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ORIGINAL PAPER

Characterization of human bone tissue for diagnosis

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Abstract.
Thermogavimetric methods and differential thermal analysis (DSC) are generally used diagnostic techniques of Materials Science. There are a particular group of specific methods for measuring the properties of a system depending on temperature. Moreover, measuring the heat released or absorbed during a reaction provides important analytical information. Applying these methods of investigation in the study of human bone has led to promising results in early diagnosis of various diseases in the bone tissue with great therapeutic importance. Preliminary study that we conducted reveals encouraging results of using these techniques in early clinical diagnosis of some bone diseases, given the complex structure of bone and the advantages of knowledge the degree of tissue damage before surgery which offers the premises for more advanced studies in this direction. Experimental results will be shown to assess the suitability of the pointed out characterization method , and to allow the definition of parameters to evaluate the hallux valgus and coxarthrosis diseases.

Key words: Bone tissue, thermal analysis, termogravimetry, differential thermal analysis

Introduction

One of the most remarkable tissues of the human body, bone, is the material which makes vertebrates distinct from other animals. Far from inert and lifeless, bones are living, dynamic structures, with same strenght as cast iron, meanwhile light as wood. Bones serve a wide variety of very diverse functions within us: structural support for heart, lungs and marrow; protection for brain, uterus and other internal organs; attachement sites for muscles allowing movement of limbs; mineral reservoir for calcium and phosphorus; defense against acidosis; trap for some dangerous minerals such as lead. Noted for their strength and resiliency during life, it is light, strong, can adapt to its functional demands, and repair itself, bones will remain after we are long gone.

Bone origin - The origin of the bone cells are mesenchymal cells which were within the embryo during early development; some of them remain in the bone

marrow but do not form blood cells. The hematopoetic cells from the liquid part of the bone marrow, and some of them circulate with the blood.

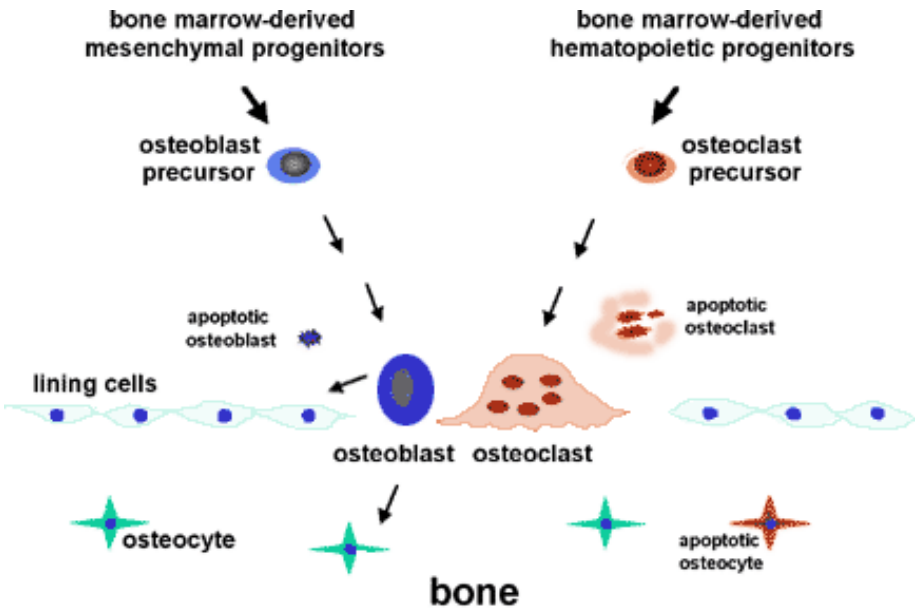


Fig.1 Diagram of the origins and fates of the bone cells (by Merry Jo Oursler and Teresita Bellido 2003) .

We can distinguish two major kind of bone: trabecular (spongy) and cortical (solid). Bone are build by 10% cells and 90% matrix. There are two categories of bone cells: osteoclasts which resorb (dissolve) the bone and osteoblast family which consists of osteoblasts that form bone, osteocytes that help maintain bone and lining cells that cover surface of the bone.

Osteoclast are large multinucleai cells able to resorb the bone. They form sealed compartments next to the bone surface, secrete acids and enzymes able to degrade the bone. Finishing the resorbing bone proces, they undergo apoptosis.

Osteoblasts, cuboidal and columnar in shape with a centra nucleus on the bone surface, come from bone marrow precursor cells. Osteoblasts job is to make the proteins that will form organic matrix of the bone and to control mineralization of the bone . Osteoblasts have receptors for hormones like vitamin D, estrogen and parathyroid hormone. They secrete PHEX, a protein that regulate the amount of

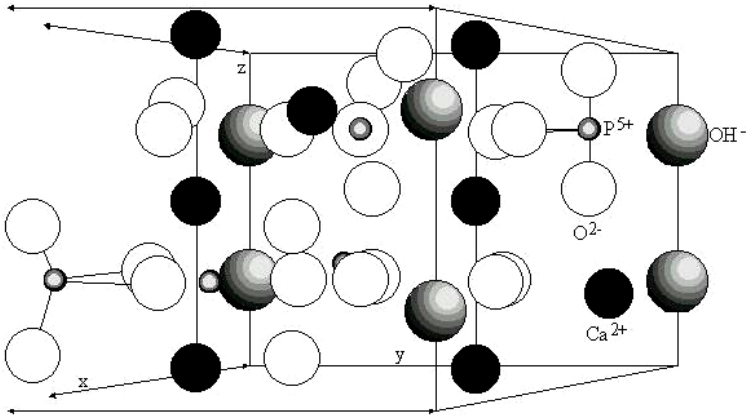
phosphate excreted by the kidney. When the team of osteoblasts has finished making new bone, some become sorrounded with matrix and differentiate into osteocytes, others will remain on the surface of the new bone and differentiate into lining cells. The remains undergo apoptosis and desintegrate.

The mature bone is always remodeling: the old bone is resorbed and replaced with new bone. A team of osteoblasts and osteoclasts move along the bone, dissolving and rebuilding. Factors wich could modify apoptosis play the role in treating and preventing osteoporosis.

Bone structure - Mature bone is composed of proteins and minerals. Approximately 60% the weight of the bone is mineral, manly calcium and phosphate. The rest is water and matrix, which is formed before the mineral is deposited, and can be considered the scaffolfing for the bone. About 90% of the matrix proteins are collagen, which is the most abundant protein in the body. Collagen is very strong and forms bone, cartilage, skin and tendons.

Besides its mechanical role, the bone is a reservoir for minerals (metablic function). The bone stores 90% of the body's calcium and 85% of the phosphorus, that's why the blood level of calcium is very important to stand within a narrow range. If blood calcium gets too high or to low, the muscles and nerves will not function. In times of need (during pregnacy) calcium can be removed from the bones process carefully regulated by hormones.

Inorganic matrix (60%) of the matrix, is mostly hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ which fills in holes of collagen. We can find different type of calcium phosphates in the composition of bone sturvcture such as: Monocalcium phosphate, E341(i): $Ca(H_2PO_4)_2$; Dicalcium phosphate (dibasic calcium phosphate), E341(ii): $CaHPO_4$; Tricalcium phosphate (tribasic calcium phosphate or tricalcic phosphate), E341(iii): $Ca_3(PO_4)_2$, sometimes referred to simply as calcium phosphate or calcium orthophosphate; Hydroxyapatite $Ca_5(PO_4)_3(OH)$; Apatite $Ca_{10}(PO_4)_6(OH, F, Cl, Br)_2$; Octacalcium phosphate $Ca_8H_2(PO_4)_6.5H_2O$; Biphasic calcium phosphate (not to be confused with dibasic calcium phosphate) is a biomaterial that is biphasic. It is an alternative to bone grafting that is used around osseous implants (such as dental implants). It gradually resorbs and becomes substituted by new vital bone (via bone regeneration). In addition to the above, of the compounds occurring in the $CaO\text{-}H_2O\text{-}P_4O_{10}$ phase diagram, $Ca_4P_2O_9$ (probably $Ca_3(PO_4)_2.CaO$) is notable.



Crystal Structure of Calcium Hydroxyapatite Powders Synthesized in SBF at 37°C
(Hexagonal, *P6 3/m*, a = 9.4125, c = 6.8765 Å)

Fig.2 Crystal structure of calcium hydroxyapatite powder synthesized in SBF at 37° C.

Biomechanics - Sciencists figured out how to design this wonderful material, bone, appreciating how nature has achieved a solution to a demanding task, such as holding up a one ton animal who runs at a very high speeds.Due to the forces which are applied to the bones, deformation like compressive strain an tensile strain give rise to shear strain. Strain can be expresses as a percentage (100x change in length/original length). To have stretched or compressed the bone, a force had to be applied to it. The force per unit area is called stress measured in Newtons per square meter or Pascals.

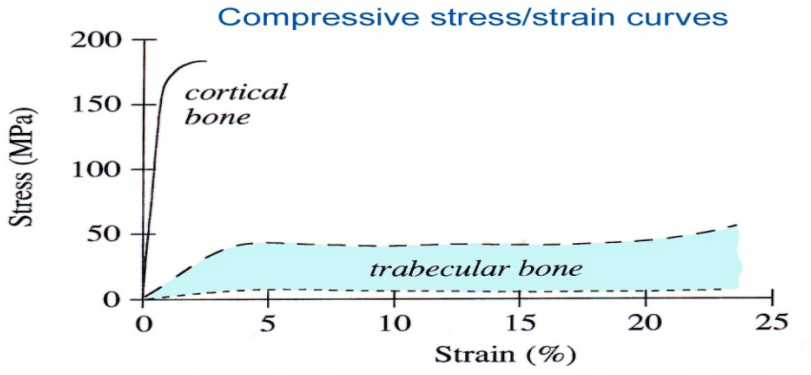


Fig.3 Diagram of compressive stress/strain for cortical and trabecular bone.

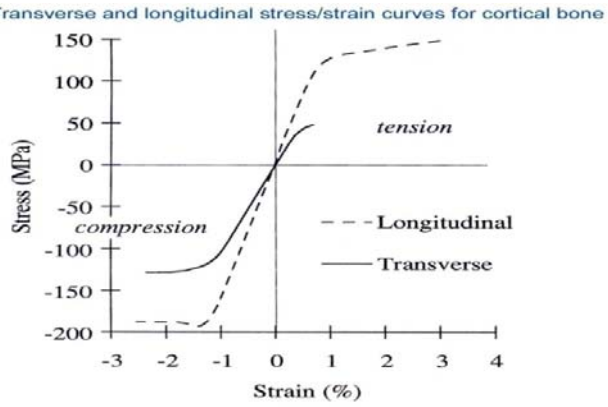


Fig.4 Diagram for transverse and longitudinal stress/strain curves for cortical bone.

In order to investigate the bone structure and changes produced in this structure by different deseases, we can use a series of investigation methods wich provide cantitative and qualitative measures that can be a good indicator for the pathological stage of its structure.

Measurement Methods

Thermal Analysis (TA):Thermo Gravimetric (TG) analysis is a technique in which the mass of the substance under investigation is monitored as a function of temperature or time as the sample specimens are subjected to a controlled temperature program in a controlled atmosphere[4].
A TG consists of a sample pan that is supported by a precision balance. That pan resides in a furnace and is heated or cooled during the experiment. The mass of the sample is monitored during the experiment. A sample purge gas controls the sample environment. This gas may be inert or a reactive gas that flows over the sample and exits through an exhaust[4].

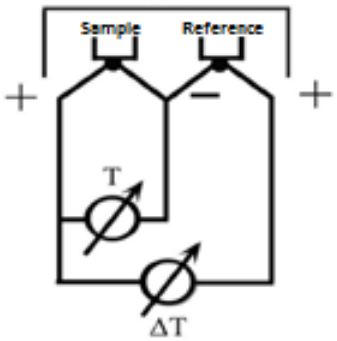
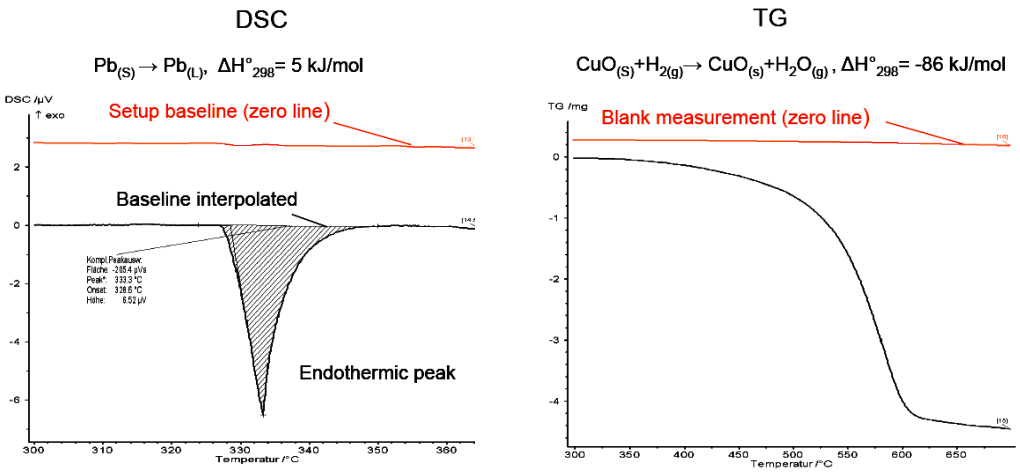


Fig. 5 Principle of combined thermocuple able to register the temperature of an object and the temperature difference between sample and reference.

Depending on the engineering design, measuring cell constructuction and the way of the data representation, variety of methods has been arisen: DTG, DTA, DSC, STA, etc.

Thermal Analysis (TA): Derivative Thermo Gravimetric (DTG) analysis is an undirected thermal analysis in which the rate of material weight change upon heating versus temperature is plotted. It is obtained as the first derivative of the TG. It is used to simplify reading of weight-versus-temperature thermogram peaks that occur close together[4].

Thermal Analysis (TA): Differential Scanning Calorimetry measuring principle is to compare the rate of heat flow to the sample and to an inert material that is heated or cooled at the same rate. Generally, the temperature program for a DSC analysis is designed so that the sample holder temperature increases linearly as a function of time. The reference sample should have a well-defined heat capacity over the range of temperatures to be scanned. Changes in the sample, which are associated with absorption or evolution of heat, cause a change in the differential heat flow, which is then recorded as a peak. The area under the peak is directly proportional to the enthalpic change and its direction indicates whether the thermal event is endothermic or exothermic. For proteins, the thermally induced process detectable by DSC is the structural melting or unfolding of the molecules. The transition of protein from a native to a denatured conformation is accompanied by the rupture of inter- and intra-molecular bonds, and the process has to occur in a cooperative manner to be discerned by DSC[4].



a) Shift of the baseline could appear due to change in thermal resistance of the setup. Position is depending on measuring-history.

b) Measurements may have a significant change in weight due to chandes in gas density and viscosity.

Fig.6 Diagram resulting from DSC (a) and TG (b)

X-Ray power diffraction - X-rays are electromagnetic waves with a wavelength in the range of interatomic distances (0.1-10 Å). This match of length scales makes them suitable for the study of crystalline materials. For single-phase materials the crystal structure can be obtained directly using X-Ray powder diffraction (XRD). With the help of a database of known structures XRD can be used for phase identification[12].

When X-ray radiation is directed on a sample, the X-rays are scattered ("diffracted") by electrons present in the material. If the atoms in the material are arranged in a regular structure, i.e. if the material is crystalline, this scattering results in maxima and minima in the diffracted intensity. The signal maxima follow Bragg's law $n\lambda = 2d\sin\Theta$. Here n is an integer, λ is the X-ray wavelength, d is the distance between crystal lattice planes and Θ is the diffraction angle (Fig.7). Thus, for each lattice spacing d, Bragg's law predicts a maximum at a characteristic diffraction angle Θ. During an XRD measurement the angles of incidence and detection are scanned. When the intensity of detected X-rays is plotted as a function of angle Θ, an X-ray powder diffraction pattern is obtained, which is characteristic for the sample material.

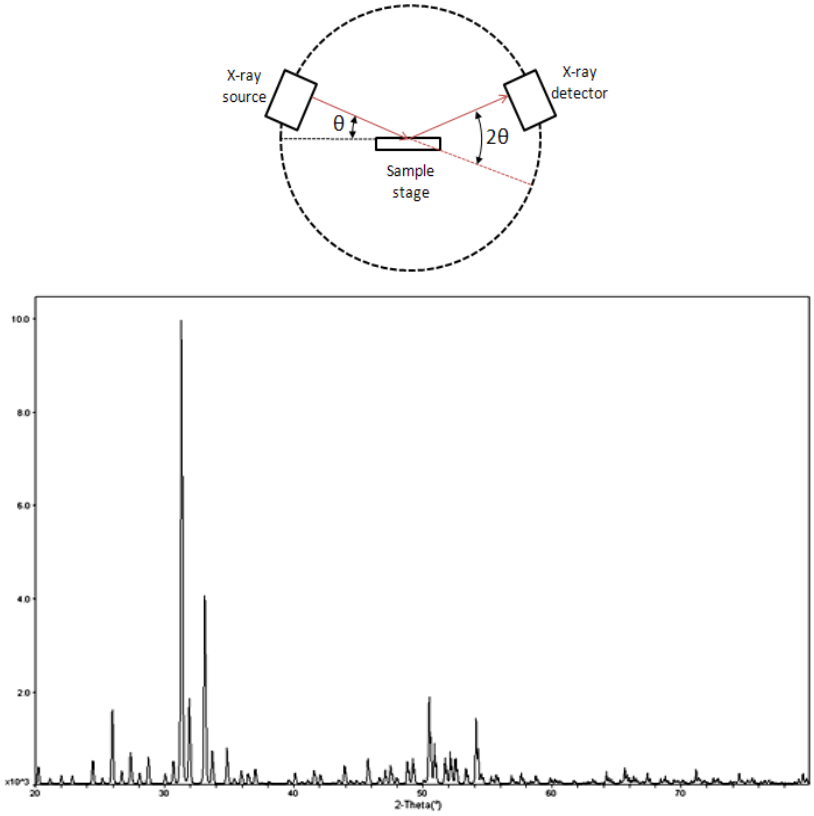


Fig.7 Principle of X-ray diffraction and resulted spectum.

Energy-dispersive X-ray spectroscopy (EDX) is an analytical measurement technique used for the local elemental analysis or chemical characterization of a sample. The sample characterization is possible because each element has a unique atomic structure allowing a unique set of peaks on its X-ray spectrum[7]. At rest, an atom within the sample contains ground state (or unexcited) electrons in discrete energy levels or electron shells bound to the nucleus. To stimulate the emission of characteristic X-rays from a specimen, a high-energy beam of charged particles (electrons or protons), or a beam of X-rays, is focused into the sample. The incident beam may excite an electron in an inner shell, ejecting it from the shell while creating an electron hole where the electron was. An electron from an outer, higher-energy shell then fills the hole, and the difference in energy between the higher-energy shell and the lower energy shell may be released in the form of an X-ray. The number and energy of the X-rays emitted from a specimen can be measured by an energy-dispersive spectrometer. As the energy of the X-rays is characteristic of the difference in energy between the two shells, and of the atomic structure of the element from which they were emitted, this allows the elemental composition of the specimen to be measured[7].

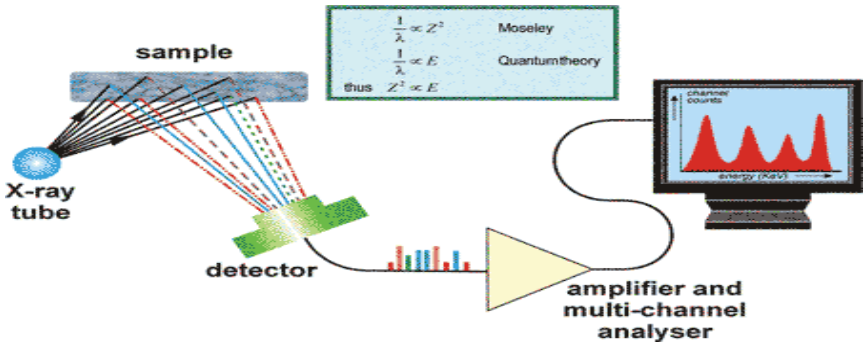


Fig. 8. Principle of working of X-ray spectroscopy.

Our study takes in consideration two major deseases of bone structure, with a high incidence in population, with a major impact on their lifestyle and the quality of their life. The study has been performed on samples collected from pacients under observation with ages between 54 and 73, male, samples beeing removed by surgery. Two specific deseases have been taken in consideration in this study: hallux valgus and coxarthrosis.

Case study 1: Hallux Valgus

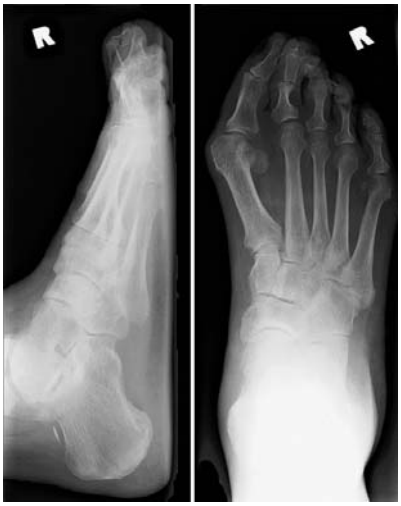


Fig.9. Preoperative radiograph of foot affected by hallux valgus. Picture reported by MEDSCAPE[5]

Case study 2: Coxarthrosis

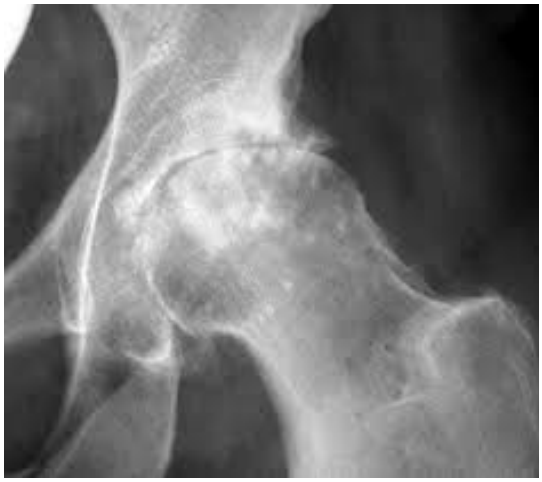


Fig.10. Coxarthrosis

Materials and experimental setup

Case study 1: Hallux valgus

The analyses were carried out on 6 different samples of human bones of the first metatarsal head as reported in Tab.1. One of them is extracted from a healthy foot, the others from feet affected by hallux valgus. From each patient a sample of about 600-700 mg is extracted in order to perform all the proposed measurements. The samples are extracted during surgery sections. After the surgical removal, the samples of bone were stored under acetone to prevent the degradation of organic liquids and the collagenase effect due to contact with air. Each sample was washed with acetone to remove the lipidic phase, crushed and washed again in acetone and distilled water. Then, the powder was dried at about 80°C to remove the residual acetone and water adsorbed on the bone powder. Finally, the samples were normalized at room temperature.

Table 1. Characteristics of the surgical removed samples.

	Age	sex	Bone	pathology	Weight of the sample [mg]
Patient#1	54	Male	first metatarsal head	healthy	607
Patient#2	57	Male	first metatarsal head	hallux valgus	654
Patient#3	55	Male	first metatarsal head	hallux valgus	606
Patient#4	67	Male	first metatarsal head	hallux valgus	702
Patient#5	75	Male	first metatarsal head	hallux valgus	600
Patient#6	73	Male	first metatarsal head	hallux valgus	635

NETZSCH STA 409 - permits to evaluate at the same time: (i) the TG curve; (ii) the DTG curve, and (iii) the DSC. The advantage of this instrument is that it provides the possibility of easily comparing the three curves by assuming the same environmental conditions.

Concerning the thermal analysis, preliminary studies were carried out to optimize the experimental conditions regarding the weight of the samples, the speed of the heating, and the rate of heating

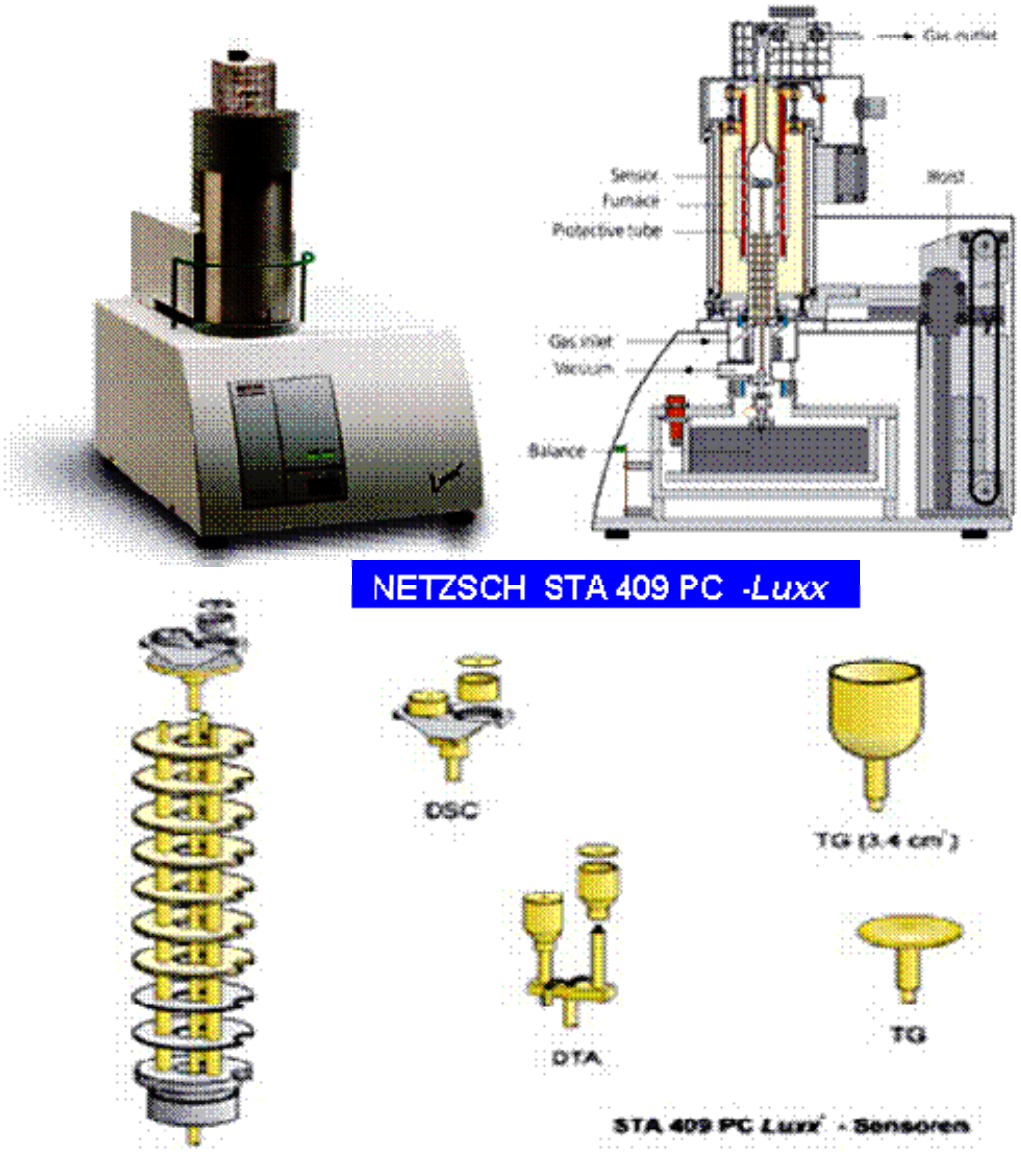


Fig. 11. NETZSCH STA 409 device used for thermal analisys.

Experimental Conditions: reference sample is 20 mg of calcined coalin, maintained in the environment of air, at a flow rate 15 ml/min, heating rate of 10°C/minute, initial temperature 20°C, final temperature 800°C, crucible of Al_2O_3.

Case study 2: Coxarthrosys

In order to define a procedure based on TGA for the diagnosis of primary coxarthrosis we have analyzed three bone samples affected by primary coxarthrosis in the temperature range [20, 800] °C. These samples are furnished by: Armsav, Gentilor, Grato with weight varying in the range (22–30) mg.

The experimental conditions used in the experiments are the following: weight of sample 20mg, flow speed 15ml/minute, environment air, temperature step 10°C/minute, initial temperature 20°C, final temperature 800°C.

Results and discussion

Thermal Analisys performed with the device NETZSCH STA 409 for healty bone extracted from patient #1 (fig.12) proved the diagram below for DSC, TG and DTG. By comparison we can see in fig.12 changes in TG curves for patients affected by hallux valgus with different stages of the desease.

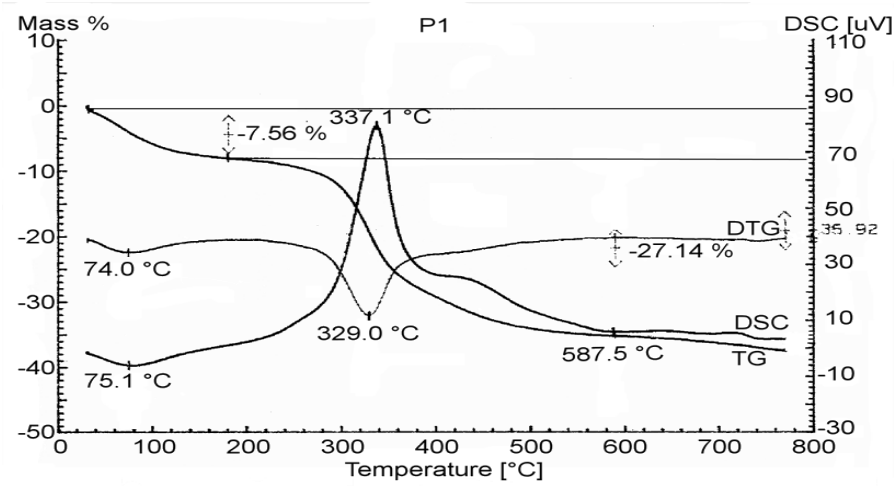


Fig.12. DSC, TG and DTG curves of a healthy human metatarsal head, extracted by Patient#1

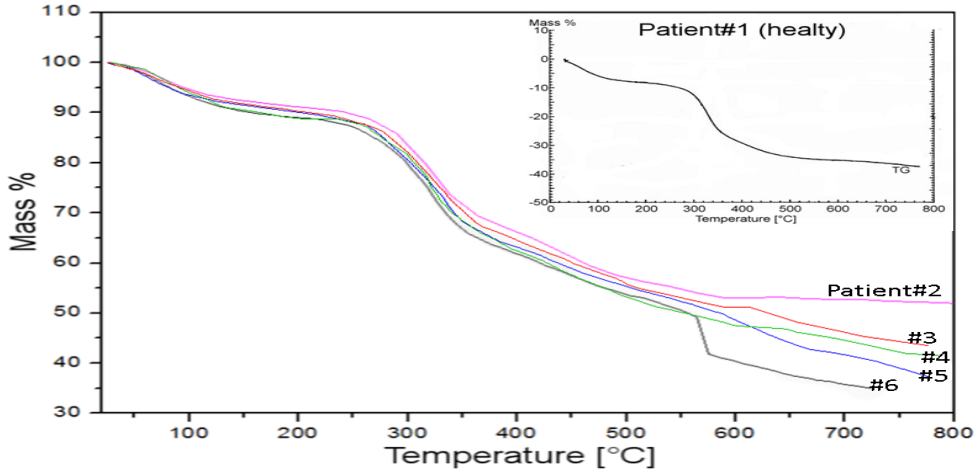


Fig.13. TG curves of the samples of bones affected by hallux valgus.

Table.2 Main weight losses detected by using the TG.

Bone Sample	Pathology	Age	H_2O loss (%)	organic loss peak I (%)	organic loss peak II (%)	organic loss peak III (%)	Total loss (%)
Patient#1	healthy	54	7.56	27.14	///	///	34.70
Patient#2	hallux valgus	57	8.71	23.50	11.53	4.27	48.01
Patient#6	hallux valgus	73	10.92	26.95	8.00	1.,24	65.11

TG curves for patients affected by coxarthrosis with different stages of the desease can be seen in fig.14. The chart shows a higher weight loss function of temprature for Gentilor sample.

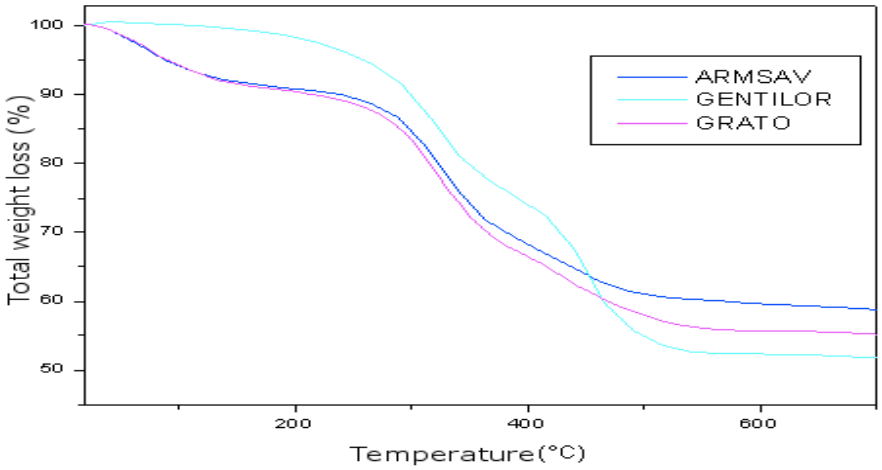


Fig.14. Total weight loose of the samples of bone under examination versus the temperature

Table 3. Related and total weight losses of bone's samples

Bone's sample	Age	H_2O Loose (%)	Organic loose I peak (%)	Organic loose II peak (%)	Organic loose III peak (%)	Total loose (%)
Healthy	/	7,56	27,14	/	/	34,70
Gentilor	60	3,00	21,29	11,56	11,56	47,41
Armsav	65	9,47	21,35	8,70	8,15	47,67
Grato	72	9,07	20,96	6,89	4,28	41,20

The DTC curves shows aditional organic weight loss (peak II and III), the amplitude of thise peaks being heigher as the stage of the desease is more advanced.

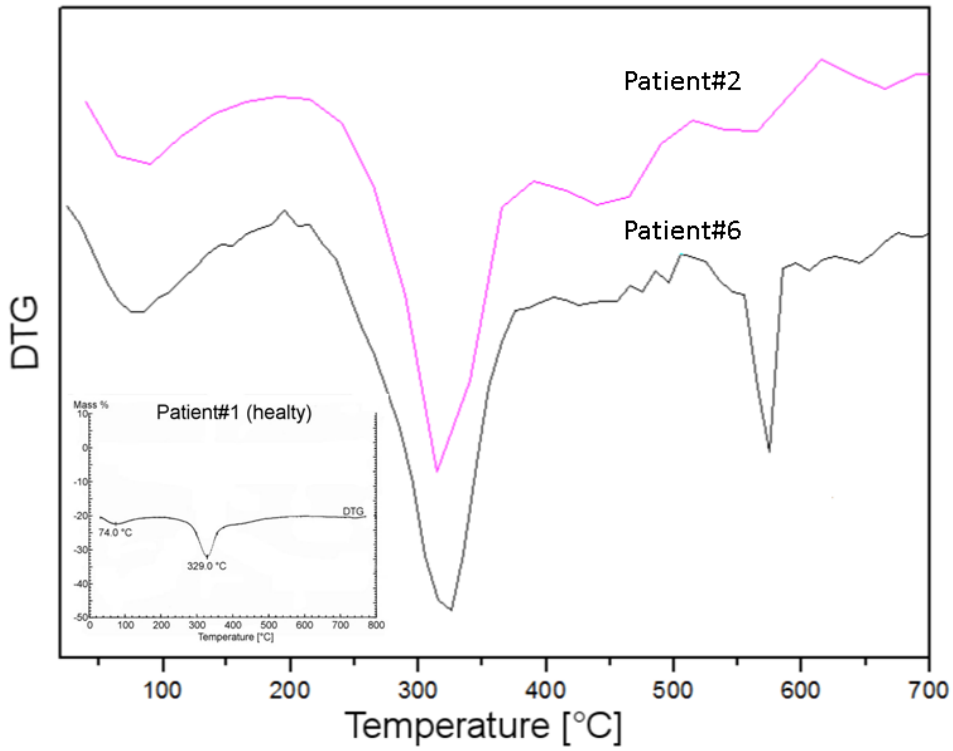


Fig.15. DTC curves of the samples of bones affected by hallux valgus.

DTG curves undeline the degree of desease mure advanced for Gentilor sample affected by primary coxatrosis.

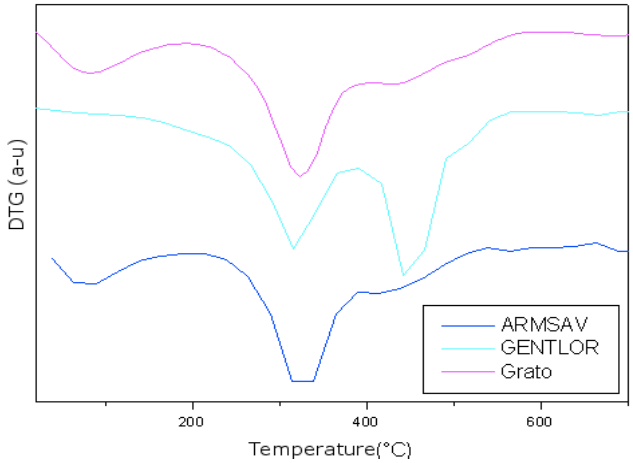


Fig.16..Differential Thermo Gravimetric curve of the bone samples affected by primary coxarthrosis.

Analysing the results of DSC curves we can see that the ratio of weight loss to calcium phosphate of the bone structure affected by hallux valgus is in a reverse proportionality with the degree of degeneration.

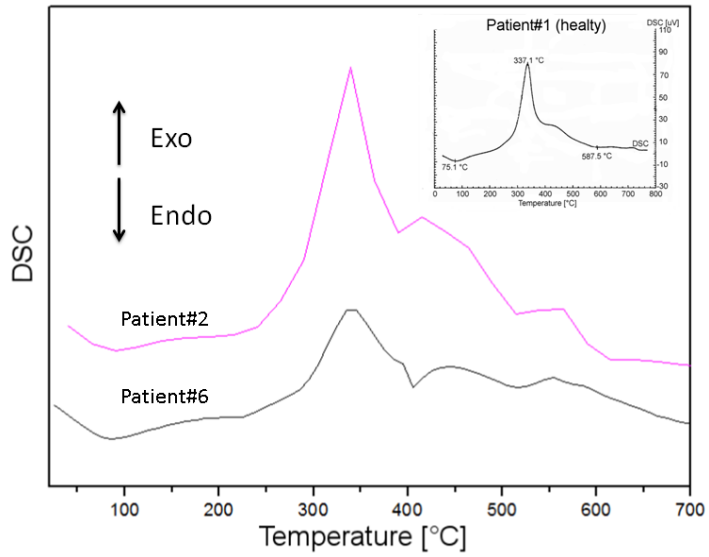


Fig.17. DSC curves of the samples of bones affected by hallux valgus.

Table.4 Temperature of the DSC peaks for each bone sample affected by hallux valgus.

Bone Sample	**Pathology**	**Age**	**Temp.H_2O Loss [°C]**	**Temp. peak I [°C]**	**Temp. peak II [°C]**	**Temp. peak III [°C]**
Patient#1	healthy	54	75.1	337.1	440.0	///
Patient#2	un-healthy	57	88.9	336.4	440.8	552.0
Patient#6	un-healthy	73	87.7	340.6	444.2	552.7

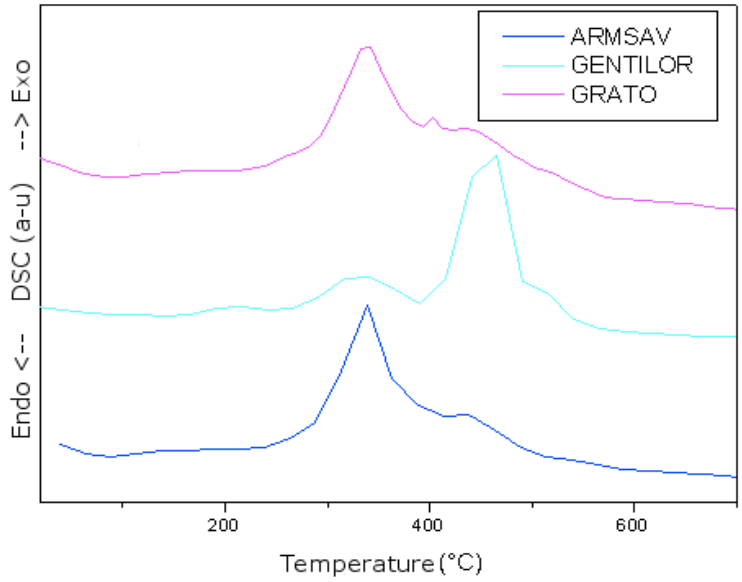


Fig.18. Differential Scanning Calorimetry curve of the bone samples affected by primary coxarthrosis

Table 5.Temperature of the DSC peaks for each bone samples affected by coxarthrosis.

Bone's Sample	**Age**	**Temp.H_2O Loose [°C]**	**Temp. I peak [°C]**	**Temp. II peak [°C]**	**Temp. III peak [°C]**
Healthy	/	75,1	337,1	440,0	/
Gentilor	60	98,0	319,2	456,9	501,6
Armsav	65	84,4	336,9	436,2	541,9
Grato	72	90,8	338,1	403,9	436,4

Using energy dispersive X-ray spectroscopy, by comparison, we have investigated the X-ray spectrum of healty bone and comercial hydroxyapatite, and also with the bone affected by hallux valgus.

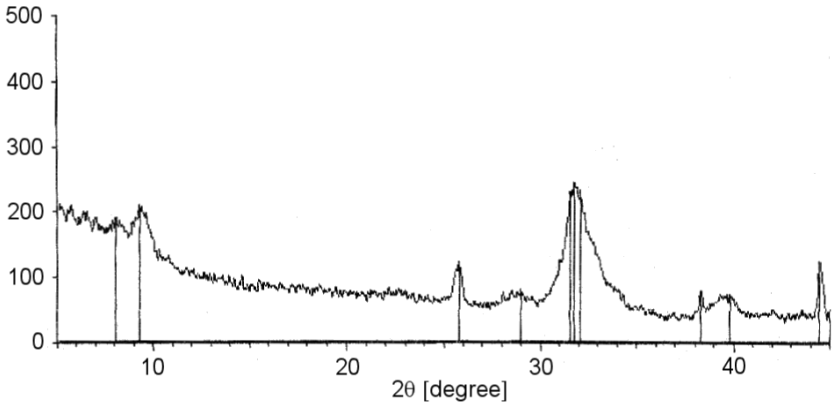


Fig. 19. X-ray spectrum of a sample of healthy bone tissue.

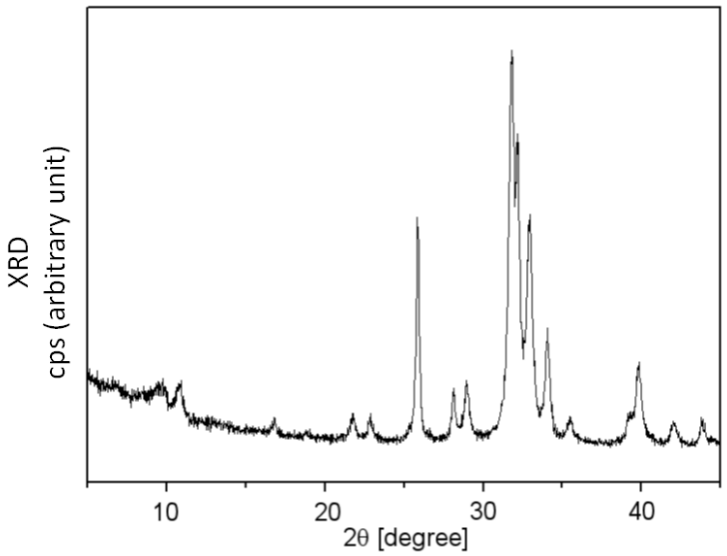


Fig. 20. X-ray spectrum of commercial hydroxyapatite.

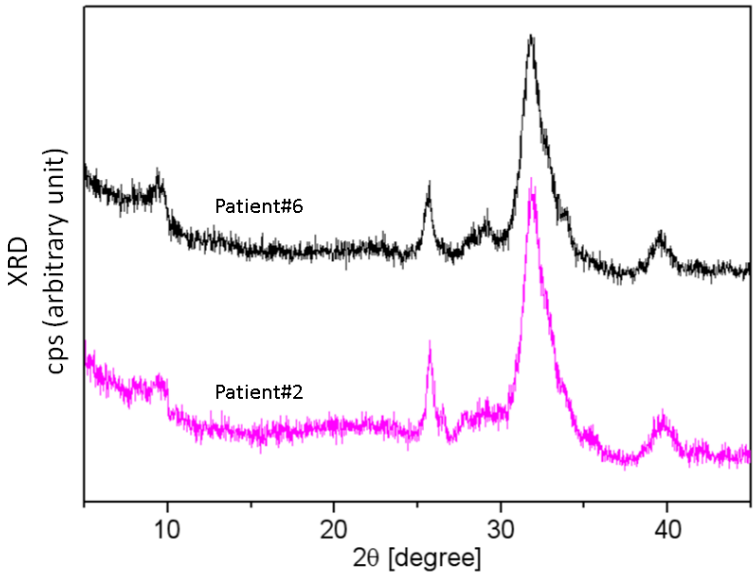


Fig.21. X-ray spectrum of the samples of bones affected by hallux valgus.

Table 6. Number of Counts per Second (cps).

			cps (arbitrary unit)	
Sample	Pathology	Age	2q=26°	2q =32°
Patient#1	healthy	54	122.4	228.9
Patient#2	un-healthy	57	151	279
Patient#6	un-healthy	73	100	361

X-ray spectrum and number of counts per second proves the Ca/P ratio for advanced stage of coxarthrosis as being less than one of healty bone.

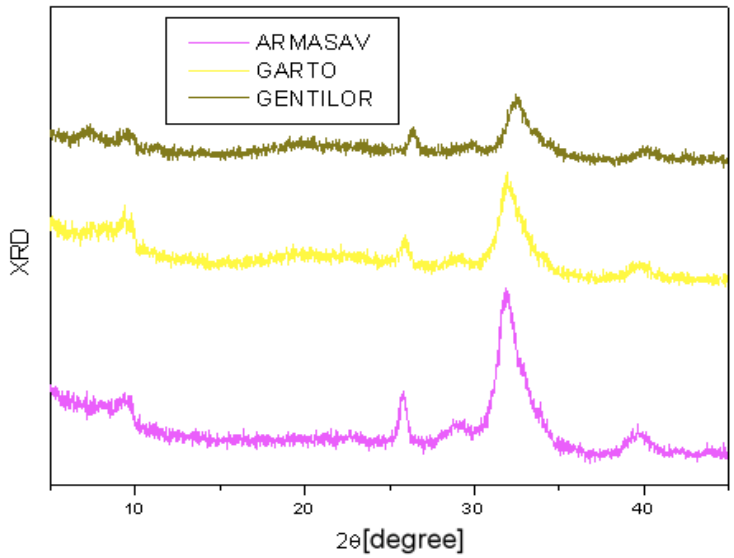


Fig.22. X-ray spectrum of the samples of bones affected by primary coxarthrosis.

Table 7 . Average intensity value for bone samples affected by primary coxarthrosis

Bone's Sample	**Age**	**2q=26 (cps)**	**2q =32 (cps)**	**Average intensity**
Healty	/	122,4	228,9	176
Gentilor	60	52	119	86
Armsav	65	128	128	128
Grato	72	56	204	130

The chemical analysis of the pathologic bone showed Ca/P ratio equal to 1.78. This value is higher with respect to the typical stoichiometric value of the pure hydroxyapatite (1.67) and different from the ratio of healthy bone (normally less than 1.67 [14]). More investigations are in progress to well define these values and the role of the Ca/P ratio.

Table 8. Percentage of the elements detected by EDS.

Sample	Age	Na	K	S	Al	Si	Ca	Mg	P	O	Total	Ca/P
Gentilor	60	0,10	0,00	0,26	0,00	0,31	25,16	0,06	11,97	62,13	100	2,10
Armsav	65	1,20	0,15	1,14	0,00	4,80	15,26	0,13	5,70	71,61	100	2,68
Grato	72	0,52	1,91	6,13	0,00	0,08	14,07	0,00	6,60	70,69	100	2,13

Conclusions

Our research presents a new possible thermoanalytical diagnostic measurement techniques to characterize bone tissue. Due to the reduced quantity of bone sample, the proposed methodologies can be performed by simple biopsy (20mg) of the first metatarsal bone. The proposed methodology is based on measurement techniques and instrumentations typically applied to study engineering materials. The proper combination of well-known measurement techniques and commercial measurement instruments allow assessing the traceability of the measurement. Experimental tests are carried out by performing thermal, X-ray powder diffraction and X-Ray dispersive analysis of samples extracted from healthy metatarsal bone head, and the ones affected by hallux valgus. The experimental results highlight a different behaviour of the healthy human bone tissue with respect to the one affected by hallux valgus pathology. All the analyses point out that the intensity of the pathology in Patient#6 is higher with respect to Patient#2. But, while the Thermo Analysis can be performed on 20 mg of bone sample (that can be easily extracted by a biopsy), the X-ray powder diffraction analysis requires a larger quantity of bone (at least 500mg), while the scanning electron microscopy analysis requires high cost measurement instrument.

An extended investigation and then validation of the proposed measurement techniques could make them a routine investigation for the objective and early diagnosis of hallux valgus disease allowing its prevention and giving an answer to the new clinical requirements highlighted in the recent literature.

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