

UNIVERSITA' DEGLI STUDI "G. d'Annunzio"
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SCUOLA SUPERIORE "G. d'Annunzio"

PhD IN

Medical Biotechnologies

CYCLE

XXXVII

**ARTIFICIAL INTELLIGENCE IN DENTISTRY:
INNOVATIVE TOOLS AND ADVANCED ANALYSIS
OF BONE AND SOFT TISSUES**

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ACADEMIC YEARS 2021/2024

Table of Contents

Abstract

1. Introduction

2. Artificial Intelligence in Dentistry

2.1. Machine Learning

2.2. Artificial Neural Networks

2.2.1. Deep Neural Networks

2.2.2. Convolutional Neural Networks

3. High-resolution X-Ray Tomography

3.1. Micro Computed Tomography

3.2. Synchrotron Radiation Phase Contrast Tomography

3.3. Cone Beam Computed Tomography

4. Results

4.1. Bone Tissue: Osteocyte Lacunae AI detection and Bone Mineralization evaluation

4.2. Peri-Implant Connective Tissue: Deep Learning for Microstructural

Characterization of Synchrotron Radiation-Based Collagen Bundle Imaging

4.2.1 Case Report: Dental Implant with Conometric Abutment–Prosthetic Cap Connection

4.3. Artificial Intelligence in CBCT: New Frontiers in Radiology

4.4. Mechanical Testing Dental Implants: Performance for Lasting Reliability

4.5. Decoding Periodontal Health: A Machine Learning Approach for defining staging and grading of periodontal diseases and conditions

4.6. Oral Pathology: Multidisciplinary-based evidence that biomolecular alterations temporally anticipate microarchitectural modifications of collagen in oral tongue squamous cell carcinoma

5. General considerations, Limits of the Methods and Future Developments

Bibliography

Appendix

Acknowledgements

Abstract

This dissertation will show the potential applications of Artificial Intelligence (AI) in the dental field, the rationale behind its introduction, the methodologies employed, and the quantitative analyses conducted. AI-integrated analysis was implemented on both clinical and high-resolution tomography data. It was observed that Artificial Neural Networks (ANN) can accelerate the processing of clinical image segmentation tasks and that machine learning (ML) algorithms can make predictions based on numerical data. The dental field was explored through the analysis of Synchrotron Radiation Phase Contrast High Resolution Tomography (PhC-microCT) images of bone and non-mineralized connective tissue surrounding dental implants to assess the health status and healing processes after insertion. Furthermore, the properties of conical dental implants were verified through mechanical testing and 3D analysis. Subsequently, studies with a wide field of view were conducted by segmenting the regions imaged by Cone Beam Computed Tomography (CBCT) images. Then, the focus was on pathologies that can impact periodontal regions, developing an integrated ML application capable of predicting the staging and grading levels of periodontitis, incorporating clinical data from patients. Finally, a multidisciplinary examination of the collagen structure of oral squamous cell carcinoma in the Intra - Peri - Extra tumor zones was carried out. This analysis highlighted the central role of the extracellular matrix in the biomechanical and pathophysiological kinetics of tumor tissues.

1. Introduction

This doctoral research aimed to apply Artificial Intelligence (AI) in different areas of dentistry: Artificial Neural Networks (ANNs) were exploited in implantology to analyze bone and connective tissue around dental implants to monitor their performances and the regenerative properties of the tissues surrounding them; Machine Learning (ML) algorithms were used also in periodontology to make a classification on the progression of periodontal diseases; moreover, ANNs were also applied in surgery in order to segment images to evaluate dental prostheses and their correct positioning.

The research activity was carried out in collaboration with the Polytechnic University of Marche and ELETTRA Synchrotron in Basovizza (Trieste, Italy), where the Phase Contrast Tomography (PhC-microCT) experiments were performed. Furthermore, AoN Implants from Grisignano di Zocco (Vicenza, Italy) participated in the research on AI-guided cone beam computed tomography (CBCT) image segmentation and micro-gap investigation using micro-computed tomography (micro-CT). Finally, during my PhD period abroad, the School of Dental Medicine at the University of Belgrade collaborated with us on several studies, including the analysis of data from patients with periodontal disease.

In this framework, after the present brief introduction of the overall PhD activity (Chapter 1), the Chapter 2 provides a detailed analysis of the principles that underlie the AI algorithms that were employed, including Machine Learning (ML) and Deep Learning (DL). Additionally, it provides a detailed description of ANN, including Convolutional Neural Networks (CNNs) predominantly applied in the field of image analysis. The high-resolution X-ray tomography techniques employed for the research cases discussed in this thesis are the subject of Chapter 3: the contents covered focused in particular on the physics underlying the micro-CT, PhC-microCT and CBCT methods. Subsequently, after having described the image analysis and data acquisition techniques, we moved on to the discussion of the applied experiences in research, which are the subject of Chapter 4. In Chapter 4, several manuscripts, submitted for review or already published in international scientific journals, constitute the content of each subchapter: (4.1) in this study, synchrotron radiation-based imaging was used to study bone tissue, with osteocyte lacunae segmented using artificial intelligence and several morphometric and mineralization parameters analyzed by multivariate statistical analysis; (4.2) here, images

of collagen bundles, acquired by PhC-microCT, were segmented in peri-implant connective tissues using CNNs to differentiate them in the two primary orientations (longitudinal and transverse), in order to generate a quantitative analysis distributed according to the orientation of the bundles; (4.3) in this work, CBCT images were analyzed using CNNs to differentiate between teeth, bone tissue and dental implants; (4.4) here, the presence of micro-gaps in the implant-abutment interface was determined by mechanical testing and micro-CT imaging of dental implant samples provided by AoN Implants; (4.5) in this study ML was applied to clinical data provided by the School of Dental Medicine, University of Belgrade with the aim of developing an application that can be used by dentists to classify the staging and grading of periodontal diseases; (4.6) finally, multidisciplinary techniques were employed to analyze oral diseases, especially oral tongue squamous cell carcinoma. In the final Chapter 5, the critical issues identified in the various studies were discussed and possible future developments were presented.

2. Artificial Intelligence in Dentistry

Artificial Intelligence (AI) aims to develop computer systems that mimic human behavior by constructing machines capable of replicating human thought processes and actions. Recent advancements in the field have encouraged clinicians to provide data to computer scientists, facilitating the creation of validated technologies for healthcare staff [1,2]. This approach allows for a digital second opinion that assists doctors during diagnosis, drawing on the collective expertise of multiple clinicians who trained the machine in its early phase. AI spans a wide range of developing technologies that have an impact on several aspects of society, including the field of Healthcare and Dentistry [3,4]. Particularly, in the field of periodontology and implant dentistry, AI is still in the early stages of development, but new discoveries have been made in recent years [5].

Therefore, over the last few years, Artificial Intelligence (AI) has been applied to different types of images [6,7] supporting clinical decision, assisting human doctors [1,8,9], personalizing medicine and more: MRI [10], X-ray tomography [11,12], Ultrasound [13] for different purposes such as detail detection, object recognition and noise reduction. This allowed the various disciplines to interface with new methodologies and to analyze images and data with a support they had not previously considered. Recently, AI has been used to improve image interpretation and facilitate diagnosis in 2D and 3D dental radiology [14], identifying dental caries [15] and automatically segmenting anatomical structures such as maxilla, mandible and teeth [16,17].

Deep learning is a subset of artificial intelligence, more specifically it refers to the capacity of a machine to learn like a human being from experience without using instructions, thus it can make predictions or take decisions based on the large amount of data entered into the training phase. More complex is the algorithm deeper its knowledges are and more it can predict. In the orthodontic field, exploiting deep neural networks, it is possible to separate the different scanned regions and process them separately in order to extract useful information in the phase of preparing the patient for surgery [16]. Furthermore, thanks to image segmentation technique, AI image processing techniques can identify details that could escape human analysis, as well as plan interventions and give advice to the doctor in the diagnostic phase: such as the identification of caries and restorations [2,15,18].

A fundamental fact is linked to the increasingly widespread digitization in the dental sector, through the use of implant planning AI-integrated software such as Navident [19], useful for correctly positioning the implant and evaluating the quality of the underlying bone in order to decide the correct implant to use [20,21].

2.1 Machine Learning

The term “Machine Learning” (ML) defines that branch of computer science dedicated to the study of AI, in which automatic algorithms for computational learning of a certain input pattern are used. The basic idea is to train the algorithm starting from available data, the so-called *training data*, and then ensure that it acquires the ability to predict new information on *testing data*, based on what it has learned. Once the basic model is set, training is done automatically based on observations. Furthermore, the computational times are quite short (a few hours) and can be applied in many fields: search engines, optical character recognition, computer vision, recognition of images such as faces or, as in our case, medical images [22]. Every type of algorithm must have a goal. The criterion used is based on the minimization of a mathematical function, generally defined as *loss function*. The loss functions are chosen based on the type of training, in particular the two main classes are the functions used in *regression* and those used in *classification*. In regression problems, the input data are interpolated to provide a continuous numerical value in the output; in fact, the output domain itself is continuous and not determined by a discrete set of possibilities. An illustration is forecasting the future value of a currency's exchange rate based on its recent historical values.

While in classification problems the task is to assign to each input a label that corresponds to the class it belongs to. It can be either binary (0 or 1) or if there are more than two labels, it refers to multi-class classification and the system must develop a model that assigns labels to previously unknown inputs.

The most commonly used method for finding the minimum of the function is the *gradient descent*, an algorithm based on local search. One limitation is the dependence on the initial condition, i.e., the first point of the objective function at which the gradient is calculated, because it is on this that the direction of movement to reach the minimum will depend. Considering the loss function as a curve, it will be made by a series of local minima and a global minimum, which is the one sought. The limitation of this algorithm

is that it often does not find the global one but the local one because, by always accepting the best solution, it may fall into one of them and consider it as the absolute one. Another limitation is the dependence on the size of the *neighborhood*, the range of observation around the point considered at each iteration; this affects the *learning rate*, which is a parameter that defines the step size of each iteration along the progression towards minimizing the loss function. It determines the degree to which newly obtained information supersedes existing knowledge, figuratively symbolizing the rate at which a machine learning model "learns".

There are three variants of the Gradient Descendent:

- *Batch gradient descent*: the gradient is computed over the entire dataset by executing a summation of all observations to produce a single parameter update in the direction opposite to the gradient. The disadvantage of this variant is related to the slowness of the algorithm and the computational costs of iterating over the total dataset at each *epoch*, which is the hyper-parameter defining the number of times the learning algorithm updates internal parameters, which can also be defined as the number of iterations of the algorithm. The advantage is that the Batch gradient descent guarantees convergence to the global minimum for convex error functions and to the local minimum for nonconvex error functions. In the following equation θ corresponds to the parameters to be updated at each iteration, η to the learning rate, and ΔJ to the gradient of the cost function.

$$\theta = \theta - \eta \Delta J(\theta)$$

(2.1.1)

- *Stochastic gradient descent*: the updating of the loss parameters occurs at each training example; this promotes greater speedup than Batch but also greater fluctuations in the objective function that give the algorithm a chance to jump to end up in a better local minimum. Nevertheless, this can complicate the final convergence and make the representations noisier. It guarantees convergence to the global minimum for convex functions and to the local minimum for nonconvex ones.

$$\theta = \theta - \eta \Delta J(\theta; x(i); y(i))$$

(2.1.2)

- *Mini-batch gradient descent*: divides the dataset into a number n of batches (usually powers of 2) and updates the weights at each iteration, thus reducing the variance of the updated parameter and ensuring more stable convergence.

$$\theta = \theta - \eta \Delta J(\theta; x(i:i+n); y(i:i+n))$$

(2.1.3)

The selection of a loss function is contingent upon various criteria, including the existence of outliers, the selected ML algorithm, the computational efficiency of gradient descent, the simplicity of derivative calculation, and the reliability of predictions. For this reason, some of them are shown below.

Square Error Loss:

$$MSE = \frac{1}{n} \sum_{i=1}^n (y_i - y_i^*)^2$$

(2.1.4)

Function mainly used in ML; the advantage is linked to the safe convergence of the algorithm even with a fixed learning rate. The term n represents the number of observations, while y and y^* are respectively the predicted value and the desirable value.

Absolute Error Loss:

$$MAE = \frac{1}{n} \sum_{i=1}^n |y_i - y_i^*|$$

(2.1.5)

The module enhances the algorithm's resilience to outliers; minimizing the MSE yields the average of all targets, whereas minimizing the MAE results in the median of the observations, providing a more robust estimate than the mean. The primary drawback is that its gradient remains substantial, even with minimal loss values. A dynamic learning rate that diminishes with each iteration can resolve this issue, facilitating the algorithm's convergence.

Generally, other types of loss functions are used for classifiers, since the objective is to separate the inputs into a predetermined number of output classes, obtaining a discrete value as a result:

Binary cross entropy:

$$H(p, q) = -(p(x) \log(q(x)) + (1 - p(x)) \log(1 - q(x)))$$

(2.1.6)

Function deriving from information theory that expresses the distance between the probabilities of the input belonging to class 0 or class 1. The term p represents the "target" while q the "prediction" for each class.

Categorical cross entropy:

$$H(p, q) = - \sum_{i=1}^n p_i \log(q_i)$$

(2.1.7)

Function similar to the previous one on which it calculates the probability of the input belonging to a class among the n established. Likewise, the term p represents the "target" and q the "prediction" of the n possible values of the input.

ML tasks are often categorized into four primary types based on the characteristics of the input data utilized for learning or the "feedback" accessible to the learning system:

- *Supervised learning:* the training dataset is known and contains both the inputs and the labels, i.e. the associated categories for identifying the class to which it

belongs. The objective is to derive a general rule that links the input to the accurate label. Typical applications include classification and regression.

- *Unsupervised learning*: the training dataset contains exclusively the inputs without labels, consequently the algorithm cannot make associations but will instead tend to group the data based on similarity criteria, such as Euclidean or Probabilistic distance. The typical example is *clustering*.
- *Reinforcement learning*: a model engaging with a dynamic environment to attain a specific objective (such as operating a car), receiving feedback solely from an instructor regarding the achievement of that goal. Another instance is acquiring the skill to play a game through competition with an adversary.
- *Semi-supervised learning*: the training dataset is incomplete; it contains labeled and unlabeled data. It is a hybrid technique between supervised and unsupervised learning [23].

2.2 Artificial Neural Networks

Artificial Neural Networks (ANN) are algorithms for supervised learning inspired by the functioning of biological neural networks, both from a structural and functional point of view. It is a multi-layered method with a path made up of arcs and nodes. The arcs represent artificial synapses, the weights of the network updated at each iteration, its true source of learning. The nodes represent the neurons at each layer, characterized by their *activation function*, which is a mathematical function that calculates the output of the nodes based on its individual inputs. This model has a network of linkages via which information flows from input to output unidirectionally (*forward phase*). During each iteration, the output generated by the network may vary in similarity to the actual output, necessitating the computation of an error that is "propagated back" to update the weights to facilitate learning. The method is iterative until the loss is reduced, and a high degree of accuracy is achieved (*backward phase*).

The *Perceptron* is the basic ANN characterized by a sequence of inputs, each multiplied by its appropriate weight and subsequently summed, which then proceeds to the activation function to produce the expected binary output. This is typically employed for simpler assignments, but for more complex tasks, such as multi-class classifications or using substantial input data *Multi-Layer Perceptron* must be used (Fig.2.2.1) [24].

The distinction is that an additional layer of intermediate "hidden layers" of neurons is incorporated into the model, enhancing network depth and improving classification accuracy. The output of a binary categorization is done using Binary Cross Entropy, since the result must be 0 or 1. While, for multi-class classification we utilize Categorical Cross Entropy, where the labels are represented as vectors containing a 1 for the corresponding class and 0 elsewhere.

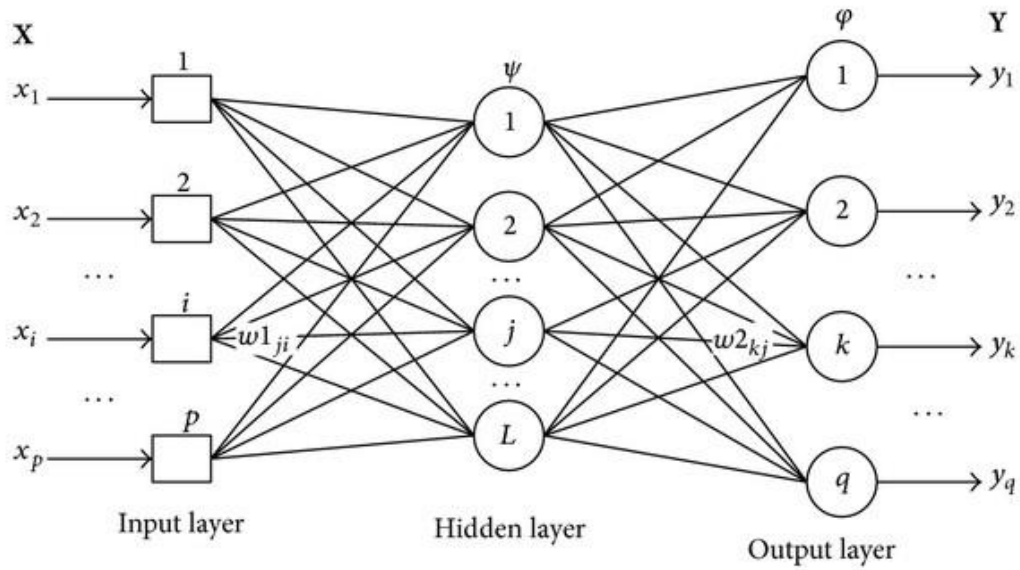


Figure 2.2.1 Multi-layer Perceptron ([24], CC BY 3.0).

The main limitation of ANN is that learning occurs in a "black box", meaning it is impossible to know how the network learns and interprets individual weight updates. Each neuron represented has its own processing capacity and transforms input into output based on the chosen activation function:

$$output = f(\sum weights * input) + bias$$

(2.2.1)

Activation functions can vary in type, with the most common being:

- *Threshold function*: function that returns 1 if the summation of the inputs is greater than or equal to zero while returning 0 in other cases.

- *Sigmoid function*: function with continuous range between 0 and 1, but the output is no longer 1 but the probability that the output value is equal to 1. The same applies to zero.
- *Rectifier function*: function that returns 0 when the summation of the inputs is less than or equal to 0, while it returns the summation of the weights for the respective inputs in all other cases. With a range from 0 to infinity and commonly defined as ReLU (Rectified Linear Unit), it is applied in convolutional networks in order to accelerate gradient descent, exploiting an inexpensive function from a computational point of view.
- *Hyperbolic Tangent function*: similar to Sigmoid function but with a range from -1 to 1, a useful feature in certain applications.
- *SoftMax function*: function applied in multi-class classifications; its aim is to highlight the maximum value and hide the smaller ones, which will be given less weight.

A prevalent issue seen in multi-layer ANN is overfitting, which transpires when the network excels at recognizing training samples but fails to generalize to test examples. In certain instances, it is essential to stop learning and identify the best number of epochs that yield a significant reduction in the loss function while ensuring the network's capacity to generalize remains intact. Nonetheless, determining an ideal number of epochs is challenging; thus, "regularization techniques" are employed to introduce "noise" into the network's input during training, ensuring that examples are not learned optimally, a situation that may arise with increased network depth. Some regularization techniques are as follows:

- *Dropout*: consists of the elimination of some neurons in the training step according to a certain rate (probability of "drop"). Therefore, the network will be forced at each step to make predictions in the absence of some neurons, this will allow the network to be less sensitive to specific weights, improving the ability to generalize. Each neuron will be forced to uniquely learn the most robust features and then classify them in the correct way in testing.
- *Batch-Normalization (Added Normalization Block)*: allows to improve the speed, performance and stability of the network by means of two techniques: "Re-center" and "Re-scaling" applied to groups of inputs. This makes it possible to solve the

problem of the internal covariate shift, the shift of the distribution of inputs during learning. This problem causes training to slow down and thus would require lower learning rates for the network to still succeed in learning. Batch-Normalization goes to normalize the mini-batches by subtracting their mean and dividing by the standard deviation of each data set [25].

- *L1(sparsity) and L2(weight decay)*: the L1 and L2 regularization techniques go to apply a norm to the vector of weights the former goes to sum the moduli of the elements while the latter does the square root of the sum of the elements squared. This is done to make the coefficients of the model small by going to reduce its complexity. The input parameter (around 0.001/0.005) corresponds to the regularization rate i.e., that compromise between finding sufficiently small weights and minimizing the cost function [26].

2.2.1 Deep Neural Networks

Another networks' characteristic is linked to depth: the "deeper" the network, the more complex the structure becomes. Increasing computational time by building more layers becomes necessary to have greater accuracy of the results. Deep Neural Networks (DNN) have been generated for this purpose. Deep Learning allows computational models, composed of multiple layers, to autonomously learn representations of data with multiple levels of abstraction. These methods have greatly improved the state of the art in speech recognition, visual object recognition, object detection, and many other areas such as drug discovery and genomics. DNN are concerned with subjecting data for more processing, on multiple levels, thus creating a deep network that can extract features from the inputs. Features are the characteristics of the dataset extracted by the algorithm automatically. This is made possible by the addition of numerous hidden layers of neurons. The pivotal operating principle is analogous to the classical ANN in which the gradient method and back propagation are used to update the weights, with the difference being the fixing of hyperparameters, such as the size of the input data, the size in terms of the number of layers of the hidden layers, the learning rate, the initialization of the initial weights, and the optimization operation. Thus, to achieve better accuracy in the results, it is necessary to vary these parameters and control the *vanishing gradient problem* arising from the deep

structure of the network. It is also called “gradient degradation” because the activation functions produce very low values in output (-1;1) that, multiplied together, due to chain computation, are propagated back without producing a consistent update in the weights. If the weights are not updated, therefore, the network fails to learn [27].

2.2.2 Convolutional Neural Network

Convolutional Neural Networks (CNNs) are types of deep networks particularly suited to images and specialized in classifying and segmenting objects by extracting features that then need to be classified [16,28]. However, being a subclass of DNN, they will also be organized hierarchically with at least two hidden layers. The advantage of this setup is the sharing and reuse of information across multiple layers, going to select features and discarding unnecessary details. This idea is inspired by the neurons in our visual system, which compose a complex cellular network. Cells can be of two types: (1) simple cells, sensitive to small subregions of the visual field (receptive field), which act as feature detectors behaving as local filters on the input space (input image map). They exploit the spatial correlation present in natural images; (2) complex cells, which merge the outputs of the simple cells into a surround, accomplishing the pooling operation. Even the number of layers used (about ten) is inspired by those between the retina and the actuating muscles, which if they were in greater number would be too slow to respond to stimuli. As the name suggests, the convolution of the image with a fixed kernel consists of making element-by-element products at each shift and then summing them up obtaining a feature map as output. Other parameters to be controlled will therefore be the size and number of kernels defined in the network architecture. To detect particular aspects of the image, for example edges, it will be necessary to use kernels much smaller in size than the image; therefore, it is possible to analyze millions of pixels by detecting only a few significant features, thus reducing memory usage.

The convolution operation for continuous input is as follows:

$$s(t) = \int_{-\infty}^{+\infty} x(a)w(t-a)da$$

(2.2.2.1)

But since images are maps of integers, discrete convolution can be defined as:

$$s(t) = \sum_{-\infty}^{+\infty} x(a)w(t - a)$$

(2.2.2.2)

Three different types of layers can be considered in the architecture of neural networks: convolutional layers, max pooling layers and dense layers.

In *convolution layers*, the filter flows over small segments of the image map; *max pooling layers* serve to diminish the dimensions of the feature maps. In this scenario, the filter, possessing a defined size, retains only the maximum value from each applied window; hence, an increase in filter size results in a reduction of the output feature map.

The primary purpose of employing such actions in the network is to expand its receptive field, enabling each output neuron to perceive a broader segment of the input. Furthermore, down sampling allows more feature maps to be employed without increasing the computing weight of the method.

A fundamental concept of CNN is weight sharing, which establishes a dependency among layers. A weight is not restricted to a single layer; it can be repurposed in other contexts, hence avoiding the need to compute additional kernels that would prolong the system's computational duration. In addition to assuring efficiency, this technique allows convolution to acquire the 'translation equivariance', which is the property of creating a dependency between input and output such that the convolution of a translated input is identical to the translation of the convolution of the same input. Reducing the number of weights and connections also significantly reduces the computational costs of the algorithm, as different neurons of the same hidden layer perform the same type of processing (same weights) on different portions of the input.

Dense layers are utilized for classification, where each neuron is interconnected with every neuron in the subsequent layer, hence the designation 'completely linked.' This segment of the architecture features similar characteristics to traditional neural networks, employing bias and weights to classify inputs, which is the primary goal of the network.

When the input dataset is insufficient for training a network or lacks sufficient diversity the *dataset augmentation* process is implemented: the objective is to obtain a more

substantial and comprehensive dataset by eliminating duplicates that may result in overfitting. The fundamental concept involves “deforming” the data by geometric methods such as *flipping, rotation, scaling, zooming, and cropping*, as well as image improvement techniques like *gamma correction* and *histogram equalization*. Consequently, both the original and altered images are produced. The neural network will not perceive this distinction and will regard the two groups as distinct, generating predictions for each.

Data augmentation approaches can be divided into two types: (1) pre-processing the data prior to inputting it into the network model, hence expanding the initial dataset size; (2) implementing it on a mini batch immediately before the training begins. The first method, called “offline augmentation”, is favored for smaller datasets, as it increases the size of the dataset by a factor corresponding to the number of changes applied. The second method, referred to as “online augmentation” or “on-the-fly augmentation”, is preferred for extensive datasets, as it mitigates the prohibitive expansion in size and is executed solely on mini-batches. For each implemented technique, a factor is outlined to increase the size of the dataset to ensure a regulated expansion [29,30].

3. High Resolution X-Ray Tomography

3.1 Micro Computed Tomography

Micro Computed Tomography (micro-CT) is an advanced imaging modality that employs X-rays to produce high-resolution three-dimensional images of small objects or specimens. Conventional micro-CT methods predominantly utilize absorption-based imaging, employing variations in X-ray attenuation to differentiate among various materials or structures inside the sample. This system is exploited for in-vitro studies of biomaterials, scaffolds, bones, teeth etc. as well as for organs and small animals to discover its morphology and functionality [31].

However, widely applicable approaches for non-destructive analysis of different sample types are limited. Quantitative studies necessitate precisely aligned and calibrated volumetric images of structures [32].

Image Acquisition Process

Referring to Figure 3.1.1, during a micro-CT scan the specimen is positioned in the trajectory of the x-ray beam, where it is illuminated by the source, thereby creating a projection picture on the scintillator or another x-ray-sensitive detector array. The object is rotated at a fixed angular rotation increment; for each angle a bi-dimensional projection image is created and “back projected” to reconstruct the x-ray absorption. The overall rotating angle is contingent upon the system's geometry, generally measuring 180° for parallel beam configurations or 360° for cone-beam geometries. On the other hand, in clinical CTs, source and detector are rotated around the patient to produce the different projections.

In micro-CT the resultant 3D output volume comprises a series of reconstructed cross sections perpendicular to the axis of rotation [31].

Typically, micro-CT systems utilize digital flat 2D detectors: (1) Charged Coupled Devices (CCDs), which employ scintillator screens connected through fiber-optic bundles to convert x-rays into visible photons [33]; (2) Complementary Metal Oxide

Semiconductor (CMOS), a technology recently emerged and employed in systems for in vivo imaging of tiny animals [34].

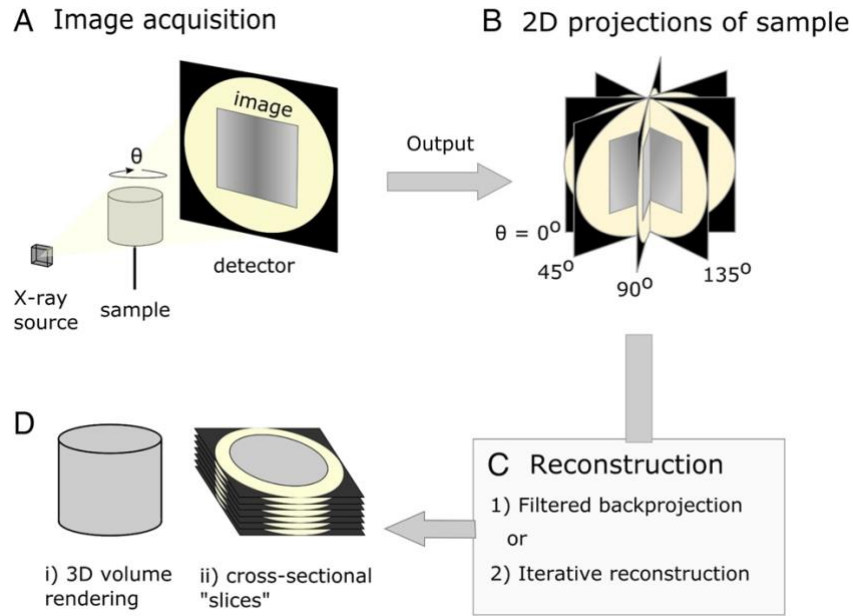


Figure 3.1.1. Scheme of the X-ray micro-CT process. (A) Image acquisition with the X-ray source that cross the sample during its rotation in increments of θ while the detector captures the projections; (B) sequence of projections of the sample captured at various angles; (C) reconstruction step done by two prevalent methods: filtered back projection and iterative reconstruction; (D) sample observed in a virtual environment ([35], CC BY 4.0).

Absorption law

To explain image creation, one must transition from the interaction processes of an individual x-ray photon to the quantitative attenuation of an x-ray beam, considering both absorption and scattering. The mechanics underlying x-ray imaging can be elucidated through the complicated refractive index of the specimen. At the macroscopic level, the absorption of a monoenergetic incident x-ray beam in a homogeneous material (characterized by uniform density and atomic number Z) can be described as follows:

$$I = I_0 e^{-\mu \Delta x}$$

(3.1.1)

Where I represents the intensity of the beam after traversing the material, while I_0 denotes the incident intensity; Δx signifies the thickness of the material; μ is referred to as the linear attenuation coefficient, determined by a linear combination of the photoelectric effect, Compton scattering, and coherent scattering. The equation 3.1.1 is referred to as the Lambert–Beer law [36].

A high value of μ indicates that materials can attenuate x-rays more effectively than those with low μ values. In medical imaging, x-ray photons experience greater attenuation by bones (high μ) compared to soft tissues (low μ). In the case of a non-uniform object, composed of many smaller homogeneous elements with varying absorption coefficients, the incident intensity of one element is determined by the outgoing intensity of the preceding element [31].

X-ray interaction with matter

At the microscopic level, the interaction between X-rays and matter transpires through three primary mechanisms: the *Photoelectric effect*, the *Compton effect*, and *Coherent scattering*.

- The *Photoelectric effect* occurs when an incident x-ray photon, possessing a specific energy E (Formula 3.1.2), strikes an atom where the binding energy of the electrons is inferior to E , resulting in its absorption by the atom. The interacting electron is elevated to a state within the continuous spectrum, meaning that an electron from a lower shell is ejected from the atom and moves through the material as a free photoelectron. Subsequently, the photon is absorbed. An outer-shell electron occupies the vacancy in the deep shell. *Characteristic radiation* is emitted because the outer shell is at a higher energy level than the inner shell.

$$E = h\nu$$

(3.1.2)

Where h represents Planck's constant, and ν denotes frequency.

- The *Compton effect* occurs when the energy of the x-ray photon exceeds the binding energy of the electron. The x-ray photon liberates the electron from the atom and is scattered, losing a portion of its initial energy. This type of interaction generates a dispersed photon and a positive ion. The photon may be deflected at any angle between 0° and 180° , contingent upon a certain angular distribution influenced by photon energy and sample composition.
- The *Coherent scattering* does not involve an ionization process. The incident electromagnetic wave, characterized by its oscillating electric field, generates a transient vibration in the target atom, causing the oscillating electrons to release radiation at the same wavelength (energy) as the incident wave. Consequently, the scattered photon possesses identical energy to the initial photon.

Image Reconstruction Process

Reconstruction programs calculated numerical value for each voxel, which, for the absorption method, represents a linear x-ray attenuation coefficient at the respective location within the sample volume [32]. Indeed, in the conventional x-ray, imaging formation is based on the differences in x-ray absorption of the sample details. The attenuation depends upon the composition and density of the objects.

Moreover, by employing filters as *Smoothing*, *Ring Artifacts Reduction (RAR)* and *Beam Hardening (BH)*, a satisfactory reconstruction can be achieved: (1) The goal of Smoothing or “Blurring” filter is to reduce image noise, caused by any micromovements and instrumental technology, *Gaussian* is an example algorithm; (2) Ring artifacts are a prevalent characteristic of tomographic reconstruction, arising from the non-uniformity of the background image captured by the x-ray sensor. In fact, an object in the camera image which remains in the same place in all projection images of the rotating sample is interpreted as a ring by the tomography reconstruction. To mitigate this problem RAR filter is introduced; (3) BH is a tomographic artifact that causes the object to appear artificially denser at or near its surfaces, while appearing less dense in its core regions. This happens when x-rays source is *polychromatic*, thus x-rays with a lower energy (“soft”) will be attenuated more rapidly through the sample than the higher energy x-rays

(“hard”). Due to this soft depletion, the beam “hardens” along the beam path, which causes more energetic x-rays to traverse a longer path.

At the end of all processes, the tomographic output volume image is represented as a three-dimensional matrix of luminance values, equivalent to a stack of aligned two-dimensional digital images (i.e. the slices) [37].

X-ray micro-CT is able to efficiently image small biological specimens; nevertheless, its extensive application for non-mineralized tissues is challenging because of insufficient x-ray contrast. Conversely, the advancement of novel x-ray production methods utilizing particle accelerators (synchrotrons) has facilitated the generation of sources with enhanced characteristics, specifically high spatial coherence and brilliance, thereby enabling the application of innovative imaging methodologies known as phase-sensitive techniques, which were recently implemented to increase contrast and signal-noise ratio.

3.2 Synchrotron Radiation Phase Contrast Tomography

Since its initial use nearly three decades ago, phase-contrast X-ray imaging has gained more and more attention. Studies that employ this method to examine soft tissues, which are normally impossible to examine in three dimensions and at high resolution using traditional absorption-based settings were given special attention. Indeed, researchers from a variety of fields, including botany, zoology, and human physiopathology, are becoming more interested in using it due to its consistency and discrimination power in low-absorbing samples combined with the fact that it is a non-destructive analysis. Based on the special properties of synchrotron radiation phase-contrast microtomography (PhC-microCT), this difficult technique for 3D imaging and quantitative analysis of collagenous tissues has gained interest recently [38].

Image Acquisition Process

Synchrotron radiation (SR) is produced when an electron, moving at relativistic velocities (2-2.4 GeV), alters its trajectory. SR encompasses a broad range of electromagnetic waves, spanning from infrared to hard X-rays. The primary properties include high intensity (3-4 orders of magnitude greater than typical x-ray tubes), high brilliance, and

low divergence emission. Furthermore, SR is generated at extensive accelerator facilities that utilize various magnetic configurations, including bending magnets and insertion devices, optimized to enhance radiation output for diverse experimental objectives and user specifications (Fig. 3.2.1) [31]. Once the electrons are accelerated, they are deflected at research stations (*beamlines*), and it is from this impact that X-rays are generated.

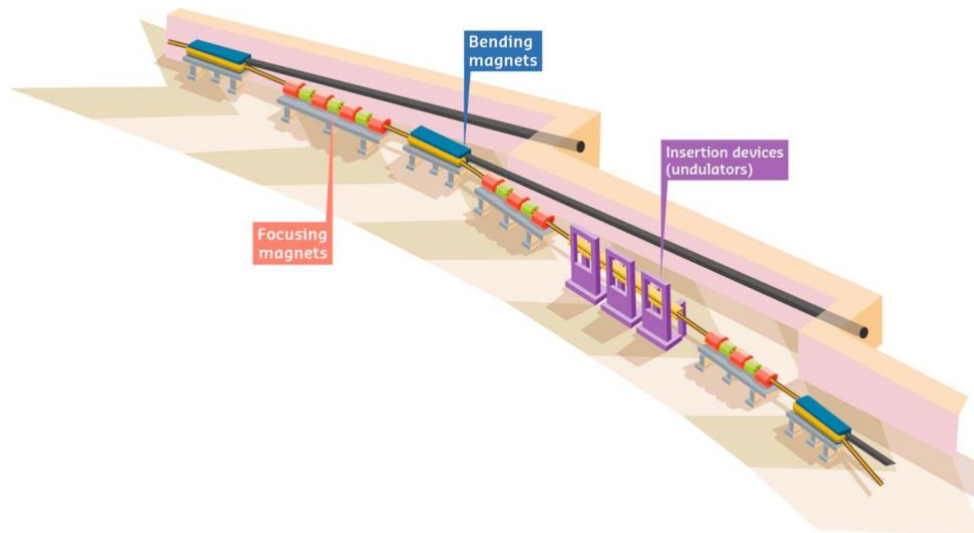


Figure 3.2.1. Illustration of a curved segment depicting a bending magnet and an insertion apparatus. Reproduced from www.esrf.eu.

Imaging beamlines consist of several components utilized for beam shaping, focusing/defocusing, filtering, and monochromatizing. At hard x-ray imaging beamlines experimental stations are often situated at considerable distances from the source and are designed to accommodate certain investigations. The photon flux, energy range, collimation, and focus of X-rays are adjusted based on the materials being analyzed.

The SR beam exhibits a high degree of spatial coherence, enabling the utilization of phase shift effects in transmitted x-ray waves as an additional mechanism for image creation.

In contrast to x-ray tubes, synchrotrons inherently offer laminar, almost parallel geometry, resulting in small object amplification on the detector and negligible scatter contribution to image formation. The utilization of monochromatic x-rays minimizes beam hardening artifacts, which is essential for obtaining quantitative information about the sample, including composition, density, and morphology [31].

Phase-based Techniques for x-ray Imaging

PhC-microCT utilizes the coherence of the X-ray beam to implement experimental methods that not only differentiate the varying attenuation characteristics of the elements within the photographed object but also address the phase contrast among its internal phases (Figure 3.2.2). The contact of an object with x-rays results in attenuation of x-ray intensity and a change in the direction of x-ray propagation by a refraction angle α . Conventional tomography methods solely capture the x-ray intensity for each pixel in the detector, thereby measuring only the attenuation signal. The PhC-microCT method also captures the local refraction angle α , which provides information on the phase-shifting intensity. The complex refractive index is expressed by the equation:

$$\begin{aligned}\eta &= 1 - \delta + i\beta \\ \delta &\gg \beta \\ (3.2.1)\end{aligned}$$

Example: $\delta=10^{-6}$, $\beta=10^{-9}$

In the region where the photoelectric effect predominates over absorption, away from absorption edges, δ and β can be articulated as functions of electron density ρ and radiation wavelength λ as follows:

$$\begin{aligned}\delta(\lambda) &= \rho \frac{r_e \lambda^2}{2\pi} \\ \beta(\lambda) &= \mu(\lambda) \frac{\lambda}{4\pi} \\ (3.2.2)\end{aligned}$$

where r_e is the electron radius.

β is intricately associated with the linear attenuation coefficient μ as depicted in traditional tomography, whereas the phase-shifting intensity is represented by δ , the real component of the index. Consequently, the refraction angles are contingent upon δ and, for X-rays, are around $\alpha \cong 10^{-5}$ degrees.

Consequently, utilizing phase information in tomography can augment image contrast and enhance the perception of intricate details, particularly in samples exhibiting poor X-ray absorption contrast.

PhC-microCT techniques employ phase retrieval algorithms to derive phase information from the acquired intensity data, alongside the absorption information. Incorporating phase information into the reconstruction process enables PhC-microCT to yield more detailed and precise pictures, especially for samples exhibiting small density fluctuations or weakly absorbing materials. Phase information can disclose supplementary structural details, including borders, interfaces, and interior features, that may not be distinctly observable in absorption-based micro-CT images [38].

Indeed, to obtain quantitative data on the absorption and phase shift induced by the item on the beam, a range of algorithms have been analyzed. A streamlined technique utilizing the transfer of intensity equation (TIE) and a singular propagation-based distance is extensively employed in the near-field region for homogeneous samples to separate phase from absorption effects [39]. This approach is utilized to enhance picture contrast and aid analysis in propagation-based images, where the resulting edge enhancement improves the visibility of structural details but impedes further quantitative analysis dependent on threshold-based segmentation of the data sets.

Phase retrieval is employed across multiple disciplines, such as X-ray imaging, electron microscopy, and optical imaging, to augment picture contrast and refine the viewing of intricate features. Furthermore, the integration of PhC-microCT with sophisticated phase retrieval and reconstruction methods enhances imaging of biological specimens, materials research, and various other applications.

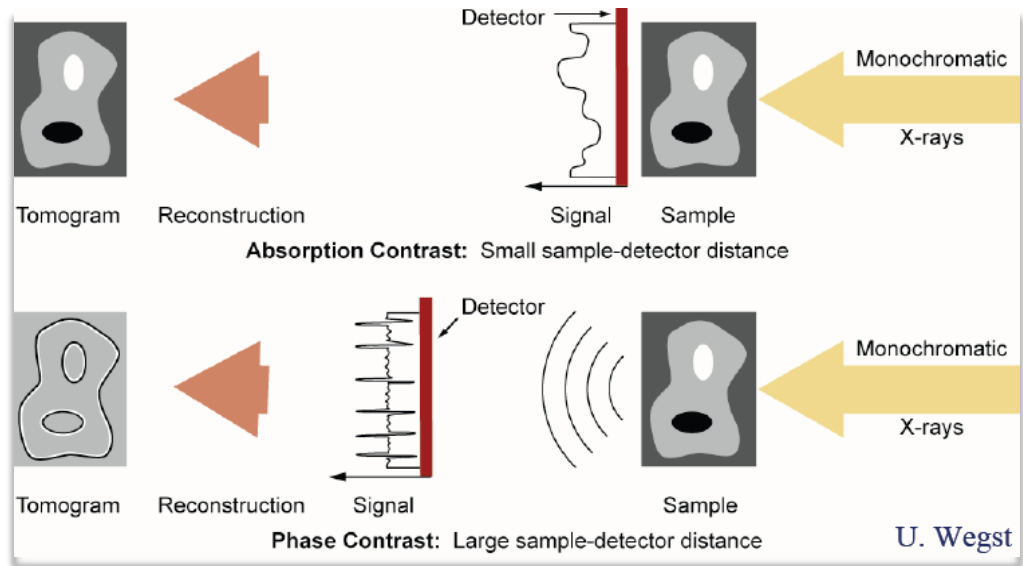


Figure 3.2.2. Schematics of the dual modality for absorption and phase contrast imaging.

Phase retrieval method

Phase retrieval (PhR) is a technique employed in imaging to derive phase information from intensity data for the reconstruction of phase contrast images. It facilitates the retrieval of phase information, which encompasses critical details regarding the object's structure and characteristics. PhR generally entail resolving a mathematical problem to extract phase information from the acquired intensity data. This can be accomplished by numerous algorithms:

- The Phase-Attenuation Duality (PAD) approach is a prevalent methodology for phase retrieval, relies on the correlation between phase and attenuation in the Fourier domain [40].
- The Contrast Transfer Function (CTF) method, which entails examining the intensity distribution of holograms to reconstruct phase information [41].
- Convolutional Neural Networks (CNNs) directly determine the correlation between intensity data and phase information. These approaches have demonstrated encouraging outcomes regarding precision and velocity [42] .
- The Ptychography, which involves scanning a focused beam across the sample and recording the diffraction patterns. This facilitates the acquisition of both amplitude and phase data, resulting in enhanced reconstruction quality [43] .
- Non-linear phase-retrieval (NLPR) techniques are employed to circumvent restrictive approximations, providing precise and clear reconstructions. Upon

retrieval of the phase information, it is utilized to recreate the phase contrast image [44].

Frequently employed methods for reconstruction encompass filtered back-projection and iterative algorithms. Filtered back-projection is an efficient technique that employs a Fourier-based filtering method for picture reconstruction. Iterative techniques enhance the reconstruction by minimizing a cost function derived from the obtained data and the reconstructed image [45].

Regarding reconstruction, a recent technique involves the utilization of model-based iterative reconstruction (MBIR) algorithms. These algorithms integrate pre-existing knowledge regarding the photographed object, such its shape or material characteristics, to enhance the quality of the reconstruction [46]. Ultimately, during the reconstruction phase, neural networks are contributing by being included into filtered backpropagation algorithms to enhance their efficiency or by establishing an entirely new paradigm in reconstruction [47–49].

SYRMEP beamline at ELETTRA Synchrotron

I must be grateful for having participated in the experiments at ELETTRA Synchrotron in Basovizza (Trieste), without which I would not have been able to acquire the images for the projects presented in the chapters below. SYRMEP (SYnchrotron Radiation for MEDical Physics) is the beamline that provides beamtime via a proposal process and permits access for specified time shifts of eight hours each.

Throughout the Doctoral Course, I utilized it for a total of 27 sessions distributed across 3 rounds, facilitated by two successful proposals and access granted via the EuroBioimaging network (refer to <https://www.eurobioimaging.eu/> for details).

In each experiment, the parameters were adapted based on the type of tissue investigated, hard tissues (bone tissue) and soft tissues (collagen). The SYRMEP beamline allows for operation under both absorption and phase contrast settings; in these experiments the latter was utilized (Figure 3.2.2).

Samples exhibiting a vertical dimension exceeding the frame's field of vision were obtained through sequential repeated scanning at varying z locations, facilitating the comprehensive acquisition of the entire sample.

Since the beam is fixed in SR, an image is acquired by rotating the sample between beam and detector. In particular, if the object is cylindrical, the axis of rotation coincides with the axis of the sample. If this axis is not perfectly orthogonal to the plane containing beam and detector, artifacts and “ghost” images can be created, suggesting the presence of detail that does not actually exist. Therefore, it is critical to pay attention to alignment during sample placement (Figure 3.2.3).

Once the acquisition parameters have been decided, it is necessary to use the motorized remote control for the focusing operation. To do this, a small sphere of a few mm³ positioned in the center of the field of view is used.

Subsequent to the acquisition, each sample volume was promptly reconstructed utilizing the STP software (SYRMEP Tomo Project), an open-source application provided by the beamline team, featuring a user-friendly interface that facilitates the entire phase-retrieval and reconstruction process, allowing for the adjustment of appropriate parameters for each sample.

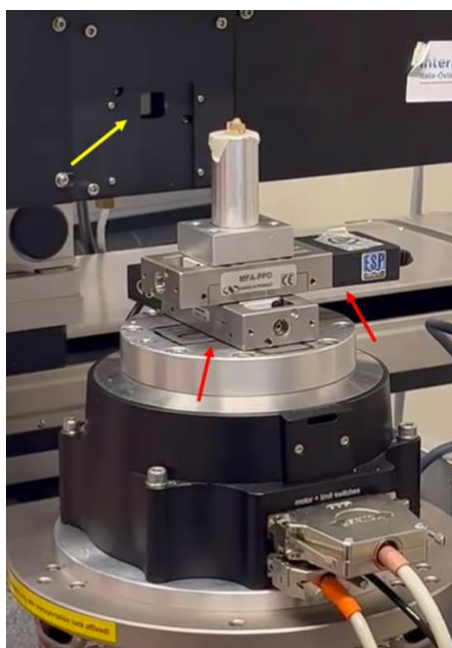


Figure 3.2.3. Image captured at the SYRMEP beamline: sample positioned on its support, preceding the tomographic acquisition procedure. The image depicts the detector, the small spot in front of the sample (yellow arrow), and the motorized remote control for precise motions in the x and y directions (red arrows), enabling precision positioning of the sample inside the field of view.

3.3 Cone Beam Computed Tomography

On the other hand, the Cone Beam Computed Tomography (CBCT) uses a cone-shaped beam and a reciprocating solid-state flat panel detector that spins from 180 to 360 degrees around the patient covering the defined anatomical volume (Fig. 3.3.1B). It provides three-dimensional analysis and superior imaging accuracy for oral and maxillofacial applications rather than slice-by-slice imaging found in conventional CT.

This singular scan (rotation) acquires intended data, 180-1024 2D images, similar to lateral cephalometric images, in contrast to stacked axial slices in CT (Fig. 3.3.1A), hence diminishing the absorbed x-ray dose by a factor of 6 to 15 relative to CT [50].

The scanning duration of CBCT equipment ranges from approximately 5 to 40 seconds, contingent upon the manufacturer and the exposure settings. The divergent X-rays produced by the X-ray tube are typically collimated using a lead alloy with a rectangular aperture, the dimensions of which can be modified according to the size of the exposed volume, or field of view (FOV). Collimation produces a conical or pyramidal beam, unlike traditional CT machines that emit a fan beam from the collimator. A multitude of projection images is captured by the detector during the rotation of the X-ray source and the detector. In contrast to traditional CT scans, where the patient table continuously moves during capture, each slice must be individually reconstructed to generate the whole volumetric image data [51].

The acquired 2D pictures are promptly transmitted to the computer, which reconstructs them using a modified Feldkamp-Davis-Kress (FDK) algorithm [52] into an anatomical volume for visualization at a 1:1 ratio in axial, coronal, and sagittal planes (orthogonal planes). The reconstruction software allocates a grayscale value on a 12-bit scale, and in certain instances a 16-bit scale, contingent upon the level of attenuation (typically, the lowest grayscale value corresponds to air) to each intensity value obtained from the linear attenuation of X-rays traversing the tissues on the detector's pixel matrix. The FDK technique reprojects these values to each virtual component of the image, namely to each voxel. The reconstructed image will then be presented on the monitor as an authentic volumetric dataset [53].

Finally, data is formatted in Digital Imaging and Communications in Medicine (DICOM), facilitating communications and compatibility with third-party imaging tools.

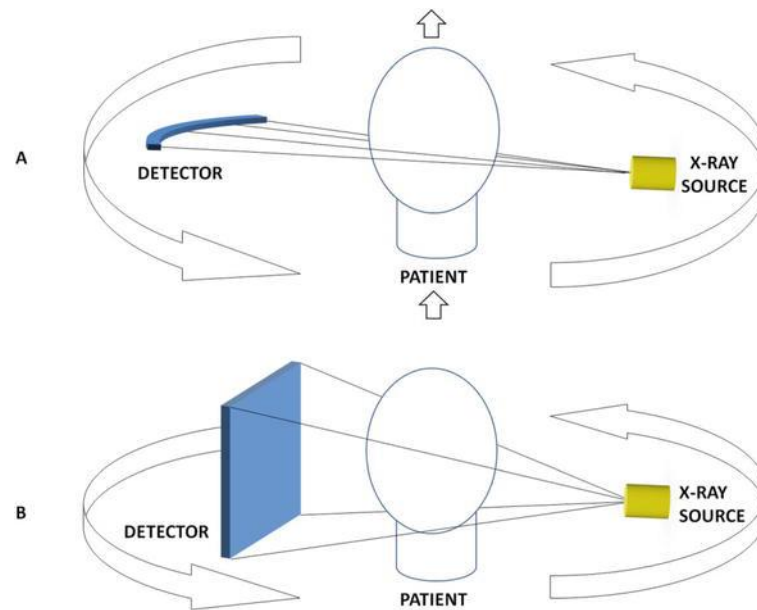


Figure 3.3.1. (A) Conventional CT device, X-ray beam with a fan-shaped and detector elements arranged in an arc rotating in the opposite direction from the source; (B) CBCT, cone-shaped beam with a flat-panel detector ([51], CC BY 3.0).

4. Results

4.1 Bone Tissue: Osteocyte Lacunae AI detection and Bone Mineralization evaluation

Mechano-sensing refers to the tissue's capacity to respond and adjust to environmental stimuli. The investigation of cellular activity in response to mechanical stimulation has been a longstanding research topic [54]. Some cells respond to external stress by generating contractile forces that are influenced by the mechanical characteristics of the extracellular matrix. Therefore, the significance of mechano-sensing is equal to that of chemical processes.

Bone tissue shows a special anisotropic morphology which is generated from the alignment of collagen fibers and the orientation of apatite crystals. In addition, the bone structure undergoes constant remodeling through the actions of osteoclasts, which break down bone, and osteoblasts, which build new bone. These processes are controlled by osteocytes. Thus, it is extremely interesting to study the osteocytes function as mechano-sensors in bone, because they generate signaling molecules that impact the activity of osteoblasts and/or osteoclasts [55].

The osteocyte activity in terms of adaptation to mechanical loading is a clear illustration of how the entire tissue undergoes global adaptation; osteocytes secrete nitric oxide (NO) in reaction to stress, which controls the functioning of other cells involved in bone formation or breakdown. Thus, osteocytes serve as the bone's mechano-sensors and maintain their round shape within ellipsoidal lacunae [56]. Mechanical loading is expected to impact the deformation of the lacunae and the subsequent ability of the osteocytes to sense mechanical forces. Two relevant studies showed that osteocyte lacunae are aligned to collagen: while the early work received little attention [57], a more recent study again demonstrated this correlation [58].

Since collagen fiber orientation was shown to be affected by loading mode [59], this alignment may help to understand how mechanical loading shapes the osteocyte lacuna. The presence of longitudinal collagen has been linked to tensile loading, while the presence of transverse collagen has been linked to compressive loading. The collagen fibers in the osteoid layer of growing osteons align in a way that matches the direction of

stretch (positive strain) caused by various loading modalities. The morphology of osteocytes may therefore mirror the extent of stretching in the osteoid layer during its creation, in accordance with the orientation of collagen fibers [60].

One widely accepted idea suggests that osteocytes detect mechanical forces through the movement of fluid caused by strain in the lacuna-canalicular network. In the jaw district, significant differences in the osteocyte surface area and orientation seem to exist locally, which were related to the tensile strain magnitude and orientation. This might reflect local differences in the osteocyte mechanosensitive and bone quality, suggesting differences in dental implant success based on the location in the jaw [61].

Matsugaki et al. established that the arrangement of collagen and apatite in a matrix is influenced by the extent to which osteoblasts align themselves on orientated collagen substrates. The recently produced matrix was found aligned with the cells, suggesting a reciprocal connection between collagen and osteoblast orientation [62].

Furthermore, variations in the geometry of lacunae can impact the mechanical stimulus perceived by osteocytes: lacunae that have diverse shapes or orientations are likely to experience varying volumetric deformation when subjected to a specific weight. For instance, altered lacunar shape could potentially contribute to osteoporosis either by modifying mechano-sensitivity or because of an increased matrix strain in an osteoporotic bone under normal loads. It was also supposed that altered lacunar shape shares a common cause with osteoporosis, such as hormonal changes that affect osteoclastic/blast activity and induce osteocytes osteolysis [60].

Therefore, it was widely proved that mechanical stimuli have the potential to impact the osteocyte activity, the alignment of osteoblasts and the structure of collagen. Considering these premises, further studies on the connection between lacunar and collagen orientation may provide insight into how mechanical loading influences the morphology of the osteocyte lacuna. This is especially true when referring to dental sites and to the jawbone osteocyte lacunae network. In this direction, Wu et al. examine how the levels and direction of tensile strain relate to the shape and direction of osteocytes at various dental implant sites in the upper jawbone [61]. The alignment of osteoblasts was found to be effectively controlled by the orientation of the collagen substrate, resulting in a preferential alignment along the direction of the collagen fibers.

Thus, it is necessary to develop a suitable model to study these morpho-functional aspects in the jaw because, to date, it is lacking in the literature. In this direction, a good study

model consists in investigating the jawbone in two conditions: namely, edentulism - i.e. bone not subjected to masticatory load - and dental implant - where the peri-implant bone is subjected to high mechanical forces during mastication.

This study aims to explore the relationship between mechanical stimulation, bone mineralization distribution and lacunar morphology by means of synchrotron-based phase-contrast microtomography (PhC-microCT) and artificial intelligence (AI) image segmentation techniques. In addition, histological and histomorphometry investigations were performed to confirm the results obtained by synchrotron analysis and complement them with additional information on soft tissues.

Materials and Methods

Samples

A total of 19 samples were analyzed, including 12 samples taken from edentulous ridge before implant placement and 7 implants removed because they could not be maintained. The edentulous bone samples were collected from patients who underwent implant placement according to the prosthetic planning. Six bone samples were removed from the mandible and 6 from the maxilla, the samples were harvested at the time of implant placement after a healing period ranging from 1 to 6 years.

All the 7 implants were removed after a period of functional load ranging from 2 to 10 years due to several reasons: recurrent peri-implant infection (3 implants), implant collar fracture (3 implants) and mispositioned/misplaced implant (1 implant). Four implants were removed from mandible and 3 from maxilla. The study was conducted in full accordance with the ethical principles of the Declaration of Helsinki (version, 2013) and was independently reviewed and approved by the Ethical Committee of University G. d'Annunzio, approval n. 22 -10 July 2022. All patients signed an informed consent form. The complete set of demographic data is reported in Table 4.1.1.

Table 4.1.1. Demographic data of the study. ED: edentulous bone sample; Peri-I: peri-implant bone sample.

Sample	Gender	Age	Extraction site	Edentulism time	Masticatory loading time
Peri-I1	M	71	#33	-	7 years
Peri-I2	M	77	#43	-	8 years
Peri-I3	M	69	#16	-	10 years
Peri-I4	F	78	#43	-	12 years
Peri-I5	F	81	#33	-	9 years
Peri-I6	M	43	#12	-	2 years
Peri-I7	F	71	#16	-	7 years
ED1	NS	NS	#46	5 years	-
ED2	F	53	#27	3 years	-
ED3	M	49	#46	5 years	-
ED4	F	66	#14	3 years	-
ED5	F	55	#17	7 years	-
ED6	M	31	#16	1 year	-
ED7	F	80	#36	+ 10 years	-
ED8	F	50	#47	2 years	-
ED9	F	54	#46	1 year	-
ED10	F	75	#26	6 years	-
ED11	F	75	#27	6 years	-
ED12	F	82	#44	5 years	-

Clinical Procedure

All patients screened and enrolled in the study were treated by the same clinician following standardized clinical procedures. All the patients received 2 gr. of amoxicillin (or 600 mg. of clindamycin for those who were allergic to penicillins) as a pre-medication one-hour before surgery. All patients rinsed for 1 minute with 0.2% chlorhexidine mouthwash (and twice a day for the following 3 weeks) and received local anesthesia.

Patients who had dental implants deemed hopeless received a mid-crestal incision with mesial and distal releasing incisions (when considered necessary) to raise a full thickness flap and to access the implant platform. The prosthetic rehabilitations -if present- were removed and bone-implant biopsies were harvested using a trephine bur under copious irrigation with sterile saline solution. The implants were removed selecting the most appropriate trephine bur according to the minimal diameter required. The harvested

specimens were immersed immediately in 10% neutral buffered formalin and sent to the laboratory the histologic and micro-CT analysis.

Patients who were planned to receive an implant supported rehabilitation received a mid-crestal incision to raise a full thickness flap and to access the edentulous ridge, while preparing the osteotomy implant site a bone biopsy was harvested using a trephine bur with an external diameter of 2.5 mm and internal diameter of 1.5 mm. Once the bone core sample was collected and stored in 10% neutral buffered formalin, the implant site preparation was undertaken according to the implant manufacturer.

Synchrotron Radiation-Based Phase-Contrast Microtomography

The microtomographic imaging was conducted at the SYRMEP imaging beamline of the Elettra Synchrotron Facility in Basovizza (Trieste, Italy) [63]. The experiment was set with the following parameters: the imaging system captured a total of 1800 projections over 180° sample rotation, with exposure time per projection of 0.6 s; the white beam was filtered with 1 mm thick silicon (Si) plate leading to a peak energy of 19.3 keV; sample-to-detector distance was set to 100 mm in order to enable phase contrast imaging. The raw images were collected by using an Orca Flash 4.0 CMOS camera (2048 × 2048 pixels, physical pixel size 6.5 μm × 6.5 μm) coupled with a 17 μm GGG scintillator screen. Thanks to the optical magnification system used in combination with the detector, the effective pixel size selected for imaging was 0.9 μm x 0.9 μm, yielding to a field of view of 1.8 mm x 1.8 mm.

After the acquisition, each sample was reconstructed using the STP software (SYRMEP Tomo Project) [64]. The parameters selected for the slice's reconstruction were Rivers ring removal (wd=3) for ring removal correction, Paganin TIE-Hom algorithm [65] for the phase retrieval step and Filtered Back Projection algorithm, in combination with Shepp-Logan filter, for the ultimate reconstruction step. In detail, phase retrieval was performed by applying Paganin's algorithm with a fixed δ/β ratio of 100. Specifically, in the X-ray regime the refractive index of a material is a complex number ($n = 1 - \delta + i\beta$) where δ represents the phase shift, whereas β is directly proportional to the absorption index. For this application, the δ/β ratio was empirically chosen in order to better visualize distinct sample phases such as collagen, vessels, etc. Finally, each reconstructed stack

comprising 2048 images was converted in 8-bit images, with the whole 32-bit images remapped using the selected real range [0.0001÷0.008].

Image Analysis with AI Segmentation Techniques

The image processing involved data elaboration using the Dragonfly software [66] (Dragonfly 2022.2, Comet Technologies Canada Inc., Montreal, Canada). We exploited a deep learning tool of Dragonfly, which applies artificial intelligence to the segmentation process. Furthermore, the Scalar Generator tool was applied to precisely extract quantitative information on the specific region of interest (ROI) previously segmented by the neural network. This process involved the computation of the morphometric and shape complexity parameters described in the next paragraphs.

A sub-volume of 1 mm³ was virtually extracted from each sample; hence, the use of a specific neural network enabled the precise detection of the osteocyte lacunae in an automatic way and short time [67]. It was achieved using a Pre-Trained U-Net, one of the AI tools of the Dragonfly software. Table 4.1.2 displays the parameters selected during the training phase.

Table 4.1.2. Selected training parameters to maximize the CNN performance.

U-Net Training Parameters	
Class count	3
Depth level	5
Initial filter count	64
Patch size	32
Stride ratio	0.5
Batch size	32
Epochs number	40
Loss function	Categorical Cross-Entropy
Optimization algorithm	Adadelta
Metrics	Categorical Accuracy
Early stopping	NO
Reduce learning rate on plateau	YES
Training/Validation	90/10

Class count refers to the number of components that the network must discriminate; the *depth level* is the number of layers that capture contextual information in the two phases

of encoding and decoding; the *initial filter count* represents the filters count at the first convolutional layer; the *patch size* is the number of sub-sections in which the dataset will be cut; the *stride ratio* specifies the overlap between adjacent patches; the *batch size* determines the number of patches in a batch; the *epoch's number* indicates the number of iterations; the *loss function* measures the error between the neural network's prediction and reality; the *optimization algorithm* is used to update the parameters of the model, to minimize the prediction errors; the *metric function* is used to judge the model's performance; the *early stopping* is used to stop iterations once the estimation error drops below a certain threshold; the *learning rate* is a parameter which controls the step with which we arrive at the final network optimization result. Generally, this last parameter reduces as one gets closer to optimal performance; we chose to have it adapted during training and not to keep it fixed. The percentage division of the dataset is 90% for training and 10% for testing.

The network performance was evaluated by applying the training to each sample and visually verifying that it accurately segmented each osteocyte lacuna. Specifically, once the sub-volume of 1 mm³ was selected, the neural network was applied to it, distinguishing the osteocyte lacunae from both the background and the bone matrix. The region of interest (ROI) of the lacunae (representative case in Fig. 4.1.1A) was selected and the Scalar Generator tool was applied; it considered each lacuna individually (Fig. 4.1.1B); afterwards, morphometric parameters were measured in each osteocyte lacuna.

Morphometric and Shape Complexity Analysis

According to literature [60,61], only lacunae within the volumetric range of [80-980] μm³ can be considered functional. Indeed, to verify the accuracy of this selection, the distributions of the lacunae volumes were plotted for each sample. It was confirmed that the greatest concentration was precisely in this range (data not shown). Consequently, morphometric and shape complexity parameters were only computed for lacunae with these specific dimensions.

The following morphometric and shape complexity parameters were evaluated: the mean lacuna volume (LacV - μm³); the 3D aspect ratio (LacAR), which returns the ratio between the lacuna longest and shortest axes; the Sphericity (LacS), which quantifies the extent to which the lacuna resembles a sphere by calculating the ratio of the surface area

of an equal-volume sphere to the actual surface area of the lacuna; the specific number of lacunae (LacNr - mm^{-3}), which refers to the number of lacunae within a 1 mm^3 volume of bone matrix.

Bone Mineralization Analysis

Starting from the reconstructed complex refractive index n , that is linearly related to the mass density (mg/cm^3), the apparent bone mineralization distribution (BMD^r) of the ROIs was calculated in each sample, following the Roschger approach [68]. The BMD^r parameters were calculated within the mineralized domain; since the reconstructed complex refractive index might be biased due to the constant ratio δ/β and the use of a white beam, absolute values of bone mass density (calcium concentrations – Ca weight %) could not be retrieved, hereinafter the superscript r denoting relative values for all the parameters. Indeed, the Paganin phase retrieval algorithm [65], which is based on the assumption of a monochromatic beam, works as an approximation in our analysis. For this reason, the analysis was performed with reference to the distribution of pixel frequencies in the 0-255 range of 8-bit. The average opacity values were then compared relatively between the different samples, leading the opacity distribution to more or less mineralized bone tissue levels. Moreover, since ROIs in the different samples were similar in terms of size and composition, relative difference in bone mineralization distribution between them were appreciated.

Three bone mineralization parameters were extracted using the PeakFit software (Systat Software, San Jose, CA): the mean relative mass density (BMD^r - mean), the full width at half maxima of the distribution (BMD^r - fwhm), and the most frequent relative mass density value (BMD^r - peak).

Spearman Rank Analysis

Correlation analysis between osteocyte lacunae morphometric and shape complexity data, mass density distribution and clinical data (including gender, age, dental location, and edentulism time or masticatory loading time for peri-implant bone) was performed using the Spearman rank analysis. The correlations were classified “very weak” ($\rho = 0 \div 0.20$), “weak” ($\rho = 0.20 \div 0.40$), “moderate” ($\rho = 0.40 \div 0.60$), “strong” ($\rho = 0.60 \div 0.80$) and

“very strong” ($p = 0.80 \div 1.0$). “Strong” and “very strong” correlations with $p < 0.05$ were considered statistically significant.

Histological Analysis

The same retrieved samples (i.e. Peri-I and ED groups) were also investigated by optical microscopy.

They were fixed in a 10% formalin solution and processed to obtain thin, polished sections using a Precise 1 Automated System (Assing, Rome, Italy). Specifically, the samples were dehydrated through a series of alcohol rinses with increasing concentrations and embedded in a specialized glycol methacrylate resin (Technovit 7200, VLC, Kulzer, Wehrheim, Germany). After polymerization the samples were sliced using a high-precision diamond disc to a thickness of approximately 150 μm and further polished down to about 30 μm . Subsequently, they were stained with acid fuchsin and toluidine blue and observed at optical microscope (Laborlux S, Leitz, Wetzlar, Germany).

Histomorphometry was employed to determine the percentages of mature bone, newly formed bone tissue, total bone, marrow spaces, and bone to implant contact (BIC). In addition, at higher magnification, the surfaces occupied by osteocyte lacunae were measured in single regions of interest (0.03 mm² wide) selected randomly in bone biopsies and close to the interface with dental implants. These analyses were conducted using a standard light microscope connected to a high-quality video camera (3CCD, JVC KY-F55B, JVC, Yokohama, Japan). The camera system was interfaced with a monitor and a computer (Intel Pentium III 1200 MMX, Intel, Santa Clara, CA, USA). This entire optical setup was further enhanced with a specialized digitizing pad (Matrix Vision GmbH, Oppenweiler, Germany) and histometric software equipped with image capturing capabilities (Image-Pro Plus, Media Cybernetics Inc., Immagini e Computer Snc, Milano, Italy).

Polarized light Imaging

Birefringence was exploited as an indicator of transverse-to-light collagen orientation using polarized light. Collagen fibers were imaged by placing the thin sections of bone under an Axiolab light microscope (Laborlux S, Leitz, Wetzlar, Germany) equipped with

two linear polarizers and two quarter wave plates arranged to have a transmitted circularly polarized light. Collagen fibers aligned perfectly transverse to the direction of the light propagation (parallel to the plane of the section) appeared bright due to a change in the refraction of existing light, whereas the collagen fibers aligned along the axis of light propagation (perpendicular to the plane of the section) appeared with different colors.

Statistical Analysis

The statistical analysis of PhC-microCT data was performed with the software package Prism 6.0 (GraphPad Software, San Diego, CA). All data were presented as mean $\pm$ SD. Statistical significance among Peri-I and ED groups was evaluated using unpaired t-test because data distributions were verified for normality, by Kolmogorov–Smirnov and Shapiro–Wilk tests, and for homogeneity of variances, by Levene test.

Correlation analysis between microarchitectural indices, BMD^r parameters, and clinicopathological data were explored by Spearman rank correlation analysis. A P-value of <0.05 was accepted as statistically significant for all tests. *P < 0.05 ; **P < 0.01 ; ***P < 0.001 ; ****P < 0.0001 .

Results

Neural Network Segmentation

The neural network detected and classified each lacuna using the knowledge acquired during the training phase (learning process). In a subsequent step, to specifically select only the lacunae in the size range of $[80-980] \mu\text{m}^3$ (functional osteocyte lacunae), all the lacunae detected by the algorithm that fell outside this range were excluded from the quantitative analysis. Figure 4.1.1A depicts the neural network's identification of the osteocyte lacunae and their further segmentation as a single region of interest (ROI). In Figure 4.1.1B, each osteocyte lacuna is assigned a distinct color, forming a multi-ROI. This is essential for applying the subsequent statistical analysis method of the Scalar Generator, which allows for the quantitative measurement of volume, aspect ratio, and sphericity for each individual element (Figure 4.1.2).

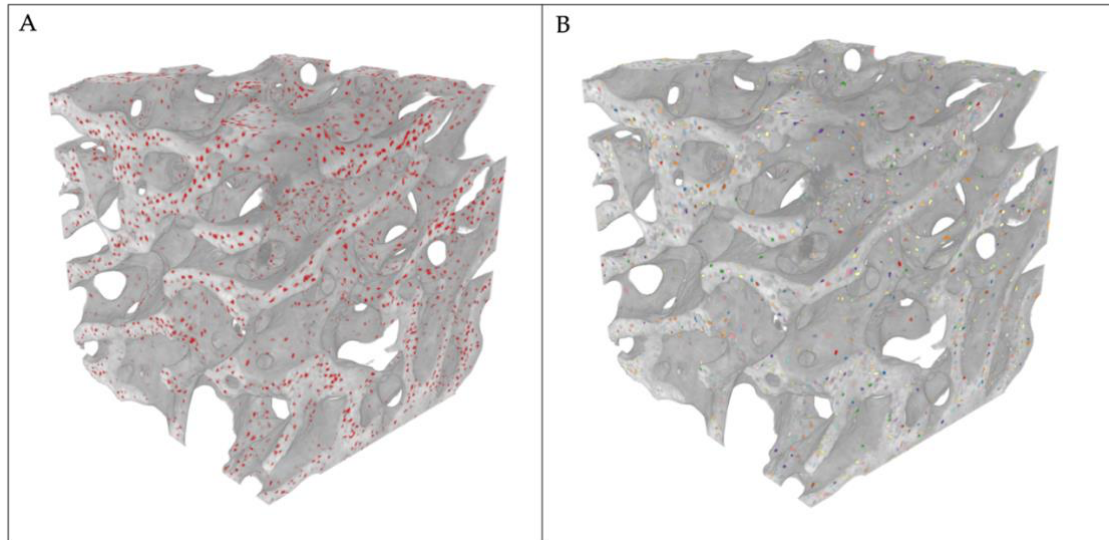


Figure 4.1.1. Box of 1 mm³ within a representative sample (ED2). **(A)** Red ROI included all the osteocyte lacunae segmented by the neural net. **(B)** Multi-ROI selected by the Scalar Generator algorithm and analyzed individually: each osteocyte lacuna was assigned a distinct color.

Morphological and shape complexity lacunar parameters

Figure 4.1.2 displays the quantitative microarchitectural parameters that were evaluated by the synchrotron analysis: namely LacVol, LacAR and LacS. These values were extracted using the Dragonfly tool's Scalar Generator. The density of the lacunae (LacNr) was estimated by dividing the total amount of lacunae by the total bone matrix volume for each sample.

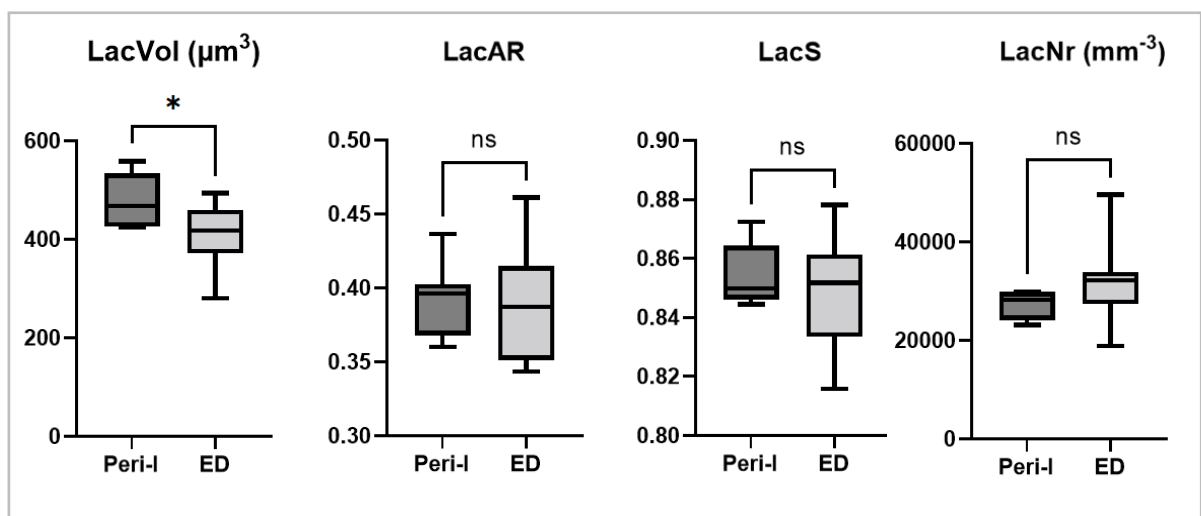


Figure 4.1.2. Box plots of the osteocyte lacunae microarchitectural parameters: mean lacunar volume (LacVol – expressed in μm^3), the lacunar aspect ratio (LacAR), the lacunar sphericity (LacS) and the specific number of lacunae (SpNr - mm^{-3}). The upper and lower ends of boxes represent 75th and 25th percentiles. The median value is shown with a solid line. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Number of investigated samples: 19, one for each biopsy.

The quantitative analysis of the selected morphometric and shape complexity parameters revealed that only the mean lacunar volume showed significant mismatches ($p < 0.05$) between the two experimental groups, with larger values for the Peri-I group – mainly in the range $[400-600] \mu\text{m}^3$ – than for the ED group. The ED group also revealed a greater variability for all the investigated parameters. Figure 4.1.3 shows, in two representative samples - one for each experimental group (edentulous and peri-implant bone volumes)-, the difference in the size of osteocyte lacunae. The osteocyte lacunar volumes are primarily clustered within the range from 300 to 620 μm^3 for the representative sample of the edentulous group (Fig. 4.1.3A), with a noticeable lack of uniformity among the different parts of the sample being examined. For the representative sample of the peri-implant bone group (Fig. 4.1.3B), most of the lacunae have a size ranging from 540 to 700 μm^3 , uniformly distributed among the different areas of the sample.

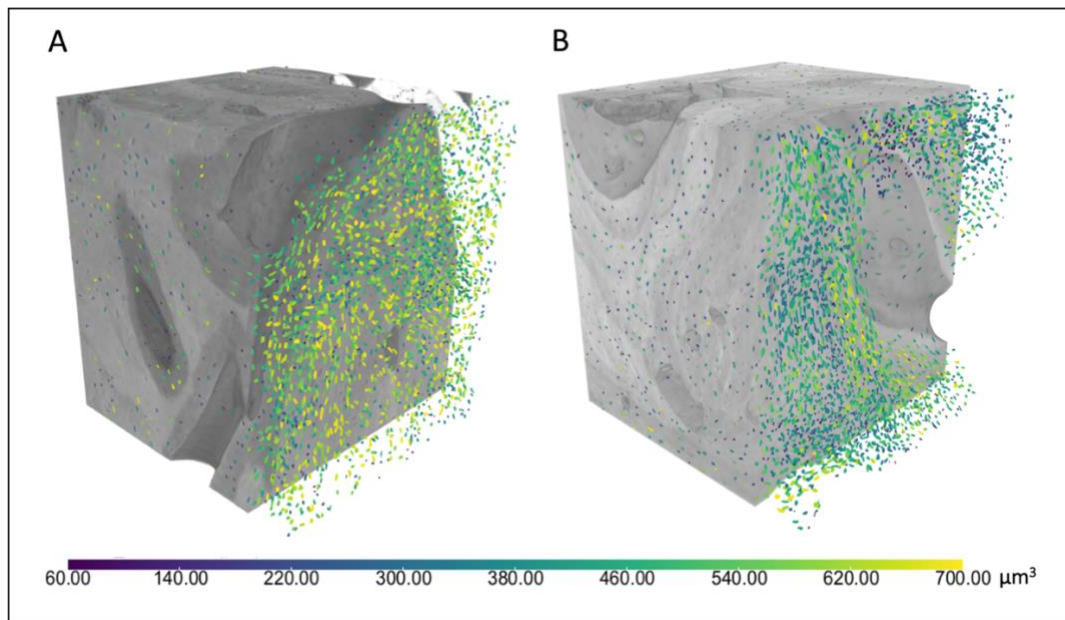


Figure 4.1.3. Osteocyte Lacunar Volume distribution analysis in representative peri-implant bone (Peri-I1) (A) and edentulous bone (ED3) (B) samples. Larger lacune were detected in the Peri-I group than in the ED group. Bottom: volumetric color scale bar in the range of 60-700 μm^3 .

Bone Mineralization Distribution Analysis

BMD^r profiles of Peri-I samples and of the ED controls are shown in Figure 4. Significantly higher ($p < 0.0001$) BMD^r_{mean} and BMD^r_{peak} values were obtained in the Peri-I group when compared to the ED group. Furthermore, although the mean BMD^r_{fwhm} values did not differ significantly between one group and another, unlike the peri-implant sites, a non-homogeneous BMD^r_{fwhm} was found among the edentulous biopsies.

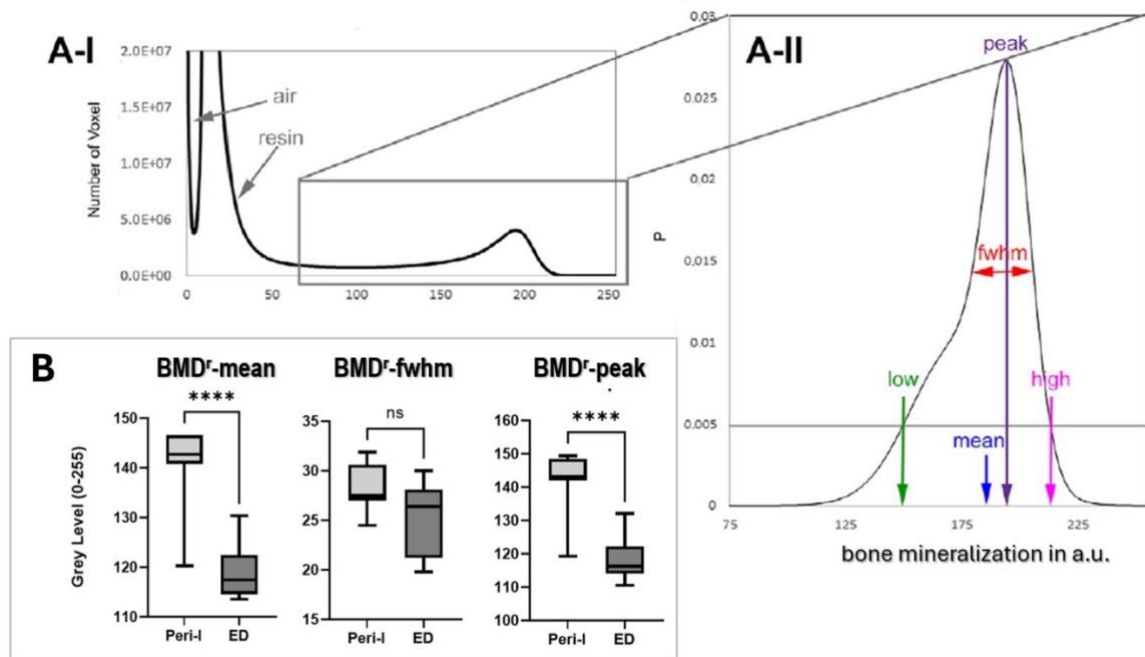


Figure 4.1.4. (A) Relative Bone Mineralization Distribution (BMD^r). (A-I) The peaks indicated by the arrows are referred to the air outside the sample and to the resin used to include the biopsy. (A-II) The parameters derived from the segmentation are indicated. (B) Box-and-whisker diagrams of the distribution of PhC-microCT based BMD^r parameters: the mean relative mineralization (BMD^r_{mean}), the full width at half maxima of the distribution (BMD^r_{fwhm}), and the most frequent relative mineralization value (BMD^r_{peak}). Upper and lower ends of boxes represent 75th and 25th percentiles. The median value is shown with a solid line. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Number of investigated samples: 19, one for each biopsy.

Spearman Correlation Analysis

The correlation analysis (Figure 4.1.5) among lacunal microstructural indices, bone mineralization distribution parameters, and clinical data in the Peri-I and EC groups

suggests the following general observations: (1) there are “strong” and “very strong” (direct/indirect) correlations between the studied parameters only with reference to the Peri-I group; (2) limited to the parameters studied, the correlation of the Region parameter with the other parameters is not relevant (“strong” or “very strong”), neither with reference to the Peri-I group nor with reference to the EC group; (3) while the loading time (Load_Y) presents a correlation with the other parameters that is not relevant in the Peri-I group, for the EC group it was found a “strong” direct correlation of the edentulism time (ED_Y) with patient age.

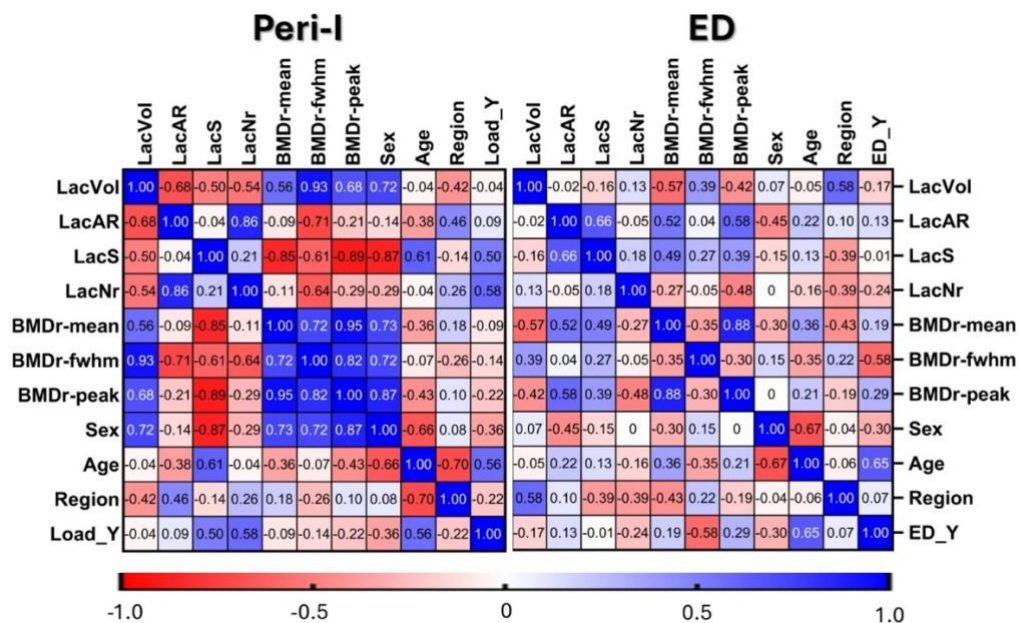


Figure 4.1.5. Spearman coefficient analysis of microarchitectural, bone mineralization distribution and clinical data in peri-implant (**Peri-I**) and edentulous (**ED**) groups. The correlations were classified “very weak” ($\rho = 0 \div 0.20$), “weak” ($\rho = 0.20 \div 0.40$), “moderate” ($\rho = 0.40 \div 0.60$), “strong” ($\rho = 0.60 \div 0.80$) and “very strong” ($\rho = 0.80 \div 1.0$). Bottom: color map of the correlation levels: “strong” and “very strong” direct (color map: bright blue) and inverse (color map: bright red) correlations, with $p < 0.05$, were considered statistically significant. Sex: 1 = man, 2 = woman; region: D1=1, D2=2, D3=3, D4=4; Load_Y= years of implant loading; ED_Y = years of edentulism.

To the best of our knowledge, the Spearman correlation analysis also suggests that in the Peri-I group:

- (a) The LacVol correlates to the LacAR (inverse: $\rho = -0.68$), $BMD^{r_{fwhm}}$ (direct: $\rho = +0.93$), $BMD^{r_{peak}}$ (direct: $\rho = +0.68$), and sex (direct: $\rho = +0.72$, i.e. with larger lacunar volume in male than female patients);
- (b) The LacAR correlates to the LacNr (direct: $\rho = +0.86$), and $BMD^{r_{fwhm}}$ (inverse: $\rho = -0.71$);
- (c) The LacS correlates to the $BMD^{r_{mean}}$ (inverse: $\rho = -0.85$), $BMD^{r_{fwhm}}$ (inverse: $\rho = -0.61$), $BMD^{r_{peak}}$ (inverse: $\rho = -0.89$), sex (inverse: $\rho = -0.87$, i.e. with lacunae more resembling spheres in female than male patients), and age (direct: $\rho = +0.61$, i.e. with lacunae more resembling spheres in older than younger patients);
- (d) The LacNr correlates to the $BMD^{r_{fwhm}}$ (inverse: $\rho = -0.64$),
- (e) The $BMD^{r_{mean}}$ correlates to the $BMD^{r_{fwhm}}$ (direct: $\rho = +0.72$), $BMD^{r_{peak}}$ (direct: $\rho = +0.95$), and sex (direct: $\rho = +0.73$, i.e. with higher mineralization values in male than female patients);
- (f) The $BMD^{r_{fwhm}}$ correlates to the $BMD^{r_{peak}}$ (direct: $\rho = +0.82$), and sex (direct: $\rho = +0.72$, i.e. with larger mineralization in male than female patients);
- (g) The $BMD^{r_{peak}}$ correlates to the sex (direct: $\rho = +0.87$, i.e. with higher peak mineralization in male than female patients);
- (h) Finally, the sex inversely correlates to age ($\rho = -0.66$, i.e. with female patients undergoing implants at a younger age than male patients).

Histological evaluation

- Peri-Implant (Peri-I) Bone – Loaded Condition

Seven peri-implant bone samples were investigated, harvested from human retrieved implants, after in vivo loading periods ranging from 2 to 10 years.

Overall, the samples have shown different structural organization of the peri-implant bone and varying degrees of bone to implant contact (BIC). Histological observations showed a continuous bone remodeling and well-organized lamellar bone around the dental implant surface. Close and tight contact between bone and implant surface was observed in all specimens, and no gaps or connective, fibrous tissue was found at the

bone-implant interface (Figure 4.1.6a-d). The osteocyte lacunae retained their ellipsoid shape, with large and contained osteocyte within them (Figure 4.1.6e).

Two years of loading: in detail, after this loading period, bone formation was observed in proximity to the implant surface. The bone showed numerous areas of remodeling and the portion in direct contact with the implant surface appeared to be more recently formed than the more distant areas. The implant exhibited signs of resorption in the coronal portion, with initial bone-implant contact observed at the level of the second thread. The bone edge displayed irregular margins, a characteristic feature of bone resorption, resembling Howship's lacunae even if multinucleated osteoclast-like giant cells were absent. Lymphocytes and small blood vessels were observed near this area. Additionally, numerous large osteocyte lacunae, typical of new bone, were found near the implant surface in many areas. (Figure 4.1.6f).

Seven years of loading: mature bone with osteons were present inside some threads, near the interface (Figure 4.1.6g). No signs of inflammation or resorption were detected in either the apical or coronal portions. Presence of remodeled bone were observed near the implant surface (Figure 4.1.6h). Furthermore, osteoblasts actively depositing osteoid matrix were noted near the implant surface, indicating continued bone formation even after prolonged loading (Figure 4.1.6i).

Ten years of loading: lamellar bone was present near and far from the implant.

Among the samples examined, two implants exhibited initial contact at the level of the first thread, while one displayed slight coronal resorption. Circularly polarized light analysis of the peri-implant bone of loaded implants shows the presence of transverse collagen fibers under the lower flank of the implant threads. In contrast, at the apex of the implant threads, the fibers were parallel to the long axis of the implant (Figure 4.1.7a-b). In addition, lamellar bone showed collagen fibers oriented parallel to each other (Figure 4.1.7c-d)

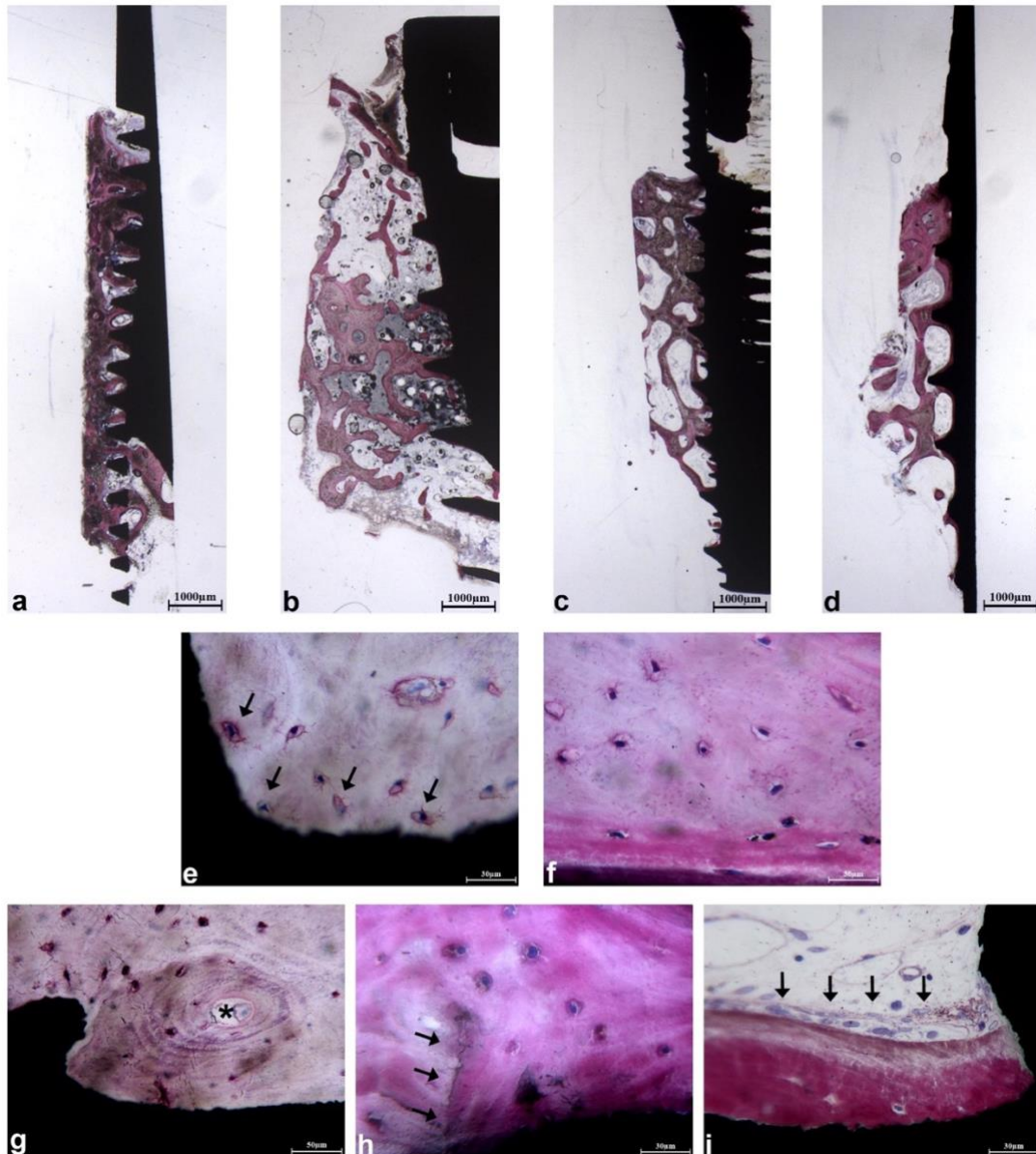


Figure 4.1.6. Histology images of the peri-implant bone. (a) Peri-I6. Peri-implant bone after 2 years. Compact bone with marrow spaces was observed in contact with implant surface. (b) Peri-I7. Peri-implant bone after 7 years. Trabecular bone with large marrow spaces, typical of maxillary bone, was detected both at coronal and apical portions. (c) Peri-I2. Peri-implant bone after 7 years. Close contact between bone and implant was observed both at coronal and apical portions, with a thin layer of new bone surrounding the implant surface. (d) PeriI5. Peri-implant after 10 years. Lamellar bone was evident near and away from the implant. (e-f) Peri-I3, Peri-I6. The large osteocyte lacunae containing osteocytes were in close contact with the implant surface (black arrows) (g) Peri-I3. Presence of mature bone with osteon were detected (*) (h) Peri-I7. Presence of remodeled bone with reversal lines (black arrows) were noted near the implant surface (i) Peri-I3. Osteoblasts (black arrows) engaged in the deposition of osteoid matrix were detected in close contact with the implant surface. Magnification 6x (a-d), 200x (g) 400x (e-f, h-i).

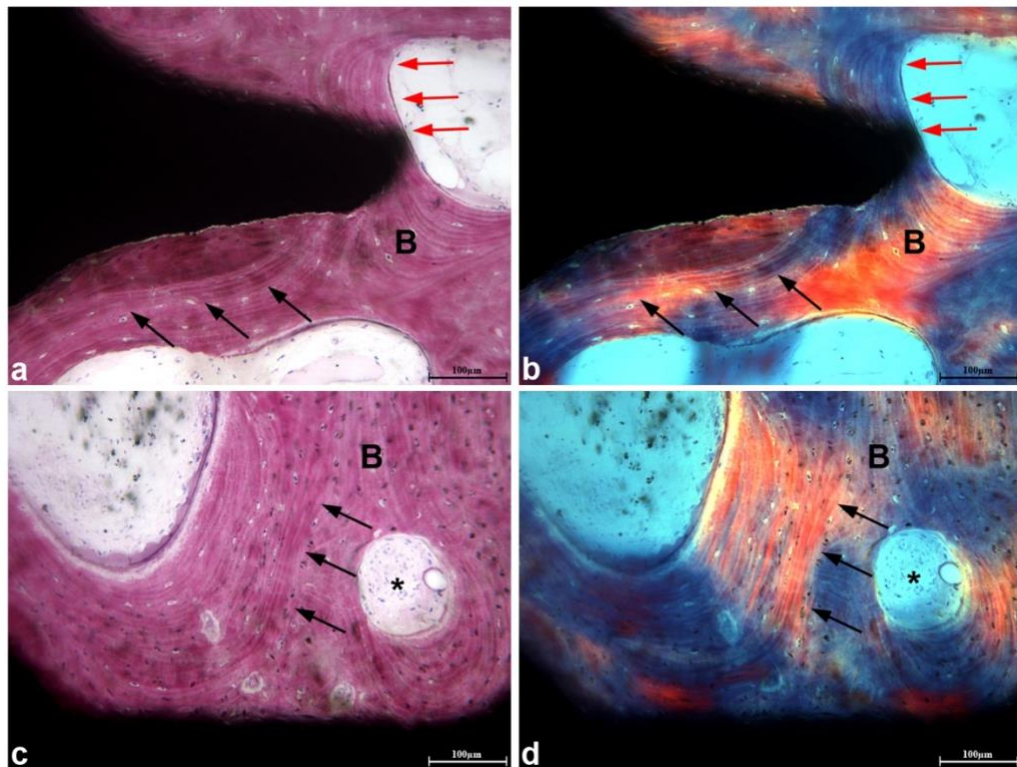


Figure 4.1.7. (a) Peri.I4. Acid fuchsin-Toluidine blue staining. Mature bone was observed in close contact with the implant thread. (b) Peri.I4. Polarized light. Under the lower flank of the implant threads, collagen fibers were arranged transversely (red arrows). In contrast, at the apex of the implant threads, the fibers were parallel to the longitudinal axis of the implant (black arrows). (c) Peri.I4. Acid fuchsin-Toluidine blue staining. Mature bone (**B**) was noted within the implant thread. (d) Peri.I4. Polarized light. Lamellar bone showed collagen fibers oriented parallel to each other (black arrows). An osteon was detected within the thread (*) (Magnification 100x).

- **Edentulous Bone (ED) – Unloaded condition**

Twelve edentulous bone samples were collected (#6 in the mandible and #6 in the maxilla). In general, histological analysis revealed that with increasing edentulous time, i.e. the absence of masticatory load, a mosaic structure of the bone was observed. It was characterized by well-defined regions of lamellar bone alternating with few areas that had lost their lamellar structure, with the osteocyte lacunae appearing empty.

Analyzing the samples in relation to the period of edentulism, the following histological findings emerged:

One year of edentulism: the trabeculae were composed of an internal portion of mature bone surrounded by newly formed bone in which large lacunae containing osteocytes were present. Furthermore, small and large blood vessels within the marrow spaces were evident (images not shown).

Two years of edentulism: trabecular architecture was preserved, with trabeculae evenly distributed and interconnected. Bone remodeling was present in areas where lamellar bone predominated, showing primary and secondary osteons (images not shown).

Three years of edentulism: trabecular bone with large marrow spaces and areas of bone remodeling was observed. In some areas, the mature bone showed a lower staining affinity for acid fuchsin and appeared more disorganized with smaller and sometimes empty lacunae (Figure 4.1.8a). However, in areas where remodeling was present the lacunae contained osteocytes. Small and large blood vessels were observed in the marrow spaces. In addition, some areas showed signs of bone resorption (irregular margins), indicative of Howship lacunae (Figure 4.1.8b). The specimens showed a mosaic structure characterized by well-defined regions of lamellar bone alternating with areas that had lost their lamellar structure (Figure 4.1.8c-d).

Five to seven years of edentulism: mature bone was observed with areas of remodeling that were not always well demarcated by reversal lines (Figure 4.1.8e-f). Osteocyte lacunae were observed and were irregular in shape and distribution (Figure 4.1.8g). No areas with osteoblastic cells or osteoid matrix were detected (Figure 4.1.8h).

Ten years of edentulism: The specimens exhibited characteristics very similar to those observed in the 7-year edentulous group, however reduced bone remodeling was evident (Figure 4.1.8i).

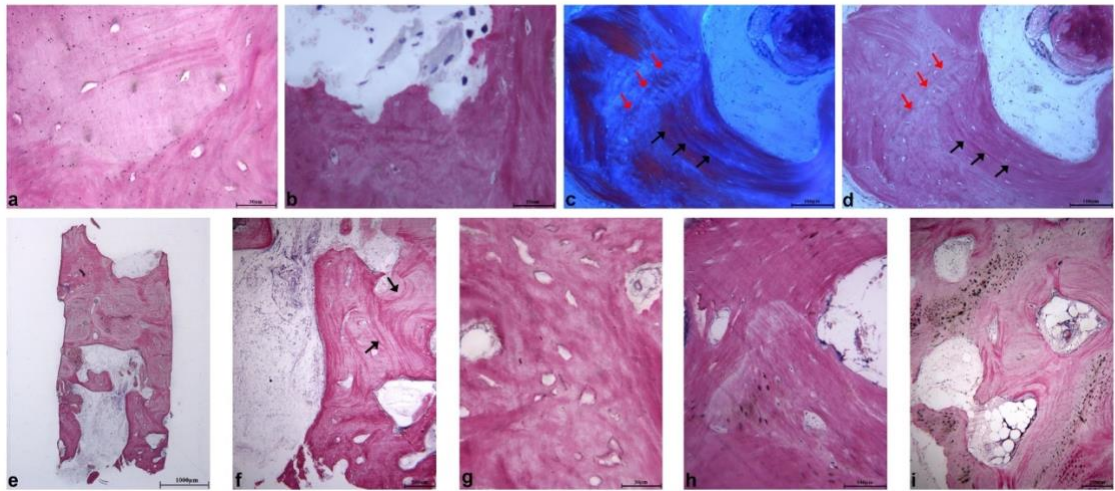


Figure 4.1.8. Histology images of the edentulous bone. (a) ED4. Edentulous bone after 3 years. Presence of empty and small osteocytes lacunae was detected. (b) ED2. Edentulous bone after 3 years. Presence of bone reabsorption (irregular margins) indicative of How ship lacunae was noted. (c-d) ED4. Area characterized of mosaic structure, that presented well-defined regions of lamellar bone (black arrows) alternated with areas that lost their lamellar structure (red arrows), were observed with polarized light (c) and Acid fuchsin-Toluidine blue (d). (e) ED3. Edentulous bone after 5 years. Compact bone with large marrow spaces was noted. (f) ED3. Presence of mature bone with reversal lines (black arrows) was detected. (g) ED3. Osteocyte lacunae appeared irregular in shape and distribution. (h) ED5. Edentulous bone after 7 years. No areas with osteoblastic cells or osteoid matrix were detected. (i) ED7. Edentulous bone more than 10 years. Presence of trabecular architecture with lamellar bone was observed. Magnification: 12x(e), 40x(f,i), 100x(c-d,h), 200x(b), 400x(a,g).

Histomorphometric analysis

The same Peri-I samples analyzed by synchrotron light were evaluated by histomorphometry for BIC percentages analysis; results are reported in Table 4.1.3.

Table 4.1.3. BIC evaluation performed in Peri-I group samples. Number of investigated samples: 7, one for each biopsy. Data are expressed in percentages as Mean (SD).

BIC in mandible (%)	BIC in maxilla (%)	Total BIC
76.11(20.75) %	49.89(17.70) %	64.87(22.72) %

The BIC for the four mandibular Peri-I samples was found to be higher compared to the three maxillary Peri-I samples. However, due to the high SD, mismatches were not statistically significant ($p > 0.05$).

Histomorphometric analysis was also conducted on both groups to evaluate the percentages of mature bone, new bone, total bone, and marrow spaces (Table 4.1.4).

Table 4.1.4. Histomorphometric analysis (Area %) of the ED and Peri-I groups. Number of investigated samples: 12 for ED and 7 for Peri-I, one for each biopsy. Data are expressed in percentages as Mean (SD).

Group	Mature bone	New Bone	Total Bone	Marrow spaces
Peri-I group	23.69 (13.10) %	20.89 (4.97) %	44.57 (11.72) %	55.43 (11.72) %
ED group	37.47 (25.21) %	13.44 (11.76) %	50.91(15.37) %	49.09(15.37) %

Additionally, the section area (μm^2) of the osteocyte lacunae was evaluated in both the Peri-I and the ED groups (Table 5).

Table 4.1.5. Section area of the osteocyte lacunae in the Peri-I and the ED groups. Number of investigated samples: 19, one for each biopsy. Data are expressed in μm^2 as Mean (SD).

Group	Area (SD) [μm^2]
Peri-I group	111.31 (54.64)
ED group	37.94 (10.98)

In agreement with PhC-microCT 3D data and histological images evidence, the mean section area (μm^2) of the osteocyte lacunae in the Peri-I samples was found to be significantly wider ($p=0.0003$) than in the ED group.

Discussion

An effective dental implant process necessarily requires the use of biomaterials, methods and procedural timing that ensure the quality and quantity of the peri-implant bone is as similar as possible to that of the functional periodontal bone. One of the patient-related prognostic factors is linked to the bone mineralization distribution (BMD) of the jaw. BMD has been shown to be influenced by physical muscle activity on skeletal bone [69]

which may suggest that dentate patients should have more mineralized jawbone than edentulous patients due to their greater bite force capacity. However, the effect of masseter activity on the alveolar bone is still under study and, to date, the research results are very controversial, with some literature supporting the presence of jawbone BMD mismatches between dentulous and edentulous patients [70–75]. These conflicting data are presumably due to the heterogeneity of the human sample (gender, age, clinical conditions, region, etc.) as well as to the different experimental methods used and they're not comparable resolution levels for the investigation of the bone structures.

Indeed, the remodeling process favored by the mechanical loading is a challenging case of study because it was shown to produce changes not only in the bone microarchitecture but also in the BMD - i.e. in terms of calcium concentrations- and in the osteocyte lacunar network settings. Synchrotron radiation-based X-ray microtomography (micro-CT) has already proven to be an efficient technique, capable to achieve the analysis of the 3D bone microarchitecture and of local mineralization at different hierarchical length scales, including the imaging of the lacuno-canalicular network [76–78].

In this context, it was set an interesting and contributing study referred to comparisons between peri-implant and edentulous bone tissues, to understand whether the masticatory load (i.e. the mechanical stimulus) can in any way influence the microarchitecture and the BMD of the jawbone.

In the present study - by employing AI-based segmentation technique to synchrotron PhC-microCT 3D reconstructions of human peri-implant and edentulous bone tissues – we rapidly and accurately detected each individual lacuna in every human biopsy. Compared to conventional segmentation techniques, the neural network achieved the identification of structures with mismatches in both photonic signal contrast and pure morphology, allowing a discrimination between different tissues and between these and the background.

We have shown by synchrotron imaging that the osteocyte lacunar volume was significantly bigger in the Peri-I group than in the ED group; this was confirmed also by histomorphometry evidence, showing that the mean section area of the osteocyte lacunae in the Peri-I biopsies was found to be significantly wider than in the ED group. Indeed, the smaller lacunae of the ED group appeared devoid of osteocyte; therefore, they could be considered dysfunctional. The absence of osteocyte, key regulators of bone remodeling, can lead to an imbalance in bone resorption and formation, contributing to the accelerated bone loss often seen in edentulous patients. Furthermore, osteocytes play

a vital role in maintaining bone strength and integrity by responding to mechanical loads, therefore without these cells, the bone tissue may become more susceptible to fractures or micro-damage [55]. The correlation between the size of lacunae and the functionality of bone tissue is significant, as larger lacunae appear to enhance the mechano-response of osteocytes. Literature indicates that osteocytes in enlarged lacunae, such as those found in lactating mice, exhibit a stronger response to mechanical loading compared to those in smaller lacunae, possibly because of an increased expression of β -catenin and decreased sclerostin in response to loading [79].

Furthermore, our study also revealed a wide distribution of the Aspect Ratio and Sphericity values across edentulous samples, while this variation was not observed among peri-implant bone samples. This evidence could suggest that loading affects the peri-implant osteocyte lacunae, making them comparable in shape and size. These lacunae should fall within a specific range to be considered functional, meaning they should not be excessively thin (sagging) or too spherical. Indeed, several investigations [57,58] have observed that the osteocytes lacunae align with the primary load direction and extends across it acquiring an ellipsoid shape, while yet preserving its dimensional integrity and avoiding excessive sagging, i.e. excessive variations of the Aspect Ratio and Sphericity values.

The microarchitectural analysis of the osteocyte lacunae network also showed that the number of value of lacunae per unit volume was similar between the two groups, even if the edentulous group revealed a wider variability between samples.

Overall, the edentulous group showed a wider variability of the studied lacunar microarchitectural parameters with respect to the peri-implant group, confirming once again the cause-and-effect relationship between the masticatory mechanical loading and the lacunar morphology guided by mechano-transduction processes. This result is corroborated by histology observations that showed – in the edentulous group - the presence of mosaic structure, characterized by regions with lamellar bone alternated with areas that lost this structure. This aspect was less evident in the cortical region, while the reabsorption process was observed in deeper portions. The presence of both lamellar and non-lamellar bone suggests a dynamic process of bone remodeling [62]; however, the areas without the typical lamellar structure indicate bone resorption. These histological findings support the well-established fact that edentulism leads to significant bone loss. Referring to Bone Mineralization Distribution analysis, significantly higher ($p < 0.0001$) values were obtained in the peri-implant group than in the edentulous one. These data

indicate that the loaded peri-implant sites have higher calcium percentages than the edentulous sites, that were unloaded for variable years. Indeed, cyclic mechanical loading – as jawing – was proved to stimulate bone cells, resulting in the mechano-adaptive osteogenic response of bone [80,81].

In general, the Spearman correlation analysis among lacunal microstructural indices, bone mineralization distribution parameters, and clinical data has brought extremely interesting results; in particular, a low correlation was found between the different parameters within the edentulous group, while several strong or very strong correlations were found in the peri-implant group. This finding is also in agreement with the wide variability found in several parameters of the edentulous group. This confirms that the most important functions of the osteocyte are orchestrated by the masticatory load, from the osteocyte lacunae network microarchitecture to the mineralization distribution of the extracellular matrix surrounding each cell.

Another extremely interesting data obtained from these analyses is the fact that the specific dental region (R1÷32) with its bone volume density (D1÷D4) does not significantly influence or is not influenced by any of the other investigated morphometric/mineralization parameters both in the Peri-I and in the ED group; this evidence seems to indicate that the different hierarchic levels of organization - the cortico-trabecular microarchitecture and the finer organization of the osteocyte lacunae network and of the surrounding mineralized extracellular matrix - do not correlate with each other. Interestingly, years of loading were not strongly correlated with either any lacunar morphometric parameter or with the mineralization indices of the surrounding matrix in the Peri-I group; this supports the idea that loading orchestrates the functional behavior of bone tissue from the beginning of the implant's service life during mastication, as recently suggested by Peñarrocha-Oltra et al. [82] who demonstrated that immediate loading for partially or totally edentulous jaws showed survival rates comparable to those obtained with conventional long-term loading protocols. This was confirmed by histological observations that showed a well-organized lamellar bone both near and far from the implant surface. This implies a mature and stable bone structure, suggesting successful osseointegration. Lamellar bone is characterized by its organized structure, which provides optimal mechanical properties. The lacunae retained their ellipsoidal shape, which was large and contained the osteocyte; in fact, the presence of osteocytes indicates a functional bone environment [83]. These histological findings strongly correlate with successful implant osseointegration and long-term stability. Moreover, the

formation of mature lamellar bone around the implant is a key indicator of osseointegration. In addition, in peri-implant bone an average BIC of 64.87% was observed, suggesting a reasonable level of osseointegration for the implants studied. The slightly higher (not significant) BIC values for mandibular implants compared to maxillary implants are consistent with previous studies [84–86], this could indicate that mandibular bone might offer a more favorable environment for implant osseointegration. Interestingly, it has been observed, for the edentulous group, a good inverse correlation ($\rho = -0.64$) of the years of edentulism with the BMD full-width-at-half-maximum parameter, indicating that prolonged absence of load modifies the mineralized bone matrix making it more uniform compared to what happens for mineralized bone tissues subjected to functional loading, where the composition of the mineralized bone is varied. Histologic observation supported this data: indeed, even if it showed similar percentages of total bone and marrow spaces respect to the peri-implant bone, it revealed a predominance of old bone compared to newly formed bone, suggesting that bone turnover and regeneration in these edentulous sites might be limited.

Within the Peri-I group, Spearman correlation analyses also indicated that, where the lacunae assume a more elongated ellipsoidal shape, the concentration of lacunae increases a well; this condition is expressly functional because of loading, as documented in the recent literature [87].

On the contrary, Spearman correlation analyses in the Peri-I group also tell us that a more spherical shape of the lacuna is instead correlated with a lower and more uniform level of matrix mineralization. Furthermore, this condition - less functional than the one previously described - is more present in female patients and in older age.

The results of this study - read collegially - indicate that it is important not to leave a dental site unloaded for a long time. Therefore, it is preferable to proceed with immediate implant loading or - in patients in whom this procedure is not feasible - during the edentulous period, stimulate the site with innovative devices that reproduce the effects of loading. Indeed, studies showed that longer periods of edentulism are associated with greater alveolar bone loss [88,89]. Moreover, it was observed that immediate implants, placed soon after tooth extraction, often have higher success rates compared to those placed after a prolonged period of edentulism [90,91].

4.2 Peri-Implant Connective Tissue: Deep Learning for Microstructural Characterization of Synchrotron Radiation-Based Collagen Bundle Imaging

Wound healing is an essential process for restoring tissue integrity after trauma. While large skin wounds often heal with hypertrophic scarring, resulting in disfigurement and reduced joint mobility, these adverse healing outcomes were not generally observed in oral mucosa, which generally heals faster than skin [92]. Several studies have identified differences between oral and cutaneous wound healing [93–96]. It has been observed that the faster wound closure in the oral mucosa compared to that in skin could be due to the presence of saliva, a faster immune response, and increased extracellular matrix remodeling. In particular, during remodeling, oral wounds show less contraction and better restoration of tissue architecture in terms of collagen structure [92].

However, in comparison to skin, much less is known about the kinetics of remodeling during oral mucosa healing [97]. In particular, understanding the organizational kinetics of the area surrounding the transgingival part of dental implants would ensure more accurate monitoring of the healing process, with favorable effects in terms of implant osseointegration and long-term success.

Until now, tissue tension during wound healing has been attributed to cellular forces produced by tissue-resident (myo-)fibroblasts alone; more recently, the storage of tensile forces in the extracellular matrix has been shown to have a significant, hitherto overlooked, contribution to macroscopic tissue tension. It has been shown that the organization of collagen fibrils is related to tissue contraction, indicating a mechanical contribution of the collagen fibrils to tension in the contraction process [98,99].

In this direction, reorganization of the soft tissues in the early stages following the placement of the dental implant was recently imaged using high-resolution tomography based on synchrotron radiation, discovering how the collagen bundles of the newly formed connective tissue intertwine impart tensile strength to the entire peri-implant soft tissue. It was, therefore, hypothesized that good organization of collagen bundles may reduce the risk of early bone resorption by reducing the infiltration of inflammatory cells [100]. However, the limitation of the previous synchrotron study was related to the fact that conventional techniques of segmentation of the tomographic image are based on the discrimination of grey levels, i.e., the physical density of the tissues. Therefore,

conventional segmentation techniques fail to discriminate the orientation of the collagen fibers, as they all have the same physical density.

In this demonstrative study, for the first time to the authors' knowledge, AI was applied directly on highly resolved images acquired by synchrotron tomography to automatically segment collagen bundles of the peri-implant connective tissue. We trained the algorithm starting from available data, the so-called training data, and then we made it acquire the ability to predict new information, called test data. Once the basic model was set up, the artificial neural networks were able to distinguish the internal portions of the soft tissue not only according to the grey levels of the synchrotron image, as conventional thresholding methods do, but also according to the orientation of the collagen bundles themselves. In this way, we were able to quantitatively distinguish longitudinal and transversal peri-implant collagen bundles, with evidence of the times and modalities of connective tissue formation around the implant during the wound healing process [101].

Materials and Methods

Samples

The peri-implant soft tissues of four patients (posterior jaw) were retrieved from the archives of the Dental School of the University of Chieti-Pescara, Italy.

They were selected based on the specific characteristics listed in Table 4.2.1.

Table 4.2.1. Description of the investigated samples. *Ctrl_st_wt*: immediately loaded and retrieved after 6 weeks (*st*: short time; *wt*: immediately loaded). This specimen had already been studied [100]. *Ctrl_lt_no-wt*: the abutment was inserted subgingival, healed without loading for 6 months and then placed on the surface with the healing screw; the final prosthesis was made after another 3 months, with a total of 9 months before applying the masticatory load (*lt*: long time; *no-wt*: not immediately loaded). *Pt1_st_no-wt*: patient test 1—not loaded and retrieved after 6 weeks. *Pt2_st_no-wt_r*: patient test 2—not loaded and retrieved after 8 weeks. Right side of the jaw (*r*); *Pt2_st_no-wt_l*: patient test 2—not loaded and retrieved after 8 weeks. Left side of the jaw (*l*). ([101], CC BY 4.0)

Sample Name	Time before Retrieval	Immediately Loaded	Region
<i>Ctrl_st_wt</i>	6 weeks	YES	-
<i>Ctrl_lt_no-wt</i>	10 years	NO	-
<i>Pt1_st_no-wt</i>	6 weeks	NO	-
<i>Pt2_st_no-wt_r</i>	8 weeks	NO	right
<i>Pt2_st_no-wt_l</i>	8 weeks	NO	left

The use of these specimens for these scientific purposes was approved by the Ethical Committee of the University of Chieti-Pescara (CODE: BONEISTO, 15 September 2019). Indeed, the implants included in this study had been archived in previous years. Before being stored in the archives, the samples were fixed by immersion in 10% buffered formalin, dehydrated in an increasing series of alcoholic rinses and finally embedded in glycol-methacrylate resin (Technovit 7200 VLC; Kulzer, Wehrheim, Germany). After retrieval from the archives, they were processed according to Ref. [100]: briefly, they were sectioned along their longitudinal axis in order to obtain two portions: the first was examined by synchrotron-based microtomography after removal of the abutment, and subsequently sectioned along its longitudinal axis for histological analysis; the second portion was used to obtain histologic transversal sections of the peri-implant soft tissue.

Synchrotron Radiation-Based Phase-Contrast Microtomography

The microtomography experiment was performed at the SYRMEP beamline of the ELETTRA Synchrotron Facility (Basovizza, Italy); the following parameters were set: 1800 projections, each with 0.2 s exposure time, over a total range of 180°; peak energy at ~17 keV; sample-detector distance at 100 mm, achieving a pixel size of the projections of 890 nm. The high-resolution acquisition exploited the propagation-based phase-contrast setting, made possible by the coherence characteristics of the synchrotron beam. The refractive index $n = 1 - \delta + i\beta$ was reconstructed, where the phase shift term δ is related to the electron density of the tissues inside the sample, and β is proportional to the absorption index. The Paganin method [65] was used to retrieve the different phases (collagen, vessels, etc.), assuming a constant δ/β ratio of 100.

The next phase of image processing was the data elaboration: it was performed with the Dragonfly software (Vers. 2022.1; Object Research Systems, Montreal, QC, Canada) [66]

and in particular with the deep learning tool, able to apply artificial intelligence to the segmentation process. Moreover, the open-source Fiji software [102] was used to perform a threshold image segmentation and the following extraction of quantitative morphometric parameters. In particular, the morphometric evaluation of the collagen bundles was calculated with the BoneJ plugin [103] of the Fiji software. It was performed by exploiting the structural indices usually measured in bone samples: collagen specific volume (expressed as a percentage), the degree of anisotropy and the connectivity density (expressed as pixel^{-3}). The percentage volume is the amount of collagen tissue per unit of volume considered; the degree of anisotropy gives information on the directionality of the collagen bundles. The more anisotropic an object is, the more the parameter will tend from zero to collagen bundles. The more anisotropic an object is, the more the parameter will tend from zero to one; the connectivity density quantifies how much collagen fibers are connected one; the connectivity density quantifies how much collagen fibers are connected to each other, presenting an index of interlacing per unit of volume.

Training and Test Data

Four connective tissue volumes of $600 \times 300 \times 300 \text{ pixels}^3$ (around 0.04 mm^3), taken at the interface with the implant, were selected from each sample to form a dataset as uniform as possible so as not to create a bias within it. The Dragonfly software was used to manually segment slices with an artificial intelligence tool, which provides all the instruments required to best separate fibers (longitudinal and transversal) and background and create a network that distinguishes three classes (Figure 4.2.1).

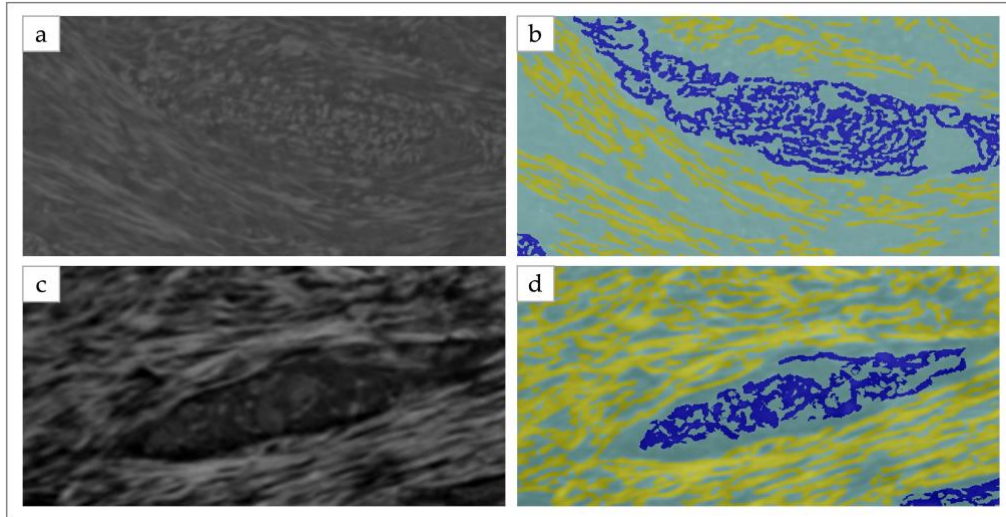


Figure 4.2.1. Manual segmentation in three classes (transversal fibers in yellow, longitudinal fibers in blue, and background in teal) with ORS Dragonfly AI tool. Ctr1_st_wt raw slice example (a), respective manual segmentation (b); Ctr2_lt_no-wt raw slice example (c), respective manual segmentation (d) ([101], CC BY 4.0).

The training dataset was formed using a total of 50 slices as input to the algorithm: 10 slices were manually segmented in each of 3 volumes of Ctr1_st_wt and in each of 2 volumes of Ctr2_lt_no-wt. This training dataset was given as input to the AI tool, which internally separates training and validation. Training parameters chosen after several tests and set before starting the algorithm are reported in Table 4.2.2. The network was tested on all slices of each volume (including the ones remaining from those used for training) in order to visualize the network's performances when applied to a 3D volume.

The graphics card of the PC on which the training was conducted was an NVIDIA GeForce RTX 3070, Version 512.59.

Table 4.2.2. Training parameters chosen after several tests and set before starting the algorithm ([101], CC BY 4.0).

U-Net	
Class count	3
Depth level	5

Initial filter count	64
Patch size	32
Stride ratio	1
Batch size	32
Epochs number	300
Loss function	Categorical Cross-Entropy
Optimization algorithm	Adadelata
Metrics	Categorical Accuracy
Early stopping	NO
Reduce learning rate on plateau	YES
Training/Validation	90/10

Due to the scarcity of input datasets, the data augmentation method [104] was applied. In fact, it is usually applied when the input data are insufficient to train the network or when the dataset is not varied enough: with this method, we wanted to obtain a more robust and wider dataset, avoiding redundancies that could lead to overfitting. The method aims to “deform” the data by means of geometric techniques such as flipping, rotation, scaling, zooming, cropping, or techniques that deal with image enhancement such as gamma correction and histogram equalization. Thus, both the original and modified images are introduced as input into the algorithm. The neural network does not notice the difference and sees the two groups as unique and makes predictions on both. This method was used in the online version [105] inside the Dragonfly AI tool to artificially increase the input dataset.

Image Segmentation

Two segmentation algorithms, aiming to separate different image features based on different properties, were used: the Otsu histogram-based threshold technique [106] and the deep learning semantic segmentation method.

The former is based on the choice of a segmentation threshold applied to the grey level histogram that separates pixels into two groups that have more similar grey levels (proportional to the physical densities—mg/cm³), usually foreground and background [107]. Otsu calculates the maximum variance between them and sets the threshold on this

value [108]. In this study, the Otsu algorithm was applied to each volume selected (consisting of 300 slices), and it chose a single threshold for each of them.

Conversely, the deep learning semantic segmentation method assigns a label to each pixel, not only relying on grey level distribution but also on the image morphometric characteristics. Thus, if objects inside the image have a different shape or direction, they will be classified as two distinct subgroups, and, therefore, associated with a different color. The use of the semantic segmentation method involves neural networks; we chose to use U-Net. It was developed by Olaf Ronneberger et al. [109] and was designed for biomedical image segmentation. The architecture is divided into two parts; the first is called encoder, which is the contracting path to capture context in the image and find key elements, while the second is the symmetric expanding path called decoder, which employs the learned pooling layers to enable precise localization of features.

U-Net Structure and Parameters

U-Net corresponds to the network predominantly used in biomedical applications; the reference model is the one proposed by Olaf Ronneberger et al. [109].

After several tests, we chose the training parameters listed in Table 4.2.2. The patch size is a subsection of the image on which the convolution kernel iterates, while the stride ratio indicates how much that kernel moves through the different patches of the image. The batch size defines the number of slices that will be propagated through the network at each training step. Categorical Cross-Entropy is the loss function chosen to understand the performance of the algorithm as a function to be minimized [110]. The optimization algorithm is Adadelta, and the metric is Categorical Accuracy. To ensure the robustness of the model and prevent overfitting, 10% of input slices were used as validation; we selected a small portion because of the limited number of images. The epochs number was fixed to 300 to guarantee the uniqueness of all evidence; consequently, Early Stopping was deactivated. Data Augmentation was chosen to secure enough input slices and to differentiate them; in that way, geometric applications such as flipping, rotation, shearing and scaling are made to the images and added to the standard dataset by convincing the network that they are new incoming images.

Comparative Light Microscopy Investigations

Longitudinal and transversal sections were obtained for histological evaluation. Histological examinations were performed using a light microscope (Laborlux S, Leitz, Wetzlar, Germany) connected to a high-resolution video camera (3CCD, JVCKY-F55B, JVC, Yokohama, Japan) and to a PC.

Birefringence was measured using polarized light microscopy to determine the orientation of the collagen bundles. An Axiolab optical microscope was used to examine thin transversal tissue sections (Laborlux S, Leitz, Wetzlar, Germany). Two linear polarizers and two quarter-wave plates were used in this instrument to transmit circularly polarized light. Because of the variation in the existing light refraction, collagen bundles aligned transversely to the light propagation direction, i.e., parallel to the section plane, appeared bright, whereas collagen fibers aligned along the light propagation axis, i.e., perpendicular to the plane of the section, appeared in a different color because refraction did not occur.

Statistical Methods

The following tests were used for statistical analysis: Welch *t*-test (deep learning vs. thresholding segmentation and transversal vs. longitudinal collagen bundles), Brown–Forsythe and Welch ANOVA tests and Dunnett’s multiple comparisons between samples for each morphometric parameter (only deep learning data). These tests were preferred to the classic ANOVA in order to also include data failing equivariance conditions. The tests were executed by GraphPad Prism 6.0 (GraphPad, Inc., San Diego, CA, USA).

Results

The aim of the semantic segmentation technique was to label each image pixel with a corresponding color that indicates the membership class of what is being represented. Thus, it was possible to quantify the collagen bundles and reconstruct the pattern of the connective tissue during the wound healing in the peri-implant site. This information is of paramount relevance in order to understand the action of forces in this site and, eventually, how the presence of the masticatory load can cause the collagen tissue to adapt to better respond to these stresses.

U-Net: Accuracy and Loss for Training and Validation

The U-Net network was trained and validated by following its performance epoch by epoch and verifying that the training was moving in the right direction, i.e., that the loss function decreases (Figure 4.2.2a), and the accuracy function (Figure 4.2.2b) increases until an acceptable level is reached. In this way, it was proved that the network learned without incurring overfitting [111].

At the end of the process (300 epochs), an accuracy of 83% was achieved on training and 87% on validation. The latter was higher most probably because of the absence of regularization techniques (that were applied in the training phase); the reason behind this choice was to avoid overfitting by making the network more generic at the expense of a lower accuracy level.

The accuracy already reached the regime in the first 100 epochs, exceeding 80%: this percentage can be considered a good level of learning, taking into account the scarcity of the samples [112].

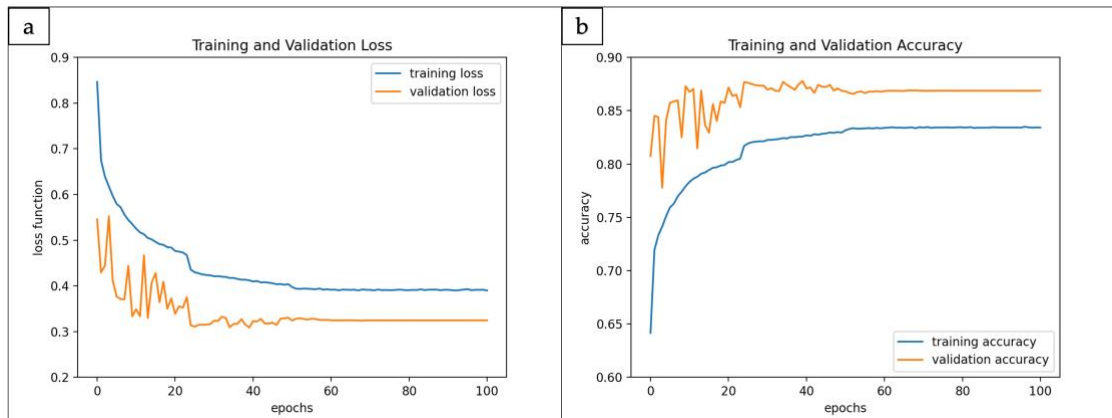


Figure 4.2.2. (a) The loss function minimization; (b) the accuracy trend; training and validation cases were plotted. Only the first 100 epochs were plotted because the network achieved its best performance around this level ([101], CC BY 4.0).

Semantic vs. Otsu Thresholding Segmentation

Semantic and Otsu thresholding segmentation were compared in order to verify if the results obtained by AI with the semantic segmentation method were superimposable with those obtained by common thresholding techniques.

The thresholding segmentation was applied using the open-source Fiji software [102]. As the first step, the Histogram Equalization Fiji plugin was used to enhance contrast in the spatial domain, adjusting the pixel intensity from 0 to 255 [113,114]. Subsequently, the Otsu segmentation process was chosen as the thresholding segmentation method (Figure 4.2.3a,c,e).

The semantic segmentation was performed with the Dragonfly software [66]. The semantic segmentation allowed us to distinguish not only background from connective tissue signals but also, unlike Otsu segmentation, transversal bundles from longitudinal bundles (Figure 4.2.4); this was achieved with a training based on three classes. However, to compare the semantic with the Otsu thresholding method, in the former, the two classes of collagen bundles (transversal and longitudinal) were grouped and just distinguished from the background (Figure 4.2.3b,d,f). The following morphometric parameters were considered: the percentage volume, the anisotropy degree, and the connectivity density. They were represented, in Python, by means $\pm$ standard deviation (Table 4.2.3) of the four volumes extracted from each sample (Figure 4.2.5).

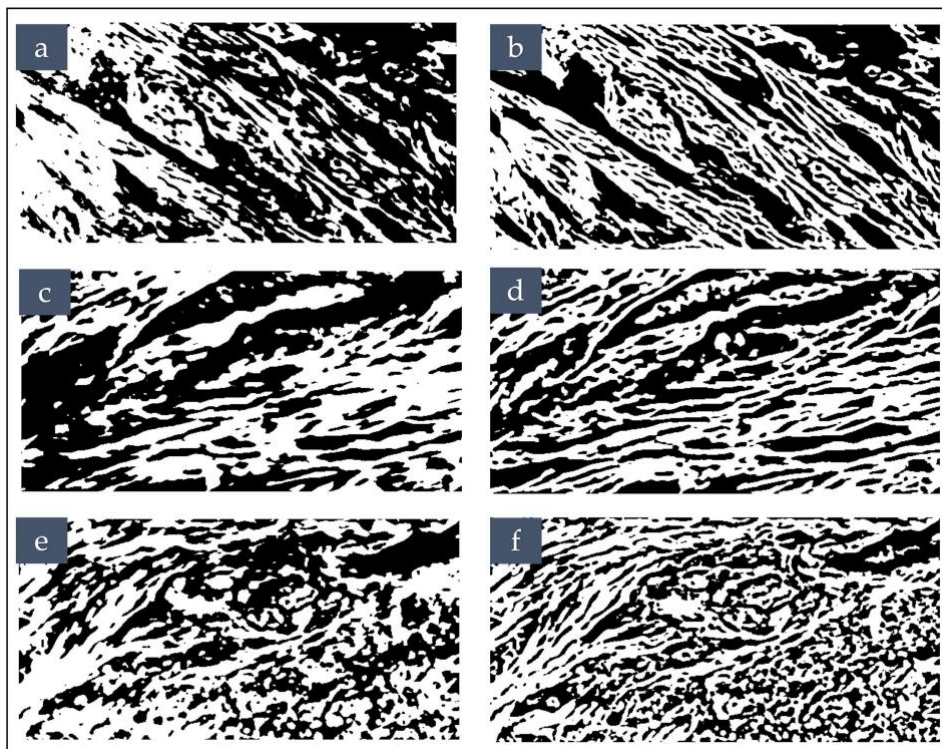


Figure 4.2.3. Thresholding (a,c,e) vs. semantic (b,d,f) segmentation methods. Examples of comparisons on homologous slices: (a,b) Ctr1_st_wt; (c,d) Ctr2_lt_no-wt; (e,f) Pt2_st_no-wt_l ([101], CC BY 4.0).

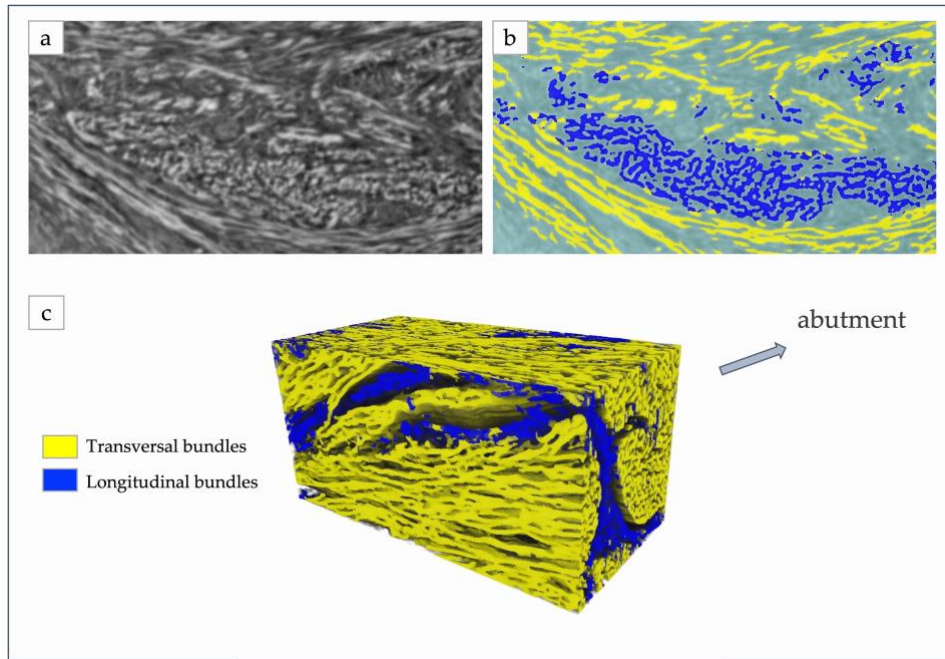


Figure 4.2.4. (a) Raw slice of a Ctr1_st_wt sub-volume, as achieved after phase-retrieval reconstruction; (b) semantic segmentation of the same slice with deep learning: transversal bundles (yellow), longitudinal bundles (blue) and background (teal) were detected. (c) Application of artificial intelligence to a Ctr2_lt_no-wt sub-volume and automatic segmentation of collagen bundles (blue: longitudinal; yellow: transversal) ([101] ,CC BY 4.0).

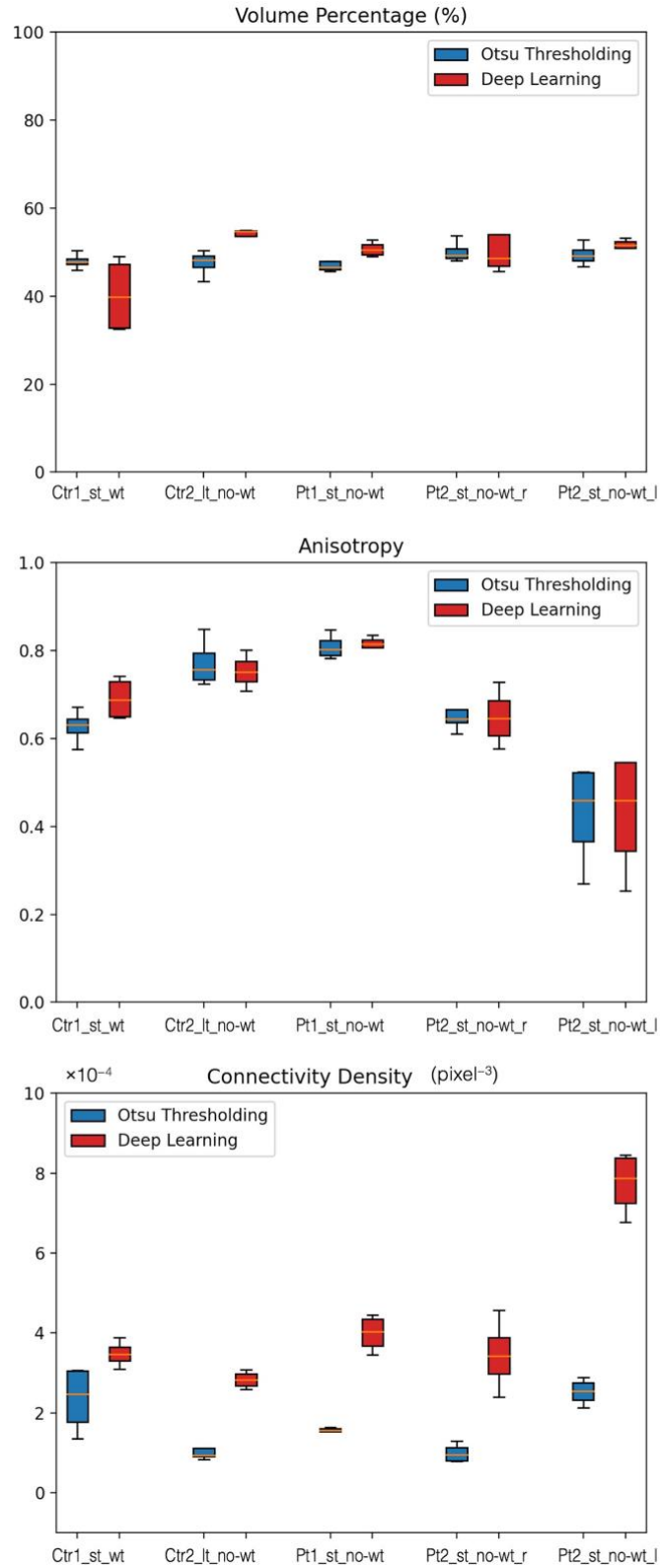


Figure 4.2.5. Box plots of volume percentage, degree of anisotropy and connectivity density calculated on each volume segmented with deep learning semantic method (red) and Otsu

thresholding (blue). For deep learning, both longitudinal and transversal fibers were considered together for comparison ([101], CC BY 4.0).

Table 4.2.3. Welch *t*-test between deep learning vs. thresholding segmentation; Brown–Forsythe and Welch ANOVA test, Dunnett’s multiple comparisons between samples per parameter (only deep learning data). Test executed by GraphPad Prism 6.0 (GraphPad, Inc., San Diego, CA, USA). c, •: $p < 0.05$; b, a, ••: $p < 0.01$; b,d, •••: $p < 0.001$ ([101], CC BY 4.0).

	Volume Percentage (%)		Anisotropy Degree		Connectivity Density ($\times 10^{-4} \text{ px}^{-3}$)	
	<i>Deep Learning</i>	<i>Thresholding</i>	<i>Deep Learning</i>	<i>Thresholding</i>	<i>Deep Learning</i>	<i>Thresholding</i>
Ctr1_st_wt	40.3 $\pm$ 8.8	47.9 $\pm$ 1.8	0.691 $\pm$ 0.049	0.627 $\pm$ 0.040	3.48 $\pm$ 0.33 ^a	2.34 $\pm$ 0.85
Ctr2_lt_no-wt	53.8 $\pm$ 1.9 •	47.5 $\pm$ 3.0 •	0.753 $\pm$ 0.040	0.771 $\pm$ 0.056	2.83 $\pm$ 0.22 •••,b	1.08 $\pm$ 0.36 ••
Pt1_st_no-wt	50.7 $\pm$ 1.7	47.4 $\pm$ 2.4	0.818 $\pm$ 0.014	0.809 $\pm$ 0.029	4.00 $\pm$ 0.47 ••,c	1.57 $\pm$ 0.05 ••
Pt2_st_no-wt_r	52.3 $\pm$ 9.5	50.1 $\pm$ 2.6	0.649 $\pm$ 0.066	0.657 $\pm$ 0.050	3.45 $\pm$ 0.91 ••,d	1.00 $\pm$ 0.24 ••
Pt2_st_no-wt_l	51.8 $\pm$ 1.1	49.5 $\pm$ 2.6	0.430 $\pm$ 0.143	0.428 $\pm$ 0.121	7.75 $\pm$ 0.80 •••,a,b,c,d	2.53 $\pm$ 0.3 •••

Referring to the volume percentage, in general, the average values obtained by deep learning through semantic segmentation were similar to those obtained by the thresholding segmentation method. Interestingly, the volume percentage distribution throughout the whole sample of Ctr2_lt_no-wt was significantly wider using semantic segmentation than in the case of the Otsu thresholding method.

The degree of anisotropy trend was found to be similar using the thresholding or the semantic segmentation techniques.

Conversely, referring to the connectivity density, the average values obtained by deep learning through semantic segmentation were significantly higher than those obtained by the Otsu thresholding segmentation method in all the samples except for the Ctr1_st_wt. This higher level of connectivity was probably due to a better discrimination of the single fibers; consequently, each intertwinement was better recognized.

Interestingly, using semantic segmentation, the connectivity density was significantly higher in Pt2_st_no-wt_l than in all the other samples. This was most likely attributable to the immaturity of the connective tissue present in this biopsy, also demonstrated by the morphological evidence shown in Figure 4.2.S1 (see Appendix). In fact, the abundant presence of micro vessels (Figure 4.2.S1a) and the presence of collagen fibers still

strongly disorganized (Figure 4.2.S1b) at a three-dimensional level suggested the presence of a connective tissue only at the beginning of the remodeling phase, with a wound not yet completely healed at the connective level.

Transversal vs. Longitudinal Bundles

Since semantic segmentation allows one to distinguish transversal bundles from longitudinal ones, the quantitative analysis of these two classes of bundles was performed on samples segmented by deep learning. Thus, the transversal and longitudinal collagen bundles, isolated by the neural network, were quantitatively analyzed in four different sub-volumes extracted from each sample. This information could not have been obtained by thresholding segmentation, which merely analyzes the image based on grey levels (i.e., on the physical density mismatches) without looking at the morphology of the tissue itself. This quantitative analysis, made possible by semantic segmentation that discriminates transversal from longitudinal bundles, was reported by mean $\pm$ standard deviation (Table 4.2.4) and by box plots (Figure 4.2.6).

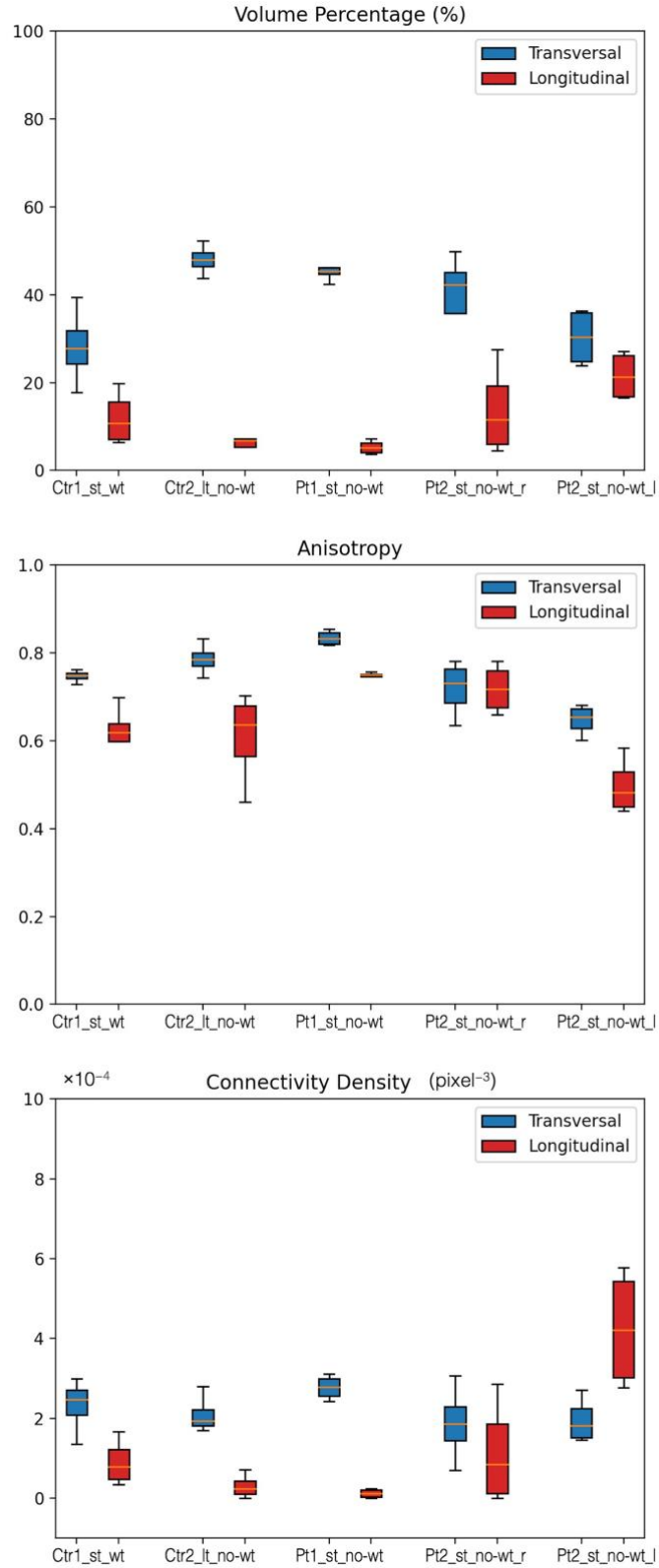


Figure 4.2.6. Box plots of volume percentage, degree of anisotropy and connectivity density calculated on each volume segmented with deep learning: transversal fibers (blue), longitudinal fibers (red) ([101], CC BY 4.0).

Table 4.2.4. Welch *t*-test between morphometric data in transversal vs. longitudinal collagen bundles; Brown–Forsythe and Welch ANOVA test, Dunnett’s multiple comparisons between samples per parameter (only deep learning data). Test executed by GraphPad Prism 6.0 (GraphPad, Inc., San Diego, CA, USA). a,b,c,d,●: $p < 0.05$; ●●: $p < 0.01$; ●●●: $p < 0.001$; ●●●●: $p < 0.0001$ ([101], CC BY 4.0).

	Volume Percentage (%)		Anisotropy Degree		Connectivity Density ($\times 10^{-4} \text{ px}^{-3}$)	
	<i>Transversal</i>	<i>Longitudinal</i>	<i>Transversal</i>	<i>Longitudinal</i>	<i>Transversal</i>	<i>Longitudinal</i>
Ctrl_st_wt	28.2 ± 8.9 ●	11.9 ± 6.3 ●	0.747 ± 0.014 ●	0.619 ± 0.065 ●	2.32 ± 0.70 ●	0.90 ± 0.60 ●
Ctrl2_lt_no-wt	48.0 ± 3.6 ●●●●	5.8 ± 2.2 ^a●●●●	0.787 ± 0.037 ●	0.609 ± 0.108 ●	2.10 ± 0.49 ●●	0.30 ± 0.31 ^d●●
Pt1_st_no-wt	45.4 ± 2.5 ●●●●	5.3 ± 1.6 ●●●●	0.834 ± 0.017●●●	0.747 ± 0.009 _{b,●●●}	2.77 ± 0.31 ●●●	0.12 ± 0.11 ●●●
Pt2_st_no-wt_r	38.6 ± 13.0 ●	13.8 ± 10.6 ●	0.720 ± 0.065	0.719 ± 0.058 ^c	1.87 ± 0.98	1.14 ± 1.34
Pt2_st_no-wt_l	30.2 ± 6.7	21.6 ± 5.7 ^a	0.647 ± 0.036 ●	0.497 ± 0.065 _{b,c,●}	1.96 ± 0.58	4.24 ± 1.52 ^d

In terms of volume percentage, the quantity of transverse bundles was always shown to be higher than for longitudinal ones. The anisotropy degree was found to be significantly lower for longitudinal bundles than for transversal ones in all of the samples but Pt2_st_no-wt_r. Interestingly, Pt2_st_no-wt_l presented the lowest degree of anisotropy for both longitudinal and transversal bundles.

With regard to the connectivity density parameter, almost all samples, with the unique exception of those referred to patient 2, showed significantly higher values in the transversal direction than in the longitudinal one. Notably, the connectivity density of longitudinal bundles in the Pt2_st_no-wt_l sample was found to be considerably larger than in the other samples.

In general, referring to Pt2 (i.e., both Pt2_st_no-wt_r and Pt2_st_no-wt_l samples), high standard deviations were found, both considering the volume percentage and the connectivity density; this was not the case with Ctrl1, Ctrl2 and Pt1 samples. As previously observed, the biopsies extracted from patient 2, in particular the one referring to the left side of the jaw, had very particular characteristics, with high volume percentages and connectivity in the longitudinal direction and low anisotropy degree. This morphometric evidence was compatible with a probable immaturity of the connective tissue (already highlighted in Figure 4.2.S1 (see Appendix) and probably linked to the patient’s clinical history).

Polarized light microscopy confirmed the semantic segmentation results referred to the detection of transversal and longitudinal collagen bundles. It showed that the peri-implant mucosa was constituted by different collagen bundles, distinguishable both in transversal (Figure 4.2.7a–e) and longitudinal (Figure 4.2.7f–j) sections.

Regarding transversal sections, it was possible to observe how the semi-circular fibers coming from different collagen bundles intersected with each other by showing the semi-circular orientation of the collagen fibers around the transversal abutment profile. In Ctr1_st_wt (Figure 4.2.7a), the bundles were better organized, and the transversal fibers were alternated with collagen fibers parallel to the long axis of the implant (dark areas), whereas in all other cases (Figure 4.2.7b–e), transversal bundles predominated.

Regarding longitudinal sections, only Ctr1_st_wt (Figure 4.2.7f) and partially Pt1_st_no-wt (Figure 4.2.7h) showed, near the surface, longitudinal bundles distributed parallel to the long axis of the implant abutment. In all other cases (Figure 4.2.7g,i,j), a network composed of woven bundles was observed.

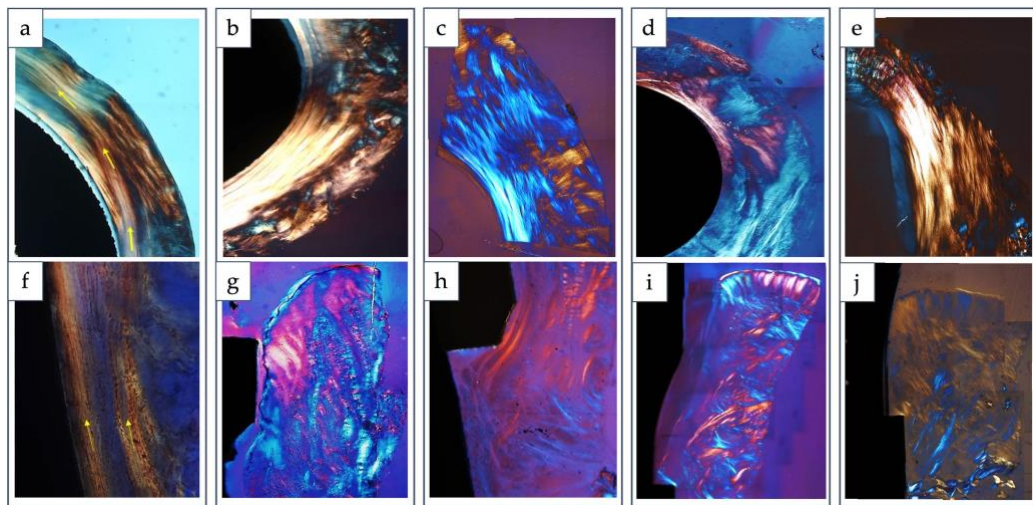


Figure 4.2.7. Polarized light microscopy. Transversal sections (a–e): (a) Ctr1_st_wt showed bundles better organized; (b) Ctr2_lt_no-wt, (c) Pt1_st_no-wt, (d) Pt2_st_no-wt_r, (e) Pt2_st_no-wt_l showed a predominance of transversal bundles. Longitudinal sections (f–j): (f) Ctr1_st_wt and (h) Pt1_st_no-wt showed fibers parallel to the long axis of the implant abutment; a network composed by woven bundles was observed in (g) Ctr2_lt_no-wt; (i) Pt2_st_no-wt_r and (j) Pt2_st_no-wt_l (a,f) ([101], CC BY 4.0).

Discussion

In this demonstrative study, artificial intelligence was used, for the first time to the authors' knowledge, to perform morphometric analysis on high-resolution synchrotron-based tomographic images of peri-implant connective tissue. The overall objective was to obtain reliable quantitative information on collagen bundles and their 3D distribution. We showed that deep learning semantic image segmentation can better identify the bundles compared to a common thresholding segmentation technique [115]. Notably, we had recently found, by synchrotron imaging, the presence of transversely and longitudinally oriented (with respect to the dental implant axis) collagen bundles in the peri-implant soft tissue; these grow and organize intertwining patterns during their development at different times and in different quantitative distributions depending on environmental conditions, to date not fully understood [100]. Therefore, discriminating and quantifying transversal and longitudinal bundles turns out to be an extremely interesting step forward in understanding the wound healing process in the peri-implant soft tissue and the role of collagen in it.

In this study, we demonstratively showed that a good way to find these features is to use the semantic segmentation method of synchrotron-based highly resolved tomographic images. With the support of neural networks and deep learning, it was possible to quantify structures in the samples that were not considered before. In particular, collagen bundles were identified by their orientation and not by their physical densities. This is fundamental in order to discriminate transversal and longitudinal bundles that, having the same physical density, could not have been discriminated using conventional thresholding segmentation techniques. In practice, we have managed to create a neural network able to separate longitudinal and transversal fibers by U-Net [108,109].

Within the limits of the extremely reduced sample size, we did not detect a statistically significant difference in volume percentage and degree of anisotropy between data obtained by conventional segmentation methods based on thresholding and the innovative ones based on artificial intelligence. On the other hand, as regards the connectivity density parameter, it was observed that the data obtained in deep learning were higher for all samples than the data obtained with conventional thresholding (statistically significant in all samples except for the Ctr1_st_wt); this fact is certainly attributable to a greater discrimination of the collagen bundles and their connectivity through artificial intelligence.

Based on quantitative comparison between transversal and longitudinal bundles, carried out in deep learning, we discovered an extremely interesting fact: considering all the parameters globally, a clear prevalence was observed of the quantity and connectivity of transversal bundles compared to longitudinal ones in all of the samples; moreover, transversal bundles were found to be more anisotropic oriented (i.e., they had a preferred orientation) than the longitudinal ones. These differences were, in most cases, statistically significant and were confirmed by polarized light microscopy observations.

This evidence could indicate that in the wound healing process in the peri-implant area, the transverse fibers are formed first, and only subsequently the longitudinal ones. Having found in the Ctr2_lt_no-wt, investigated after permanence in vivo for 10 years, the same quantitative mismatch between transversal and longitudinal fibers as in the other samples, we could reasonably exclude that the lower presence of longitudinal fibers compared to transversal ones was due to the limited healing time. Thus, in general, we can state that, even in a mature and functional peri-implant connective tissue, the number of longitudinal fibers is always lower than the number of transversal ones; this information is completely new and has never been observed in the literature.

However, in the Ctr1_st_wt, Pt2_st_no-wt_r and Pt2_st_no-wt_l, there were, in terms of volume percentages, the greatest equilibria between transversal and longitudinal bundles. This could suggest a better stability and functionality of these samples, but, as confirmed by polarized light microscopy observations, referring to the other two morphometric parameters, i.e., the degree of anisotropy and the density of connectivity, it could be observed that both samples referring to patient 2, with particular reference to Pt2_st_no-wt_l, showed high variability both in terms of volume percentage and connectivity. Moreover, Pt2_st_no-wt_l appeared to have bundles less oriented than the other samples and highly vascularized (Figure S1a), indicating that the tissue was still highly immature. Indeed, in three samples (Ctr2_lt_no-wt, Pt2_st_no-wt_r, Pt2_st_no-wt_l), the longitudinal sections obtained by polarized light microscopy showed collagen fiber orientation that was never perfectly parallel to the surface of the abutment.

In conclusion, within the limits of the sample size, this demonstrative study showed that the implant subjected to immediate loading, i.e., the Ctr1_st_wt, presented the most balanced distribution of collagen bundles (transversal vs. longitudinal). This evidence would suggest a more favorable stabilization of the soft tissues, because a better balancing between transversal and longitudinal intertwining bundles should be due to forces balancing each other in those directions. Consequently, immediate loading appears to be

a good solution, in patients where it is possible [116], since it stabilizes the connective tissue around the neck of the implant, which thus becomes an effective barrier against the apical migration of inflammatory cells towards the bone, thus preventing implant failure. The positive effect of immediate loading was observed also in previous studies [117–119], but in all these investigations the analysis was just focused on bone tissue without searching for correlations with the peri-implant connective tissue organization. Clearly, given the demonstrative nature of this study and the extremely small sample size, this new finding must be verified within a wider sample size. Thus, in the next steps of this research, we will expand the dataset and use a 3D U-Net with more complex models applicable with larger amounts of data. This way, it will be possible to support this preliminary study and validate the artificial intelligence analysis method.

4.2.1 Case Report: Dental Implant with Conometric Abutment– Prosthetic Cap Connection

Several papers have recently shown that peri-implant connective tissues' three-dimensional (3D) organization plays a key role in the preservation of peri-implant bone tissues and the long-term survival and success of dental implants [120–123]. Animal models and human studies have described the structure and ultrastructure of peri-implant soft tissues using bi-dimensional imaging methods like histology, scanning electron microscopy, polarized light microscopy [124–129], and transmission electron microscopy [130]. However, only advanced 3D imaging through high-resolution, tomography (micro-CT) has been able to give evidence and quantify the organization of connective tissue microarchitecture [100,101,131].

Until recent years, prosthetic restorations were connected to implant abutments mainly by using screws or cement. Nevertheless, both methods have some contraindications [132–135]. In screw-retained prosthetic restorations, there can be loosening or fracture of the screws; moreover, a not-so-optimal esthetic result is often obtained due to the presence of an occlusal access hole [133,135]. This access hole can also produce a weak spot, with possible chipping and fracture of the porcelain [136,137]. In cement-retained restorations, biological complications can

occur, e.g., inflammation of the peri-implant soft tissues with loss at the level of the peri-implant crestal bone due to excess cement [133–135].

In the last decade, a conometric system has been proposed, by which the prosthetic crowns are coupled to the abutment using a precise friction fit, without screws or cement [138–144]. This connection, eliminating the cement residues in the subgingival region, has been proven to reduce the risk of inflammation of the peri-implant tissues. Moreover, by not having to remove cement residues, it is possible to set the margins in more apical portions, improving the aesthetics of the restorations. It has also been proven that retentive force is adequate for fixed rehabilitation even after a high number of insertion–disengagement cycles [145].

Several studies report successful follow-up evaluations of conometric systems [145]; some of these studies are accompanied by histological investigations mainly referring to the study of the peri-implant bone. Consequently, for full acceptance and recognition of the conometric abutment–prosthetic cap connection, it is necessary to further study the peri-implant soft tissues which constitute the actual barrier to the migration of any bacteria and inflammatory cells towards the apical bone areas.

According to the present authors' best knowledge, there are no cases in the literature showing definitive results on the 3D structure of peri-implant connective tissues around conometric prosthetic restorations with proven absence of macro- and micro-gaps between the abutment and the prosthetic cap. The aim of the present case study is to shed light, through the use of micro-CT, on possible 3D defects in conometric abutment–prosthetic cap connections and, via histology, polarized light microscopy, and synchrotron-based micro-CT, the organization of peri-implant connective tissues around a retrieved human dental implant with conometric connection used for restoration.

Materials and Methods

Samples

A 61-year-old female patient who had undergone implant rehabilitation received three (nr = 3 samples) dental titanium implants with conometric connection (AoN

Implants, Grisignano di Zocco, Vicenza, Italy). The patient received a clear explanation of the study and signed an informed consent form. A cone-beam study was conducted before the surgery for the planning of implant rehabilitation (NewTom Giano with field of view (FOV) $11 \times 5 \text{ cm}^2$ and resolution $300 \text{ }\mu\text{m}$) (Figure 4.2.1.1). Implant rehabilitation was performed in the premolar–molar region of the left maxilla (sites 25-26-27). The three implants (AoN Implants, Grisignano di Zocco, Vicenza, Italy) were placed 1 mm subcrestally, and the conometric abutments, measuring 3 mm, were inserted in all sites and tightened at 25 N (Figure 4.2.1.2A,B). The drilling protocol involved the use of a 2 mm cylindrical dental drill and a dedicated $8 \times 3.3 \text{ mm}^2$ conical drill at 50 rpm without irrigation. The implant was inserted into soft bone (type IV) at 15–18 N. The retrieved implant was an AoN Is-Four implant, measuring 3.3 mm in width by 8 mm in length. It consisted of a titanium, conometric straight abutment (5 mm high) and a conometric cap made of PEEK. The PEEK cap was used over a metal one to offer a less bulky alternative during this temporary phase.

After suturing, wound healing remained undisturbed for 45 days. Therapy included clavulanic acid and amoxicillin at 1 g for 6 days, prednisone at 5 mg for 4 days, and paracetamol at 1 g if necessary. At the end of the operation, dexamethasone at 5 mg was administered to contain post-operative swelling. After implant rehabilitation, a radiographic investigation was conducted using orthopantomography (OPT) and cone-beam study (Figure 4.2.1.3). After a period of 45 days, one of these implants—site 27—together with the peri-implant tissues was retrieved. Indeed, it was positioned distally far, causing discomfort and interfering with her speech and tongue movement. Therefore, a change in prosthetic rehabilitation was necessary (Figures 4.2.1.2C and 4.2.1.4). In detail, implant retrieval was performed via a parallel incision to the implant axis at 2 mm from the abutment in the mucosal tissue, using a magnetic mallet for the bone tissue. This achieved a cylinder core of tissue of 8–10 mm thickness in the axial direction. In addition, a gingivectomy was necessary near to where the implant was located.

The Ethics Committee of the University of Chieti-Pescara approved the use of this specimen for research purposes (CODE: BONEISTO, 15 September 2019). The implant used in this work was delivered in 2019 to the Dental School of the University of Chieti-Pescara, Italy, to be stored. The specimen was already fixed with 10% buffered formalin. Then, dehydration was carried out with increasing

concentrations of alcohol. Finally, the sample was embedded in glycol–methacrylate resin (Technovit 7200 VLC; Kulzer, Wehrheim, Germany) before being stored in the archives. The specimen was processed after being retrieved from the archives in 2023, in accordance with Ref [100]. The synchrotron and histological analyses were performed on the same sample. In brief, it was sectioned into two parts along its longitudinal axis. The first part was used to examine the implant’s peri-implant tissues using synchrotron micro-CT after abutment removal; after micro-CT, this part was further sectioned along the transversal axis for histological analysis. The second portion of the sample was used to obtain histological longitudinal sections of the peri-implant tissues. This sample was already included in a prior study to validate an artificial intelligence (AI) module, not for diagnostic purposes [101]. Two other (nr = 2 samples) identical titanium implants with conometric connection were received by AoN Implants (Grisignano di Zocco, Vicenza, Italy) for implant characterization using laboratory-based micro-CT. The implants had the abutment fixed in a plexiglass support and the conometric cap was pre-assembled.



Figure 4.2.1.1. Cone–beam study (CBCT) (FOV $11 \times 5 \text{ cm}^2$). The resolution was $300 \text{ }\mu\text{m}$ for planning implant rehabilitation ([146], CC BY 4.0).

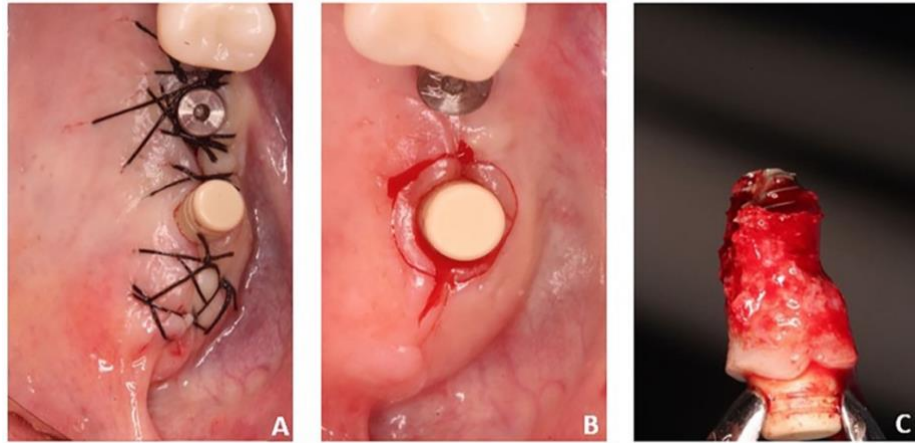


Figure 4.2.1.2. (A) Implants in sites 26 and 27 after insertion; (B) mucotomies around the implant after 45 days of healing; (C) implant and peri-implant soft tissues retrieved ([146], CC BY 4.0).

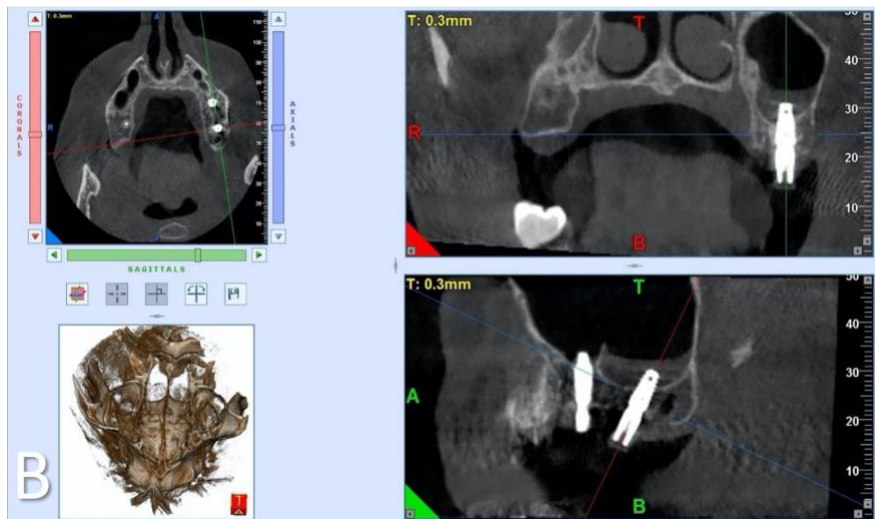


Figure 4.2.1.3. (A) OPT study after 45 days of healing and (B) cone-beam study after 45 days of healing: blue: axial planes; red: coronal planes; green: sagittal planes; T: top; B: bottom; R: right; L: left; A: anterior; P: posterior ([146], CC BY 4.0).

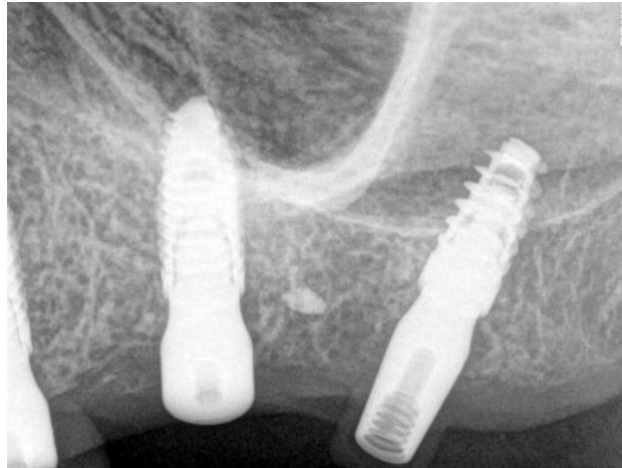


Figure 4.2.1.4. Radiography of the implant after 45 days of healing ([146], CC BY 4.0).

Laboratory-Based Micro-CT Investigation of the Implant

The X-ray micro-CT analysis was performed on nr.2 commercial implants as such using a Bruker Skyscan 1174 tomographic system (BRUKER, Antwerp, Belgium). In order to carry out the volumetric analysis, the two systems, taken from two different productions to verify their reproducibility, were pre-assembled with the abutment in the plexiglass support and already connected with the conometric cap. The analysis was performed to detect eventual defects (gaps) in the conometric abutment–prosthetic cap connection.

The micro-CT system was set with a power voltage of 50 kV and a beam current of 800 μ A. Parameter settings: pixel size = 6.5 μ m; 1.5 mm Al filter on the source X-ray beam; and exposure time = 17.5 s. The samples were scanned over 180° using a 0.3° scan step. After the tomographic scan, the reconstruction phase was carried out

with the Bruker NRecon software (v. 1.7.3), setting the smoothing algorithm (3.0), ring artifact reduction (5.0), and beam hardening correction (10%).

Synchrotron Light-Based Phase-Contrast Micro-CT of Peri-Implant Connective Tissue

The sample retrieved from the 61-year-old female patient 45 days after implantation surgery was investigated via synchrotron-based micro-CT.

The micro-CT experiment was conducted at the SYRMEP beamline of the ELETTRA Synchrotron Radiation Facility (Basovizza (TS), Italy). The parameters were set as follows: 1800 projections, each with an exposure time of 0.2 s; a sample–detector distance of 100 mm; a total angular range of 180°; peak energy at 17 keV; and a pixel size of 890 nm. The coherence characteristics of the synchrotron’s light allowed for use of a propagation-based phase contrast setting at high resolution. The index of refraction $n = 1 - \delta + i\beta$ was reconstructed; in this formula, the phase shift term (δ) is proportional to the tissue electron density, whereas the complex part β is proportional to the tissue density. The δ/β ratio was established at 100, after several tests. To recover the various phases (vessels, collagen, etc.), the Paganin method was utilized [65]. Data processing was carried out using ORS Dragonfly software (Version 2022.1; Object Research Systems, Montreal, QC, Canada) [66]: indeed, its anisotropy and deep learning tools are capable of employing different algorithms to analyze and segment images with artificial intelligence. The collagen bundles were subsequently analyzed morphometrically with the BoneJ plugin [103] within the Fiji platform [102]. These software tools computed several structural indices: the specific volume of collagen (expressed as a percentage), i.e., the amount of collagenous tissue per unit volume; the density of connectivity (expressed as pixel^{-3}) that quantifies how many collagen fibers are connected to each other, presenting as a connectivity index per unit volume; and the degree of anisotropy (DA), which provides information on the direction of the collagen bundles (the more anisotropic the object, the greater the DA value).

Four volumes of $600 \times 300 \times 300 \text{ pixels}^3$ (approximately $40 \times 10^{-3} \text{ mm}^3$) were chosen close to the implant interface in order to generate a dataset that was as uniform as feasible and did not induce internal distortions. The artificial intelligence module of the Dragonfly 2022.1 software was used to manually segment slices, as

it contains all of the necessary tools to best isolate fibers (transverse and longitudinal) from the background. As described elsewhere [101], a network that employed a semantic segmentation of three classes was trained; this deep learning method allowed us to label each pixel based on the morphometric properties of the image. Therefore, if some objects in the foreground had a different orientation or shape, they were categorized into two distinct subgroups with two distinct labels. The neural network U-Net, devised by Olaf Ronneberger et al. [109] and specifically designed for biomedical image segmentation, was used in the semantic segmentation method.

Histological Investigation

For the histological investigation, the specimen was sectioned, along its longitudinal and transversal axis, with a high-precision diamond disk at about 150 μm and ground down to about 30 μm with a specially designed grinding machine, Precise 1 Automated System. The obtained slice was then stained with acid fuchsin and toluidine blue. Histological analysis was carried out using a light microscope (Laborlux S, Leitz, Wetzlar, Germany) connected to a high-resolution video camera (3CCD, JVCKY-F55B, JVC, Yokohama, Japan) and interfaced with a monitor and PC (Intel Pentium III 1200 MMX, Intel, Santa Clara, CA, USA). This optical system was associated with a digitizing pad (Matrix Vision GmbH, Oppenweiler, Germany) and a histomorphometry software package with image-capturing capabilities (Image-Pro Plus 4.5, Media Cybernetics Inc., Immagini & Computer Snc, Milano, Italy). The histological findings were reviewed by a single, highly qualified expert (G.I.), who was not engaged in the surgical procedure.

Polarized Light Microscopy

Using polarized light microscopy, the transverse orientation of collagen bundles was observed as a result of birefringence. The collagen fibers were viewed using an Axiolab light microscope (Laborlux S, Leitz, Wetzlar, Germany) equipped with two linear polarizers, two quarter wave plates, and circularly polarized transmitted light. As a result of a change in the refraction of existing light, the collagen fibers (bright

fibers) were precisely aligned perpendicular to the direction of light propagation (parallel to the section plane). In contrast, the different-colored collagen fibers were aligned along the axis of light propagation (perpendicular to the section plane) and no refraction occurred.

Results

Laboratory-Based Micro-CT

The micro-CT analysis carried out on nr.2 commercial implants as such (and in static conditions) revealed the absence of macro- and micro-gaps for both samples studied; in fact, as shown in Figure 4.2.1.5, the connection interface (indicated with the yellow arrows) cannot be viewed in any of the three axial, sagittal, and frontal perspectives. This result is extremely satisfactory in order to guarantee the in-service stability of the implant, for the benefit of the peri-implant bone, and to prevent bacterial infiltration.

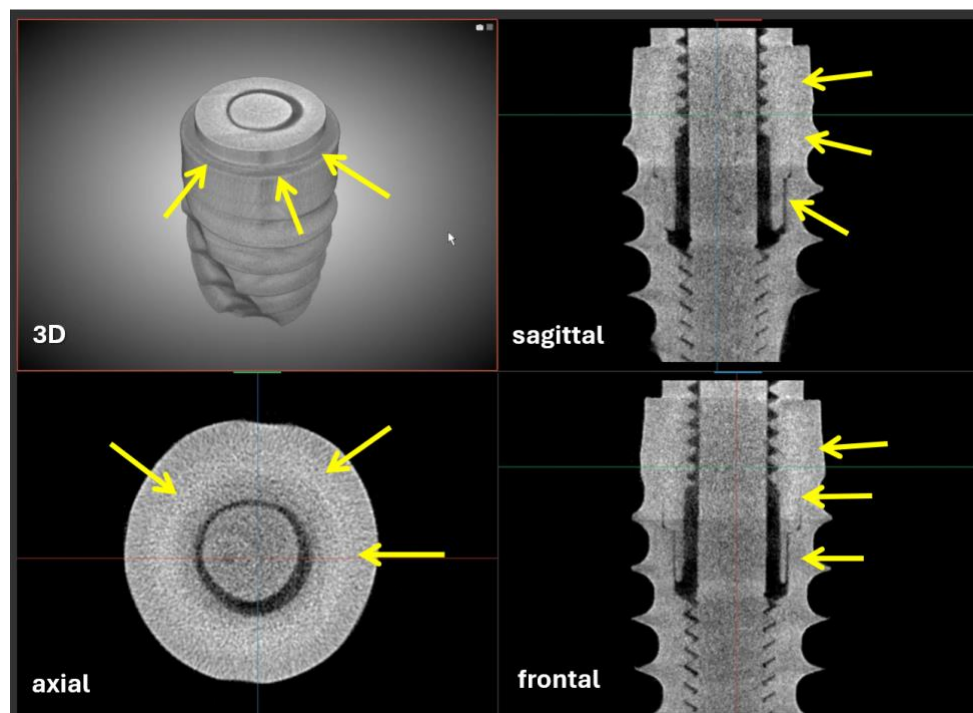


Figure 4.2.1.5. Dental titanium implant with conometric connection: 3D reconstruction and sampling sagittal, axial, and frontal slices. Freeze frame from Supplementary Video S1. The connection interface (yellow arrows) is free of macro- and micro-gaps ([146], CC BY 4.0).

Synchrotron Micro-CT

The analysis of the degree of anisotropy (DA) provided us with a comprehensive examination of the extent to which the collagen bundles were oriented. The eigenvectors depicted in Figure 4.2.1.6 serve to differentiate between regions characterized by low DA (0–0.30), regions with medium DA (0.30–0.60), and regions with high DA (0.60–1.0). The number of eigenvectors was notably greater within the maximal discriminant analysis range; this observation indicates that the collagen bundles had a distinct orientation and were highly aligned. As a result, with the assistance of artificial intelligence, it became feasible to discern the primary orientations and quantify them. The semantic segmentation of the images obtained from the synchrotron analyses allowed us to discriminate the transverse from the longitudinal bundles, as shown in Figure 4.2.1.7, adding more quantitative information for these two classes that could not be distinguished with the common thresholding techniques. The quantitative analyses of the four different sub-volumes segmented by the U-Net neural network in the deep learning tool allowed us to achieve the quantification of transverse bundles that were found to be significantly higher than the longitudinal ones ($45.4 \pm 2.5\%$ vs. $5.3 \pm 1.6\%$, respectively; $p < 0.0001$). The anisotropy degree (DA) was found to be significantly lower for the longitudinal bundles than for the transversal ones (0.834 ± 0.017 vs. 0.747 ± 0.009 , respectively; $p < 0.001$). Moreover, with reference to the connectivity density parameter (Conn. D), it showed significantly higher values in the transverse direction than in the longitudinal one (2.77 ± 0.31 vs. $0.12 \pm 0.11 (\times 10^{-4}) \text{ px}^{-3}$, respectively; $p < 0.001$).

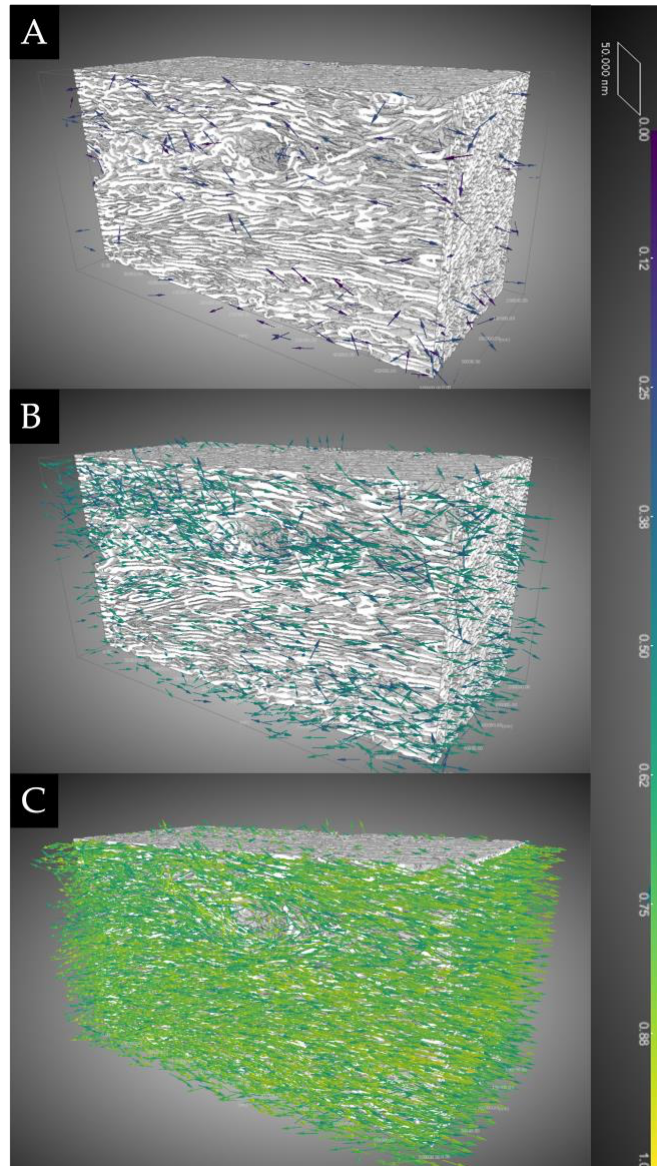


Figure 4.2.1.6. Qualitative analysis of the degree of anisotropy (DA). The depicted eigenvectors serve to differentiate between (A) regions characterized by low DA (0–0.30), (B) regions with medium DA (0.30–0.60), and (C) regions with high DA (0.60–1.0) ([146], CC BY 4.0).

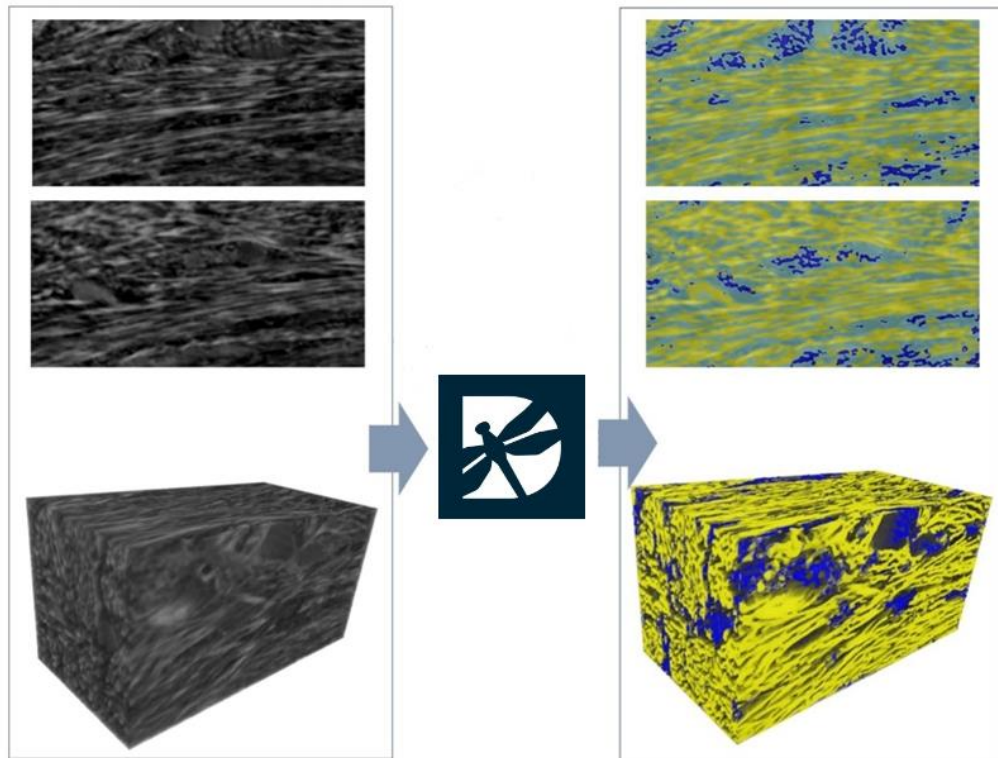


Figure 4.2.1.7. Segmentation achieved by artificial intelligence algorithms. Left panels: two raw axial slices and a 3D rendering of a subvolume as achieved after phase-retrieval 3D reconstruction. Right panels: semantic segmentation of the same slices and 3D rendering with deep learning. In semantic segmentation, the transversal bundles were rendered in yellow, the longitudinal bundles were rendered in blue, and the background was rendered in teal. Data were processed with Dragonfly software (Vers. 2022.1; Object Research Systems, Montreal, QC, Canada); semantic segmentation was performed by the U-Net neural network ([146], CC BY 4.0).

Histology and Polarized Light Microscopy

At low magnification, soft tissues and newly formed bone were observed around the implant abutment. The peri-implant soft tissues adhered to the abutment surface and there were no gaps at the interface. They were composed of sulcular epithelium (SE), junctional epithelium (JE), and connective tissue (CT). The JE was composed of a layer of epithelial cells in close contact with the surface of the transgingival collar.

In the apical portion, the CT was close to the surface of the stump. It was 610 μm high and characterized by the presence of some small blood vessels. At higher

magnification, areas of newly formed bone, up to 2080 μm above the implant shoulder, were found. Specifically, newly formed trabecular bone grew over the implant shoulder and had a direction up to the abutment surface. A layer of connective tissue was present between the newly formed bone and the surface of the abutment; no epithelium was observed in this portion. The tissue height from the implant shoulder (IS) to the margin of peri-implant mucosa (MP) was approximately 5080 μm .

The peri-implant mucosa included several collagen bundles, which were visible in both longitudinal and transverse sections, as shown during polarized light microscopy. As far as the longitudinal section was concerned, in the portions close to the new bone, longitudinal bundles were observed parallel to the profile of the implant and the abutment in the long axis (Figures 4.2.1.8 and 4.2.1.9A). Regarding the cross-section, predominantly semicircular fibers could be observed around the abutment (Figure 4.2.1.9B).

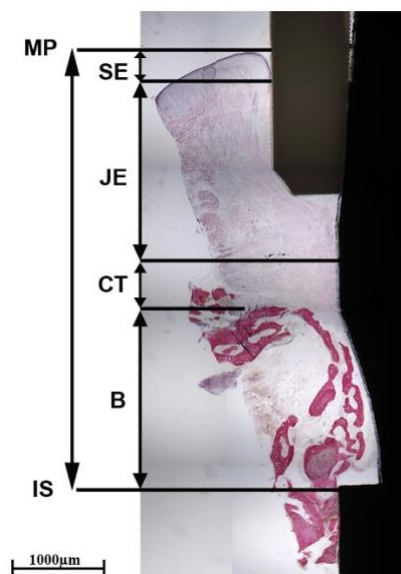


Figure 4.2.1.8. Histological section of the implant–abutment unit. Peri-implant soft tissues (MP) were composed of sulcular epithelium (SE), junctional epithelium (JE), and connective tissue (CT), which were in close contact with the abutment surface. The new bone formation (B) had grown above the implant shoulder (IS) (acid fuchsin–toluidine blue 20 $\times$) ([146], CC BY 4.0).

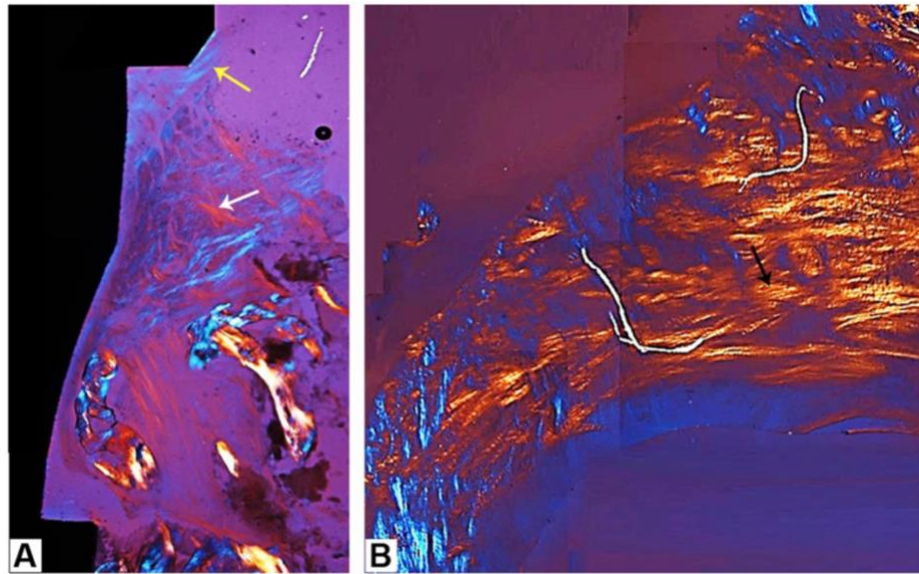


Figure 4.2.1.9. Polarized light microscopy of the peri-implant soft tissues: **(A)** longitudinal bundles distributed parallel to the surface of the abutment (yellow arrow) in the coronal portion, while in the apical portions—and far away from the implant surface—there was a lattice composed of interwoven bundles (white arrow); **(B)** the transverse section with the majority of semicircular collagen bundles (black arrow) around the abutment (acid fuchsin–toluidine blue 40×) ([146], CC BY 4.0).

Discussion

The causes of dental implant failure can be divided into two broad categories: biological (for instance, peri-implantitis) or mechanical (due to fracture of the implant, fracture of the abutment, loosening or loss of the connecting screw, and fracture of the prosthetic structure). The presence of an incorrect coupling, i.e., misfit between the implant and the abutment, is known to be the basis for an increase in mechanical stress on the connection structures and the surrounding bone tissue. This condition can lead to biological consequences due to bacterial penetration into the gap that occurs between the fixture and the abutment.

While many authors have reported on the importance of the characteristics of the fixture–abutment interface on the level and loss of peri-implant bone tissue [147], few studies have examined the structure and ultrastructure of human peri-implant soft tissues [100,101,125,129,131,132]. The 3D networks of collagen bundles that compose the connective portion have been found to be transversely and longitudinally oriented in relation to the dental implant axis. During the growth of these bundles, they organize themselves into intertwining patterns [100,101] with

different quantitative distributions depending on time from surgery, environmental conditions, and implant macro- and micro-geometry; these boundary conditions are currently the focus of several studies [100,101,125,129,131,132].

A conometric prosthetic restoration system has been reported by some authors as a means to fix the prosthetic restoration to the abutment, obviating the use of retention through the use of screws or cement. The use of this technique offers very good clinical results; however, while we were able to find relevant histomorphometry evidence of the good performance of peri-implant crestal bone [145], no definitive evidence of the 3D structure of peri-implant connective tissues around conometric prosthetic restorations has been documented.

In the present case study, through the use of micro-CT, the total absence of 3D gaps in the investigated conometric abutment–prosthetic cap connection was verified. This is extremely relevant for the *in vivo* service of the implant because it protects the implant from bacterial contamination.

Histological analysis of the peri-implant tissues around this human-retrieved conometric connection implant showed a lack of inflammation, most likely due to a reduced percentage of bacterial leakage when using this type of implant–abutment connection. This is in agreement with previous studies [125,126]; indeed, the few studies that have reported on bacterial contamination at the abutment–prosthesis coupling level demonstrate either an absence of a complete bacterial seal or no bacterial infiltration into and from this coupling. One of these studies reported an *in vitro* investigation of the microleakage of bacteria in three different implant connections for a period of 14 days [148]; in this case, 60 dental implants were distinguished into three groups, according to the type of connection: external hexagon, internal hexagon, and conometric connection. All implants were immersed in a bacterial suspension into the surrounding solution. Less bacterial leakage and a lower rate of infiltration were found in conometric connections when compared to hexagon connections. Moreover, other microbiological studies confirmed a very low percentage of bacterial leakage in conometric connection Cone Morse implants [149,150]. These results could, most likely, be related to a very precise fit between the two components, as was shown through the use of micro-CT in the present study. Furthermore, in the present investigation, with reference to the sample implanted *in vivo* for a period of 45 days, growth of supracrestal bone over the shoulder of the implant was observed. This was also observed in another study on dogs [151] aiming

to evaluate the histological and histomorphometric differences at the marginal bone level when using two different implant–abutment assembly designs (namely the traditional external hexagon and the Morse Cone tapered connections). In this case, it was shown that subcrestal placement had a positive impact on crestal bone remodeling in Morse Cone implants.

In the sample implanted in vivo for a period of 45 days, a large amount of correctly organized peri-implant connective tissue was also observed through synchrotron investigation and subsequent analysis of 3D morphometric parameters. It was found that the quantity and connectivity of transverse bundles were greater than those of longitudinal bundles; furthermore, it was found that the transverse bundles were better aligned than the longitudinal ones. This is in line with a previous study that found that even in mature and functional peri-implant connective tissue, the proportion between longitudinal and transverse fibers is always against the former [101]. Despite the promising results obtained in this single case study, it is necessary to carry out further tests in a statistically significant number of conometric prosthetic connections, both in static conditions—to confirm the present evidence—and in cases subjected to chewing load. Indeed, as suggested in previous studies [152,153], including synchrotron-based ones [154,155], elucidation of the mode of mechanical behavior of the implant–abutment connection under various loading scenarios will provide information to enhance the design and function of the connections and minimize the in-service failure sometimes encountered to date.

According to the authors' best knowledge, this clinical case constitutes the first quantitative analysis of the 3D microarchitecture and properties of the peri-implant connective tissues surrounding a conometric prosthetic connection with proven absence of macro- and micro-gaps in static conditions.

The peri-implant soft tissues, as revealed by cross-linked light microscopy and synchrotron-based imaging, appeared to be rich in collagen bundles, with a functional intertwined organization, and tightly adhered to the abutment surface. Thus, this intertwined organization of the collagen bundles, already after just 45 days from implant surgery, seems promising and worthy of further study with the objective of searching for a favorable stabilization of the soft tissues, which could thus constitute an effective barrier against the apical migration of the inflammatory cells towards the bone.

4.3 Artificial Intelligence in CBCT: New Frontiers in Radiology

For the purpose of these analyses, Cone Beam Computed Tomography (CBCT) images are often used, which are very common in 3D analysis in dental offices and therefore easily available in the formation of an input dataset for artificial intelligence. The use of convolutional neural networks in particular has also made it possible to automate the diagnosis of pathologies such as inflammatory lesions and oral cancer [11,17,156,157]. In addition, following an intraoral scan it is also possible to exploit the learning capacity of deep neural networks in order to manufacture customized hybrid abutments. In particular, they would identify the margin line and then define the CAD design project [158]. Segmenting teeth or subcrustal regions individually may also be another way of applying these new techniques in dentistry. In fact, by segmenting the first molar it would then be possible to identify the length and position of the root canal [159].

Moreover, Xi R et. al. used artificial intelligence (AI) to analyze alveolar bone for periodontitis in a mouse model. Their goal was to develop an automatic deep-learning segmentation model that allows researchers to examine alveolar bone from micro-computed tomography (μ CT) data without prior machine learning knowledge [160].

The aspect that will be covered in this article is firstly how the semantic segmentation of Cone Beam images takes place aided by Artificial Intelligence, particularly Deep Learning algorithms; secondly what quantification approaches can be applied to the different segmented regions. Such as bone analysis, bone density and verification of bone loss, teeth structural evaluation and position relative to sub-crestal zone, which is crucial for the correct positioning of implants.

Materials and Methods

Cone Beam Computed Tomography Samples

3D images of CBCT (*Cone Beam Computed Tomography*) of 15 patients were selected. Stacks of images of patients before and after implant surgery were taken for the network to learn to recognize both teeth and implants and devitalization. In this way, the algorithm will be able to generalize and thus recognize as many structures as possible. Each stack consists of 165 axial slices, each at a resolution of 300 μ m. The Field of View (FOV) is

11×5 cm. The maximum X-ray source reaches 90 kV with a current of 3 mA, the Time of Exposure is 3.6 s.

Training and Test Data preparation

The cone beam images were first normalized, converting them from unsigned 16 bits to float and then, since they presented countless artifacts, they were filtered in order to reduce the noise. The Mean Shift with kernel at 5 was selected as the smoothing filter and then the CLAHE was applied for contrast enhancement [161].

Each image was cut into boxes with the same size as the condition of input for the Deep Learning tool. The training size for each image is 200×225×50 pixel for jaws and 260×225×50 pixel for mandibles.

Only the essential part of all Cone Beam images was selected to be focused on the features that will be learned by the neural network and don't lead it into mistakes.

Image segmentation technique

125 slices in total were manually segmented among the 15 patients.

Dragonfly's segmentation tool was crucial to being accurate at this stage of the work process [Dragonfly 2022.2 [Computer software]. Comet Technologies Canada Inc., Montreal, Canada; software available at <https://www.theobjects.com/dragonfly>]. The semantic segmentation method applied to images has the task of assigning a label to each group of pixels based on its intensity characteristics (grey tones) and morphometric aspects. Thus, it will be possible to distinguish gutta-percha, implants, teeth, and bone tissue and assign to each of them a different color.

U-Net was chosen to achieve this goal. It was developed by Olaf Ronneberger et al. [109] and was particularly designed for biomedical image segmentation. [108] The architecture is composed of two parts: the encoder, a contracting path that extracts context from the image and locates key elements, and the decoder, a symmetric expanding path that makes use of learned pooling layers to enable accurate localization of features.

U-Net structures and parameters

U-Net was the neural network model used for training, in which 125 CBCT slices were selected as input and segmented manually. The subdivision between training (80% of the entire dataset) and validation (20% of the entire dataset) set was done internally by the Dragonfly AI tool [66].

Four classes were chosen to be segmented: bone, teeth, implant and background. Training parameters chosen after several tests and set before starting the algorithm are reported in Table 4.3.1.

Table 4.3.1. Setting of training hyper-parameters.

U-Net	
Class count	4
Depth level	4
Initial filter count	64
Patch size	64
Stride ratio	1
Batch size	32
Epochs number	100
Loss function	Categorical Cross-Entropy
Optimization algorithm	Adadelta
Metrics	Categorical Accuracy
Early stopping	NO
Reduce learning rate on plateau	YES
Training/Validation	80/20

Data augmentation method was applied to further differentiate the dataset and make the network more generalizable. It is often used to obtain a wider dataset, trying to avoid overfitting and redundancies. Deforming images by geometric techniques allows to increase dataset and give greater strength to the network. Therefore, both the original and augmented images are entered as input into the algorithm, which not notice the difference between them. The Online version [105] was used inside the Comet Dragonfly AI tool.

Results

U-Net Training Accuracy

To evaluate the neural network performances and to validate the training results some parameters were extracted from the Dragonfly evaluation tool. The Accuracy which assesses whether a series of measurements are correct on average, the DICE coefficient which is used to compare the pixel-wise agreement between a predicted segmentation and its corresponding ground truth. It was calculated as the 2 times the area of overlap divided by the total number of pixels in both the images. False Negative (FN), False Positive (FP), True Negative (TN) and True Positive (TP) rates define how many pixels can be considered respectively as false negative, false positive, true negative and true positive. The True Positive Rate (TPR) or Recall is also known as Sensitivity and measures the proportion of actual positive instances that are correctly predicted as positive; while the True Negative Rate (TNR) is also known as Specificity and measures the probability that an actual negative will test negative. All these parameters are reported in Table 4.3.2, in which firstly all parameters were calculated on the amount of segmented dataset. Subsequently, measurements were repeated for each class.

Table 4.3.2. Statistical rates for comparing image segmentation prediction and the ground truth.

	<i>Accuracy</i>	<i>DICE</i>	<i>FN*</i>	<i>FP*</i>	<i>TN*</i>	<i>TP*</i>	<i>TPR</i>	<i>TNR</i>
Total	0.990	0.980	121802	121802	17833198	5863198	0.980	0.993
Bone	0.981	0.916	83636	30399	5252312	618653	0.881	0.994
Implant	0.990	0.963	1497	1104	5948227	34172	0.958	0.998
Teeth	0.997	0.949	11394	7835	5788108	177663	0.940	0.999
Background	0.982	0.989	25275	82464	844551	5032710	0.995	0.911

**Number of pixels*

Segmentation CBCT Images

The U-Net neural network already shows in initial training, with 125 slices in total, very good performance in segmenting the different anatomical components found in CBCTs

(Fig. 4.3.1C). The purpose of this segmentation is also to be able to measure thicknesses in separate locations of bone, teeth and implants in a fully automated way. This is done both to determine the diameter of each tooth at the subgingival level of implant placement in the pre-surgical stages and to ascertain the correct placement of implants in the post-surgical stages. For this purpose, the minimum, average and maximum Feret Diameter can be useful parameters. It is used to calculate the diameter of nongeometric figures by measuring the distance between two parallel planes tangent to the lateral extremes of the figure. On each tooth therefore this parameter can be calculated, once the segmented 2D slice is selected (Fig. 4.3.2).

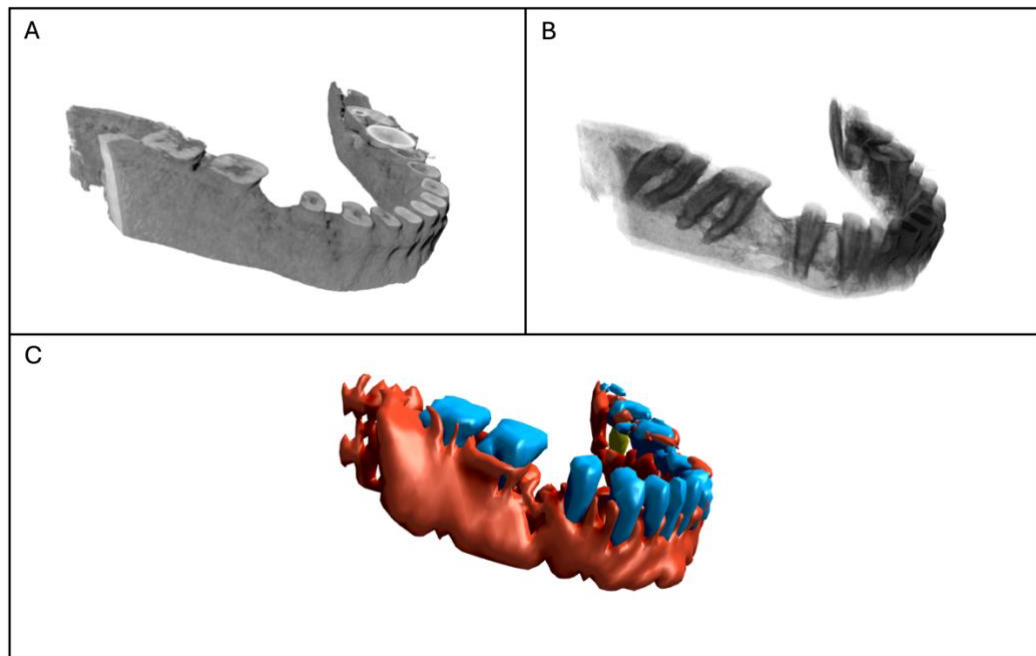


Figure 4.3.1. Example of original dataset trimmed (A), its visualization with Edge Contrast filter (B) and its corresponding segmented in the different classes: bone (red), teeth (blue), implant (yellow) (C).

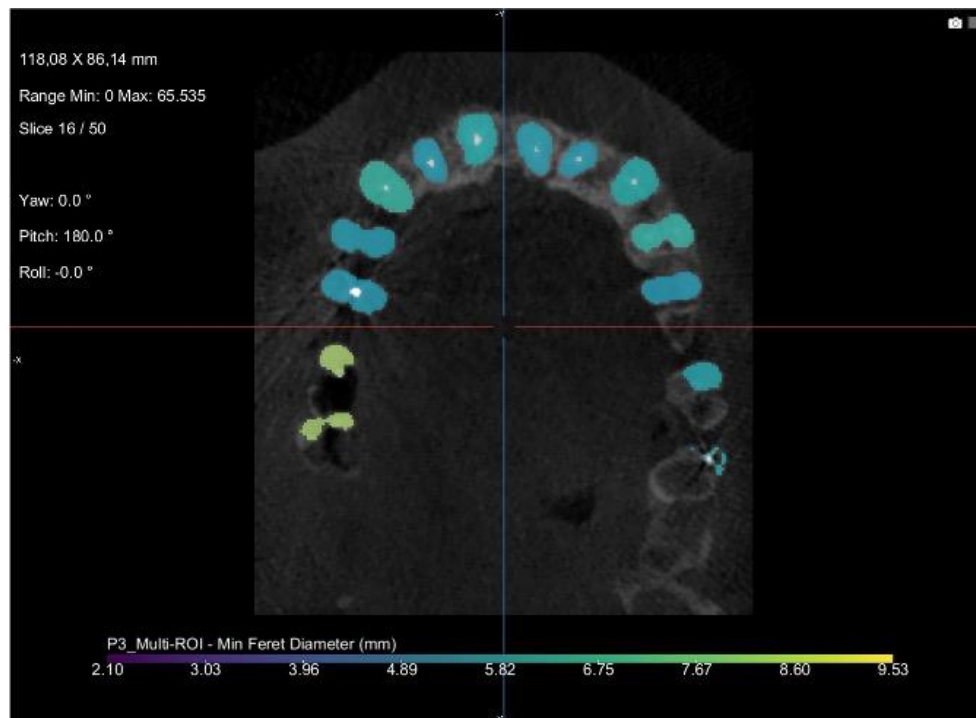


Figure 4.3.2. 2D slice selected in which only the class of teeth was shown, in particular *Min Feret Diameter* was calculated for each tooth and its dimension is reported in mm.

Bone Analysis

In addition, it is essential to analyze the state of the patient's bone. In this case, the Dragonfly software also allows for a Bone Analysis by inputting the bone Region of Interest (ROI), segmented by the network before, as input and providing a bone status report. It includes the common bone morphometric measurements (Table 4.3.3). In a whole-bone region of interest, this module can independently detect cortical and trabecular bone before computing the global measurements. Examples include area and perimeter fractions, which can differentiate the proportions between cortical and trabecular bone, mean thickness, anisotropy, which can help investigate the relationships between observed structural features and a bone's mechanical function, and volume thickness, which can offer specific local information.

Figure 4.3.3 shows an example of the 3D Anisotropy rendering map, which reports the measures of the different orientations in the segmented trabecular bone. Thus, it is possible to understand how masticatory forces act along the perimeter of the trabecular bone. In fact, where the regions are more oriented (closer to 1), it stands to indicate a

constant presence of load that is well oriented in one direction. Whereas the more isotropic regions (closer to 0) go to indicate a situation where the tissue is not well oriented and therefore the chewing forces affect it weakly. In particular, it can be seen that in bone-deficient regions the arrows are bluer, thus tending toward 0 and therefore less anisotropic. This indicates that in the less affected areas masticatory load the trabecular bone is less oriented.

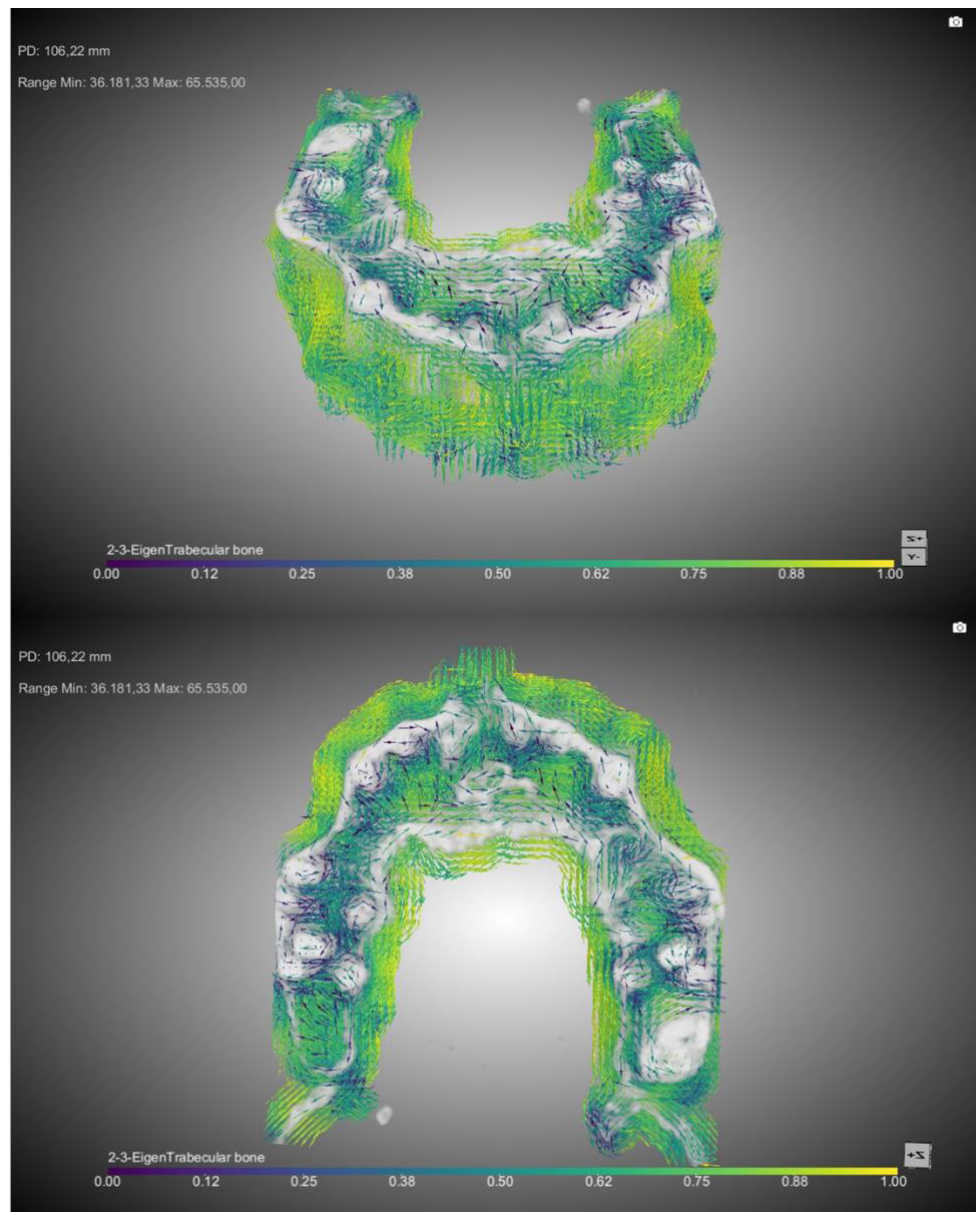


Figure 4.3.3. Degree of Anisotropy in Bone Analysis tool. The arrows show the different orientations of bone boundaries along the jaw.

Table 4.3.3. Example of bone analysis' report for one patient extract from Bone Analysis tool.

Abbreviation	Title	Value
Ani. MIL	Anisotropy (MIL)	0.39
Ani. SVD	Anisotropy (SVD)	0.25
BV	Bone Volume	7.794 mm ³
BV/TV	Bone Volume fraction	0.69
Ct. Ar	Average cortical area	487 mm ²
Ct. Ar/Tt. Ar	Average cortical area fraction	0.64
Ct. Th	Average cortical thickness	2.01 mm
Ec. Pm	Endocortical perimeter	257.30 mm
Ec. S3D	Endocortical surface (3D)	5550.22 mm ²
Ma. Ar	Average marrow area	269.84 mm ²
Ps. Pm	Periosteal perimeter	254.55 mm
Ps.S3D	Periosteal surface (3D)	14203.09 mm ²
TV	Total volume	11354.71 mm ³
Tb. Sp	Average trabecular separation	1.72 mm
Tb. Th	Average trabecular thickness	1.23 mm
Tt. Ar	Average total (cortical + marrow)	756.98 mm ²

Discussion

Nowadays, several studies have presented artificial intelligence applied in the dental setting and particularly in implant surgery [4,162]. Through image analysis using segmentation techniques and image quality improvement, artificial intelligence (AI) was applied to radiographic pictures, particularly in the field of oral and maxillofacial radiology, to aid in diagnosis and the development of treatment regimens [101,146,163]. In addition to planning therapies and offering guidance to the physician during the diagnostic phase, AI image processing systems can detect features that may elude human analysis, such as the presence of cavities and restorations [164]. Viewing these works, in this preliminary study, it was shown how it is possible to segment different maxillofacial districts, by means of a convolutional neural network [16], in order to help the dentist with information related to tooth position and bone health status. In addition, analysis of the bone demonstrates how forces can change the morphology of the tissue itself and how

they go to influence the Anisotropy parameter. All these steps of tissue analysis using neural networks could be integrated into a single user-friendly support tool with a graphical interface that allows the dentist to view CBCT images, segment them, extract parameters and make considerations.

In the subsequent stages of the study, an artificial intelligence method will be used to help the development of a system that will be proposed to determine the optimal amount of implant implantation. With this information, the dentist will be able to provide a recommendation on the depth of the implant placement. One of the benefits of digitization in the field of dentistry is that it allows for more accurate detection of potential diseases, more precise implant placement, automatic identification of cavities, and evaluation of bone tissue. In addition, evaluating the latter becomes extremely important when selecting the implant itself, as the structure of the implant is dependent not only on the tooth that is being replaced but also on the density of the bone that is underneath it.

The objective of this study was to emphasize how the utilization of innovative methodologies using AI can assist doctors in diagnostic and treatment planning. Specifically, one can identify the individual tooth requiring treatment, assess its dimensional attributes, and thereafter select the suitable implant or intervention. In this way it will be possible to minimize the patient's discomfort and also facilitate the rehabilitation processes in the phases following the insertion of the dental implant.

4.4 Mechanical Testing Dental Implants: Performance for Lasting Reliability

A mismatch between fixation and abutment in dental implants can have two main types of implications: biological complications, such as peri-implantitis, and mechanical complications such as loosening or loss of the fixation screw and fracture of the prosthetic superstructure [165]. Therefore, a good fixation-abutment fit can determine the success of the implant and a good osseointegration [166]. Otherwise, the presence of the micro-gap can induce implant failure due to possible bacterial penetration and therefore create an inflammatory infiltrate between the components.

In this context, the availability of a peri-implant mucosa of adequate size and organization certainly plays a primary role, which would allow a correct continuum between epithelial and connective tissue, avoiding bone resorption [167,168]. Furthermore, the implant-abutment interface also controls the degree and location of peri-implant inflammatory cell accumulation, which can contribute to the extent of implant-associated alveolar bone loss [169]. Another key role seems to be played by the presence of micro-gaps between fixation and implant [170,171]. According to Schmitt et al. the conical connection provides an ideal fit between the implant and the abutment, resulting in isolation of the internal part of the implant from the oral cavity. In this way, microleakage and the risk of rapid loosening are reduced. Furthermore, the load of the conical connection is transferred along the two constructions, ensuring an even distribution of forces and providing greater stability also from a mechanical point of view [172,173]. Moreover, in a literature review on different implant connections, Vinhas et al. showed that Cone-Morse connections are less prone to mechanical complications, such as fractures and screw loss [174].

Despite the success demonstrated by conical connections in the previous studies, the incidental presence of micro-gaps in conical connections continues to be repeatedly investigated within different frameworks and at different resolutions. Among these, worthy of mention is a study that used synchrotron radiation to analyze, during compression tests up to 100 N, the mechanical behavior in the coupling area of conical implants, also attempting to detect micro-movements [175]. Another study recorded, by means of a high-resolution and high-speed X-ray camera, a chewing simulator with dynamic and static loading up to 200 N on twenty implants, some with conical implant-abutment connections and others with flat connections; the conical connections did not

show micro-gaps, while some micro-movements were found for the flat connections both in static and dynamic loading conditions [176]. However, the role of the micro-gap size is still controversial in literature: indeed, despite the above findings, other studies showed that crestal bone alterations around titanium implants are considerably influenced by potential movements between implants and abutments but not by micro-gap size [177]. Furthermore, some groups investigate preload loss and bacterial penetration at the implant-abutment interface of conical and internal-external hexagon connection systems exposed to temperature cycling and mechanical wear, in order to determine the influence of implant geometry on bacterial penetration and load deformations as a result of testing the different models [178–181].

Micro-computed tomography (micro-CT) has allowed to study the effectiveness of the seal between implant and abutment in different contexts [182–185]; in fact, there are several advantages in using this imaging method for its non-destructive, non-invasive, three-dimensional approach, without requiring any sample preparation. Researchers demonstrated optimal adhesion between the abutment and the prosthetic coping in a conometric system, moreover histological examination and polarized light microscopy revealed abundant connective tissue surrounding the abutment obtained from the patient. In addition, to demonstrate implant effectiveness, a quantitative assessment of the connective tissue utilizing synchrotron imaging, enhanced by artificial intelligence, indicated that this tissue was abundant in mature collagen, featuring interlaced longitudinal and transverse collagen bundles [146].

This study aims to evaluate and measure the possible presence of micro-gaps in an innovative Morse cone connection, before and after having subjected it to a series of two successive load tests, the first up to 150 N and the second up to 300 N.

Materials and Method

Experiment Workflow

Tests to detect the possible presence of micro-gaps were carried out by micro-CT analysis.

In the first phase of the work, a total of 7 implant samples were acquired (Figure 4.4.1b). Each of them encased in plexiglass in the basal part (Figure 4.4.1c). On 2 of these (group

1) the cup was inserted and therefore only the apical portion was acquired to evaluate the junction with the underlying abutment. On the remaining 5 samples (group 2), the connection portion between the fixture and the abutment was analyzed.

In the second phase of the work, 2 implants from group 2 were selected to be subjected to mechanical tests.

In order to be mechanically tested, these samples were encapsulated in plexiglass at an angle of inclination of 60° relative to the horizontal plane (Figure 4.4.1c).

The compression test was performed twice on each sample: the first test was conducted up to a load of 150 N, while the second up to 300 N. After each load test, a micro-CT scan was performed to evaluate any morphometrical mismatches between pre-compression, post-compression at 150 N and post-compression at 300 N.

Micro-CT images allowed us to analyze the samples in three dimensions, so that we could evaluate all the internal connections and interfaces between the different components in subsequent implants. The acquisition pixel size was set to 7.93 µm, which means that any micro-gaps with dimensions <10 µm are not detectable in this type of investigation. However, the detection of micro-gaps up to this level of detail still allows us to evaluate the quality of the manufacturing processes and the robustness of the materials. In particular, static acquisitions were necessary to evaluate the manufacturing methods while acquisitions following the mechanical tests allowed us to ascertain the quality of the product in its actual application.

Implants' Manufacturing

The investigated biconometric dental implants (AoN Implants Srl, Grisignano di Zocco (VI) - Italy) were endo-osseous screws designed for dental surgery; they can be implanted in previously prepared sites of edentulous areas, according to specific operative sequences. A biconometric connection (Figure 4.4.1a) is made of three components: (1) the implant (or fixture); (2) the abutment, which is coupled to the implant via a Cono Morse connection; (3) the cap, coupled by friction to the abutment via a 4° conometric connection. The material used for the implant production was cold-drawn Gr. 4 Titanium; it was selected for its strength and because it is not contaminated by other elements. The machining process was carried out through a numerically controlled machinery, nominally maintaining control down to 1 µm details. During production, the produced components were checked by the systems VICI Vision (Vici & C S.p.A., Santarcangelo

di Romagna (RN), Italy) and Hexagon OPTIV M (Hexagon Metrology Spa, Grugliasco (TO), Italy). The body of the implant was structured with a two-main loop so that, with each revolution, the screw advances twice as much as a classic pitch implant, without creating stress in the recipient bone. Afterwards, in the post-production checks, a thorough cleaning was performed to completely degrease the parts with an ultrasonic metal washer (ILSA-MC Srl, San Pietro in Casale (BO), Italy); moreover, each component was checked for quality by sight and centesimal calipers, micrometers, pin calipers and internal and external thread calipers. Subsequently, a final cleaning was performed, and the product was bagged in medical paper. The abutment, made of Gr.5 Titanium, was washed and checked as described for the implant; furthermore, the prosthetic cap, also made of Gr. 5 Titanium, was checked with the previously described instruments. The Abutment-implant coupling was performed by fixing the screw at 30 Ncm, as from manufacturer's instructions, while the cap-abutment coupling was performed using a calibrated instrument by applying a force of 30 Ncm axial to the head of the cap, as previously reported.

X-ray Micro-CT Analysis

Metro-Tom 1500 ZEISS (Carl Zeiss Industrielle Messtechnik GmbH, Oberkochen, Germany) was used for three-dimensional (3D) micro-CT analysis, setting the x-ray source with a voltage at 200 kV and the current at 40 μ A; 1.5 mm Al filter; pixel size = 7.93 μ m and 180° rotation in 0.18° steps with an exposure time of 7s per projection. Two hours of scanning time were spent for each specimen, with a total of about 2050 projections acquired per sample.

Image segmentation and Image Registration

All images were elaborated with the Dragonfly software (Dragonfly 2022.2 [Computer software]. Comet Technologies Canada Inc., Montreal, Canada; software available at <https://www.theobjects.com/dragonfly>) [66]. The software was used in the image processing stage, which allowed the segmentation of the different internal components of the implant samples and the detection of any internal change caused by the compression tests.

Registration between the 3D reconstructions performed after the 150 N load and the one after the 300 N load was performed using the Dragonfly Image Registration tool. This software-module can automatically register datasets by applying scaling, rotations, and translations required to match features between two datasets. Two matching processes are made available by the software for registering datasets: the Mutual Information and the SSD (sum of squared differences). These methods are based on different concepts to quantify the degree of similarity between images and to apply the required linear transformations. Results may differ significantly: the performance of each algorithm is also dependent on the input data and the mode (Basic or Advanced) selected for registration. After several trials, we chose the SSD method in the Advanced mode.

Mechanical Compression Tests

The compression tests were performed on an INSTRON 5567 machine (Instron, Norwood, United States) equipped with a load cell with a maximum load capacity of 1kN. The speed of the crosshead was kept constant at a value of 5 mm/min during the test. Both samples were tested twice: a first compression test up to a maximum load of 150 N and a second compression test up to a maximum load of 300 N. At the end of each test, the sample was unloaded immediately after reaching the maximum load. The load was applied directly to the upper part of the implant in a direction perpendicular to the base of the sample (Figure 4.4.1c). The curves of the tests were determined as compressive displacement (x-axis) and compressive load (y-axis) for both tested samples.

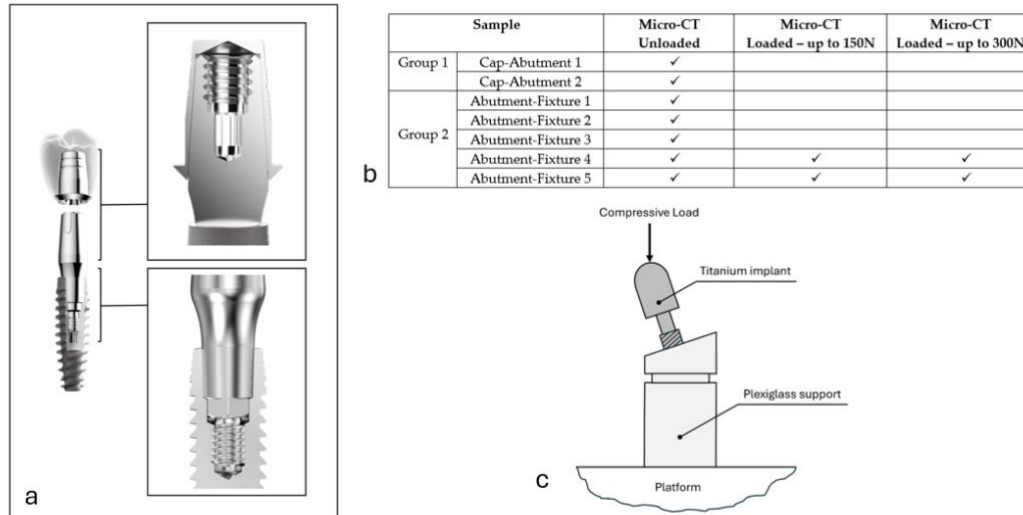


Figure 4.4.1. (a) Biconometric connection made of three components: fixture, abutment, and cap; (b) table summarizing groups and sample size; (c) scheme of the titanium implant screwed in the plexiglass support and subjected to an external compressive load. The arrow indicates the direction in which the load was applied.

Results

Micro-CT Analysis

Figure 4.4.2 shows the different portions acquired in static conditions before the mechanical compression tests with the ZEISS Metro-Tom 1500. In the apical portion (Figure 4.4.2a, b), the positioning of the cup on the abutment is studied, while the implant-abutment junction is observed in the lower part (Figure 4.4.2a, c). The portions rendered in yellow or red represent the structural voids inside the sample, respectively in the apical portion or in the implant-abutment junction.

The sharp difference in grey tones between the background (i.e., voids) and the titanium material allowed segmentation of the brightness histograms using the thresholding method. From the highlighted voids, there was no evidence of micro-gaps either between the implant and the abutment or between the abutment and the surrounding cup. Therefore, at the resolution determined by the pixel size (i.e., 7.93 μm), the presence of micro-gaps of size $> 10 \mu\text{m}$, resulting from the manufacturing process of the implant, can be excluded.

In summary, all micro-CT analyses performed on the post-production samples (static conditions) excluded the presence of micro-gaps both in the 2 samples of group 1 (referring to the study of the cup/abutment interfaces) and in the 5 samples of group 2, referring to the study of the fixation/abutment interfaces.

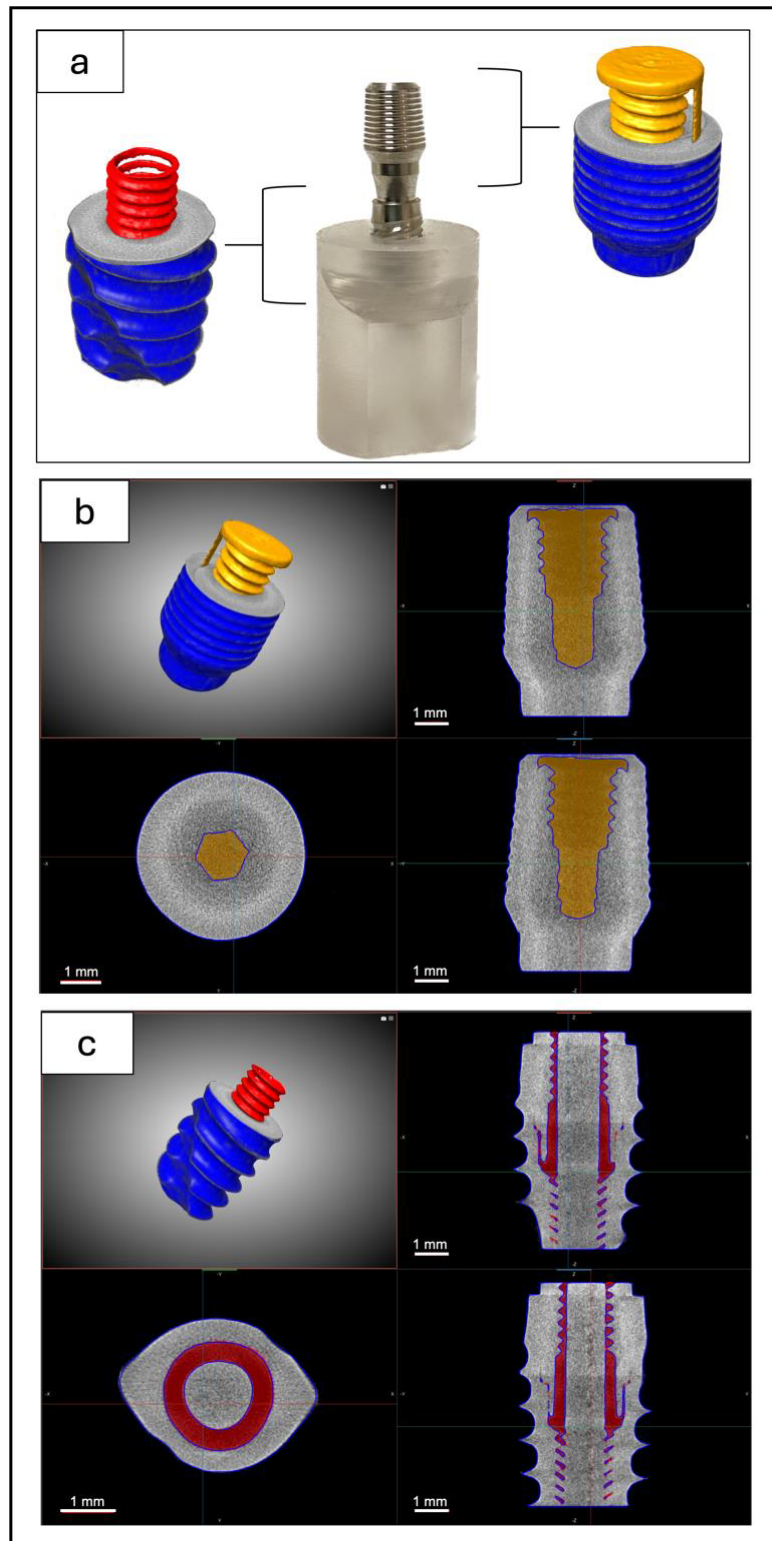


Figure 4.4.2. (a) Micro-CT 3D reconstructions: different portions in representative samples imaged in static conditions; (b) apical portion: positioning of the cup on the abutment; (c) implant-abutment junction. The portions rendered in yellow or red represent the structural voids inside the samples, respectively in the apical portion or in the implant-abutment junction.

As a second experimental step, the 3D scans were repeated on two samples of group 2, both after compression up to 150 N and after compression up to 300 N load.

The vertical compression on the implant did not show evident signs of fractures. In fact, no micro-gaps were recorded in the junction between the implant and the abutment in either of the two samples, neither after reaching the load of 150 N nor after loading up to 300 N. To further confirm this result, a registration was performed between the scan after the 150 N-load and that after the 300 N-load using Image Registration Dragonfly's feature-based registration workflows. It can automatically register datasets by applying the scaling, rotations, and translations required to match features between two datasets.

Two matching processes: *Mutual Information* and *SSD* (sum of squared differences) are available for registering datasets. These methods are based on different concepts to quantify the degree of similarity between images and apply the required linear transformations and that results may differ significantly. The performance of each algorithm is also dependent on the input data and the mode (*Basic* or *Advanced*) selected for registration. After several trials, we chose *SSD* method in the *Advanced* mode.

In the superposition of the outputs, no evident signs of internal fractures at the fixture-abutment interface were found, as shown by white arrows in Figure 4.4.3.

In particular, no micro-gaps are evident for all samples at different compression levels, in the overlying apical junction as displayed in the magnifications of Figure 4.4.3.

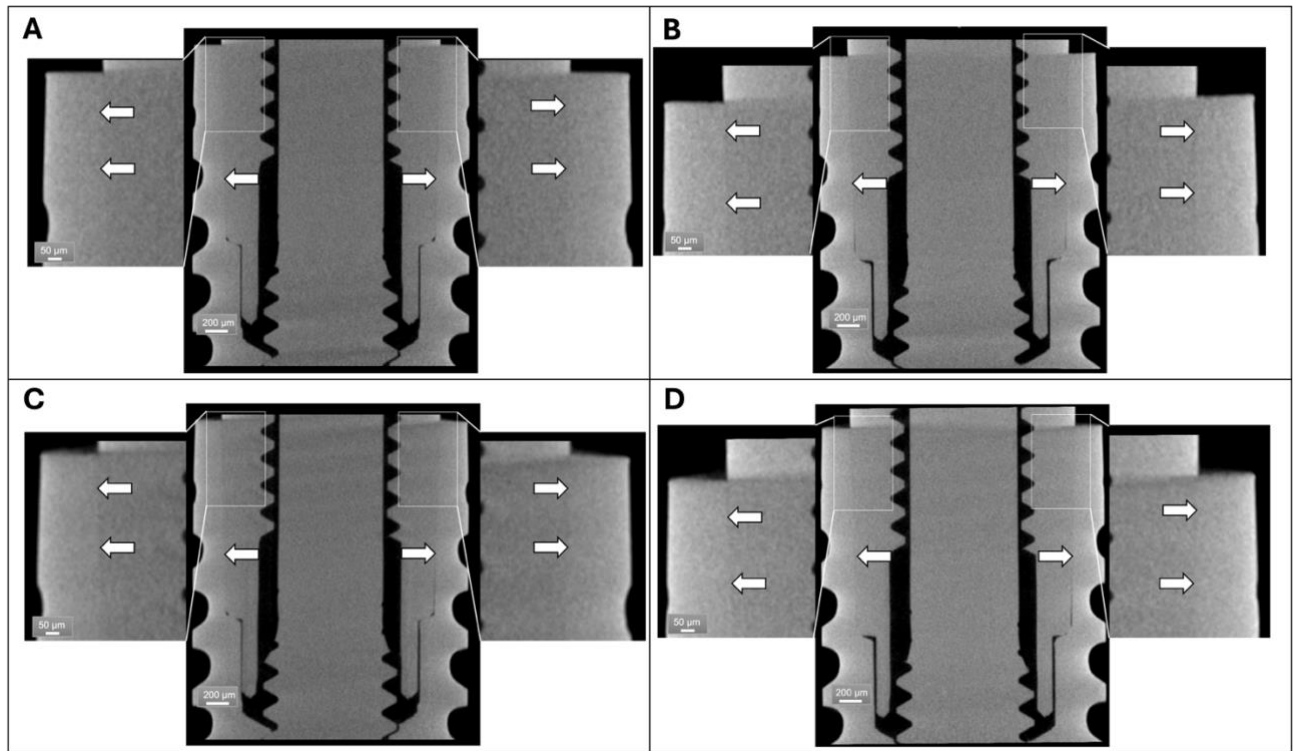


Figure 4.4.3. Micro-CT reconstruction in the sagittal section of (A) Sample 1 compressed at 150N; (B) Sample 2 compressed at 150N; (C) Sample 1 compressed at 300N; (D) Sample 2 compressed at 300N.

Mechanical Tests

The load-displacement compression curves were registered for each test performed (Figure 4.4.4). The curves revealed, in both samples, that the tests performed at the maximum load of 300 N have a greater slope than the first tests carried out at the maximum load of 150 N. In addition, for both applied loads, a deviation was observed between the curves of the two samples tested, greater than the possible uncertainty of the machine. This phenomenon could be explained by the fact that the preparation of the samples is a manual process, which can lead to slight differences between each sample. Finally, in all the tests performed, the components did not reach their compressive strength and remained structurally intact, with no visible cracks or damage after the load has been removed.

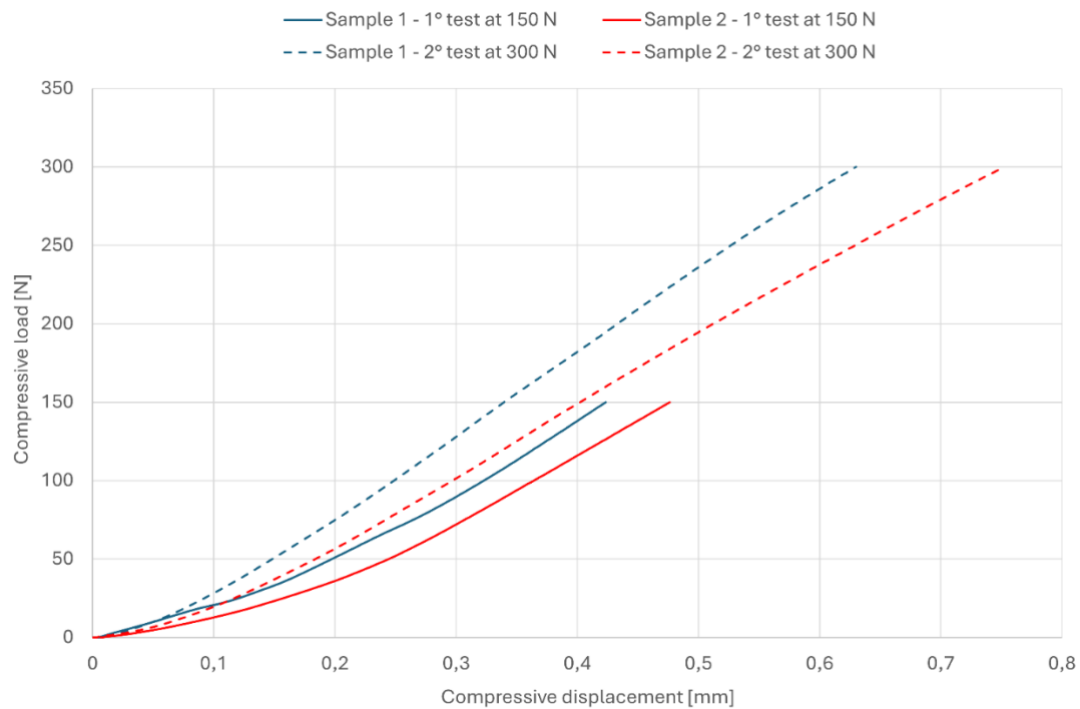


Figure 4.4.4. Load-displacement compression curves. The curves are shown for each samples tested, up to a maximum load of 150 N and 300 N, respectively.

Discussion

Several studies reported that the functional load transmission to the fixture and connection screw is influenced by the abutment connection area's design [186–189].

In particular, the type of material used, and the production process of the implant can influence the presence of micro-gaps [190,191]. They can lead to bacterial infiltration or structural fracture of the implant with subsequent implant failure.

Thus, innovative conometric dental implants, characterized by the selection of Gr. 4 Titanium, the processes controlled at each step at the μm of detail and the application of Osteo-Pore treatment, were analyzed, through the micro-topographic technique, to check their behavior under stress and to ascertain that their structural characteristics remained unaltered. In this context, micro-CT technology has frequently proven to be the ideal method to carry out high resolution surveys in a non-destructive and non-invasive way, without any sample preparation.

Our investigation showed that, although the minimum load needed to bridge the internal implant space was within the range of human biting strength, the conical connection demonstrated greater resistance against the creation of micro-gaps at the implant–abutment contact than the external hexagonal connection [192]. These results support the outcome of a previous study where different connections were compared, and the conical connections resulted in less microleakage from the implant-abutment interface than internal parallel connections [180].

Recent study has led to the identification of various connection types to determine the optimal geometry of the implant. Consequently, the transmission of occlusal load to the bone was enhanced, and micro-gaps at the implant-abutment contact were minimized, thereby preventing bacterial colonization. The review [193] determined that conical connections appear to be more beneficial, exhibiting less peri-implant bone loss, thus crestal bone levels are more effectively preserved.

Our investigation also confirms the suggestion for the studied samples. For these reasons, the Cono-Morse connections were also evaluated from the perspective of micro-X-ray tomography, trying to detect the possible presence of micro-gaps through advanced image processing techniques.

The curves documented following mechanical tests suggested that, in both samples, the tests conducted at the maximum load of 300 N exhibited a steeper slope than the initial tests performed at the maximum load of 150 N. However, they have not exhibited any fractures, obvious cracks, or damage after the weight was removed. The components did not attain their compressive strength and remained structurally intact.

Furthermore, upon three-dimensional dissection of the specimen images, no indications of fractures or micro-gaps were observed at the apical regions, considered most critical for bacterial infiltration due to their increased exposure at the implant's apex. Consequently, at the experimental resolution of 7.93 μm , it can be deduced that conical implants, perhaps owing to their geometry, facilitate increased contact between the fixture and the abutment, thereby mitigating bacterial infiltration and potential implant failure.

The absence of micro-gaps, as here demonstrated, in combination with the renewed structural typology of the plant prove the stability and in-service functionality over time of the proposed plants.

4.5 Decoding Periodontal Health: A Machine Learning Approach for defining staging and grading of periodontal diseases and conditions

Periodontitis ranks as the sixth most widespread disease globally. It is a chronic multifactorial inflammatory disease caused by dental biofilm periodontopathogens, alongside with an altered host's immune response. Untreated periodontal disease leads to the progressive destruction of periodontal supporting tissues, eventually resulting in tooth loss [194,195]. To develop a treatment plan and prognosis for each patient, it is mandatory to establish a clear and objective diagnosis based on defined criteria. The latest classification of periodontal and peri-implant diseases and conditions was developed and adopted by the American Academy of Periodontology (AAP) and the European Federation of Periodontology (EFP) at the joint World Workshop in 2017. Periodontal disease is defined as periodontitis, which is further characterized by a staging and grading system, to define the condition in greater detail. The severity of the disease and treatment complexity are determined by its stage, while grading represents a risk for disease progression and possible treatment outcomes. Four stages (I, II, III, and IV) and three grades (A, B, and C) are distinguished [196]. Accurate classification of periodontal diseases is crucial for dental professionals, enhancing diagnosis, prognosis, and treatment planning. The staging and grading system has improved risk assessment and treatment effectiveness, but it is complex and time-consuming. Implementing novel technologies, such as machine learning (ML), can aid dentists in providing better care while reducing treatment duration and costs [197,198]. Various data types, including radiographs, photographs, ultrasound images, clinical data, Electronic Health Records, and saliva markers, have been utilized to form comprehensive databases [5]. Kim et al. [199] have investigated the possibility of predicting periodontal disease severity using salivary biomarkers, while Rana et al. developed an automated system that conducts pixel-level segmentation of intraoral images to identify gingival inflammation and prevent periodontal disease progression [200]. Many ML algorithms have been developed over time and those used for classification have been elaborated and tested in this study. Few researchers already trained and studied some of these models in the periodontology field utilizing different data sets [201–203].

Based on current findings, this research aimed to identify the most suitable ML mathematical model for classifying this particular type of input data, choosing between seven algorithms: Nearest Neighbors [204], Linear Support Vector Machine (Linear SVM), Radial Basis Support Vector Machine (RBF SVM) [205], Decision Tree [206], Random Forest [207,208], Neural Networks (NN) [209] and AdaBoost [210]. Nearest Neighbors, which is based on the association, at the feature level, of the points that are closest to a specific group. Linear Support Vector Machine (Linear SVM) and Radial Basis Functions Support Vector Machine (RBF SVM), which categorize the distinct attributes of each sample into clusters according to their placement on a 2D or 3D hyperplane. Decision Tree are models that incorporate a series of simple tests in a logical order and find the error classification rate by dividing the total number of misclassified points by the total number of data points. Random Forest (RF) is similar to decision trees but is less affected by input data variations, resulting in improved generalization skills. Artificial Neural Networks (ANN) or Neural Networks (NN) are learning algorithms that draw inspiration from the structure and function of biological neural networks. This paradigm consists of a network of links through which information flows from input to output in a unidirectional manner. The primary drawback is the difficulty in comprehending how NN work internally, due to the black box nature of learning. AdaBoost is an ensemble learning algorithm that creates H hypotheses based on a training set of N samples. Subsequent iterations involve expressing error percentages to guide the method in forming the decisive hypothesis H, which classifies data into their respective classes.

In order to get a high level of accuracy in all these machine learning models, used for classification processes, it is necessary to have a large dataset sample size. Additionally, this model should undergo rigorous validation before being applied to a larger population to ensure its reliability. Feres et al. in 2018 trained a Support Vector Machine model to understand the difference between the bacterial community's composition in periodontal health and in the disease (Chronic Periodontitis and Aggressive Periodontitis) [201]. In another study, a multiclass non-clinical screening tool was developed using Logistic Regression and Random Forest algorithms for the diagnosis of periodontal disease and its precision was evaluated in distinguishing between periodontal health, gingivitis, and various stages of periodontitis [211]. In addition, images as intra-oral scanner files and radiographies could be integrated into the diagnostic process to be used as input for the Convolutional Neural Networks (CNN). Through this Deep Learning strategy, it is

possible to create a computer-assisted detection system and evaluate the possible efficacy of predicting periodontally compromised teeth (PCT) [203]. The primary testing procedures employed are F1 and F2 scores, sensitivity, specificity, accuracy, precision, and Area Under Curve (AUC) [212,213]. This facilitated a significant statistical comparison of outcomes, particularly if one desires to assess various models and select the most optimal one. Upon selecting the optimal model, it will be incorporated into a specialized web application designed for direct use by dentists. This is the final goal: to integrate the selected model into a user-friendly web application that can serve as a diagnostic support tool for dentists in distinguishing different staging and grading levels of periodontitis.

The advantages of ML algorithms over the common selection codes dictated by standard rules include: the ability to generate new predictions of periodontitis stages and grades based on additional patient-specific clinical information (such as sex, age, etc.) to explore potential new classification criteria. Additionally, these algorithms provide an essential second opinion to support the clinician in the decision-making phase. Furthermore, they enhance efficiency by reducing prediction times through an automated algorithm in which data are entered and an answer is obtained instantaneously. This capability helps reduce both the time and cost involved in clinical assessments.

Methods

Classification and data collection

According to staging, the severity of the disease is assessed by measuring the interdental clinical attachment loss (CAL), radiographic bone loss (RBL), and the number of teeth lost due to periodontitis. Additionally, treatment complexity depends on periodontal pocket probing depth (PPD), alveolar bone resorption pattern (horizontal or vertical), furcation defect involvement, and the need for complex rehabilitation. The staging is furthermore described with the extent and distribution of the disease as localized (< 30% teeth involved), generalized ($\geq 30\%$ teeth involved), or molar/incisor pattern. On the other hand, the grading system complements staging by providing information on biological attributes, risk assessment, and treatment risk, aiding in therapy outcome determination. Grades are based on primary criteria and grade modifiers. Direct evidence of progression includes CAL or RBL, while indirect evidence involves bone loss related to age or case

phenotype. Grade modifiers, such as smoking and diabetes, significantly impact periodontal disease [197]. All features that determine the staging and grading of periodontitis are listed in Table 4.5.1.

The input dataset for ML included 282 patients while an additional 50 were used to test the application. Data used in this study were collected from patients included in previous investigations conducted at the Department of Periodontology and Oral Medicine, School of Dental Medicine, University of Belgrade. The inclusion criteria were patients older than 18 years; patients with diagnosed periodontitis (all stages and grades); periodontally healthy patients; both, systemically healthy patients and those with diseases that can affect bone metabolism; smokers and non-smokers. Pregnant and lactating women were excluded. Clinical periodontal parameters collected as input data, such as PPD, CAL, bleeding on probing (BOP), and plaque index (PI), were measured in previous studies using a periodontal probe (UNC-15 Hu-Friedy, Chicago, IL) at six points around each tooth (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual, and disto-lingual). All clinical examinations and measurements were conducted by a senior periodontist with over 10 years of experience in the field. The examiner underwent the calibration process to ensure consistency with the study protocol. The clinician performed baseline measurements on a set of 10 patients with varying periodontal conditions. These measurements were cross verified with previously established datasets to ensure alignment with the study's objectives. The intra-examiner reliability was notably high, as previous studies conducted by the clinician demonstrated consistent accuracy in periodontal measurements. The examiner's involvement in numerous clinical trials further underscores the reliability of the data collected for this study. The combination of the senior periodontist's extensive experience and adherence to a standardized calibration protocol ensured that the clinical measurements were both precise and reliable, thereby enhancing the validity of the study's findings.

Additionally, RBL was meticulously assessed by the same clinician using panoramic radiographs. For all patients' images, the periodontal bone level (PBL) and the cemento-enamel junction level (CEJ) of the teeth were determined. The method involved measuring the distances from the PBL and CEJ to the root apex of each tooth. The percentage of RBL was calculated by dividing the intersection length of the PBL by the intersection length of the CEJ. The average RBL scores were calculated for the total dentition. RBL percentage was then classified according to the criteria established during the 2017 World Workshop on the Classification of Periodontal and Peri-implant Diseases

and Conditions, as proposed by Tonetti et al. (2018). Based on these classifications, the periodontal bone loss was staged and graded, providing a standardized framework for evaluation.

Table 4.5.1. Classification of periodontitis - Staging and grading features.

I – Initial periodontitis; II – Moderate periodontitis; III – Severe periodontitis with a potential of additional tooth loss; IV - Severe periodontitis with potential for loss of the dentitions; CAL – clinical attachment loss; RBL - radiographic bone loss; mm – millimeters; PPD – periodontal probing depth; BR – bone resorption; FUR – furcation; *m* – mostly; A – Slow rate of progression; B – moderate rate of progression; C – rapid rate of progression; CAL in mm/y - clinical attachment loss in millimeters per year; RBL in mm/y - radiographic bone loss in millimeters per year; %RBL/age – radiographic bone loss expressed as a percentage of root length divided by the age of the patient; Biofilm/P.D. – relation between biofilm amount and periodontal tissue destruction level (P.D.); cig./ – cigarettes per day; HbA1c – glycated hemoglobin.

STAGING				
Disease severity	I	II	III	IV
Interdental CAL (mm)	1 – 2	3 – 4	≥ 5	≥ 5
RBL	< 15% in coronal third	15%–33% in coronal third	≥ mid-third of root	≥ mid-third of root
Tooth loss	/	/	≤ 4 teeth	≥ 5 teeth
Disease complexity				
PPD (mm)	≤ 4	≤ 5	≥ 6	≥ 6
BR (<i>m</i>)	Horizontal	Horizontal	Vertical	Vertical
FUR defects	No	No	Yes	Yes
Complex rehabilitation	No	No	Moderate need	Severe need
GRADING				
	A	B	C	
CAL or RBL loss (mm/y)	No loss over 5y	< 2mm loss over 5y	≥ 2mm loss over 5y	
%RBL/age	< 0.25	0.25 – 1.00	> 1.00	
Biofilm/P.D.	Biofilm > P.D.	Biofilm = P.D.	Biofilm < P.D.	
Smoking (cig. /day)	No	< 10	≥ 10	
Diabetes (HbA1c %)	No	< 7	≥ 7	

The final database consisted of patient clinical data, information obtained through a questionnaire, and RBL data taken from X-rays. Each item was thereafter assigned a distinct label, to indicate the categorization result and guide the machine's learning. For each patient, the same examiner assigned labels for staging and grading, with the decision double-checked. The study was approved by the Ethics Board of the School of Dental Medicine, University of Belgrade (No 36/8).

Machine Learning approach

Upon gathering all available data for each patient, they were categorized according to the staging and grading rules [194]. Consequently, certain traits were employed in the staging classification (stage I, II, III, IV, healthy patient), while others were utilized in the grading classification (grade A, B, C, healthy patient). The objective was to initially identify the optimal method by analyzing a range of commonly employed ML algorithms in the context of data classification: Nearest Neighbors, Linear SVM, RBF SVM, Decision Tree, Random Forest, NN, and AdaBoost. The next step was to develop an application that may be readily utilized by clinicians to categorize novel patients throughout the diagnostic stage, so assisting them in making informed clinical judgments. In this study, the Python programming language has been utilized to accomplish this objective, particularly the *scikit-learn* library.

Data preprocessing

Initially, it was ensured that the dataset had no missing values. If any such values were found, the proper procedure in the code was employed to fill them in. Any missing data would be replaced by the arithmetic mean of the other data, so as not to create outliers in the group. Afterward, all category factors are transformed into numeric values before picking the variables that will be utilized for the staging classification and those for the grading classification. Due to the input dataset's imbalanced nature, it was imperative to employ rebalancing procedures to prevent the algorithm from exhibiting a bias towards one class over another. Hence, oversampling was selected as a result of the limited quantity of input data and to prevent data loss [214]. Following that, a 3D Principal Component Analysis (PCA) [215,216] was conducted on the complete database to

visually represent the features in a three-dimensional space and see if there were different groupings based on the staging and grading labels.

Training and Validation phases

After completing data preparation, it was essential to specify the seed, which is a number that initializes the random generator to produce the same series of integers. By following this method, the experiment can be replicated consistently whenever the simulation is executed. Subsequently, the data of 282 patients is partitioned into training (80%) and test (20%) sets, accompanied by their respective labels, this partition being the most followed strategy. After being scaled, they were prepared for input into the algorithms [217]. The implemented loop facilitated the generation of a report for each classifier, together with their corresponding confusion matrices, at every iteration. Consequently, it was feasible to establish a comparison among the models and facilitate the selection of one. The validation of each model was conducted using the 5-fold cross-validation technique [218]. The comparison of data pertains to the following parameters: Training MSE (Mean Squared Error), Training Accuracy, Test MSE, Test Accuracy, and ROC-AUC score.

Application creation

The Python *Streamlit* library was utilized to develop the application. An interface was developed to enable clinicians to submit an Excel file containing data on the new patient under analysis. This interface provides a prompt response, within a few seconds, by classifying the pathology level as both staging and grading. In addition, the app provides the option to manually adjust the parameters used to train the model. This allows for a deeper understanding of the model's sensitivity and whether some patient features have a greater impact than others. Finally, to test the application 50 more patients were inserted as input, and the app's answers were compared with the doctors. This additional testing was conducted to further validate the application's performance in its final stage and to benchmark its accuracy against expert human judgment.

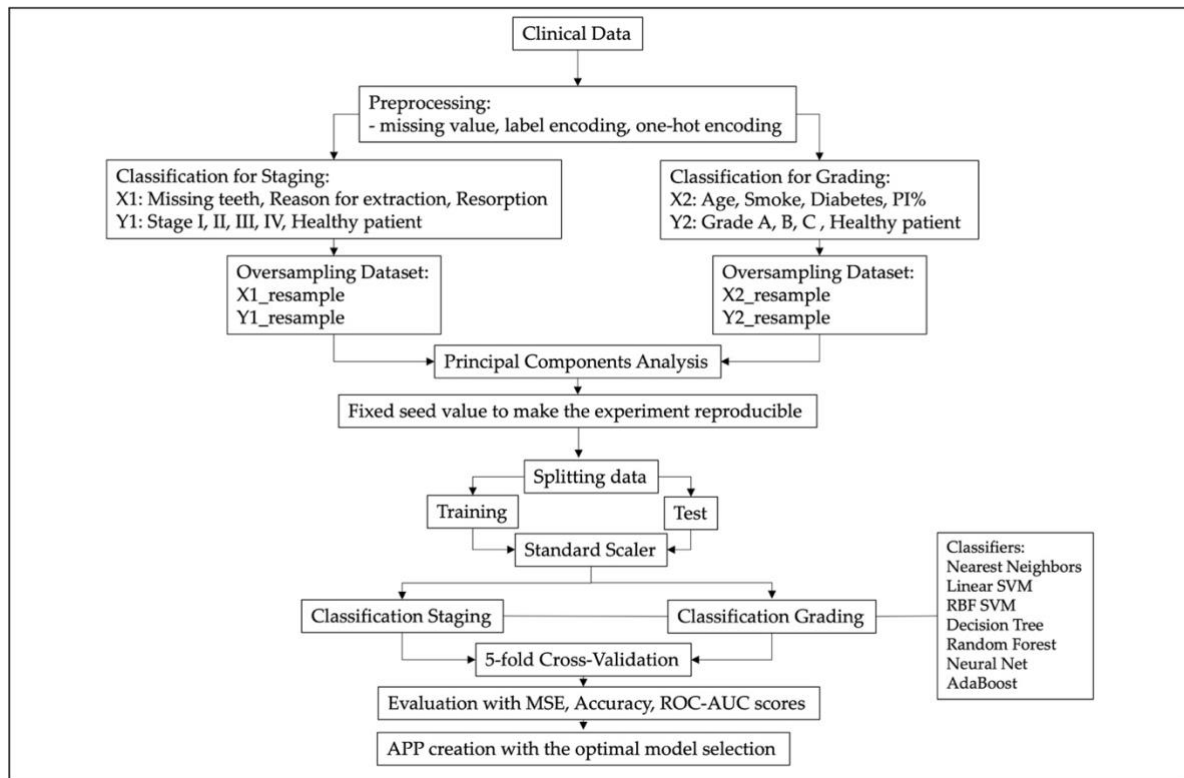


Figure 4.5.1. All the data analysis steps have been reported in the diagram, from preprocessing to the creation of the algorithm using Python code and concluding with the creation of the web application in order to make the service accessible to clinicians.

Results

Data Balancing

After the original data analysis, it was observed that the classes were very imbalanced. To ensure a consistent amount of data for training a model and to avoid loss of information, we decided to employ an oversampling strategy [219]. All classes were reported to 88 samples in the case of the staging categorization, with Stage III being the class with the highest value. Regarding the grading categorization, it is important to note that the reference class for oversampling consisted of Grade B, which had a total of 142 samples. The number of the original dataset consists of 282 patients, while after oversampling the staging input dataset consists of 440 samples, whereas the grading input dataset consists of 568 samples. Afterwards, in the Python code (Figure 4.5.1) the data

was divided into training and test sets, with 80% allocated to the Training set and 20% allocated to the Test set.

PCA – Principal Component Analysis

Following that, Principal Component Analysis (PCA) was conducted to visually demonstrate the separability of the classes and assess the correctness of the oversampling (Figure 4.5.S1, see Appendix). The criteria exhibit a noticeable distinction, resulting in the formation of different groups between healthy individuals and those who have a specific degree of periodontitis. These findings inspire optimism that artificial intelligence will be able to accurately differentiate between these categories and generate an accurate classification.

Comparison reports between trained models

The built Python code may automatically generate classification reports. Different classifiers were placed into the loop and the final report was drawn out for each one so that they could be easily compared (Table 4.5.2). The most effective methods for the staging classification were the RBF SVM, the Decision Tree, and the NN. This was determined primarily by observing the accuracy levels on both the test and training sets, as well as the ROC-AUC score (Receiver Operating Characteristic - Area Under the Curve). It quantifies the ability of a model to accurately differentiate between positive and negative instances across different categorization levels. Afterward, the MSE value in the Training set was determined for these best models to confirm that the mean square error is minimized. The models with the best accuracy and ROC-AUC score values in the grading table were Linear SVM, RBF SVM, and NN. This indicates that NN and RBF-SVMs exhibited favorable characteristics in terms of categorization. Despite this, all algorithms, save for AdaBoost, demonstrate strong performance with training accuracy levels surpassing 85% in staging and 90% in grading. Additionally, testing accuracy levels remain around 80% in staging and reach 90% in grading.

Table 4.5.2. For staging RBF SVM model presents around 91%, 82%, and 98% respectively of training accuracy, test accuracy, and ROC-AUC score; the Decision Tree model shows 94%, 85%,

and 95%, and the Neural Net model 97%, 81% and 98% for the same parameters. For grading Linear SVM model shows around 92%, 95% and close to 100% respectively of training accuracy, test accuracy and ROC-AUC score; RBF SVM around 94%, 93% and 99% and Neural Net around 96%, 94%, 99% for the same scores.

STAGING							
Classifiers	Training MSE	Training R2	Training Accuracy	Test MSE	Test R2	Test Accuracy	ROC-AUC score
Nearest Neighbors	0.1364	0.9341	0.9233	0.2273	0.8658	0.7727	0.9429
Linear SVM	0.1847	0.9108	0.8636	0.2614	0.8457	0.7727	0.9751
RBF SVM	0.0994	0.9520	0.9091	0.2500	0.8524	0.8182	0.9754
Decision Tree	0.0710	0.9657	0.9375	0.1818	0.8927	0.8523	0.9514
Random Forest	0.2244	0.8916	0.8750	0.6023	0.6445	0.7045	0.9426
Neural Net	0.0312	0.9849	0.9688	0.2614	0.8457	0.8068	0.9750
AdaBoost	0.3778	0.8174	0.7585	0.4318	0.7451	0.6364	0.8941
GRADING							
Nearest Neighbors	0.0485	0.9620	0.9581	0.1754	0.8406	0.8772	0.9520
Linear SVM	0.0903	0.9291	0.9229	0.0789	0.9283	0.9474	0.9957
RBF SVM	0.0727	0.9429	0.9405	0.0702	0.9362	0.9298	0.9900
Decision Tree	0.0374	0.9706	0.9626	0.1228	0.8884	0.8772	0.9593
Random Forest	0.1300	0.8980	0.9097	0.2018	0.8167	0.8509	0.9740
Neural Net	0.0374	0.9706	0.9626	0.0614	0.9442	0.9386	0.9937
AdaBoost	0.2533	0.8011	0.7467	0.2719	0.7529	0.7281	0.9158

Cross Validation scores

Following the Leave-One-Out approach with 5-fold (k-fold), k-1 groups of equal size were selected for training and the out one for prediction as a test. This technique was employed to mitigate the issue of overfitting on the test set. The NN and Linear SVM methods demonstrate the greatest values for both staging and grading classifications, indicating their robustness. Therefore, the preferred choice was the NN approach, which is further supported by the prior scores (Table 4.5.3).

Table 4.5.3. 5-fold cross-validation applied to all models of staging and grading. 5 values, which represent the prediction scores, were calculated for each of them.

VALIDATION SCORES		
Method	STAGING	GRADING
Nearest Neighbors	[0.682; 0.807; 0.818; 0.739; 0.807]	[0.807; 0.86; 0.868; 0.903; 0.912]
Linear SVM	[0.841; 0.886; 0.898; 0.898; 0.932]	[0.939; 0.912; 0.956; 0.92; 0.947]
RBF SVM	[0.75; 0.807; 0.727; 0.795; 0.864]	[0.816; 0.737; 0.807; 0.805; 0.867]
Decision Tree	[0.807; 0.841; 0.852; 0.909; 0.955]	[0.947; 0.912; 0.904; 0.912; 0.885]
Random Forest	[0.841; 0.852; 0.784; 0.795; 0.83]	[0.825; 0.789; 0.886; 0.912; 0.841]
Neural Net	[0.795; 0.864; 0.898; 0.886; 0.943]	[0.939; 0.895; 0.956; 0.938; 0.956]
AdaBoost	[0.523; 0.534; 0.352; 0.727; 0.807]	[0.763; 0.754; 0.772; 0.761; 0.743]

Confusion Matrices

The subsequent paragraph displays the confusion matrices of the top-performing models, illustrating the output scores of the algorithms during the testing phase and assessing the agreement between the expected and actual values. Figure 4.5.2A reveals that both the Decision Tree and the NN excel at recognizing healthy patients, though they exhibited some errors in distinguishing between Stages III and IV. In Figure 4.5.2B, healthy patients are diagnosed with 0% error, and the Grade A class is almost uniformly identified by both models. Indecision is slightly more evident in Grades B and C. However, the highest scores are primarily located along the diagonal, confirming the accuracy of the predicted trends.

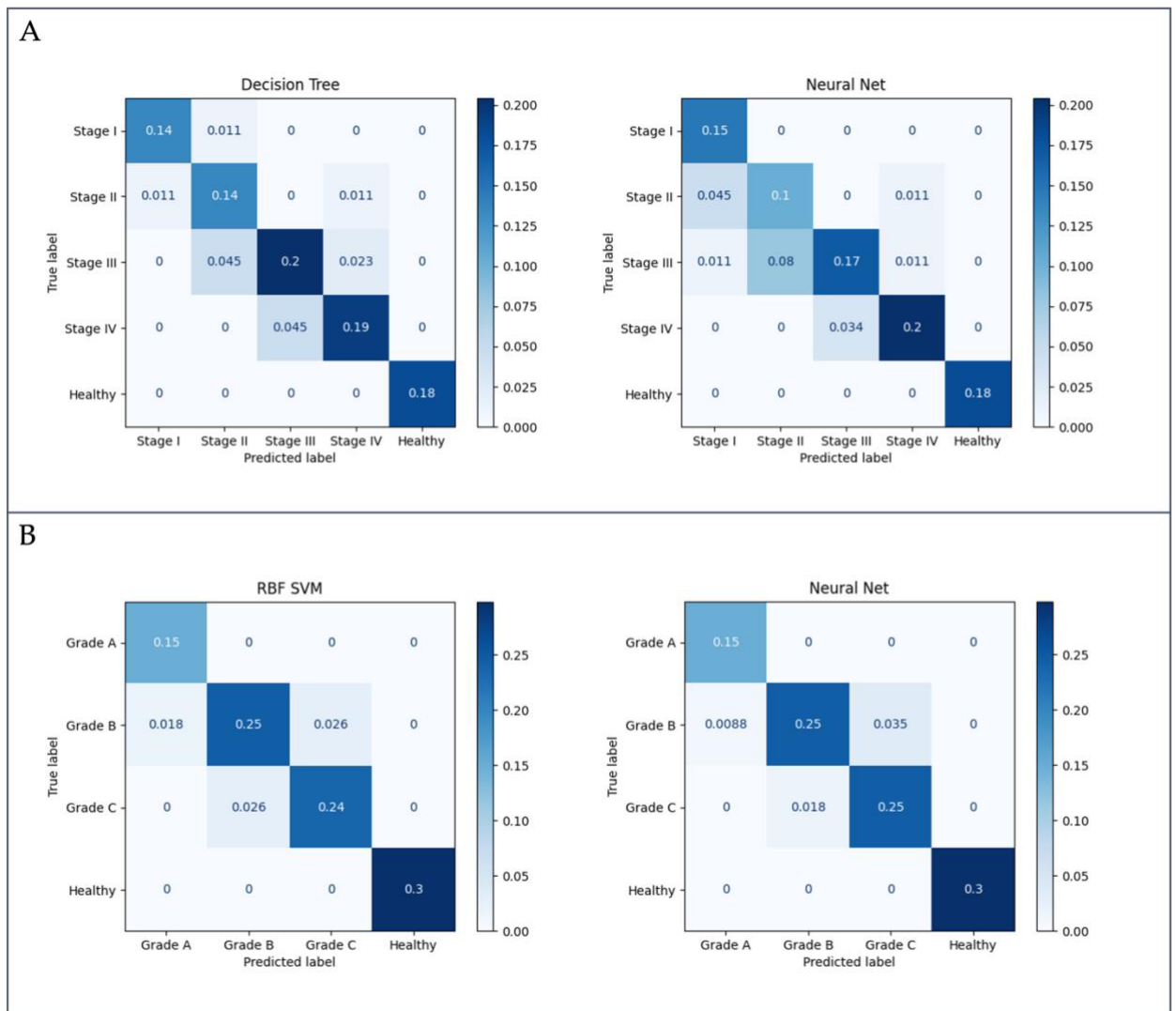


Figure 4.5.2. Confusion matrices evaluate the concordance between the expected (True label) and observed (Predicted label) values for each class of staging. The highest scores predominantly align with the diagonal for both models suggest that the prediction is quite accurate. Slight indecisions focus on stages III and IV in which percentages are also noted outside the diagonal (A). Confusion matrices evaluate the concordance between the expected (True label) and observed (Predicted label) values for each class of grading. The highest scores predominantly align with the diagonal for both models suggest that the prediction is quite accurate. Minor uncertainties revolve around grades B and C, with percentages also recorded outside the diagonal (B).

Web Application

After selecting the best-performing models, we developed a web application using Python's Streamlit module. The trained models were stored in files accessed by the application to evaluate new samples. Patient data, stored in an Excel file, ensured feature consistency with the training data. The application distinguished between parameters needed for staging and grading classifications and imported the Excel file for clinician review. It displayed classification outcomes for both staging and grading, along with prediction accuracy (Figure 4.5.3). A value of 1 indicated maximum certainty for a class. This method enabled efficient and accurate periodontitis classification, achieving approximately 90% accuracy. To compare AI with Human Intelligence (HI) 50 patients were further included in the study. Each with all the characteristics that the application requires in input. Uploading the new files (Figure 3 left column) will have the prediction of both classes of membership for each patient. In the end, these results were compared with labels assigned directly by clinicians who had previously examined and classified patients. In terms of staging, AI matched clinicians' diagnoses in 70% of periodontitis cases (35/50 patients). Minor discrepancies (one stage difference) occurred in 14% of cases, while significant discrepancies occurred in the remaining 16%. For the grading disease, AI aligned with clinicians in 48% of cases (24/50), had minor deviations (one-degree difference) in 40%, and larger deviations in 12% of cases.

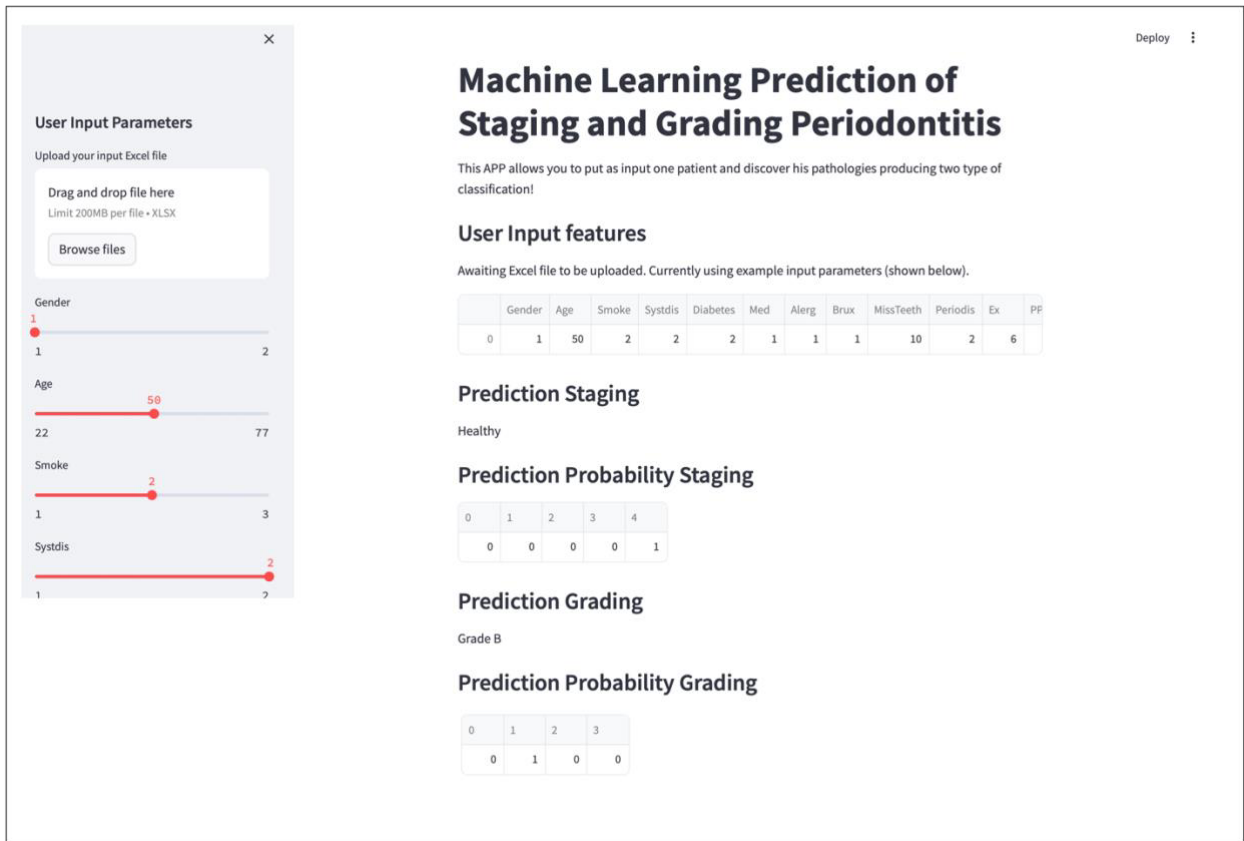


Figure 4.5.3. Web application interface.

Discussion

For several decades, AI has been a focus of medical research. Today, it is increasingly used in dentistry exploiting various algorithms for dental diagnostics [220]. In periodontology, studies have focused on classifying periodontitis using various tools and features. Park et al. employed a deep convolutional neural network (CNN) to classify teeth with periodontal diseases by optical color images [221]. Other authors have analyzed radiographs using AI algorithms [222]. Moreover, Deng et al. used a multiclass non-clinical screening tool for periodontal disease and evaluated its precision in distinguishing between periodontal health, gingivitis, and various stages of periodontitis [202]. As an AI subset, ML replicates human behavior and provides information about our uncertainties and mistakes. Therefore, even the indecisions that our study presented in the input data were then reported in the output. For this reason, referring to the

correctness of the relevant classes, we have noted how all the models displayed more indecision in separating stage II from III or III from IV for the staging classification and grade B and C for the grading classification. The connections with healthy patients and with stage I and grade A were more precise. These uncertainties in distinguishing higher stages might be connected with common features, especially with the number of lost teeth and the reason for their extraction. Even though not all patients' missing teeth were extracted due to periodontal disease, the confusion was probably caused by the fact that the cumulative number of lost teeth, encompassing those removed for periodontal and other reasons, was presented in the algorithm.

In general, all the models, except for AdaBoost, which may not be ideal for this type of inquiry probably for its computational simplicity, demonstrate strong performance, achieving training accuracy rates of over 85% in staging and nearly 90% in grading. AdaBoost was the only one not resulting in a high accuracy rate, and it might be speculated that it may not be the ideal ML system for this type of inquiry. Furthermore, the testing accuracy levels in staging consistently hover at approximately 80%, while in grading, they reach a commendable 90%. The model selected according to our results, NN, attains a test accuracy of 81% in staging and 94% in grading. Neural networks are commonly utilized in clinical scenarios [223] due to their similarity to human reasoning, as they learn from experience. The greater the number of instances they are exposed to, the better they can identify which criteria can serve as a diagnostic index and which are more significant. The lower staging percentage may be attributed to the fact that it is based on a classification of 5 classes, each including 88 samples, whereas grading is based on 4 classes, each containing 142 samples. Therefore, the quantity of input samples has a substantial impact on the system's performance. Increasing the amount of data will enhance the neural network's ability to learn from experience and identify the specific parameters that influence categorization decisions, comparable to human cognition. Similarly to our study, Ertas et al. applied ML algorithms to determine both the stage and grade of periodontitis, with the aim of achieving high reliability rates while minimizing the number of attributes used. In their study, they achieved a high classification accuracy (97.2%) using the Decision Tree algorithm with 10 features to determine the stage of periodontitis. Additionally, the Random Forest algorithm also yielded promising results, achieving an accuracy of 96.5% with only 4 attributes. For grading, the best results (97.9% accuracy) were observed using the Random Forest algorithm with 10 attributes [224]. Furthermore, Farhadian et al. also investigated the SVM model for the

classification using the patient's dataset. Results from this study showed a correct classification accuracy of 88,7%, but they only wanted to distinguish gingivitis, localized periodontitis, and generalized periodontitis [225].

In the AI and HI challenge, discrepancies were noted in the staging of classes I and II, likely attributed to limited representation in the initial dataset. To address this, it will be necessary to enhance the application by incorporating data from new patients in these categories. Moreover, in grading the APP tends to be more 'conservative'; for instance, grade A may be classified as grade B, or healthy cases may be misclassified as grade A. Consequently, the relatively high percentage of singular deviations (40%) can be attributed to this factor. Distinguishing between the stage and the grade of periodontitis is not always straightforward, even for experienced clinicians. Many factors can influence the severity and progression of periodontal disease, often resulting in a less clear picture when combined with clinical signs. These facts frequently lead to establishing a borderline diagnosis, which can explain the deviation shown in this investigation when AI and HI were compared. Whereas natural intelligence relies on the integration of various cognitive processes to adapt to new situations, AI operates on mathematical models, drawing inspiration from biological brain systems to develop neural networks capable of learning through repetition [226]. There haven't been many investigations comparing AI and HI in periodontology, although a few studies have explored this topic in the context of diagnostic dentistry. Albagieh et al. revealed an intriguing pattern in the diagnosis of oral lesions. They found that senior oral medicine residents had lower agreement levels compared to AI models [227], while You et al. found AI models showed satisfactory clinical performance in plaque detection [228]. Güneç et al. discovered AI software outperformed junior dentists in caries detection [229]. The findings from the aforementioned studies consistently favor AI over human performance in contrast to the outcomes of our study. However, it is plausible that the diversity in these results is derived from variations in the clinicians involved in diagnosis. Differences may arise, especially when less experienced practitioners are part of the diagnostic process.

The user-friendly AI application as a means of diagnostic aid and periodontal staging and grading assessment could facilitate everyday clinical life. From a future perspective, it will be incrementally enhanced with fresh data samples to improve its overall performance. Subsequently, it can offer dentists supplementary assistance in the form of a second opinion for diagnosis. It is crucial to emphasize that this technology will not replace the doctor but rather offer digital assistance to complement their work.

4.6 Oral Pathology: Multidisciplinary-based evidence that biomolecular alterations temporally anticipate microarchitectural modifications of collagen in oral tongue squamous cell carcinoma

In recent years, there has been a growing interest toward the role of the extracellular matrix (ECM) in the biomechanics of physiologic/pathologic tissues. The ECM does not only surround cells, but its rigidity stresses them mechanically, producing signals that depend on both the amount of collagen and its cross-linking and hydration. It can also induce the so-called mechano-transduction process, namely cells convert a mechanical stimulus into an electrochemical activity. Collagen remodeling, including alterations in collagen fiber orientation, cleavage, and trimer composition, exerts pronounced effects on tumor growth and immunity, some of which are mediated by specific collagen receptors [230,231]. In this light, the etiology of many types of pathologies, including tumors, could be elucidated by the characterization of the collagen bundle microstructure. Oral tongue squamous cell carcinoma (OTSCC) accounts for over 40% of all oral cancer cases [232,233]. It exhibits distinctive molecular and clinical behavior compared to oral cancer of other subsites leading to an “anatomical bias” both for research and clinical decision making [234]. The stroma represents the main component of tumor microenvironment (TME) and plays a pivotal role in cancer progression and therapy resistance [235–241]. It consists of non-malignant tumor cells and ECM. However, although stroma cells are not malignant themselves, the tumor-stroma interaction allows the acquisition of an abnormal phenotype [242].

The temporal relationship between biomolecular alteration and structural modification of tumor stroma is still largely unknown: stroma alterations seem to occur before tumor is clearly detectable and have been shown to significantly predict poor patient outcomes[239,243]. In particular, tumor stroma outgrowth is characterized by abnormal matrix secretion and collagen synthesis, attributable to hypoxia and chronic inflammation, leading to an edematous and unstable TME [244,245]. To date, OTSCC pathological staging is based on morphological features of tumor parenchyma analyzed by conventional histological techniques, overlooking the importance of tumor stroma from a prognostic and therapeutic standpoint.

Recently, a variety of experimental methods quantify tumor characteristics from molecular to tissue scales and describe how such data can be directly integrated with mechanism-based models to improve predictions of tumor growth and treatment response [246]. Moreover, engineering tools, including advanced imaging techniques, were recently shown to improve our understanding of cancer ECM biology and therapeutic development [247]. In this context, some authors of this paper recently investigated the morpho-chemical features of collagen bundles in uterine leiomyoma using an innovative synergistic approach based on two advanced imaging techniques, namely synchrotron-based phase-contrast microcomputed tomography (PhC-microCT) and Fourier transform infrared imaging (FTIRI) spectroscopy [248,249]. Compared to the conventional X-ray imaging, synchrotron-based PhC-microCT allows the three-dimensional (3D) evaluation of the ECM microarchitecture with high spatial resolution and without using contrast agents [249]. Moreover, FTIRI represents a versatile spectroscopic technique to obtain the morpho-chemical characterization of the same tissues, with additional information at macromolecular level [250,251].

Based on this evidence, in this study a multidisciplinary approach supported by synchrotron PhC-micro-CT and FTIRI spectroscopy deeply investigate the IntraT, PeriT, and ExtraT regions of OTSCC at different pathological stages, focusing on the structural organization of ECM collagen bundles at multiscale. This investigation contributes to elucidate the temporal kinetics interlacing the stroma micro-architectural modifications and biomolecular alterations.

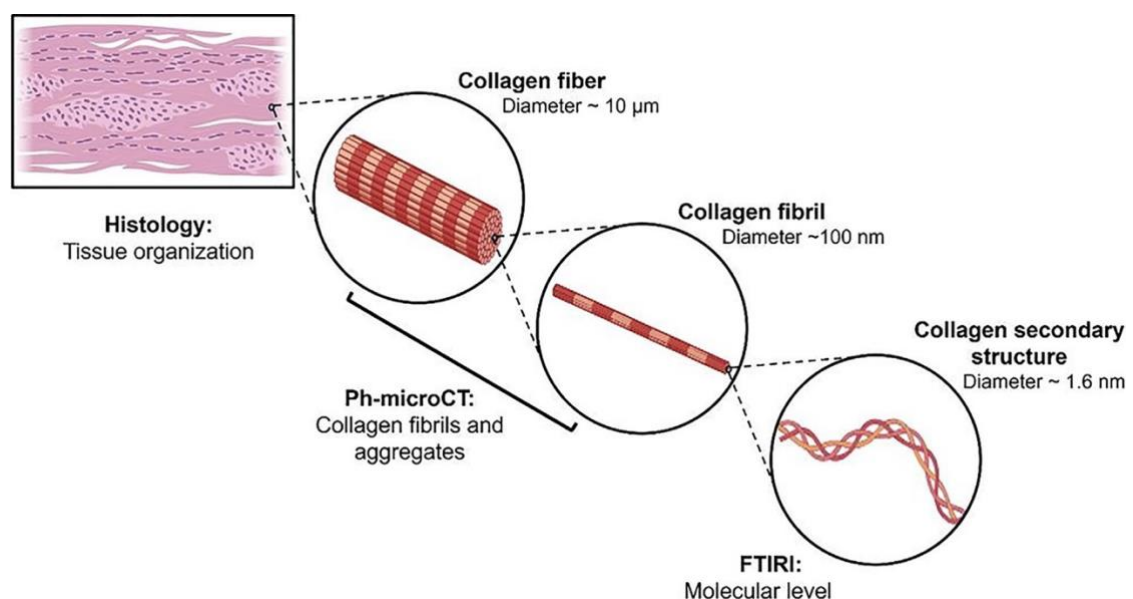


Figure 4.6.1. Structural and molecular organization of collagen. Collagen is a fibrous protein composed of three left-handed alpha chains, wound together in a tight triple helix. Collagen molecules are aggregated to form fibrils and then fibers, which subsequently form the complex tissue organization ([252], CC BY 4.0).

Results

The study was focused on biopsy samples with histological diagnosis of OTSCC at different malignancy stage (stages I–IV). Samples were investigated by PhC-microCT and FTIRI techniques. The flux of the study is described in Figure 4.6.1. The histopathological investigation of OTSCC biopsy samples was first carried out to identify specific areas containing the tumor mass (named IntraT area), the normal stroma (named ExtraT area) and the regions corresponding to the front of the tumor in each OTSCC stage (named PeriT S-I, Peri-T S-II, PeriT S-III, and Peri-T S-IV). From these areas, cylindrical portions were extracted to be analyzed by PhC-microCT and FTIRI analyses.

Clinicopathological data of the patient's cohort

A total of 35 surgical samples, corresponding to as many patients and stored in the Institutional Archive, were selected, consisting of 23 males (65.7%) and 12 females (34.3%). The mean age at diagnosis was 64.4 ± 16.1 years and the mean tumoral diameter was equal to 3.1 ± 1.6 cm. According to the 8th Edition of American Joint Committee of Cancer (AJCC) staging system, 7 patients (20.0%) were stage I, 8 (22.9%) were stage II, while 11 (31.4%) and 9 (25.7%) patients belonged to stages III and IV, respectively. According to 4th Edition of Word Health Organization (WHO) Grading (G), most of the cases (51.4%) resulted poorly differentiated tumors (G3), 14 cases (40.0%) were moderately differentiated (G2), and 3 cases (8.7%) showed a well grade of differentiation (G1).

Histological analysis investigated the biopsy samples

Biopsy samples were first submitted to histological analysis to identify and characterize the tumor regions and the areas surrounding the tumor itself; for each sample, Nr. 2 sections (4-mm thickness) were cut for hematoxylin & eosin (H&E) and Masson's trichrome staining. As an example, in Figure 4.6.2, the histologic images of OTSCC sections representative of stages I–IV are reported displaying both the tumor mass and the PeriT stroma at the tumor front. The histological Masson trichrome images highlighted the presence of different areas corresponding to the tumor (red tones) and healthy stroma (blue tones) in the PeriT region. Modifications in tissue organization in the regions surrounding the tumor mass were evidenced, as displayed by a different collagen distribution.

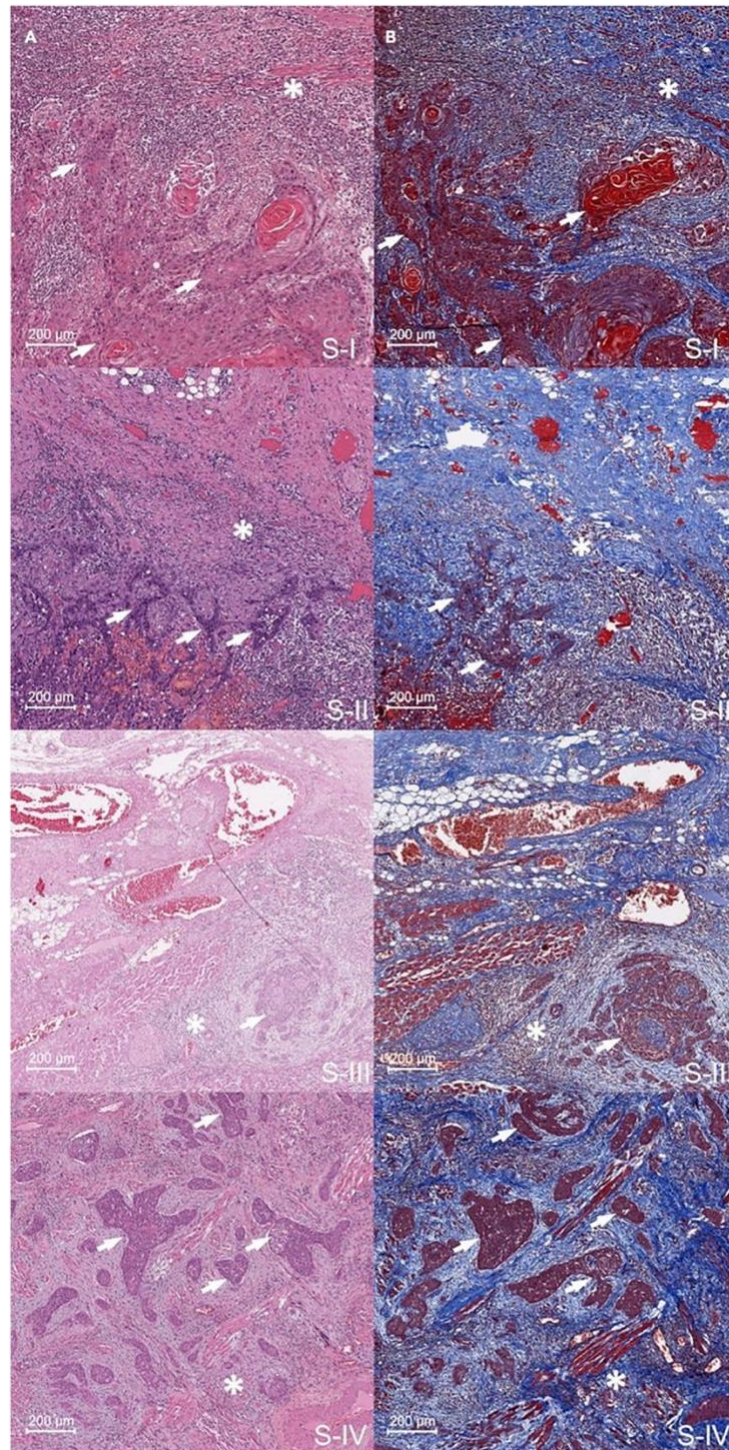


Figure 4.6.2. Examples of tumor and PeriT areas in histological sections of OTSCC:
 (A) H&E and (B) Masson's trichrome images ($\times 20$ magnification) of representative OTSCC sections showing the tumor areas (white arrows) and the PeriT stroma (white asterisks) at the tumor front in stages I (S-I), II (S-II), III (S-III), and IV (S-IV). A total of 70 histologic images (two for each biopsy) were investigated. Scale bars: 200 μm ([252], CC BY 4.0).

Synchrotron-based PhC-microCT evidenced significantly reduced and disorganized collagen in the tumor area but no significant morphometrical mismatches in the PeriT region between different OTSCC stages

All the biopsies defined previously were sectioned to obtain the same number of cylindrical samples (diameter $\cong$ 2 mm; height $\cong$ 3÷4 mm) for high-resolution synchrotron tomography (pixel size 890 nm). The 3D reconstructions were analyzed; for each sample, three sub-volumes corresponding to the ExtraT area (normal stroma), the front of the tumor (PeriT stroma) and the tumor itself (IntraT stroma) were selected and divided according to the tumor stage. As displayed in Figure 4.6.3, 3D synchrotron imaging showed the presence of a dense and well-organized stroma (yellow false color) in the ExtraT area. Conversely, both the IntraT (red false color) and PeriT (blue false color) regions seemed characterized by loosely packed and randomly arranged collagen bundles, resulting in an edematous stroma.

The statistical analysis, carried out on the complete set of samples, is reported in Figure 4.6.4, by box-and-whisker diagrams. It revealed the presence of a wide variability for all morphometric parameters. Repeated measures analysis of variance and Tukey's multiple comparisons test evaluated the differences among extratumoral (ExtraT), peritumoral (PeriT) and intratumoral (IntraT) regions.

The volume percentage significantly increased from IntraT to PeriT and ExtraT stroma. Similarly, the ExtraT bundles were significantly thicker, both respect to PeriT and IntraT ones. Limitedly to these two parameters, statistically significant differences were also detected comparing PeriT and IntraT stroma. The spacing between bundles significantly decreased from ExtraT to PeriT and IntraT stroma.

Anisotropy degree, fractal dimension, and connectivity density were significantly higher in the ExtraT stroma compared to PeriT and IntraT ones. Interestingly, no significant differences ($p > 0.05$) were found between PeriT and IntraT stroma for spacing between bundles, anisotropy degree, fractal dimension, and connectivity density.

With reference to the mean fiber length, a significant deviation ($p < 0.01$) was detected between the ExtraT and IntraT regions of the same biopsy, with longer fibers on average in the ExtraT stroma. However, this result must be taken with caution due to the very high standard deviation influencing the mean fiber length within each individual biopsy.

Indeed, the detected high standard deviation data confirm the broad heterogeneity of the tongue stroma, regardless of the presence or absence of OTSCC.

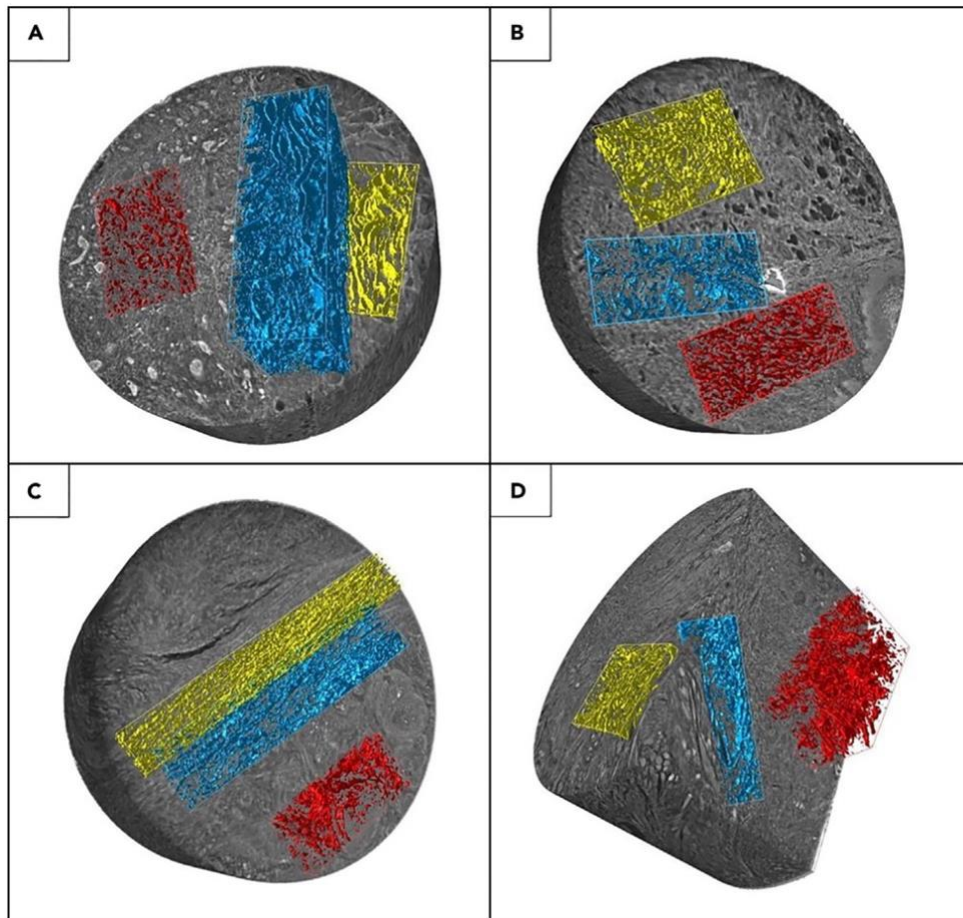


Figure 4.6.3. 3D synchrotron-based PhC-microCT reconstruction of four representative OTSCC biopsies at (A) stage I, (B) stage II, (C) stage III, and (D) stage IV. Colored boxes highlighted the distribution of the collagen bundles in the IntraT (red), PeriT (blue), and ExtraT (yellow) areas. IntraT and PeriT regions seem characterized by loosely packed and randomly arranged collagen bundles regardless of the stage. Number of biopsies investigated: 35, one for each patient. Cylindrical samples: diameter $\cong$ 2 mm; height $\cong$ 3÷4 mm ([252], CC BY 4.0).

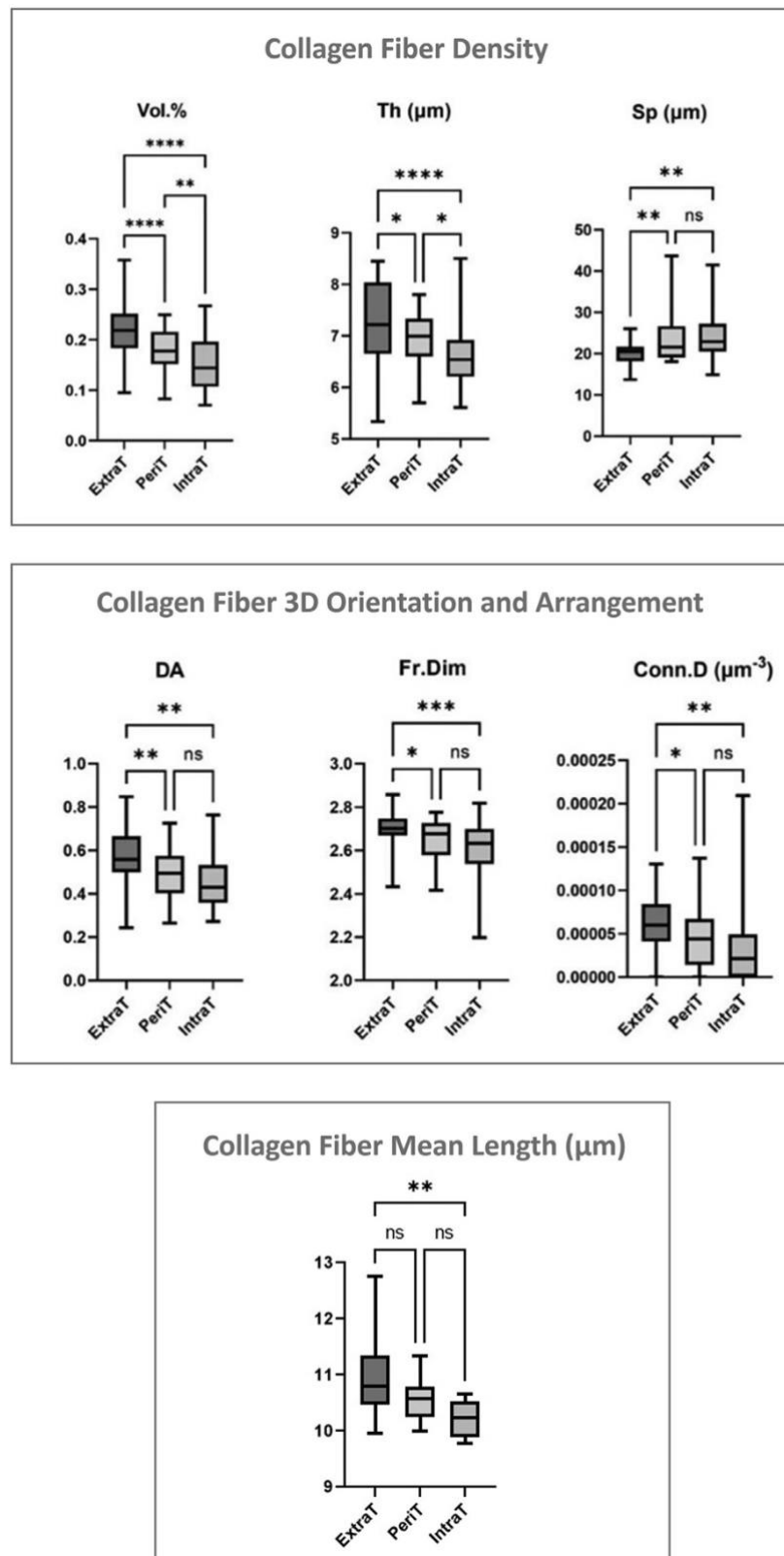


Figure 4.6.4. PhC-microCT data analysis Collagen morphometric parameters calculated in the extratumoral (ExtraT), peritumoral (PeriT), and intratumoral (IntraT) stroma of OTSCC samples. Box-and- whisker diagrams of the distribution of PhC-microCT morphometric parameters:

connective tissue specific volume (Vol. %, [0÷1]), collagen bundle mean thickness (Th, μm), collagen bundle mean spacing (Sp, μm), connective tissue anisotropy degree index (DA, [0÷1]), connective tissue 3D fractal dimension (Fr.Dim, [2÷3]), connective tissue connectivity density (Conn.D, μm^{-3}), collagen bundle mean length (mean fiber length, μm). Upper and lower ends of boxes represent 75th and 25th percentiles. Max and min values are reported. The median value is shown with a solid line. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Number of biopsies investigated: 35, one for each patient ([252], CC BY 4.0).

FTIRI evidenced a low amount of collagen—with reduced fiber organization in the tumor area as well as in the PeriT stroma of advanced-OTSCC stages

The same cylindrical samples (Nr. 35) analyzed by PhC-microCT were submitted to FTIRI analysis. In this regard, from each cylinder Nr. 2 sections (7- μm thickness) were cut and deposited onto CaF_2 optical windows for the vibrational analysis. More in detail, by using the television camera, on each section, the areas containing the tumor mass, the normal stroma, as well as the front of the tumor were selected. On these areas, by using the focal plane array (FPA) detector, infrared (IR) images of $164 \times 164 \mu\text{m}^2$ size were acquired; each image was the result of 4.096 pixel/spectra with a spatial resolution of $2.56 \times 2.56 \mu\text{m}^2$.

In Figure 5, the microphotographs of representative OTSCC sections, showing the ExtraT region (ExtraT), the PeriT areas near to OTSCC at stages I–IV (PeriT S-I, Peri T S-II, PeriT S-III, and PeriT S-IV) and the tumor mass at stage IV (IntraT) are displayed together with the false color images, and the corresponding hierarchical cluster analysis (HCA) images. False color images are useful to highlight the topographical distribution of specific biological compounds within a mapped area, in this case total proteins and collagen. To this purpose, they were generated by integrating IR images under the following spectral intervals: $1.720\text{--}1.470 \text{ cm}^{-1}$ (which includes the amide I and II bands, representative of total proteins; PROTEINS images) [253] and $1.299\text{--}1.184 \text{ cm}^{-1}$ (which includes the amide III region, mainly representative of collagen; COLLAGEN images) [254,255].

Interestingly, the hyperspectral imaging analysis was able to differentiate each pathological stage. As regards total proteins (PROTEINS images, Figure 4.6.5B), an almost similar amount was found in all the analyzed samples, even if with a different

distribution in relation with the tumor stage: in fact, total proteins displayed a more compact localization in the normal stroma (ExtraT) and in the PeriT region near to OTSCC at stage I (PeriT S-I), compared to the tumor mass (IntraT) and the PeriT areas close to OTSCC at stages II, III, and IV (PeriT S-II, PeriT S-III, and PeriT S-IV). Collagen appeared even more influenced by the pathological stage (COLLAGEN images, Figure 5C): (1) higher levels were found in the extratumoral region (ExtraT) and in the PeriT areas corresponding to early tumor stages (PeriT S-I and PeriT S-II); (2) interestingly, in these regions, a superimposable distribution of this matrix protein with total proteins was observed, allowing us to hypothesize that, in early tumor stages collagen is the main protein component like in normal stroma; (3) conversely, lower amounts of collagen were detected in the PeriT areas near to OTSCC at stages III and IV (PeriT S-III and PeriT S-IV), and in the intratumoral region (IntraT), with a different localization respect to proteins, suggesting a major contribution of tumor-related proliferating cells in advanced tumor stages.

IR images were also submitted to HCA to highlight, within the mapped areas, the presence of regions characterized by different spectral profiles and hence by a diverse macromolecular composition: in this study, HCA was useful to identify on each IR image collagen-rich clusters (orange colored in Figure 4.6.5D) from which to extract the average IR spectra (Fig. 4.6.6).

Due to the presence of convoluted bands, typical of biological samples, IR spectra were submitted to peak fitting analysis, a procedure that made it possible to obtain the precise position (centroid, cm^{-1}) of all peaks underlying a specific IR region together with the corresponding integrated areas. The analysis was focused on the amide I and II bands (centered respectively at $\cong 1.655 \text{ cm}^{-1}$ and $\cong 1.550 \text{ cm}^{-1}$), representative of total proteins, and on the amide III band (in the spectral interval $1.360\div 1.184 \text{ cm}^{-1}$), mainly diagnostic for collagen [254,256–258]. In particular, the following peaks were taken into account: $\cong 1.342 \text{ cm}^{-1}$ (CH_2 wagging of side chains in proline, the most recurrent amino acid in collagen); $\cong 1.319 \text{ cm}^{-1}$ (α helix secondary structures); $\cong 1.280 \text{ cm}^{-1}$ and $\cong 1.240 \text{ cm}^{-1}$ (collagen triple helix); $\cong 1.262 \text{ cm}^{-1}$ (random secondary structures), and $\cong 1.205 \text{ cm}^{-1}$ (amino acids' side chains) [245,256,259].

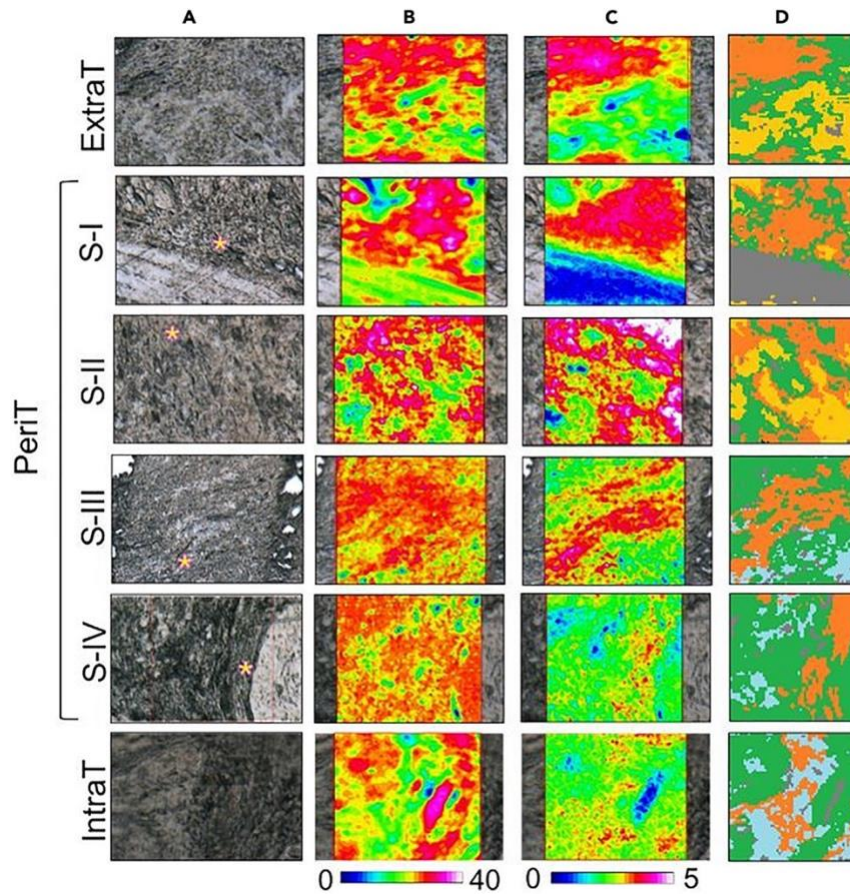


Figure 4.6.5. Hyperspectral imaging analysis of OTSCC sections representative of the extratumoral stroma (ExtraT), PeriT stroma near to OTSCC at stages I–IV (named respectively PeriT S-I, PeriT S-II, PeriT S-III, and PeriT S-IV), and IntraT stroma at stage IV (IntraT). (A) Microphotographs of the mapped areas ($164 \times 164 \mu\text{m}^2$ size, with 4.096 pixel/IR spectra; spatial resolution: $2.56 \times 2.56 \mu\text{m}^2$; pink asterisks indicate the PeriT areas).

(B and C) False color images showing the topographical distribution of (B) proteins (PROTEINS images) and (C) collagen (COLLAGEN images); false color images were built by using a specific color scale, with blue referring to areas with the lowest absorbance values, while white/pink to those with the highest ones. Due to differences in the absorbance values, different scales were used for proteins and collagen. (D) Hierarchical cluster analysis (HCA) images (green color defining the areas with higher proteins amounts, while orange specifically indicating regions richer in collagen). Number of sections investigated: Nr. 70, two for each biopsy; on each section, an IR image was acquired respectively on the ExtraT, PeriT, and IntraT areas ([252], CC BY 4.0).

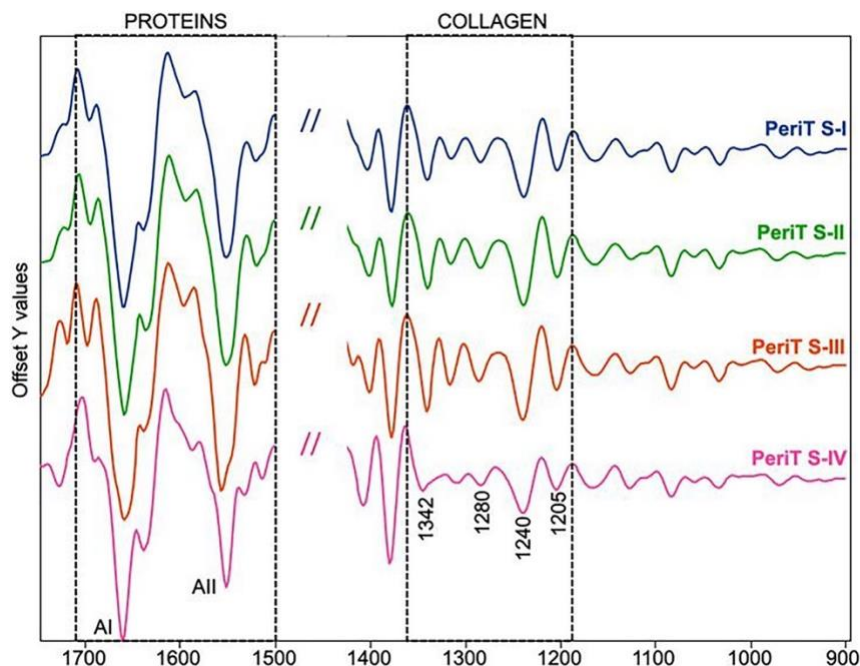


Figure 4.6.6. IR spectra of OTSCC PeriT stroma. Average IR spectra representing the PeriT stroma in OTSCC samples from stages I–IV (named respectively PeriT S-I, PeriT S-II, PeriT S-III, and PeriT S-IV). IR spectra were extracted from collagen-rich clusters in HCA images and are reported in second derivative mode in the $1720\div900\text{ cm}^{-1}$ range to better highlight small differences among groups. The regions representative of proteins and collagen are indicated by dotted boxes and the position (wavenumbers, cm^{-1}) of the main absorption peaks are reported ([252], CC BY 4.0).

With this analysis, the following spectral parameters were calculated and statistically evaluated: collagen/proteins (COLL/PRT), calculated as ratio between the area of the spectral range $1.299\div1.184\text{ cm}^{-1}$ (including the bands at 1.280 cm^{-1} , 1.240 cm^{-1} and 1.205 cm^{-1} , which form the tricuspid-shaped profile typical of collagen), and the area of the spectral range $1.720\div1.470\text{ cm}^{-1}$ (including the amide I and II bands of proteins); proline/proteins (PRO/PRT), calculated as ratio between the area of the band at 1.342 cm^{-1} (corresponding to the wagging of proline) and the area of the spectral range $1.720\div1.470\text{ cm}^{-1}$ (including the amide I and II bands of proteins); TRIPLE HELIX, calculated as ratio between the area of the band at 1.280 cm^{-1} (collagen triple helix) and the area of the range $1.360\div1.184\text{ cm}^{-1}$ (representative of the total absorption in this interval); ALPHA HELIX, calculated as ratio between the area of the band at 1.319 cm^{-1} (alpha helix secondary structures) and the area of the range $1.360\div1.184\text{ cm}^{-1}$ (representative of the

total absorption in this interval); and RANDOM, calculated as ratio between the area of the band at 1.262 cm^{-1} (representative of random coil structures) and the area of the range $1.360\div 1.184\text{ cm}^{-1}$ (representative of the total absorption in this interval).

COLL/PRT and PRO/PRT ratios can both be considered representative of the relative amount of collagen, respect to total proteins (Figures 4.6.7A and 4.6.7B). As expected, the normal stroma (ExtraT) was characterized by the highest values of these two parameters, which decreased statistically significantly from PeriT S-I to PeriT S-IV, until reaching the lowest values in the tumor mass (IntraT). A similar statistically significant decreasing trend was displayed by TRIPLE HELIX and ALPHA HELIX ratios, representing respectively the relative amount of triple helix and α -helix structures in collagen (Figures 4.6.7C and 4.6.7D). Finally, the RANDOM ratio was analyzed to evaluate the structural organization of the protein component: the lowest values were found in the normal stroma (ExtraT), with an increasing trend going from PeriT S-I to PeriT S-IV and a major extent in the tumor mass (IntraT) (Figure 4.6.7E). These findings hypothesize a higher amount of collagen, characterized also by a major structural organization in the PeriT regions near the OTSCC in the early stages, similar to what is observed in the normal stroma. Conversely, the tumor mass as well as the PeriT regions close to OTSCC at advanced stages are characterized by low levels of collagen, with a more disordered structure. p values are reported in Figure 4.6.7F; statistical significance among groups was set at $p > 0.05$.

Correlation analysis among microarchitectural collagen bundles parameters, collagen macro-molecular indices, and clinical stages revealed that biomolecular alterations of collagen in the OTSCC stroma temporally anticipate structural modifications of collagen bundle microarchitecture

Spearman rank analysis focused on the PeriT stroma, correlating clinical staging with the most relevant microarchitectural parameters (Th, Sp, and Vol. %) of collagen bundles and with two macromolecular indices (i.e., COLL/PRT and PRO/PRT) (Table 1). The correlations were considered “very weak” ($r = 0 \div 0.20$), “weak” ($r = 0.20 \div 0.40$), “moderate” ($r = 0.40 \div 0.60$), “strong” ($r = 0.60 \div 0.80$), and “very strong” ($r = 0.80 \div 1.0$). “Strong” and “very strong” correlations with $p < 0.05$ were considered statistically significant. Significant negative correlations between the pathological stage and both COLL/PRT ($r = 0.60$; $p = 0.001$) and PRO/PRT ($r = 0.77$; $p < 0.0005$) ratios were

detected. Also, the microarchitectural Th parameter showed a significant direct correlation with the COLL/PRT ratio ($r = 0.63$; $p < 0.0005$) and a significant inverse correlation with the bundle spacing parameter ($r = 0.69$; $p < 0.0005$). Notably, no significant correlation was found between any of the collagen bundle microarchitectural parameters and the clinical staging ($|r| < 0.60$, $p < 0.05$) in the PeriT stroma.

Table 4.6.1. Spearman's rank correlation analysis between the structural indices and the clinical-pathological data of the surgical samples in the PeriT stroma ([252], CC BY 4.0).

	Stage	COLL/PRT	PRO/PRT	Thickness	Spacing	Vol%
Stage	$\rho = 1$	-0.60*	-0.77*	-0.25	-0.48	0.29
	$p = 1$	0.001*	0.000*	0.188	0.009	0.132
COLL/PRT		$\rho = 1$	0.28	0.63*	-0.03	0.49
		$p = 1$	0.147	0.000*	0.883	0.007
PRO/PRT			$\rho = 1$	0.07	0.44	0.43
			$p = 1$	0.702	0.016	0.020
Thickness				$\rho = 1$	0.18	0.48
				$p = 1$	0.344	0.009
Spacing					$\rho = 1$	-0.69*
					$p = 1$	0.000*
Vol%						$\rho = 1$
						$p = 1$

(*) Statistically significant associations ($p < 0.05$) with “strong” and “very strong” correlations. (COLL/PRT, collagen/proteins; PRO/PRT, proline/proteins; Thickness, mean collagen bundle thickness; Spacing, mean collagen bundle spacing, Vol %: specific collagen volume).

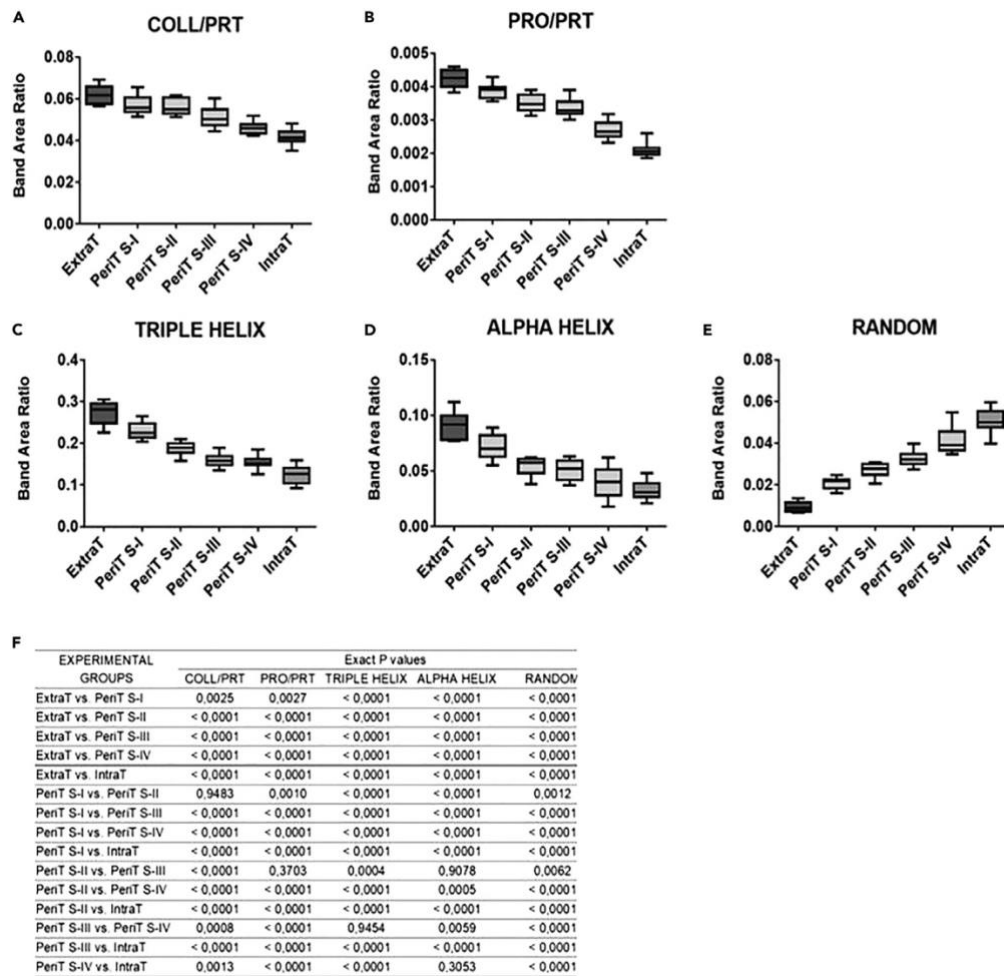


Figure 4.6.7. Statistical analysis of spectral parameters calculated in the extratumoral stroma (ExtraT), PeriT stroma near to OTSCC at stages I–IV (named respectively PeriT S-I, PeriT S-II, PeriT S-III, and PeriT S-IV), and intratumoral stroma at stage IV (IntraT). Box-and-whisker diagrams of: (A) COLL/PRT (representative of the relative amount of collagen respect to total proteins); (B) PRO/PRT (representative of the relative amount of proline respect to total proteins); (C) TRIPLE HELIX (representative of the total amount of the triple helix structures in collagen); (D) ALPHA HELIX (representative of the total amount of a-helix structures in collagen); (E) RANDOM (representative of random structures), and (F) p values. Upper and lower ends of boxes represent 75th and 25th percentiles. Max and min values are reported. The median value is shown with a solid line. Statistical significance was set at $p < 0.05$. Number of sections investigated: 70, two for each biopsy sample ([252], CC BY 4.0).

Discussion

Solid tumors like OTSCC consist of the tumor parenchyma and stroma. The traditional view is that the tumor parenchyma regulates the surrounding stroma and promotes tumor progression. However, even though the pivotal role of the tumor stroma in assisting cancer growth and invasiveness is becoming increasingly clear, the precise mechanism underlying these processes is still not fully understood. The main histological features of OSCC staging are still referred to tumor parenchyma, and the temporal kinetics interlacing structural modifications of collagen bundles in the stroma and collagen biomolecular alterations in OTSCC staging are still mostly unexplored. In oral cancer, it is known that the tumor stroma is directly involved in the biological features of neoplastic cells [260–264]. During oral tumorigenesis, an immature collagen and a higher level of proteolytic enzymes, neo-angiogenic, and profibrotic growth factors are released in the ECM, leading to the deposition of a reactive stroma [265,266]. Furthermore, the presence of a dysfunctional stroma has been significantly associated to unfavorable clinical outcomes in OTSCC patients, [267–271] especially in early-OTSCCs [272]. This could be attributable to the reciprocal cross-talking between neoplastic cells and cancer-associated fibroblast (CAFs); indeed, high levels of stromal CAFs have been associated with a high risk of metastases and poor overall survival [273–276].

However, there is still no solid evidence regarding the (temporal) cause-effect relationships linking the microarchitectural changes of the OTSCC stroma and the biochemical and macromolecular changes of the collagen protein. The main findings regarding tumor stroma in OTSCC is based on histological investigations, which are unable to quantitatively characterize the different portions of stroma due to the lack of both 3D view and high-resolution structural information of macromolecules in the IntraT, PeriT, and ExtraT areas. More reliable data can be obtained through investigations conducted on a large sample and through complementary techniques that provide significant information not only on the microstructure and 3D shape complexity of the collagen bundles but also on the macromolecular characteristics of collagen in the same analyzed tissues.

In this direction, in the present study we applied an innovative multidisciplinary and multiscale approach based on synchrotron PhC-microCT and FTIRI spectroscopy. We recently used the same cross-linked method to successfully study the massive collagen production in uterine leiomyoma [248]; hence, we proposed the same workflow, as it

proved to be highly effective to investigate collagen self-organization in pathological conditions. The PhC-microCT morphometrical data revealed the presence of wide variability for all parameters, also in the ExtraT regions: this could be related to the structural heterogeneity of the stroma between the different collected areas among the patients, confirming that the tongue is an extremely heterogeneous anatomical site from a microarchitectural point of view. For this reason, statistical analysis was performed through a repeated measures analysis of variance, comparing each ExtraT area with the corresponding PeriT and IntraT ones (Figure 4.6.4). In terms of density, we observed and quantified the presence of a reduced amount of collagen bundles, thinner and more spaced in the proximity of the tumor parenchyma. This result could suggest the presence of an immature collagen (IntraT area), which is usually observed in infiltrative tumors, while a greater amount of thicker and closer collagen bundles should be attributable to a mature and physiological collagen (ExtraT area). In this direction, other authors confirmed the prevalence of thin collagen bundles in metastatic and advanced-stage tumors [277,278]. Synchrotron imaging data also showed different arrangements of the collagen fibers between IntraT and ExtraT areas; indeed, it was demonstrated that the IntraT area has a greater isotropy, a smaller fractal dimension, and a very reduced connectivity compared to the ExtraT areas. This information, read together in the light of a biomechanical rationale, outlines evidence of compromised mechanical strength and tissue tension, and could explain the extreme dysfunctionality of the tumoral and PeriT stroma. Thus, in the PeriT and IntraT stroma, the collagen alterations could promote the clustering of the neoplastic cells in small nests, supporting the epithelial-to-mesenchymal transition (EMT). Synchrotron PhC-microCT results are corroborated by literature data; thus, it has been demonstrated that collagen bundle arrangement undergoes a progressive disorganization from clinically healthy mucosa to advanced carcinomas [279,280].

However, no significant morphometrical mismatches were found by PhC-microCT in the PeriT region between the different OTSCC stages. Conversely, FTIRI analysis showed significantly lower amounts of collagen in the PeriT stroma of advanced OTSCCs compared to early ones, characterized also by a significant reduction of folded secondary structures.

Moreover, the correlation analysis among microstructural indices, macro-molecular parameters, and clinical stage in the PeriT area of OTSCCs suggests the following observations: (1) there is a strong direct correlation between collagen bundle thickness and COLL/PRT ratio; (2) there are strong inverse correlations between stage and both

COLL/PRT and PRO/PRT ratios; (3) no relevant (“strong” or “very strong”) correlation is present between the clinical stage and the microarchitectural collagen bundle parameters (bundle thickness, inter-spacing, and volume percentage).

These data support the synchrotron results that did not detect any significant difference between the pathological stages in collagen bundle microarchitectural organization. Conversely, the FTIRI analysis showed, at a finer (macromolecular) scale level, the presence of collagen that progressively decreases with the progression of the clinical stages.

To the best of our knowledge, this evidence suggests that during tumor progression, the macromolecular deviations anticipate, at a temporal level, the coarser 3D-organization modifications of the collagen bundles present in the stroma. Indeed, collagen type I (Col-I) degradation seems to be the crucial step during the stepwise oral cancer tumorigenesis, as the main target of MMPs in desmoplastic tumors. In fact, in the PeriT stroma, the stromal progenitor cells are constantly recruited and CAFs produce a greater level of proteases, promoting the ECM degradation. In addition, MMPs can release bioactive fragments associated with other critical pathways, promoting angiogenesis, tumor proliferation, and migration. Therefore, the ECM proteolytic remodeling and the activation of a migratory cellular phenotype could represent a possible mechanism by which the MMPs were associated with poor survival outcomes.

In conclusion, these data could provide a significant contribution to understanding the TME rearrangement during oral tumorigenesis, in order to better comprehend the relationship between neoplastic epithelium and tumor stroma. Moreover, the morphometrical analysis of staging parameters and adverse risk factors could be facilitated [252].

5. General considerations, Limits of the Methods and Future Developments

Artificial Intelligence (AI) has been one of the most discussed and debated topics in recent years, raising doubts about the accuracy of the predictions and ethical issues about whether these “intelligent minds” could one day replace humankind. However, AI techniques are not yet frequently implemented in the field of dentistry, specifically dental implantology and oral pathology. Currently, in this area there is a lack of comprehensive analyses of the challenges associated with AI and its algorithms. Consequently, the objective of this research is to encourage the application of these innovative methods illustrating the feasibility of employing ML algorithms for data analysis and DL algorithms for image analysis to autonomously and rapidly segment the various tissue portions of a large number of samples, thereby expediting the diagnosis and reducing its expenses.

As we have demonstrated in the previous chapters, there is not a single AI-based method, but different types of mathematical algorithms capable of performing different functions based on the type of human need. Indeed, it is from the latter that the control over the machine starts; there are several tools, such as Dragonfly used in this research in the area of imaging, that allow one to train a process in order to accomplish a certain task. In fact, the advantage of using AI lies precisely in the effectiveness and efficiency of the processes. Moreover, in the specific case of image analysis, the added value is to be able to discriminate different tissues not only on the basis of their density (in terms of grey tones) but also on the basis of their morphology. This happens because, just like the human eye, AI can distinguish the shape and intensity of a given object that it learns to observe. Therefore, training becomes crucial to teach an algorithm which can learn from experience, as an Artificial Neural Network (ANN) can be.

However, the result of this training and subsequent tests produced on new samples/patients will always be checked by humans, who will make the final decision. As with the study on different stages of periodontitis (Chapter 4.5), the application will make a proposal based on the previously trained inputs, and the result of the classification will be a second opinion for the physician. In the future, it will be necessary to increase the number of inputs to have more case histories and for the machine learning algorithm to give more accurate results. In Chapter 4.2 likewise, it will be necessary to increase the

number of input images so that the neural network can learn to better recognize the longitudinal bundles from the transversal ones. As in the Chapter 4.3 to ensure that there is greater output accuracy and that all tissues are segmented equally across all patients, a larger dataset is needed. These studies demonstrate that the emphasis is on implementing AI more effectively in medicine and dentistry in many manners, despite the significant challenges posed by the limited availability of pictures due to privacy concerns and other clinical data accessibility issues. In this direction, effective Open Science policies could accelerate diagnoses and consequently therapies for patients.

For this reason, data repositories — systems that receive contributions of relevant information from the research community to store biomedical data in accordance with rules and requirements — are crucial for the management, preservation, and dissemination of research data, providing as vital resources for biomedical research groups. Consequently, the researchers could deposit and use the sharing data improving the AI trainings and the accuracy of the mathematical models.



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The doctoral fellowship was co-funded with resources from the National Operational Program
Research and Innovation 2014-2020, FSE resources REACT-EU
Action IV.4 “Doctorates and research contracts on innovation themes”
and Action IV.5 “Doctorates on Green themes”

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Appendix

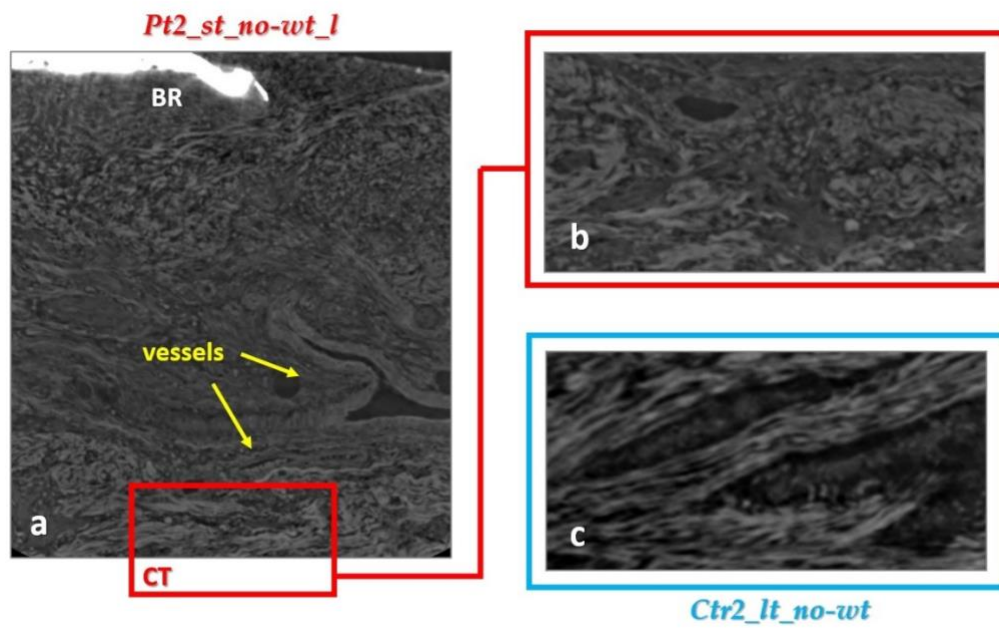


Figure 4.2.S1. *Pt2_st_no-wt_l* (a) shows the immaturity of the connective tissue: collagen fibers strongly disorganized (b) and abundant presence of micro vessels (yellow arrows); *Ctrl2_lt_no-wt* (c) differently displays a tissue more mature with collagen fibers along a preferential direction.

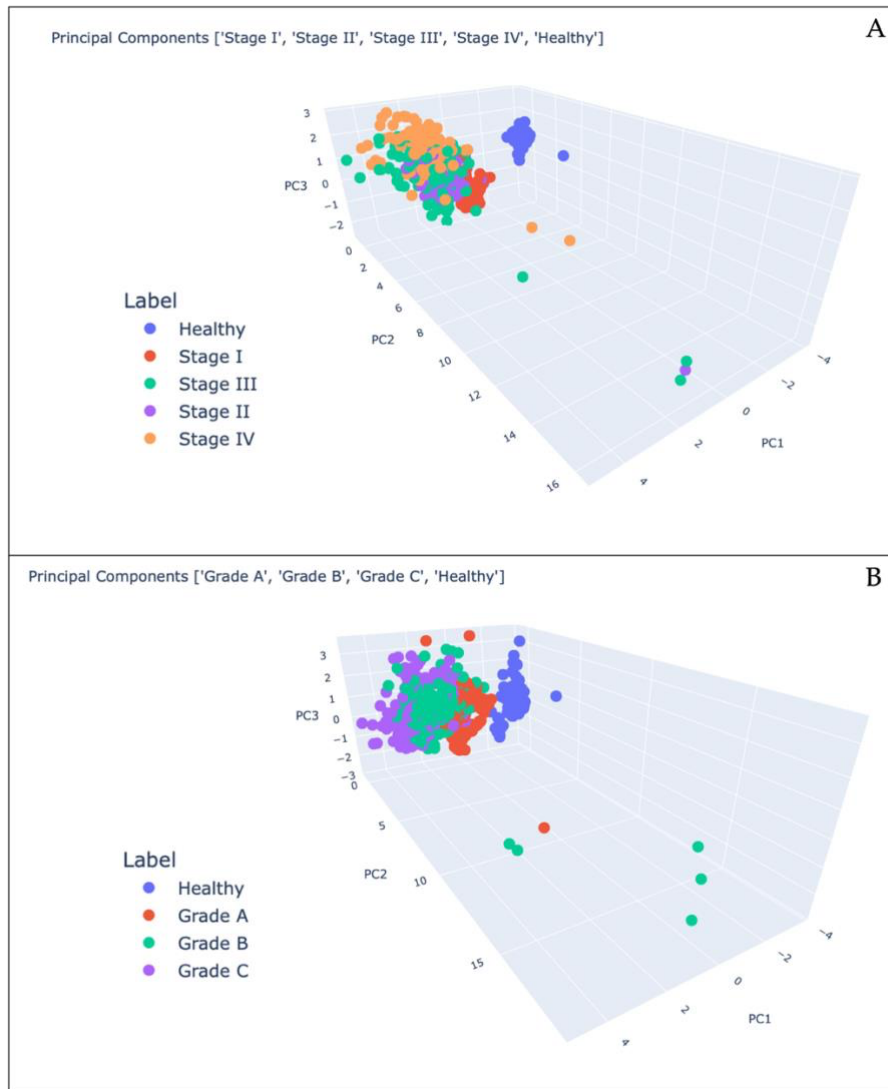


Figure 4.5.S1. PCA applied to staging data (A). PCA applied to grading data (B). The different dots in the figures stand for the main characteristics of the patients, which are associated with different colors according to their class. Therefore, since the colored groups are easily distinguishable by eye in space, it means that they are separable and consequently an algorithm will be able to easily learn to discriminate them into different cases of staging and grading, proving the divisibility of the internal classes.

Acknowledgments

My deepest gratitude goes to my supervisors Professor Di Nicola and Professor Giuliani, who guided me through this experience by instilling in me a love of research and the importance of celebrating even the smallest victories because "life is already hard in itself." Secondly, I'd want to express my gratitude to Professor Piattelli and Professor Fulle for having faith in me from the beginning of this doctoral research.

The AoN Implants industry welcomed me and is the source of a remarkable partnership; I am grateful to them.

My supervisor at the School of Dental Medicine in Belgrade, Professor Radunovic, was another invaluable resource throughout my period abroad; with her guidance, I had the opportunity to learn about a new culture and meet wonderful people who at all times made me feel at home.

For allowing me access to the ELETTRA Synchrotron facility, I would like to thank Professor Tromba and the whole SYRMEP line team.

To Michele, thank you for supporting and putting up with me in every moment of this course, for being a mentor and a friend and, most importantly, for never letting me forget how to clean a scintillator.

To my friend Tea, without whom my experience abroad would not have been the same. We found each other on our weekend trips while navigating time zones and attending baptisms.

Idemo!

Finally, I would like to thank all my friends, *Daughters of the Stars*, university colleagues and family members, my mother, brother and uncles who always believed in me and brought out the best side of me making these years unforgettable.

I would also like to thank my father and grandmother who despite their absence I could always feel close, and I believe they are with me even at this time.