Micro-CT Characterization**

The volume reconstructions of the examined samples have enabled the observation of the contact interface with the implant surface for both epoxy-embedded thin sections and bulk samples, as well as the evaluation of the 3D organization of trabecular bone tissue. Figure 6a shows a volume rendering of the control sample, sectioned and embedded into the epoxy resin,
while Fig 6b shows the volume rendering of the peri-implant bone from sample C9, also sectioned and embedded into the epoxy resin. In accordance with what was already established by SEM-EDS microanalysis and micro-ATR FTIR, the control sample has a compact bone structure, while the peri-implant sample appears to not be firmly attached to the implant. The detachment appears more pronounced, and the remaining bone is less compact.

From the 3D rendering of bulk samples in Fig 7a, it is notable that the morphology of the interface is smooth and linear in the physiologic peri-implant bone, while in the pathologic samples, a morphology altered by the presence of bone gaps larger than the intertrabecular spaces for healthy bone is evident (Fig 7b). The trabecular texture is irregular, lobular, in a context of rarefied bone appearance. This evidence is in agreement with a previous study; once the implant affected by peri-implantitis was removed, bone necrosis and chronic inflammation in the peri-implant bone and connective tissues were observed in the present study.

The presence of bone resorption is appreciable, and bone gaps resulting from this process are visible as focal points in which bone resorption is absent, or in advanced stages of regression (Fig 7b). The analysis of the results allowed the presence of these resorption gaps not only in the coronal portions of the bone/implant, but also at a more apical level to be pointed out. Unlike Howship gaps or small depressions (up to 100 microns) present in the bone due to osteoclast activity, the gaps that were observed in this study are between 1 and 1.5 mm. It is also possible that the resorption gaps are generated by monocytes-macrophages derived from osteoclast differentiation. The osteoblast chemical mediators lead the differentiation of osteoclast cells in the apical zone. These gaps of reabsorption, resistant to therapeutic procedures, could be the cause of therapeutic failures.

**DISCUSSION**

Micro-ATR FTIR, SEM-EDS, and x-ray micro-CT are techniques that have been extensively used for the characterization of bone tissues. However, to the best of the authors’ knowledge, this is the first time that their potentialities are jointly exploited to evaluate the adverse effect on bone architecture and chemical nature as a consequence of peri-implantitis.

SEM and x-ray micro-CT analyses reveal that bone affected by peri-implantitis is less compact with respect to healthy bone, and characterized by extended rarefied and demineralized areas. The spongy nature of the affected bone could also be deduced by micro-ATR FTIR data that showed a major penetration into the tissue of the embedding resin as a consequence of the demineralization, as also reported by other authors. The demineralization is very severe, as revealed by EDS analysis that showed traces of P and Ca near the detection limit in the porous areas and in proximity of the implant. The severity of
the demineralization is also confirmed by micro-ATR FTIR, which revealed a pronounced suppression of the bands related to both phosphate and carbonate moieties for the black regions of peri-implant samples affected by peri-implantitis. From here on, the yellow sample regions will be called type A and the black type B. In Table 2, the mineral to matrix ratio for all the investigated samples is reported, as derived from micro-ATR FTIR data, rationing the integrated areas of phosphate bands (1,200 to 900 cm⁻¹) to the Amide I band (1,700 to 1,600 cm⁻¹), as reported by other authors. This ratio indicates the relative amount of mineral and organic matrix content. It is directly linked to bone mineralization, and it is positively correlated with bond strength and bone mineral density.²⁷

It is evident that the mineral to matrix ratio is greatly suppressed (in sample C9-type B), if not almost nullified (in sample C6-type B, C12-type B, and C10-type B) for the samples with black regions, as a consequence of the inflammation, which compromises bone strength and osseointegration. For the yellow regions, the mean mineral to matrix value was set to 5.20 ± 0.51, comparable within the standard deviation to the control sample. It has to be highlighted that age and sex, and not only pathologic states, could affect the mineral to matrix value, and this justifies the variability within the same sample types.

Micro-ATR FTIR also allows pinpointing that the demineralization process goes with a different nature of the organic matrix. It has been reported by other authors that the ratio between the band components of the Amide I band centered at 1,660 and 1,690 cm⁻¹ correspond to the nonreducible/reducible collagen cross-links in bone.²⁸ This ratio increases with age, and it reflects the fact that divalent cross-links DHLNL diminish with maturation, since they convert to PYR ones. It is interesting to notice that the 1,660/1,690 ratio does not seem to be affected by peri-implantitis (Table 2), since it has comparable values for all the sampled points of any individual sample with a mean value of 4.44 ± 0.16. However, the spectral profile of the Amide I band of the black type-B sampled points differs from the one of the control and yellow type-A sampled points: the band peaks at 1,630 cm⁻¹ on the type-B regions, as can be clearly noted from Fig 3b. Overall, the experimental evidence demonstrated that in the bone areas mostly affected by infection, collagen content is reduced while preserving the cross-link of the non-pathologic regions. Different noncollagenous proteins are indeed present, which could be of inflammatory nature due to the massive recall of inflammatory cytokines to the region of infection.

The level of carbonate substitution in bones can be estimated by micro-ATR FTIR spectra as the area ratio between carbonate (850 to 890 cm⁻¹) and phosphate bands (1,200 to 900 cm –¹). This value, conventionally associated with the bone remodeling rate and turnover,²⁹,³⁰ increases on all type-B regions (Table 2), almost an order of magnitude at some black sampled points, and tightly relates to the occurrence of inflammatory processes.

CONCLUSIONS

The results of this study demonstrate that micro-ATR FTIR, SEM-EDS, and micro-CT are useful complementary techniques to investigate morphologic and biochemical modifications of peri-implant bone. In particular, the authors were able to conclude from this study that bone samples affected by peri-implantitis have a heterogenous morphology, characterized by regions of bone resorption and inflammation interposed between areas morphologically resembling dense and compact healthy bone. The different architecture runs in parallel with bone chemical distinctive features.

<table>
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<th>Table 2</th>
<th>Mean ± SD of the Following Band Integral Ratios: Mineral/Matrix, CO₃²⁻/PO₄³⁻ and 1,660/1,690</th>
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<tbody>
<tr>
<td></td>
<td>Mineral/matrix</td>
</tr>
<tr>
<td>Control</td>
<td>5.26 ± 0.35</td>
</tr>
<tr>
<td>C_6 (type A)</td>
<td>4.74 ± 0.28</td>
</tr>
<tr>
<td>C_6 (type B)</td>
<td>0.76 ± 0.57</td>
</tr>
<tr>
<td>C_7 (type A)</td>
<td>5.15 ± 0.59</td>
</tr>
<tr>
<td>C_9 (type A)</td>
<td>5.06 ± 0.44</td>
</tr>
<tr>
<td>C_9 (type B)</td>
<td>1.34 ± 0.88</td>
</tr>
<tr>
<td>C10 (type A)</td>
<td>4.97 ± 0.78</td>
</tr>
<tr>
<td>C11 (type B)</td>
<td>0.19 ± 0.12</td>
</tr>
<tr>
<td>C12 (type A)</td>
<td>6.07 ± 2.09</td>
</tr>
<tr>
<td>C12 (type B)</td>
<td>0.18 ± 0.06</td>
</tr>
</tbody>
</table>

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Deminerallization, increased carbonate substitution, and recall of proteins at the site of inflammation are distinctive features of bone affected by peri-implantitis. The harmonization of the results individually provided by each technique allowed a comprehensive view of the pathologic bone to be provided. The synergic correlation of micro-ATR FTIR, SEM-EDS, and x-ray micro-CT can definitely be considered a new approach to investigate bone affected by peri-implantitis that can provide new insights on the disease and help in finding strategies for increasing implant success rate.

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