REFERENCE DATA FOR BONE MATERIAL STRENGTH INDEX (BMSI) MEASURED BY IMPACT MICROINDENTATION

Farid Taymouri

Doctoral Thesis

REFERENCE DATA FOR BONE MATERIAL STRENGTH INDEX (BMSI) MEASURED BY IMPACT MICROINDENTATION

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Hacen constar:

Como codirectores, que el proyecto de tesis doctoral titulado "Reference data for Bone Material Strength Index (BMSi) measured by Impact Microindentation" elaborado por el doctorando Farid Taymouri reúne los requisitos necesarios para ser defendida ante el oportuno tribunal calificador para aspirar al Grado de Doctor.

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Justification and Acknowledgment

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Summary of the thesis

Background:
Normal reference values of BMSi by IMI in men and women of 18 years age and older have not been established.

Hypothesis to test:

A-To determine normal BMSi values in normal healthy populations of both genders i.e. Male and Female.

B- BMSi values are independent of age

C- Inter Observer Coefficient variance is less than 5%.

Objectives:
A-Establish the reference values for BMSi in normal Population from Barcelona, Spain.

B- To assess the influence of age , sex and BMI in BMSi.

C-To assess the Inter observer Coefficient variance for IMI in Hospital del Mar.

Methods:
Database of 1500 human (patients and control without disease) were gathered from internal medicine department Database system at Hospital Del Mar between 2008 -2018 from different groups of investigators in this period of time. The relevant information derived from these databases underwent the specialized biostatistical survey and statistical analysis. The inter-observer and intra-observer variability analyzed in order to validate the accuracy and precision of the IMI. This project partly concentrates on the variability from different investigators that reached results with impact Microindentation for measuring BMSi in human bones.

Results:
1-Normal range of BMSi regardless of gender is Mean=83.01 and SD=7.87.

2- In normal population aged 18 and older, BMSi value in males is: 87.80 ± 7.99, and females: 81.69 ± 6.75 (Mean ± SD).

3- BMSi values in the overall cohort as well as in both male and female are independent of age.

4- BMSi values are significantly higher in male than in female. It does mean that BMSi is gender dependent,

5-Inter Observer Coefficient of Variability is < 5%

Conclusions:

In this project BMSi regardless of gender is on average 83.01 and SD=7.87, and derived normal range of BMSi 87.80 ± SD=7.99 in men, and BMSi 81.69 ± SD=6.75 in women of the normal population aged 18 and older. Our results also show that BMSi value is independent to age but is significantly dependent to the gender.

Glossary of terms

BMD-Bone Mineral Density

BMP- Bone Morphogenetic Proteins

BMSi- Bone Mineral Strength index

DXA-Dual energy X-ray absorptiometry

Fx-Fracture

GPa- GigaPascal

IMI-Impact Microindentation

MPa-MegaPascal (In materials science and engineering, the Pascal measures the stiffness, tensile strength and compressive strength of materials. In engineering use, because the Pascal represents a very small quantity, the megapascal (MPa) is the preferred unit for these uses).

NIH –National Institute of Health

NOF-National Osteoporosis Foundation

RPI- Reference Point Indentation

TBS-Trabecular Bone Score

VFA-Vertebral Fracture Assessment

Wnt- The name “Wnt” derives from the acronym between wingless (wg) in Drosophila and Int1 (currently known as Wnt1) in mouse.

Introduction and Background

General concepts about Osteoporosis and Bone Strength

The most prevalent metabolic bone disease globally among adults is bone failure or Osteoporosis. Bone failure or Osteoporosis is a skeletal condition that comprises a diverse group of conditions that result in reduction of bone mass and microarchitectural deterioration leading to increased bone fragility and vulnerability to fractures. Indeed should be considered as a major public health problem. Osteoporosis was defined as" a disease characterized by low bone strength, leading to enhanced bone fragility and a consequent increase in fracture risk" at the 2001 NIH Consensus Development Conference [1]. Osteoporosis is the most common metabolic bone disease and 200 million persons are suffering from osteoporosis around the World. Incidence of osteoporosis related-fractures exponentially increases after the age of 50 years so that 40% of women and 13% of men will sustain at least one fragility fracture in the remaining lifetime. In the first year after hip fracture the mortality rate is about 25% in men and 12% to 24% in women [2]. It is of considerable importance for further evaluation and management of patients after sustaining a fracture because this implies an increased risk of a second fracture [3]. Incidence rates of hip fracture in the world and increasing of aging populations worldwide express indirectly that public health impact of hip fracture is on the raise globally and this implies that strategies for prevention and treatment are necessary [4]. Bone is a strong, rigid and a highly specialized form of connective tissue. Macrostructure of bone has two compartments: Cortical and

Trabecular. The ratio of cortical to trabecular compartments varies between and within bones. There are 206 bones of different shapes, sizes, and functions that form the human body skeletal system. Microscopically bone has particular cells namely osteocyte, osteoblast and osteoclast. Bone is made of an organic matrix so that deposition of calcium salts leads to its strengthening. Nearly 95% of organic matrix contains Type I collagen and the other 5% is proteoglycans and a number of noncollagenous proteins [5]. Osteocytes are the most abundant cell of bone and are 95% of bone cells (20,000 to 80,000 cells per mm3 bone tissue), so that cover 94% of bone surfaces [6].

Mechanobiology of Bone Development

Mechanostat theory of Frost established the bone formation, bone remodeling and mechanical environment relationships. According to this theory when bone sustains strains below the physiological threshold, bone resorption will happen via increased remodeling. On the other hand when strains are more than normal physiological limits, bone formation will take place by increasing modeling. Therefore, bone is intrinsically "Mechanostat-controlled" for regulating its functional adaptation [7]. Bone as a composite material consists of two compartments namely cells and matrix. By a number of pathways bone cells mechanosesors, i.e. osteocytes, sense environment signals and transduce them biochemically to other bone cells and tissues for proper adaptation, adjustment and modifications of bone microarchitecture and mass in response to mechanical loads. Aging is an inevitable process for human beings and has several challenging components like reduction in physical activity with

deleterious effects on bone. Thus, satisfactory adjustment to environmental mechanical loads by mechanosensitive regulatory signals is necessary for bone homeostasis. The most important network of mechanical sensing in bone on the basis of osteocytes "lacunar-canalicular network" is the osteoblast-osteocyte communication system and, with collaboration of osteoclasts, respond to environmental mechanical loads appropriately by alterations in microarchitecture and bone mass. Mechanotransduction process includes several pathways: Wnt signaling, integrins, COX2 and PGE2, hormones (mostly estrogen), NO2, Calcium and fluid flow forces [8].

Bone Composition

Bone as a composite tissue consists of two distinct sectors, organic and inorganic. Bone composition is as follows: mineral 60%, matrix 30%, water 10%. Mineral compartment contains calcium phosphate that leads to bone compressive strength (in cortical bone at the range of 131–224 MPa, and in trabecular bone 5–10 MPa). Matrix contains predominantly collagen fibers responsible for bone tensile strength (in cortical bone ranging from 17–20 GPa and in trabecular bone is 50–100 MPa) [9]. 70-90% of bone is mineral and 10-30% proteins, of which 90% are collagens and 10% noncollagenous. Less than 2% of bone weight is made of lipids [10].

The main component of bone mineral is hydroxyapatite and 60% to 70% of bone dry weight is minerals. There are several age-related changes in composition of healthy bone (in both cortical and trabecular compartments) that show an increase in mineral to organic matrix ratio, calcium-to-phosphate ratio and carbonate-to-phosphate ratio [11]. Conversion of flexible matrix to a very firm and stable structure is provided by mineral

component and determines most of the mechanical strength of bone. In bone, mineral crystals are comparable to geologic minerals in nature i.e. hydroxyapatite (Ca10[PO4]6[OH]2). Current evidences point to the fact that bone mineral is predominantly apatitic. Apatite crystal is a few nanometers thick and from 20 to 50 nm wide. The strength and dynamic properties of a composite material like bone is on the bases of spatial configuration of the hard component i.e. mineral to soft component i.e. bone proteins (Collagen & NCP)[12]. The main protein of bone is collagen. Twenty-nine types of collagens are known but 90 % of bone collagen is type 1 that has a mechanical function so that tensile strength of bone can be attributable to collagen type 1. The structure and organization of fibers that are made from collagens are different, e.g. Type 1 collagen fibers are long whereas fibers of type 2 form mesh-like networks. Influences of type 1 collagen and its associated proteins by mutations result in Osteogenesis Imperfecta [13].

Osteoporosis Imaging Investigation. Dual-energy X-ray absorptiometry (DXA)

Routine clinical utilization of DXA-BMD starts in 1987[14]. DXA gives low radiation dose even with VFA (1-50 mSv.), and currently is the gold standard for BMD measurement, osteoporosis diagnosis and monitoring in humans. DXA-BMD has limitations for detecting fracture risk because 54% of hip fractures in women occur without densitometric criteria for osteoporosis (they had either low bone mass or normal BMD), thus it seems that areal BMD is not the perfect way for evaluation of fragility fracture risk, and there are other factors attributable to bone strength that

are not detectable by DXA i.e. bone size, geometry, cortical and trabecular microarchitecture, matrix material, mineralization, and bone turnover [15].

Non-BMD and Non-Imaging Measurements of Osteoporosis in Clinical Practice

Current tools of clinical risk assessment have limitations and there is a need for having an assessment tool in vivo, increasing our understanding of bone fracture mechanisms. Because of bone hierarchical microstructure, bone properties as a whole are determined by bone multiscale levels structural and compositional properties. In this regard mechanical behavior of cortical bone tissue at the levels of osteonal, micro and tissue level can be investigated by atomic force microscope (AFM) cantilever-based nanoindentation and reference point microindentation (RPI) [16]. Nowadays despite the fact that BMD is a good predictor of fracture risk [17], but has the limitation of a low specificity, it is not enough as fracture risk assessment tool [18]. Fracture risk assessment clinically is provided by DXA machine (BMD measurement) as well as other factors like gender, age, and lifestyle [19]. Moreover, bone fragility and therefore fracture risk are the under influence of other factors such as microarchitecture [20]. Diseases and genetic background for example, influence bone fragility and are not dependent on bone mass [21]. Toughness and, specifically, fracture toughness of bone is determined by microstructural and compositional factors that are not attributable only to BMD [16]. In 1992 modification of classic indentation technique introduced by Oliver and Pharr is called AFM cantilever-based nanoindentation in order to measure elastic properties of materials at very small scales [22]. AFM cantilever-based nanoindentation

applies pressure on the surface of material from an AFM-cantilever as an indenter is utilized to load and indentation depth is recorded. For calculation of elastic properties of material, that are also tested, load indentation curve is used [23].

Mechanics of Materials & Plasticity and toughness of the bone

During 1870 Culmann, Meyer, Roux, and Wolff provided consideration on the relationship between bone trabecular morphology and load-bearing mechanical function of bone [24]. During 1892, Julius Wolff and others realized that mechanical loads can affect bone architecture in living beings, but the mechanisms responsible for this effect were unknown, and it had no known clinical applications [25]. Ten years after writing "Wolff's Law and Bone's structural adaptations to mechanical usage: an overview for Clinicians", enough happened to justify summarizing the updated bone physiology for clinicians [25]. Mechanical properties of bone have been measured since the late 19th century and great strides have been made in understanding factors that contribute to the outcome variables [26]. The toughest and strongest structure of the body is obviously bone because of organic and inorganic substances. The organic phase of the bone that is comprised of collagen, calcium and phosphate, constitutes about 70 percent of the bone. The strength of the bone makes adequate support and movement while its toughness gives ability to bring to existent joint surfaces which are accurately shaped to be able to tolerate loads and give rise to powerful muscle contractions leading to fast movements of extremities without bones bending. Bone is highly dense in cells and is

richly vascular, and with this high cellularity has the capability to adjust to mechanical demands alterations as well as regeneration after injury [5]. A structure can be defined as a body that resists external effects such as loads, temperature changes, and support settlements, without undue deformation. A structure that can be considered as an assemblage of members and nodes. Structures with clearly defined members are known as skeletal structures [27]. The mechanical properties of bone are defined according to type of bone, anatomic location and the applied loads direction [28]. The study of structure and function of biological system by mechanics procedures is called biomechanics. Mechanical Properties of Biological Materials that can be measured and tested are very large and may be huge, while many of these properties are insignificant. The main function of the bone biomechanically is to be stiff. The stiff bone lifts the weight. The flexible bone does not lift the weight. It is normal to imagine that the load which bone can tolerate prior to fracture is the strength of the bone and is the most important mechanical feature of the bone but certainly the bone should function for the most part as no more deformation under load. If the bones are stiff, but break, they become useless, thus the strength is of great but secondary importance. The composition and behavior of bones have been studied for many years. It is necessary to keep in mind that biological variation and environmental factors extensively have an effect on the mechanical properties of biological tissues. Bone as a biological tissue is frequently explained from structural and material properties point of view. In the undamaged bone, structural properties describe the visible shape of the bone. Relationship between force and deformation or stress and strain represents the structural properties. It is mandatory to understand this relationship for predicting tissue behavior and

its reaction in vivo [29]. The materials that make up the tissue have a behavior described as material properties that are not depending on the size of the tissue. The material properties are described as stress- strain relationship of the material. The strength of a material that is the fracturing or most extreme strength at various types of loading e.g. bending, compression, torsion or tension with exception of bending close similar to stiffness or elasticity absolute value (modulus) will be different [30]. The material properties of biological tissues are based on stress–strain relationships of the tissue substance itself. These properties are more difficult to measure than structural properties because various reasons: 1) it is difficult to grip the tissue without damaging it; 2) accurate measurement of tissue cross-sectional area is challenging; 3) strain is best measured without contacting the tissue; and 4) material properties are sensitive to external factors such as the source of the tissue and how the tissue is handled, stored, or prepared prior to testing [31]. Our bones are full of microscopic cracks, but the hierarchical character of the bones structure, from molecular to macroscopic scales, makes them remarkably resistant to fracture [32]. Within the human body, protection of major organs is the duty of the bone. And the role of bone is also to enable movement and locomotion by acting as a jointed framework to provide support and muscle attachment. By aging and disease the integrity of the bone is compromised because of diminished capability to resist crack initiation (crack initiation toughness), and propagation (crack growth toughness) and failure as result of critically large crack (fracture toughness) [33]. It is important to assess the physiological loading role of bone in the fracture resistance and strength of whole bones. Whole bone strength has been shown to

depend on the tissue mineralization measured by clinical densitometry, but also on the micromechanical properties of the hierarchical organization of bone tissue. It is thus important to evaluate bone mechanical and morphological properties on several length scales to identify structure-mechanical property relationships [135]. Bone is a heterogeneous composite material consisting, in decreasing order, of a mineral phase, hydroxyapatite (Ca10(PO4)6(OH)2) (analogous to geologic 'hydroxyapatite'), an organic phase (90% type I collagen, 5% noncollagenous Proteins (NCPs), 2% lipids by weight) and water. Proteins in the extracellular matrix of bone can also be divided as follows: (a) structural proteins (collagen and fibronectin) and (b) proteins with specialized functions, such as those that (i) regulate collagen fibril diameter, (ii) serve as signaling molecules, (iii) serve as growth factors, (iv) serve as enzymes and (v) have other functions. The relative amount of each of these constituents present in a given bone varies with age, site, gender, ethnicity and health status. The amount, proper arrangement and characteristics of each of these components (quantity and quality) define the properties of bone. The tendency of bones to fracture depends on the quantity of mineralized tissue present (size and density) often measured by clinicians as bone mineral density or BMD and several other factors, grouped together as "bone quality". "Bone quality" factors include composition (weight percent of each component), mineralization (organization of the mineral and its crystallite size and perfection), collagen content and collagen crosslinks, morphology microarchitecture and the presence of microcracks. Each of these factors varies with health, disease and drug therapies. Their distribution in the heterogeneous tissue also

varies with these perturbations [11]. Bone, like any other biological systems, has a highly hierarchical composite material and composed primarily of assemblies of collagenous protein molecules, water, and mineral nanoparticles made of carbonated hydroxyapatite, forming an extremely tough, lightweight, adaptive, and multifunctional material. Bone is actually a very dynamic organ that is constantly remodeling and changing shape to adapt to the daily forces placed upon it. Bone as a hierarchical material and because of bone heterogeneity at the different ethnicities in human has an architecture that at each level of architecture Figure 1- Hierarchical structure of the bone tissue. , has different hierarchy and their mechanical properties are notably different [35].

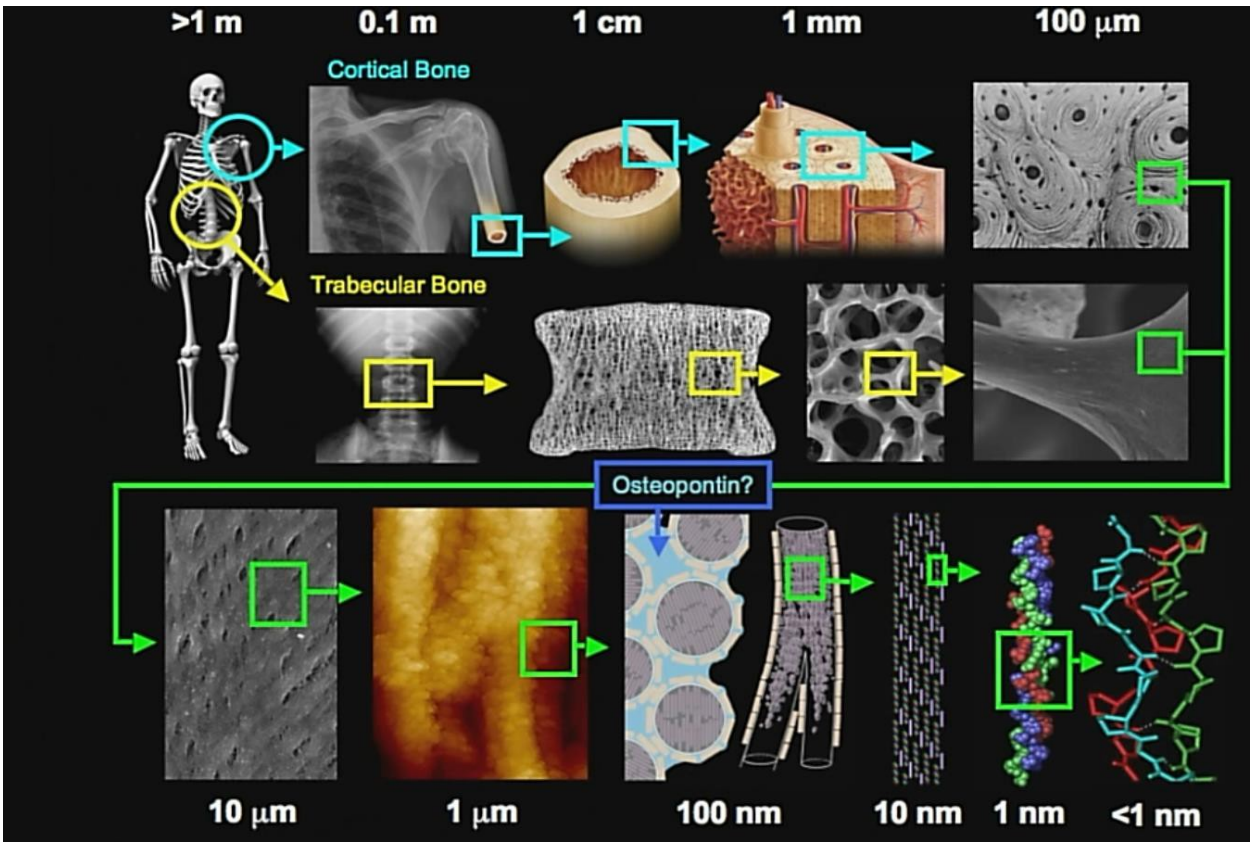


Figure 1-Hierarchical structure of the bone tissue.

Complicated and not easy understood hierarchical structures of the bone have mechanical properties with relationship with the hierarchical microstructures of bone [36]. For understanding of mechanical properties of the bone material, the understanding of complex multiscale structural geometry and complex behavior of the bone and the structural relationship between them at the various levels of Hierarchical structural organization is of utmost important Figure 2 [37].

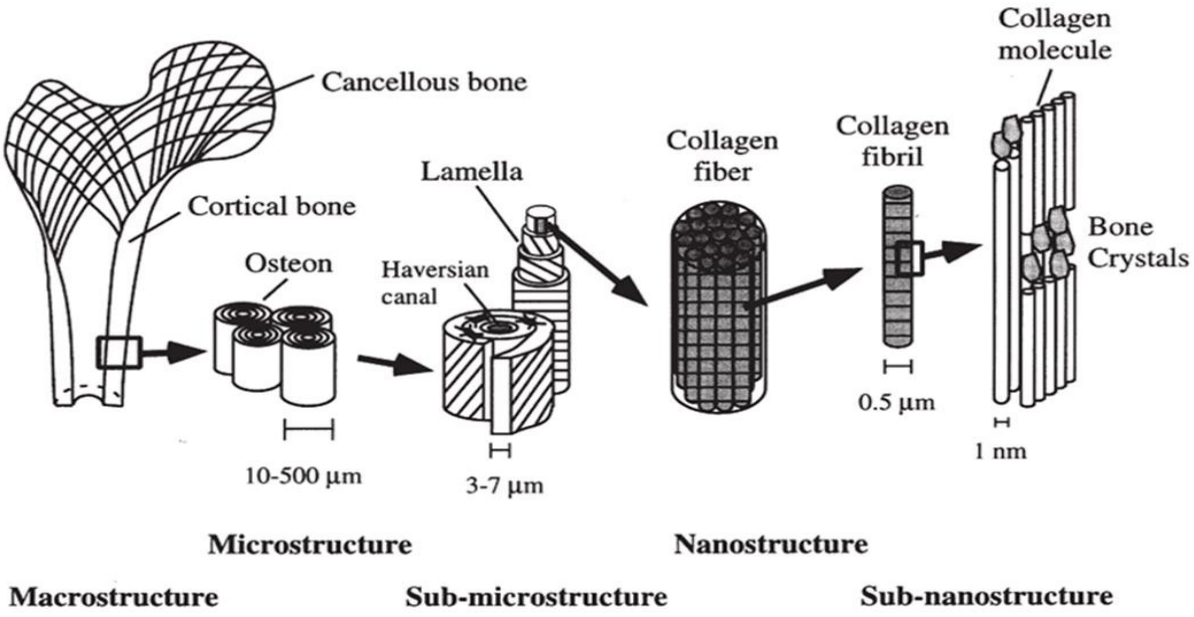


Figure 2

Figure 2. Bone hierarchical structural organization: a- cortical and trabecular bone; b- osteons with Haversian systems; c-lamellae; d-collagen fiber assemblies of collagen fibrils; e-bone mineral crystals, collagen molecules, and non-collagenous proteins.

These levels and structures are:

- Macro-structure: trabecular and cortical bone.
- Micro-structure (from 10 to 500 µm): osteons and trabeculae.

• Sub-microstructure (1–10 μm): lamellae and single trabecula.

• Nanostructure (100 nm–1 μm): fibrillar collagen and embedded mineral.

• Sub-nano-structure (<100 nm): molecular structure of mineral, collagen, and non-collagenous organic proteins. Because this, bone has the ability to adapt loadings and alterations in environment and also to repair itself. No fracture or damage failure as a result of repeated strains during normal daily activities is necessary for bone mechanical properties. On the bone, the structural and mechanical demands are completely different, e.g. bones similar to skull tolerate very limited repeated strains or stresses during daily activities in comparison to bones of the lower limbs. Thus quantity of mechanical microdamage in bones like skull in comparison to the bones of lower limbs that usually have repeated loading and unloading is insignificant. Resulting of repeated loading and unloading is microdamage and consequently macroscopic failure [38]. Skeletal bones have two portions: 1- trabecular (cancellous) portion that is the inner side of the bone and have three dimensional lattices comprising of osseous rods and plates recognized as trabeculae, and 2- cortical (compact) bone that is located in the outer side and is like a structural shell.

Inside the bone there are numerous intercommunicating pores of different dimensions created by trabeculae resulting a structure of frank density and various porosity. Porosity is the major determinant of the stiffness and strength of trabecular bone [39]. The distinction between cortical and trabecular bone can be made largely on the basis of porosity. The porosity of cortical bone ranges from 5% to 20% and is due to the Haversian and Volkmann canals and, to a lesser extent, the lacunar and canalicular spaces. Trabecular bone has another scale of porosity due to the marrow

space; typical spacing between trabeculae ranges from 100μm to 500μm.

The porosity of trabecular bone can range from 40% in the primary compressive group of the femoral neck to more than 95% in the elderly spine [40]. Structures of both trabecular and cortical bones vary with factors such are skeletal site, age, sex, physiological function, and mechanical loading [32]. It is correct to believe that toughness, and specifically fracture toughness of the bone, is the addition of compositional and structural factors and not belongs to a single factor, for instance bone mineral density. Thus understanding of precise and detailed relationships of structure-function in the bone is very important for achieving better evaluation of structural aspects of bone failure in order to predict and manage fragility fractures. The toughness is a measure of resistance to fracture [41]. Fracture toughness analysis assesses the fracture resistance of the tested material via the experimental determination of: a) the critical stress intensity factor (Kc) at the moment of failure, also referred to as "fracture toughness" and; b) the resistance of the material against the propagation of an existing crack for stress concentration below the critical point, K b Kc, referred to as "crack-growth toughness" [42].

Measurement of resistance to plastic (permanent) distortion is called strength. It is always defined in uniaxial tension, compression or bending [43]. For comprehensive review, there are standard methods for the assessment of toughness and fracture toughness in materials, such as the American Society for Testing and Materials (ASTM) standards [44].

Deformation and fracture mechanisms at each level of bone's hierarchical structure contribute to its mechanical integrity, most

significantly its strength (the resistance to inelastic deformation) and toughness (the resistance to crack growth or fracture). Strength originates from intrinsic mechanisms at small length-scales that promote plasticity and determine the inherent resistance of the material to deformation or crack initiation. Extrinsic toughening mechanisms, conversely, operate primarily in the wake of the crack tip to inhibit cracking by "shielding" the crack from the applied driving force, whereas intrinsic toughening mechanisms are effective in inhibiting both the initiation and the growth of cracks; extrinsic mechanisms, for example, crack bridging, are only effective in inhibiting crack growth Figure 3 [45]. Toughness is a competition between intrinsic and extrinsic toughening mechanisms. The intrinsic mechanisms primarily work ahead of the crack tip to develop plasticity, whereas the extrinsic mechanisms act in the crack wake (that is, after crack extension commences) to resist crack propagation through crack-tip shielding mechanisms. In human cortical bone, the extrinsic toughening mechanisms are primarily developed at large length-scales on the order of 1–100's of microns [46].

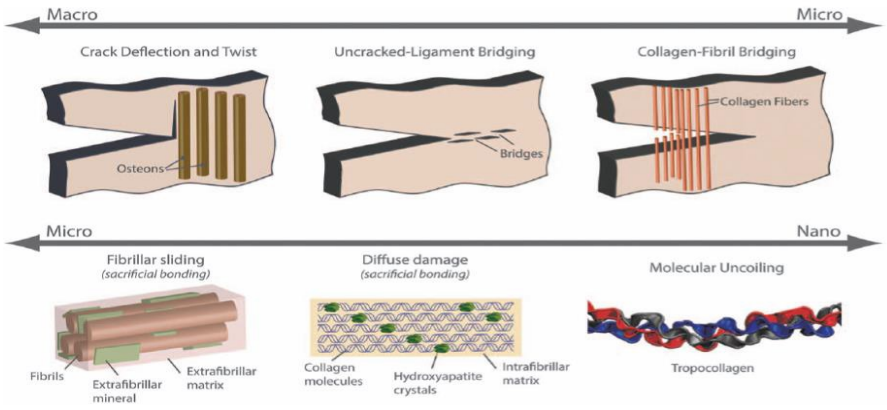


Figure 3

Figure -3. Toughening mechanisms in bone [45].

The ability of a material to undergo limited deformation is a critical aspect of conferring toughness, as this feature enables the local dissipation of high stresses that would otherwise cause the material to fracture; this is the reason why hard materials tend to be brittle and lower strength materials, which can deform more readily, tend to be tougher. The property of toughness is thus a compromise (indeed, it is a series of compromises); traditionally it is considered to represent the combination of strength and deformability (ductility) — two mechanical properties that also tend to be mutually exclusive. The attainment of both strength and toughness is a vital requirement for most structural materials; unfortunately these properties are generally mutually exclusive. The development of strong and tough (damage-tolerant) materials has traditionally been an exercise of compromise between hardness versus ductility. Interplay between the mechanisms that individually contribute to strength and toughness, noting that these phenomena can originate from very different length scales in a material structural architecture [47]. Strength (intrinsic [plasticity]) and fracture behavior (extrinsic [shielding]) can be considered toughening mechanisms associated with crack extension. Figure 4 shows the mutual competition between intrinsic damage mechanisms, which behave ahead of the crack tip to progress crack advance and extrinsic crack-tip-shielding mechanisms, which behave mainly behind the tip to delay crack advance.

Basically intrinsic toughness results from plasticity and improves quality, an inherent damage resistance of the material; in the exact sense of word it makes greater in the crack-initiation and crack-growth toughness

as well. Extrinsic toughness behaves to lower the local stress and strain fields at the crack tip; because of extrinsic toughness dependence on the presence of a crack, it has effect on the crack-growth toughness [47].

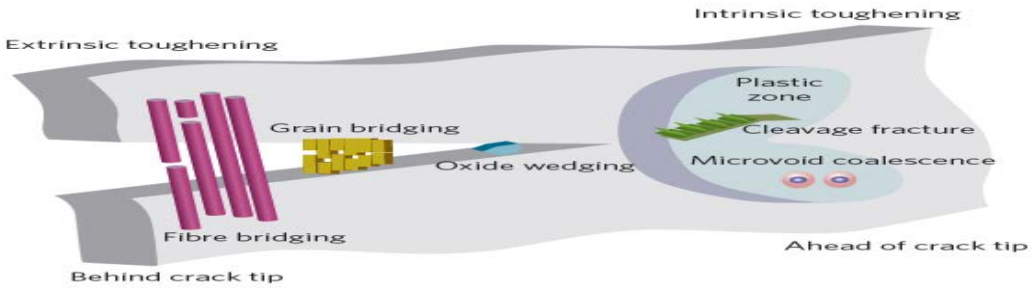


Figure 4

Figure 4- Conflicts of strength versus toughness [47].

Mechanical Properties of Cortical Bone and Trabecular Bone Tissue

Trabecular bone is a complex material with substantial heterogeneity .The directly measured trabecular thickness ranges from 82 μm to 284 μm. The thickest trabeculae are found in the femoral head (194 ± 33 μm) followed by the iliac crest (151 ± 27 μm). In the lumbar spine and calcaneal core, the distributions of the trabecular thickness are lower and similar (122–139 ± 18–28 μm). Based on the direct 3-Dimensional analysis of human bone biopsies, it appears that samples with a lower bone mass are primarily characterized by a smaller plate-to-rod ratio and to a lesser extent by thinner trabecular elements. The structure model index (SMI) is an estimation of the plate-rod characteristic of the structure Figure 5 [48]**.**

From bone hierarchical structure at micro, nano and molecular levels, bone is built from trabeculae that is comprised of thin rods and plates that are organized in 3-Dimensional patterns and slightly less regular which are

favorably heterogeneous and anisotropic constructions. Trabeculae surround an open porous space, which is 3-Dimensional and interconnected representing like a material of cellular solid type. Bone marrow and cells occupy minute openings (pores) [49].

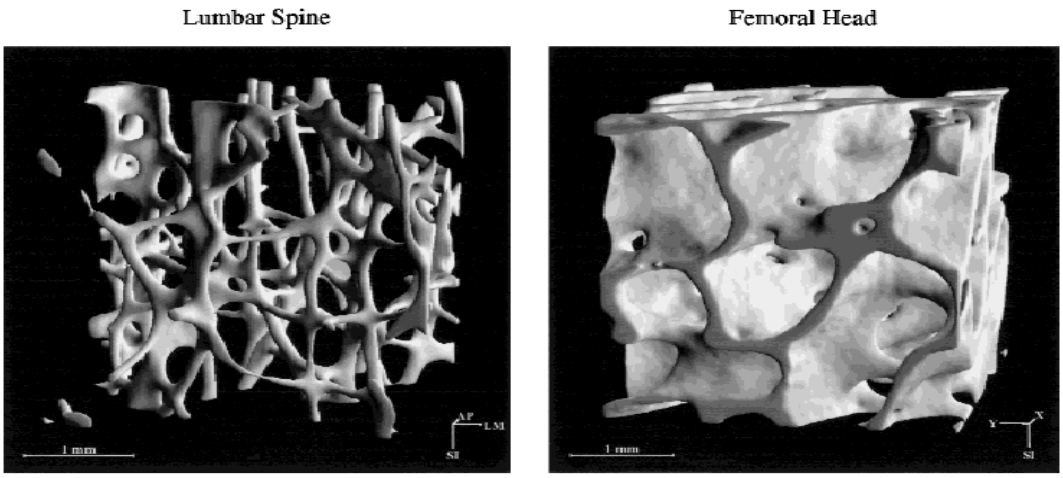


Figure 5

Figure 5 -Typical cancellous bone structures of the lumbar spine and the femoral head. In the lumbar spine rod-like trabeculae dominate (left, SMI 4 2.5).The trabecular structure found in femoral head, however, is in general more plate-like (right, SMI 4 0.16)[48].

Trabecular bone elastic and strength properties vary widely across anatomic sites, and with aging and disease. The trabecular microstructure has distinctive quality of alignment. Thus texture of arrangement is along which mechanical stiffness and strength are greatest. This specific orientation of microarchitecture has mechanical properties in trabecular bone. The cortical bone and trabecular tissue material are morphologically similar (an anisotropic composite of hydroxyapatite, collagen, water, and trace amounts of other proteins) but is arranged in "packets" of lamellar bone [50]. Obviously higher density and lower porosity in skeletal system

belongs to cortical bone [51]. The elasticity modulus and the ultimate compressive strength of cortical bone are ten times higher than trabecular bone [52]. Cortical porosity significantly affects the mechanical properties of cortical bone, and cortical macroporosity has been associated with hip fractures [53]. At the femoral neck, at least 50 % of the bone strength is the result of the cortical shell which helps the bone to maintain its critical mechanical competence [54]. Cortical bone mostly composed of the cylindrical structural units that are called Haversian systems or osteons (in more central regions of cortical bone, the lamellae are arranged in concentric cylinders around neurovascular channels called Haversian canals), they are usually in parallel position with each other.

Osteons are in parallel in long axis of the bone and because during bone remodeling, time of formation of osteons is different, adjacent osteons may encroach on each other. There are irregular gaps between osteon that are filled with interstitial lamellae, which are remnants of the older osteons, and circumferential lamellae Figure 6 .

Osteons may be spiral in shape and have arborizations and some of them may end blindly. Cross-section of osteons is round or ellipsoidal.

Depending on the site of bone, collagen fibers directions within osteons are different e.g. in long bone that are under tension collagen fibers tend to be longitudinal, but when are under compression they are more oblique [5].

Arrangement of collagen fibrils and thin and thick lamellae for orientation of trabeculae or cortices result in many toughening mechanisms and bone strength [55].

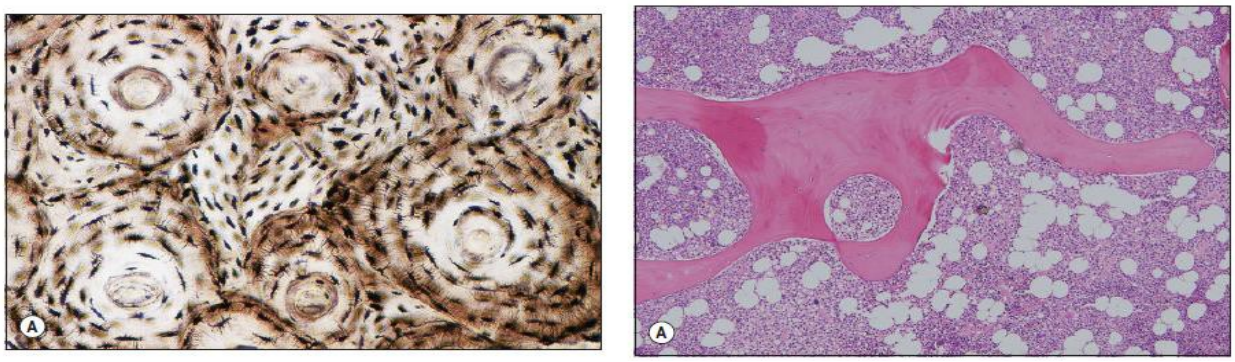


Figure 6

Figure 6 -Osteons in a dry ground transverse section of bone. Concentric lamellae surround the central Haversian canal of each complete osteon; they contain the dark lacunae of osteocytes and the canaliculi, which are occupied in life by their dendrites. These canaliculi interconnect with canaliculi of osteocytes in adjacent lamellae. Incomplete (interstitial) lamellae (e.g. center field) are the remnants of osteons remodeled by osteoclast erosion.(5).

Anisotropy is a phenomenon about parallel arrangement of collagen fibers in relation to long axis of bone resulting in bone to be stronger upon compression along its axis i.e. stronger in longitudinal axis than in transverse axis. Cortical and Trabecular bones microarchitectures are not similar, specifically in relation to density and porosity, thus their material properties are different. The ratio of bone specimen mass to its bulk volume is called bone apparent density which is 1.0-1.4 g/cm3 in trabecular bone and 1.8-2.0 g/cm3 in cortical bone [56]. With according to data from Max Planck institute in 2006, pieces of bones can stretch more than their components and this tolerability of large strains without fracturing is because of bone hierarchical structure and arrangement (despite the fact that have being made of essentially rigid units at the molecular level Figure 7 Figure 8 [57]. Essentially, from the atomic to the micro-scale level, bone

consists of rigid units joined together by a soft phase, where most of the deformation occurs. These composite structures form a single rigid unit at the next level and so on, enables the tissue to sustain large strains and create a material that is virtually unbreakable.

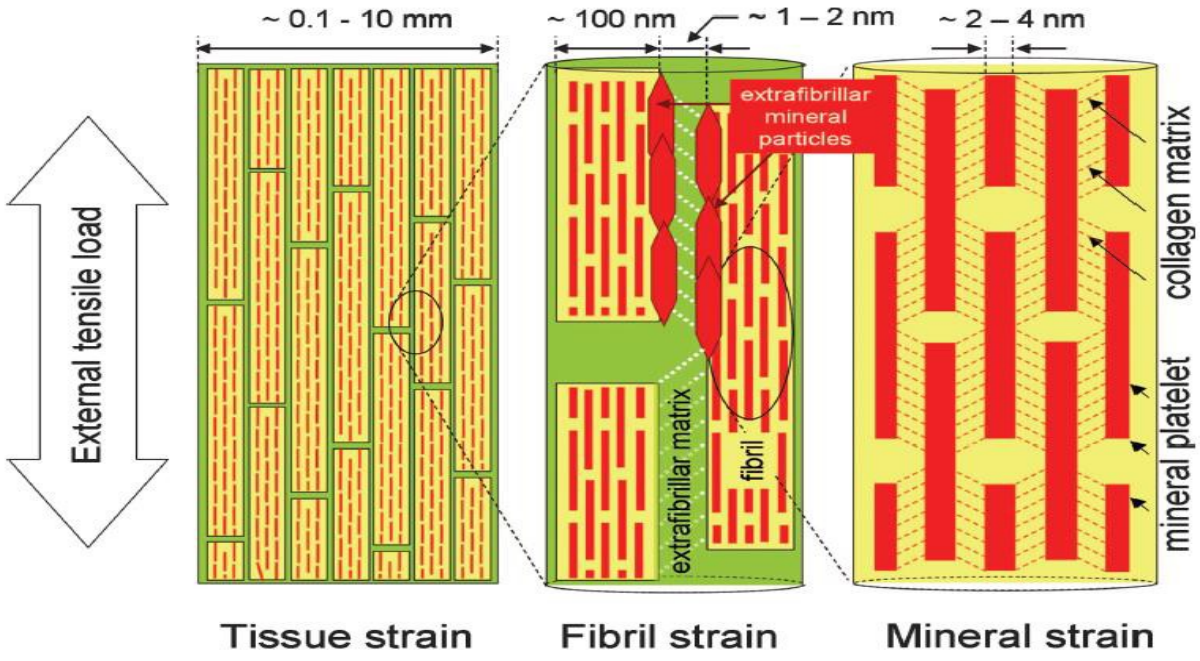


Figure 7

Figure 7 -Three level of bone deformation model in hierarchical organization in response to external tensile load: At tissue level (right side),At fibril array level(middle), At mineralized collagen fibril level(left side)[57].

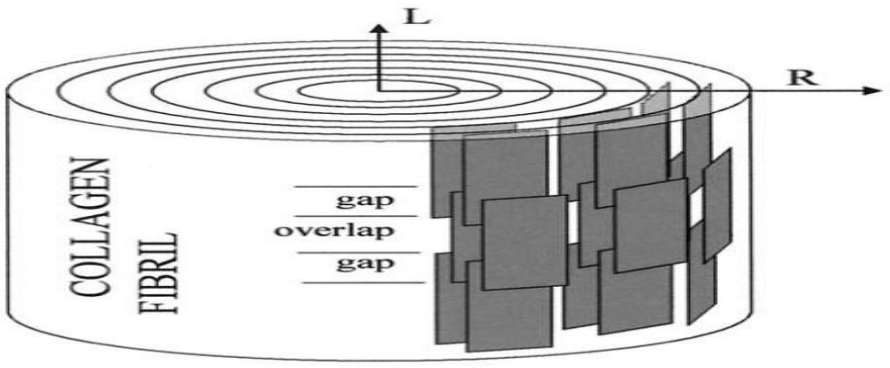


Figure 8

Figure 8-Depict of the three dimensional arranging of collagen fibril similar to fibril array level in Figure- 20[58].

Strength of Cortical and Trabecular bones

Cortical bone architecture and its mineral are important factors in bone strength [59]. Cortical porosity that is highly age-dependent is of particular importance in cortical architecture [60]. Cortical bone mechanical testing revealed increasing cortical porosity that leads to considerable negative effect on cortical bone strength [61]. Increased porosity leads to cortical bone trabecularization from endosteal aspect resulting in decreased mechanical integrity of cortical bone that in addition to cortical thinness, further compromise bone strength [62]. Cortical bone trabecularization occurs in different sites e.g. tibia and radius [63], mid–shaft of femur bone [64] and femoral neck [65]. Basically bone is comprised of collagen (fibrillar type 1 at approximately 35-45%), calcium (35-45%) and water (15-25%) [66]. Water is one of the factors that determine the nature of mechanical behavior of the cortical bone. Basically water is in two distinct compartments: matrix and within pores. Intracortical porosity mainly is representative of quantity of water within pores i.e. in the vascular-lacunar-

canalicular space and, in the exact sense of word, is inversely proportional to mechanical properties of bone [67]. In comparison to other bone constituents, the smallest degree of study has been devoted to water. In bone extracellular water exists in the intracortical porosity encompasses vascular space (Haversian and Volkmann's canals) and lacuna-canalicular network and is called pore water [68]. Water can either be loosely or tightly bound to the matrix [67]. Mineral content of bone ranges from approximately 30%/dry weight (in the skate or ray appendicular skeletal element, the propterygium) to 98%/dry weight in the stapes of the human ear. Most bones have approximately 60–70% mineral/dry weight, depending upon site, species and stage of development Figure 9 [69].

The bone structure hierarchically may be divided into three levels: macrostructure, microstructure and ultrastructure. Trabecular bone macrostructure denotes to its structure as a whole. It frequently called spongy bone or cancellous bone too. For transparency of terminology, bone containing of bone marrow space is named cancellous bone whereas for the bone tissue structure exclusively the term utilized is trabecular bone. Trabecular bone density diminish about 50% throughout the normal aging whereas bone strength becomes weaker in greater degree and arrives to 70-80% of its normal value [70].

As early as in 1876, Rauber determined the specific gravity of fresh specimens of human spongy bone as well as its compressive strengths. More extensive studies of the mechanical behavior of trabecular bone were reported by Gocke (1925, 1928), Hardinge (1949), Yokoo (1952), and Knese (1956,1958) [71].

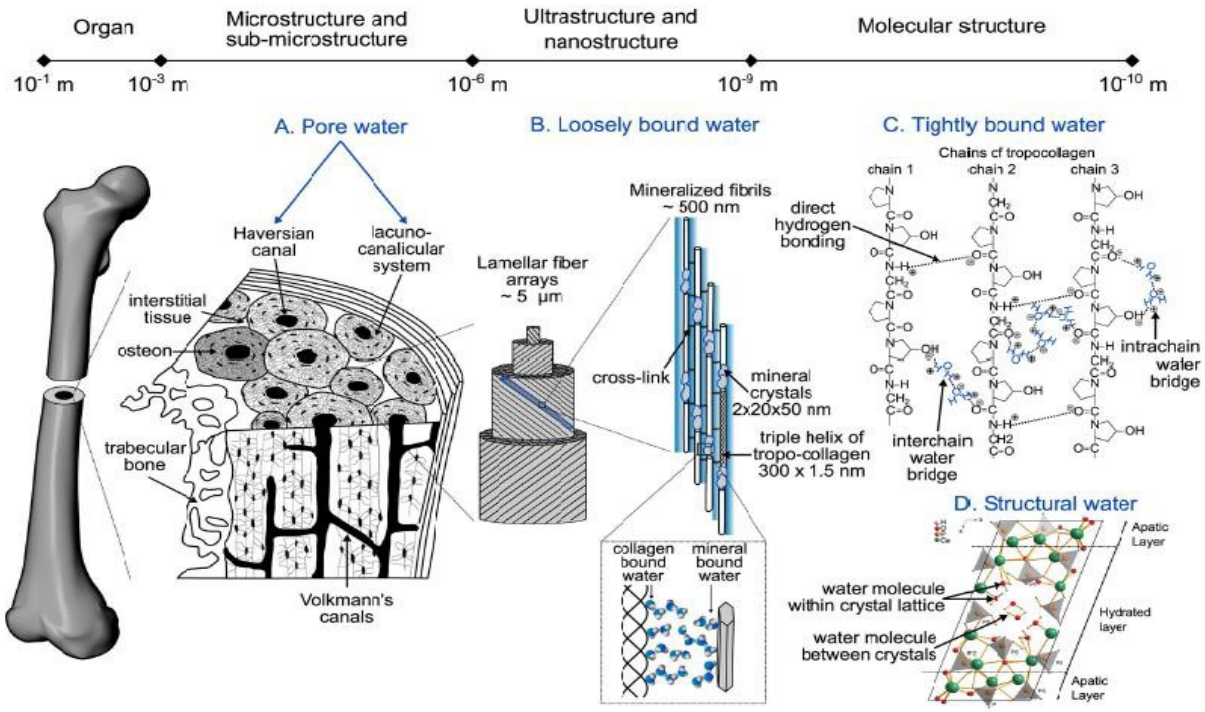


Figure 9

Figure 9-Water existence at different levels of bone hierarchical organization schematically. At cortical level there is a network of pores including Volkmann's and

Haversian canals (vascular porosity) as well as the lacuno-canalicular network. The presence of water at this complicated organization varies dependent upon the level of energy: A-free as liquid, B-loosely bound, C-tightly bound, and D-part of the mineral lattice. Water freely (A. Pore water) presents at the vascular-lacunar canalicular space at microscale. At the level of collagen fibrils surfaces, between collagen and mineral phase loosely bound water exists. Tightly bound water i.e. tightly bound water at the molecular scale. [67].

Cancellous bone at the trabecular level is known to be viscoelastic, composite and anisotropic material [72]. Bone strength is determined by three factors: the amount of tissue, the properties of the tissue, and

geometric organization of tissue in regard of loads that can be tolerated. These factors in combination specify susceptibility of bone to fracturing. Biomechanical evaluation of these factors establishes mechanical properties of bone structure. Bone structural properties defined by three biomechanical factors: strength, stiffness, and work to fracture. Quantity of these factors is measured by load-deformation curve. The maximum load prior to fracture that bone can tolerate is defined as *strength.* How easily deformation of bone under a specific load occurs is defined as *stiffness* and the amount of energy requirement for bone fracture is defined as *work to fracture* measured by total area under the load-deformation curve. By normalization of stress-strain curve for amount of bone and its distribution, tissue properties are defined. The same biomechanical features as measured structurally can be derived for the tissue—strength, stiffness, and energy to fracture— but in this case they are called ultimate stress, elastic modulus, and modulus of toughness Figure 10

[70]. There is a strong and significant link between the density of the cancellous bone tissue and that the critical stress intensity values. There is also a relationship between the critical stress intensity values and the apparent and relative density of cancellous bone tissue [72]. Bone as a composite material is comprised of collagen and an inorganic portion that mostly contains hydroxyapatite crystals. Trabecular bone quality can be under influence of hydroxyapatite crystal structure, crystallite size, hardness, and disruption in the crystalline structure of hydroxyapatite detected in osteopenic and osteoporotic bone. Microstructural characterization of hydroxyapatite crystals in dry trabecular bone can discriminate normal bone from osteopenic and osteoporotic bones and with

an emphasis on bone quality is a complementary evaluation of osteoporosis [73].

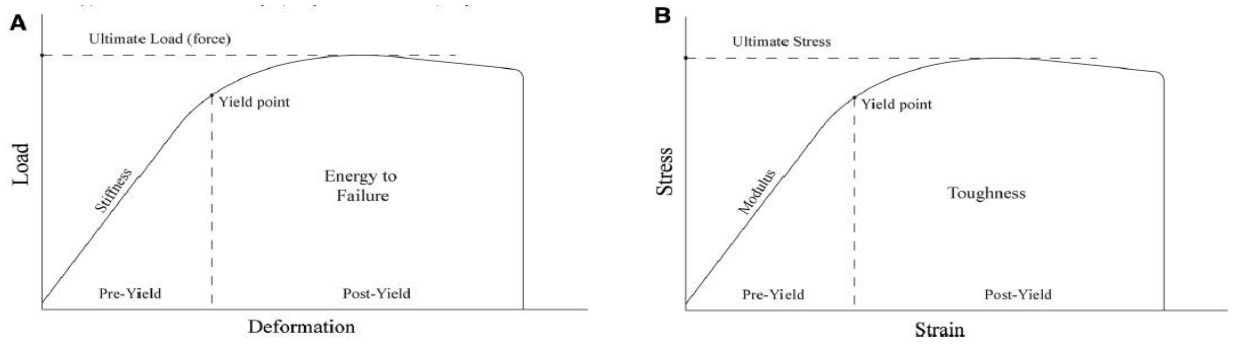


Figure 10

Figure 10 - A-Bone under load becomes deformed and extrinsic structural mechanical properties defined by relationship between load magnitude and the amount of deformation. B-Conversion of load-deformation curve to stress-strain curve independent of bone mass and geometry defines the properties of bone tissue matrix. Stress is defined as force/area and strain is the percent deformation [74].

Bone material strength components

Natural materials with a cellular structure are in large number, and an example of natural materials with polyhedral cells, include the trabecular bone. Trabecular bone is a three-dimensional cellular solid, and has a cellular structure. Its relative density is between 0.05 and 0.03 (kg/m3). Load exertion results in bone growth thus severity of loads determine trabecular bone density and loads direction determine trabeculae orientation (figure-31). Low-density trabecular bone looks like an open–cell foam whereas high-density trabecular bone has a more plate-like structure with perforation through the plates. Trabeculae have different orientations, from equiaxed in proximal femur to rectangular network in vertebral body.

There is more difference in strength and stiffness of trabecular bone due to difference in trabeculae density and architecture.

The Young's modulus of trabecular bone is plotted against relative density in Figure 11. The data lie roughly on a line of slope 2, but with substantial scatter in the data. This has been already seen for open-cell foams, with bending-dominated linear elastic behavior. In normal bone, the linear elastic deformation is bending-dominated and the Young's modulus varies as the density squared. The variation in trabecular architecture leads to large scatter in the data, even at one density. Differences in trabecular architecture can be accounted for by the inclusion of fabric in models.

There is evidence that the compressive strength of trabecular bone is related to buckling of trabeculae leading to a dependence on density squared. The strain to failure in bone is a constant for a given site with consistent trabecular architecture. In osteoporotic bone, bone loss occurs through trabecular thinning as well as resorption. The modulus and strength are more severely reduced by resorption than by uniform thinning of trabeculae [75]. Monitoring of the changes in bone mineralization, along with other parameters related to bone turnover, is essential to fully characterize bone matrix quality. Microcracks accumulate in bones with slow remodeling, i.e., when the age of the bones increases. This accumulation is associated with loss of structural properties such as bone stiffness and energy absorption [76].

Bone structural properties and bone material determine bone strength. The amount of mineralization, crystalline, collagens characteristics and

osteocyte viability of the bone material properties have considerable influence on the bone strength. On the other hand cortical thickness and

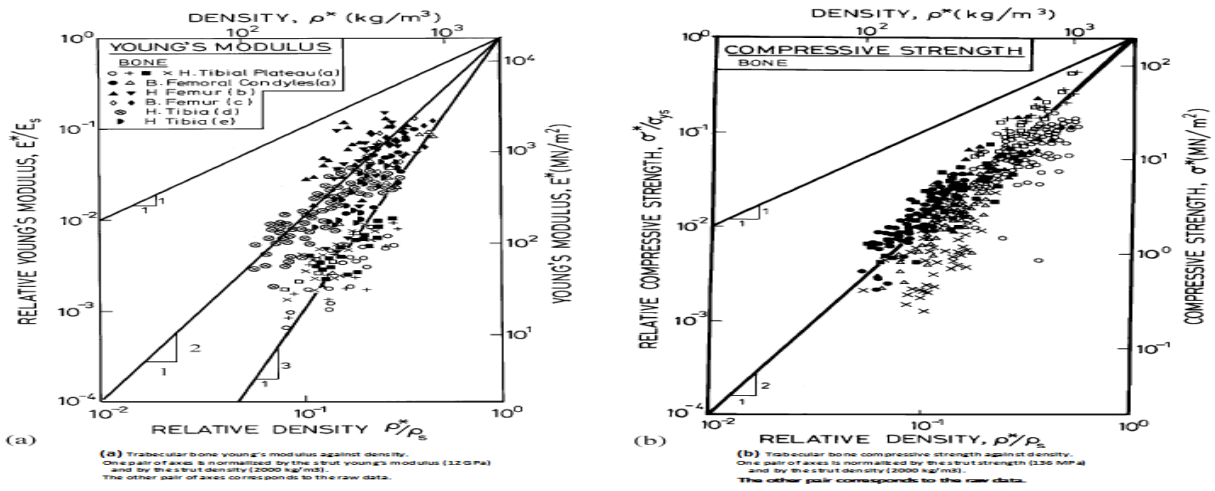


Figure 11

Figure 11 – Femur & Tibia; +Bovine femoral condyle; Tibial plateau; Vertebrae; Bovine femoral condyle; Vertebrae; Calcaneus; , Femur; B. Femur[75].

diameter, cortical porosity, trabecular network and its interconnections, trabeculae thickness and presence of microcracks also have influence on the bone strength. All of these processes are under influence of remodeling [77]. When there occurs application of an external force that is more and stronger than bone strength, a fracture supervenes. Bone capability to tolerate a loading force and resists fracturing determined by bone size, bone mass spatial distribution and bone material properties [78]. Thus determinants such as microarchitecture, geometry, mass as well as intrinsic bone tissue quality determine ability of bone to resists physiological stress. Mineralization and matrix aspects like collagen fiber orientation and chemical composition determine intrinsic bone tissue quality.

There are different techniques for evaluation and quantification of intrinsic bone tissue quality at the bone structural units (BSU) by microindentation and at lamella level by nanoindentation.

Bone Microindentation

General principles

Currently there are two types of microindentation devices for their use in clinic and in a research setting: 1- Cyclic Microindentation (cRPI or BioDent®) that mostly use in research setting for laboratory studies and 2- Impact Microindentation (IMI or OsteoProbe®) that is used mostly in clinical setting. A detailed technical note regarding recommendations about the standard procedure for evaluation of cortical bone by impact Microindentation has been recently published [79]. The major issue is the site of indentation because the most clinically relevant site of fracture is femoral neck but this site is not feasible for indentation. Comparison between BioDent® and OsteoProbe® is summarized in Table 1 [26, 80],

Table 1

Table 1-specifications of currently available devices		
Specifications	Devices of Microindentation	
Device	OsteoProbe®	BioDent®
Application by Manufacturer	Clinical	Clinical and research
Site of indentation	in vivo mostly tibia	ex vivo every site
Bone compartment	Cortical bone	Cortical bone
Application procedure	By Hand held	By set up
Size of test probe	About 370 micrometer	About 370 micrometer
Radius of probe tip	More than 10 micrometer	2.5 micrometer
Loading force	40 N	According to User (2–10 N)
Loading cycles	1	According to User (up to 20)

Loading rate	**120,000 N/s**	**40 N/s**
soft tissue penetration	**Simultaneous with loading**	**Removal by scraping before loading**
Data monitoring during procedure	**unanalyzed BMSi value**	**Force versus distance graphs**
Output parameters	**BMSi**	**IDI,TID,CID**
Interpretation of output	**Higher BMSi ,better bone mechanical properties**	**Controversy regarding IDI,TID**
IDI: the difference in depth between the first and last indentation, **TI**D: total indentation distance , **CID:** creep indentation distance		

Damage mechanisms specification in human bone with association of Microindentation

Initiation of fracture in bone is via dilatation bands that are due to osteocalcin (OC) and osteopontin (OPN) interaction. Anchorage of OC to OPN and then to mineral and by this way takes part in energy dissipation. Thus dilatation band formation by OC-OPN network for energy dissipation is mandatory. In the case of Lack of osteocalcin or osteopontin, OC-OPN network will be interrupted and consequently striking toughness loss ensues. At the higher scales, damage is associated with dilatational bands thus dilatational bands are contributing factor for bone toughness.

Therefore bone nanostructural alterations in mineral, collagen, and non-collagen proteins that mostly seen in metabolic bone diseases and osteoporosis, lead to striking decrease in bone toughness and observe as toughening alterations at nano to macro scales [81]. Dilatational bands are separate ellipsoidal spaces about 100 nm in size Figure 12, which result from non-collagenous proteins complexes deformation within rigid mineralized matrix of the bone Figure 13. Non-collagenous proteins like osteocalcin (OC) and osteopontin (OPN) are involved in bone fracture.

Osteocalcin (OC) binds with great intensity to hydroxyapatite. This complex by means of osteopontin (OPN) connects to collagen. Due to the capability

of osteocalcin (OC) to connect to bone organic and mineral components contributing it as to have a duty in mechanical function of the bone.

Osteopontin is with greater extend a companion to bone mineral, and is a participant of "rupture and reformation of Ca2+-OPN sacrificial bonds".

Osteopontin is as "bone glue" and gives to bone ability to resist crack growth, and via sacrificial bonds to repair matrix damage, and remodeling

as well [81].

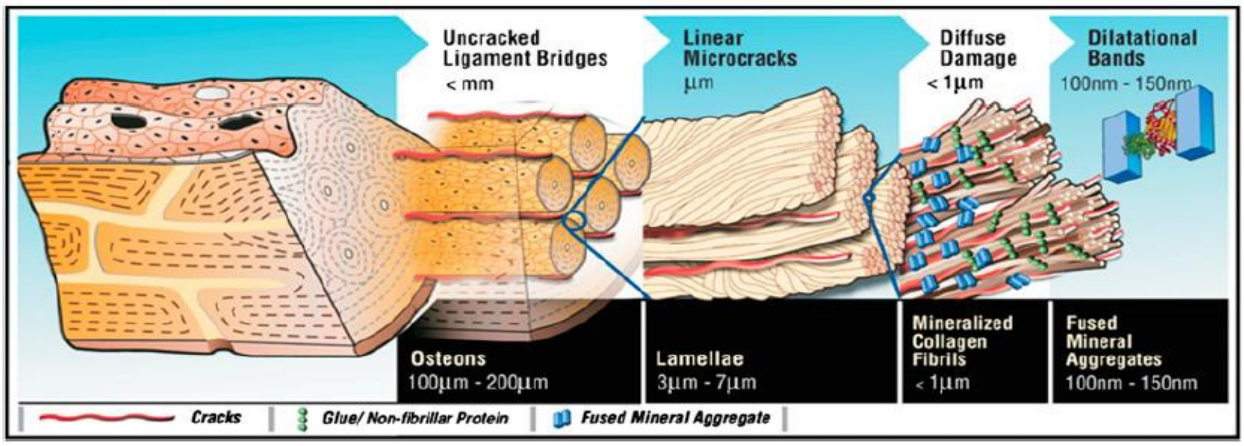


Figure 12

Figure 12- Bone hierarchy and dilatational bands. Dilatational bands and diffuse damage link to higher-level toughening mechanisms in bone at Hierarchical model of bone toughness [81].

Mechanical evaluation of bone damage for local tissue hardness can be done by microindentation technique. Reports from microindentation results in human are hopeful [82], and microindentation can be used as a diagnostic instrument for detection and predicting fragility fracture clinically

[83,84,85]. Despite microindentation determines parameters that are in connection with standard bone material properties, but basic mechanisms of damage that is measured by microindentation are not entirely described.

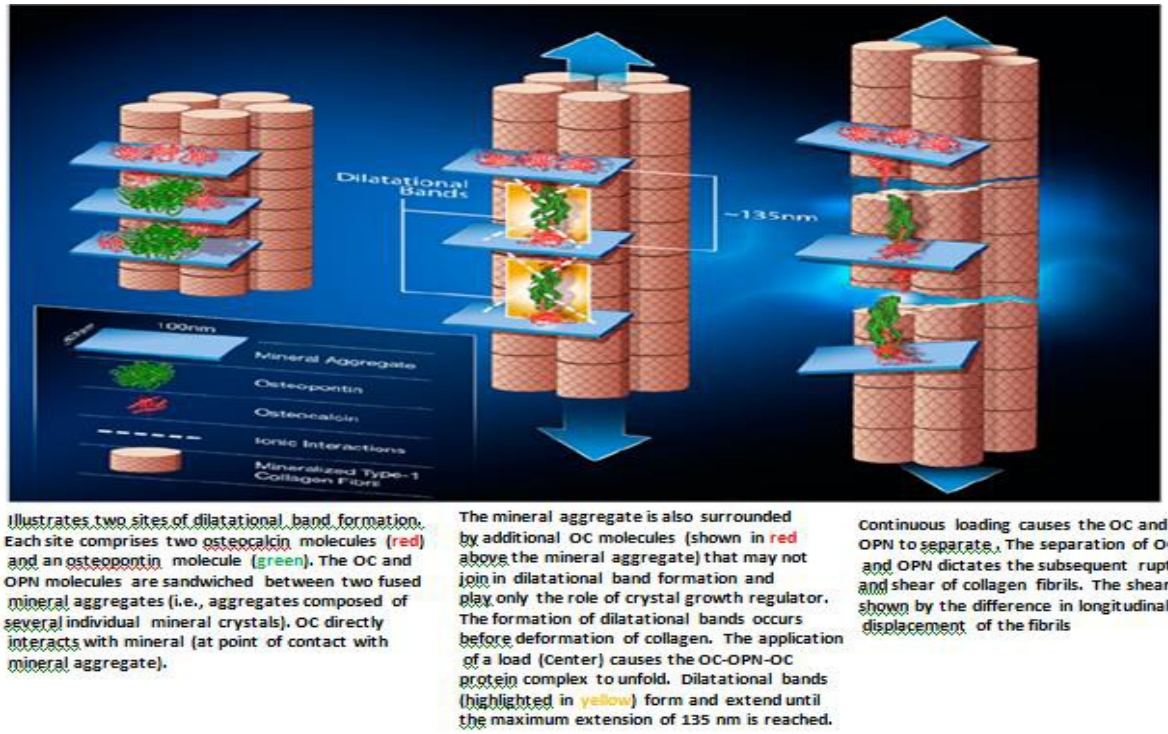


Figure 13

Figure 13-. Dilatational band formation in bone [81].

By electron microscopy an effect produced by microindentation can be detectable as crack damage and non-recoverable deformation. There are no specifications regarding damage type produced by microindentation that explain the mechanisms of these damages [86], and few studies can be found in medical literature in this regard. At this study each indentation site was located in association with damage in histological data that happened under the indenter tip at different cycle and loads levels. In the first ten

cycles of test, damage became larger at the indentation pathway and with consecutive cycles damage zone expanded in a radial direction. They concluded that RPI appears to be a promising method for collecting mechanical information directly from human bone in vivo in a relatively minimally-invasive manner [87].

Discrimination between Diseased and Healthy bone tissue by Microindentation

Bone matrix disturbance or interruption that is identified by laser scanning confocal Microscopy [88], or by light microscopy is called microdamage.

Microdamage gradually increases with aging with higher speed in females in comparison to males, and regarded as a contributing factor for osteoporotic fracture [89]. Hardness and bone quality decrease by microdamage [90]. Bone strength is predictable in 70% to 95% by bone mineral density in association with Microarchitecture; the remaining can be clarified by evaluation of microdamage as a bone quality factor [91]. For assessment of elastic properties of bone structural units (BSUs) of cortical and trabecular bones, microindentation has been utilized as a regular procedure in vitro for the evaluation of variations in different anatomical sites [92]. Microindentation was used for determining the influences of pathological processes on the mechanical properties of BSUs [93].

Microindentation seems to have ability for evaluation of microdamage extension in regard of bone mechanical properties, thus in vitro distinguishing between normal healthy form highly damaged bone tissue by microindentation is feasible. Detection of decrease in bone mechanical properties due to lower tissue resistance of mirocracks that exist under the bone surface and are unable to tolerate local pressure applied by indenter

is the capability of microindentation. So microdamage associated with bone mechanical reduction better clarify connection between microdamage and bone toughness reduction, and explain why at macro-level microdamage is a contributing factor for bone strength reduction [90]. Discrimination of normal intact bone from highly damaged bone of cortical and trabecular bone in vitro is possible by microindentation, and the ability of microindentation for disclosure of damage at physiological load in vivo required further investigation [91].

Cyclic Bone Microindentation

At lamellar level of bone, local properties can be evaluated by a novel mechanical method of testing called microindentation. In this technique the device has a tip with specific geometry like three sided pyramid that can be pushed into a smooth surface. At the same time displacement of the tip into the smooth surface and axial force that is applied can be measured. To specify the anisotropic mechanical properties of bone at the lamellar level, indentation of bone for approximately one micrometer in depth is necessary [94]. Microindentation or Reference point indentation (RPI) is this novel technique that has capability for direct evaluation of bone mechanical properties on the basis of traditional indentation principles in order to measurement of bone toughness and its elastic modulus (absolute value).

In this technique there is a probe within a sharpened hypodermic needle that remains anchored onto the sample surface and serves as reference point during measurement. During the procedure indentation probe makes a number of force cycles [83]. This instrument comprised of a reference probe that remains on the bone surface and a test probe that indents onto the bone during testing. Displacements are determined from the distance

between the test and reference probes. The test probe has a diameter of 375 micrometer with a 901 conical end and a tip radius of 2.5 micrometer.
In this method there are successive indentation cycles, in which the test probe traverses deeper into the bone with each cycle .The load function has a trapezoidal shape, with a linear increase followed by a hold time and a linear decrease. The reason for the holding is to monitor creep effects.
The indentations cause load- displacement curves. Several variables can be calculated from the RPI-generated load-displacement graphs. The indentation distance increase (IDI) is measured as the increase in an indentation distance from the first to the last cycle. The total indentation distance (TID) is measured from the initial touchdown distance to the maximum indentation distance in the last cycle. The average energy dissipated is the average of an integrated area between the loading and unloading curves. The average stiffness is the average of all slopes calculated from the top 50% of the unloading part of the force-displacement curve. The creep indentation distance (CID) is the distance traversed by the probe during the hold period for the first indentation cycle, and the average creep indentation distance is the CID averaged overall indentation cycles, whereas the total creep indentation distance is the sum of the CIDs from all indentation cycles. Definitions of these quantities are seen on the diagram in Figure 14 and the overall scheme of the device in Figure 15[95].
This TDI comprised of collection of components which by traversing through skin and its underlying soft tissue make contact with deep tissues.
In this collection of probe that is disposable and strerilizable, there are an outer reference probe made from a 23-gauge hypodermic needle and inner test probe made from stainless steel wire (its diameter from 175-300 micrometer and its length from 2-90 micrometer). Increase in length of

probe leads to accentuation of friction between test probe and reference probe, thus utilization of that amount of length for reaching to “preferred tissue site” is advisable.

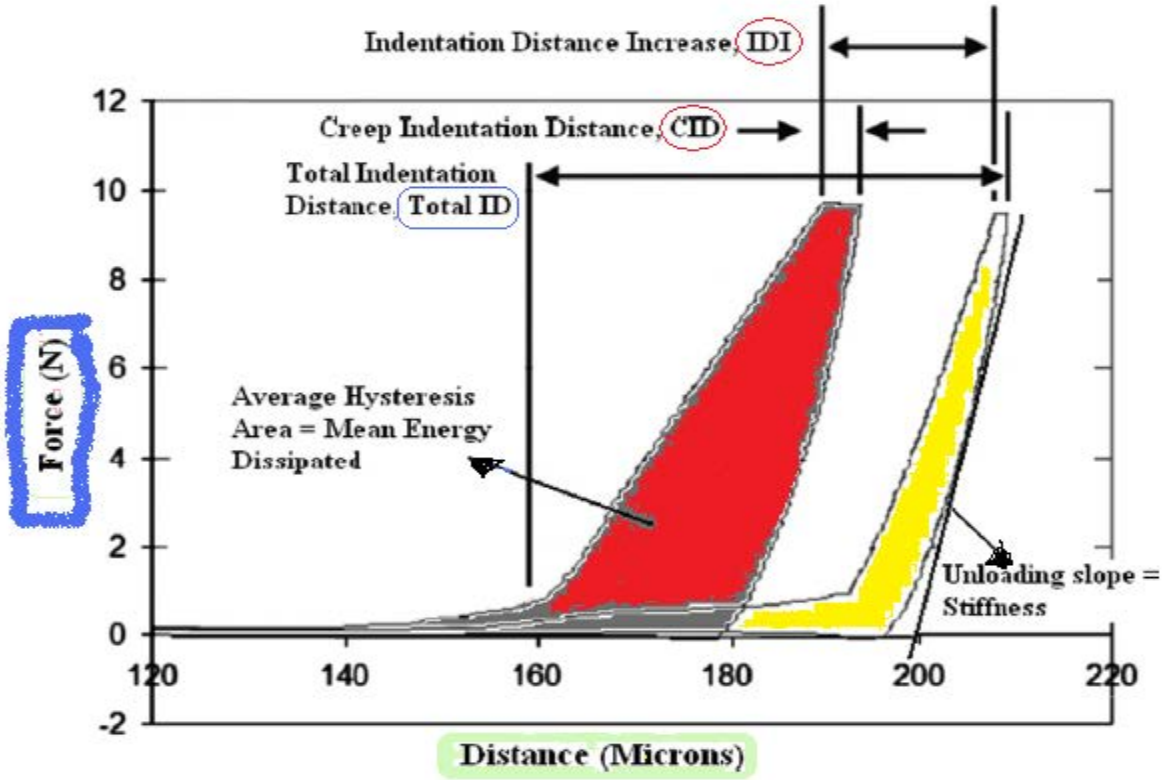
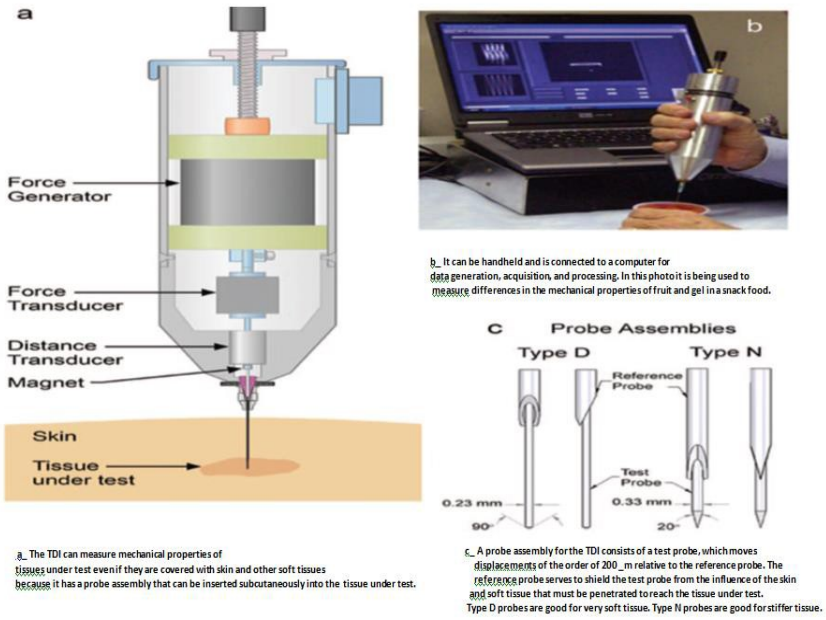


Figure 14

Figure 14- RPI analysis result with measured quantities definitions- [95]

There is a nickel tube that test tube keeps in it and associated with a magnet connect to force generator. Probe move back and forth within the desired tissue site by force generator during procedure and at the same time determine the force and displacement.



a_ The TDI can measure mechanical properties of tissues under test even if they are covered with skin and other soft tissues because it has a probe assembly that can be inserted subcutaneously into the tissue under test.

b_ It can be handheld and is connected to a computer for data generation, acquisition, and processing. In this photo it is being used to measure differences in the mechanical properties of fruit and gel in a snack food.

c_ A probe assembly for the TDI consists of a test probe, which moves displacements of the order of 200 _m relative to the reference probe. The reference probe serves to shield the test probe from the influence of the skin and soft tissue that must be penetrated to reach the tissue under test. Type D probes are good for very soft tissue. Type N probes are good for stiffer tissue.

Figure 15

Figure -15. The Cyclic indentation device

Frequency of the probe is 4 Hz during procedure and permits to keep in hand and is adequately slow for separating elastic and viscous response of tissue. The greatest amount of values of force and displacement are 12 N and 600 micrometer respectively. There is about 0.02 N frictions between reference and test probes. The relationship between force, distance, and displacement are seen in Figure 14 and Figure 16 shows the thickness of the bone in relationship with indentation distances [96].

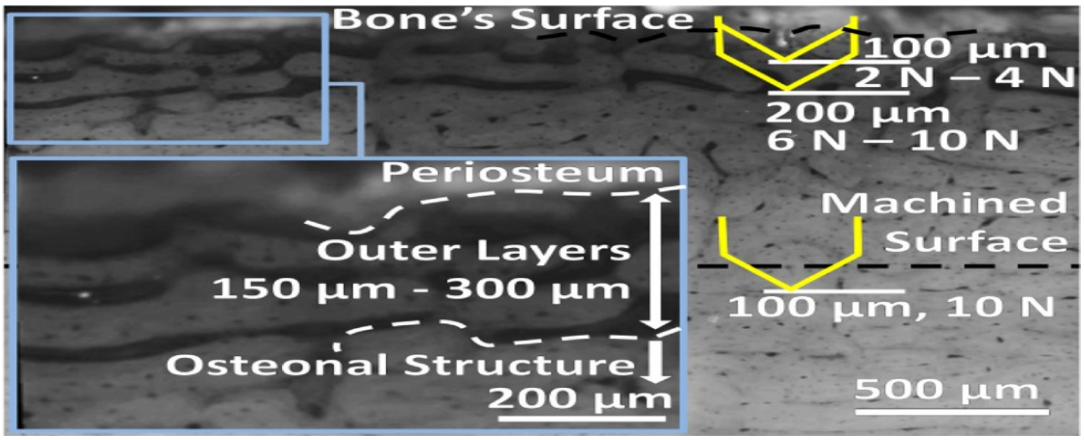


Figure 16

Figure 16- The diagram indicates thickness of the outer layers and the total indentation distance with low (2N – 4N) and high (6N – 10N) load on the natural and machined surfaces [97]

The slope of the force versus displacement curve provides a measure of elasticity: in the case of a simple spring, the slope would be the spring constant. The energy dissipation in the force versus displacement curve is the area inside the curve and is a measure of the viscous behavior.

Viscosity is absent from a simple spring yet is large for a purely viscous material such as petroleum jelly, which has an elasticity near zero [96].

There are several parameters that reveal with RPI measurement. They are the indentation distance increase (IDI) that has correlation with toughness, the Unloading Slope of the first indentation cycle (US 1st) that is indicative of bone material stiffness, creep indentation distance (CID) and energy dissipated [88]. Because of cyclic character of RPI, dissipated energy of material in response to mechanical loading can be measured [98]. Ability of RPI to distinguish more easily fractured bone from less easily fractured bone confirmed in clinical studies [82]. In microindentation technique as a

RPI, there is 20 cycles of loading on the basis of "force-control" and bone tissue properties evaluation is performed. The instrument measures the displacement (relative to the bone surface) of a stainless steel test probe (375 µm diameter, 90° cono-spherical, 2.5 µm radius tip) that indents into the bone to a given load, dwells for a short period of time (typically <200ms), and unloads to about 0 N. The total indentation increase (TID) and the indentation distance increase (IDI) are two parameters that are measured by depth of indentation. Creep indentation distance (CID), energy dissipation (ED) (i.e., the area under the load-displacement curve for a given cycle), loading slope (LS) and unloading slope (US) are calculated in each cycle Figure 17 [98].

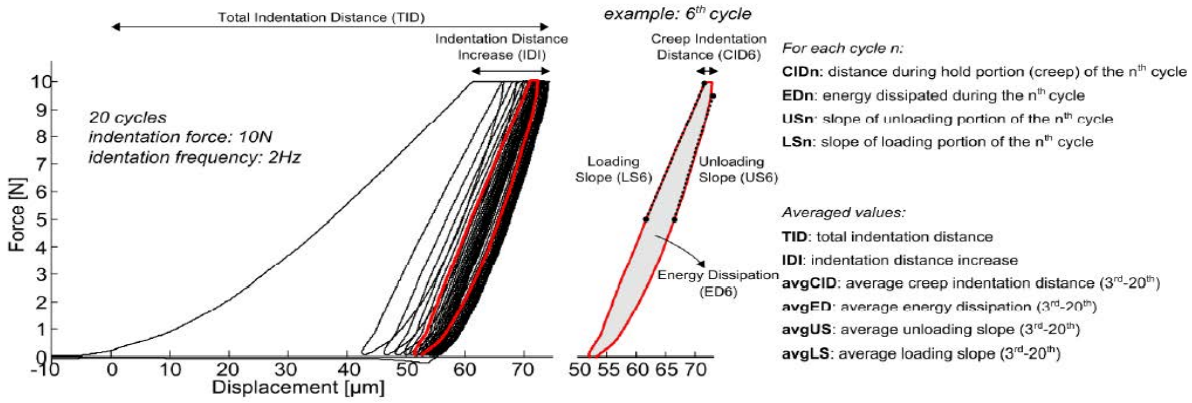


Figure 17

Figure 17-RPI measurement on human bone demonstrating Load-Displacement curve. Outcome parameters in regard of properties of bone tissue obtained from cycle-by-cycle analysis[98].

Except unloading slope (US), all other parameters have different values at

the first cycle in comparison to the consecutives cycles (indentation distance and energy is approximately 5 times more) and since third cycle till last cycle reach to a steady state Figure 18 [98].

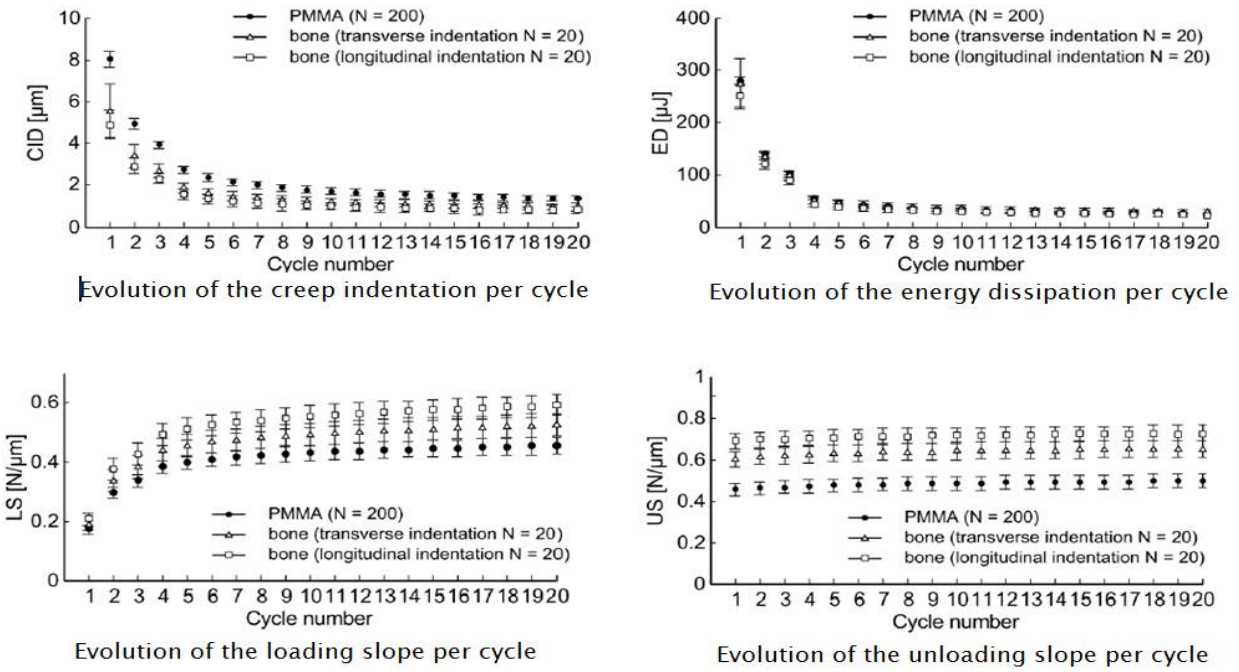


Figure 18

Figure 18-Evolution of the RPI parameters per cycle [98].

For monitoring of creep effects and for remaining of creep at the smallest degree during the linear decrease, holding at maximum current is utilized. Typically a total cycle time is 500 ms. This type of indentation analysis system with maximum load holding introduced by Oliver and Pharr in 1992 in order to measurement of elastic properties of materials at very small scales [22].

Impact microindentation

For measurement of bone material properties despite the fact that bone is concealed by soft tissues (periosteum, connective tissue and skin), an instrument has been designed and is called Osteoprobe® as a prototype.

It has: **I**- a probe assembly (a reference probe for penetration into the soft tissue up to periosteum and a test probe for insertion into the bone), **II**- an operating system for moving the test probe within and out of the bone, **III**- A sensing system for identification of test probe dynamics during insertion and within the bone, **IV**- A measurement system for recording data of sensing system during procedure [100]. The sharpening of the test probe and reference probe are not symmetrical because lateral offset between test probe tip and reference probe tip keeps in smallest possible amount Figure 19. And, this zero counterbalance reaches to the smallest possible degree in force versus distance curves, because the probe assembly axis is not totally vertical to the bone surface. Whenever fixed maximum force cycling is used instead of fixed maximum distance or when sensing distance at a fixed threshold force for insertion at a constant distance, test probes sharpening as a symmetrical are used as a routine.

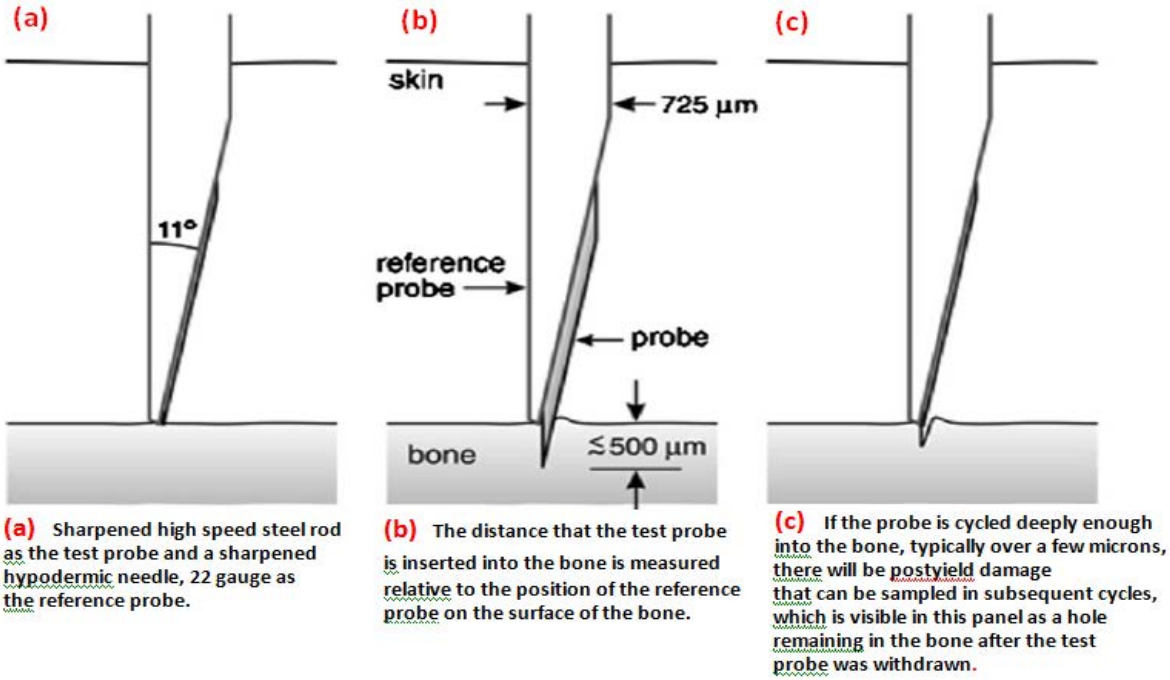


Figure 19

Figure 19.A typical probe assembly for a bone diagnostic instrument. The force to insert and withdraw the test probe is also measured [100].

The Osteoprobe II™, the **B**one **D**iagnostic **I**nstrument (BDI) was developed for measurement of human bone material properties in vivo by clinicians and investigators as well. As a successor of Osteoprobe I™, the Osteoprobe II™ **B**one **D**iagnostic **I**nstrument (BDI) is an improved version of the Osteoprobe I™ BDI. It is designed for tissue penetration and comprised of a test probe (a small diameter, sharpened rod) which slides through the skin. After probe assembly penetration through the skin, it stops at the periosteum. The distance of insertion of test probe into the bone (the indentation distance increase [IDI]) can be measured. It seems that IDI with repeated cycling to a fixed force is able to differentiate less

easily fractured bone from more easily fractured bone. By this BDI system an increased IDI is an indication of derangement of bone mechanical properties like toughness and post yield strain [101].

To perform indentation measurements by new test probe/reference probe design was the fundamental thought for manufacturing a Diagnostic measurement in vivo instrument. In the Osteoprobe II™, conical test probes have been used instead of beveled test probes that had been used in Osteoprobe I™ BDI Figure 20. Diameter of these conical test probes is 375 micrometer with curved approximately in a radius of 2.5 micrometer, and beveled to right angle. In Osteoprobe I™ BDI beveled test probes were

asymmetrical and associated with natural anisotropy of bone properties were the sources of data scattering [101].

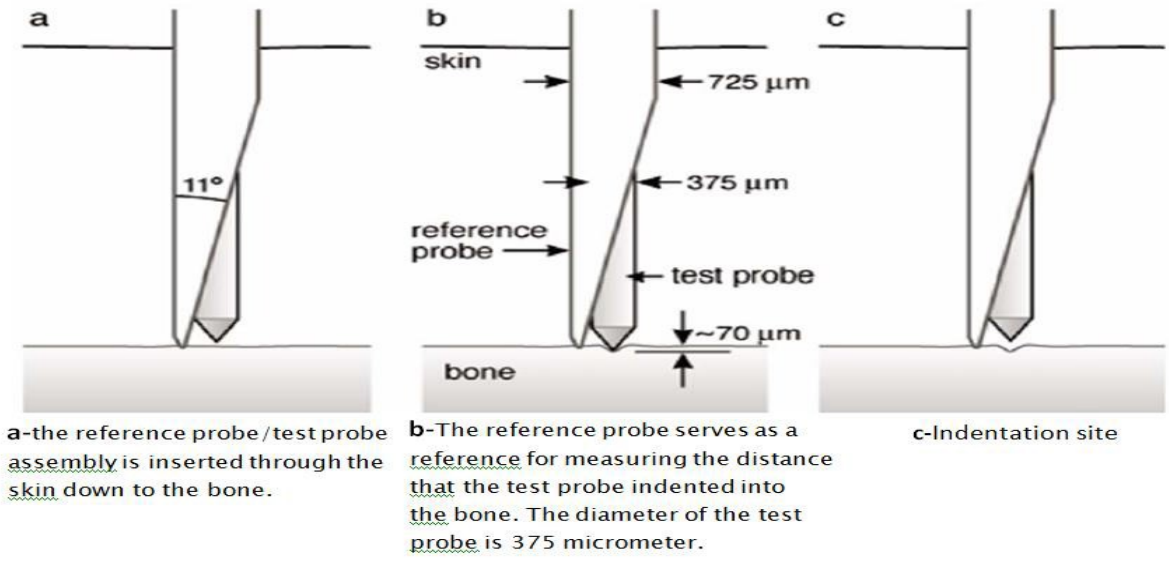


Figure 20

Figure 20-bone material properties measurement in vivo [101].

Osteoprobe® outline is seen in Figure 21[102]. An impact generation mechanism, a displacement transducer, and a probe are major components of the Osteoprobe®. The displacement transducer consists of a custom full bridge strain gage pattern mounted on a flexure and connected as a Wheatstone bridge. The probe is manufactured out of hardened stainless steel and has a 90 degree conical tip with a tip diameter of approximately 375 μm and a tip sharpness radius less than 10μm [102].

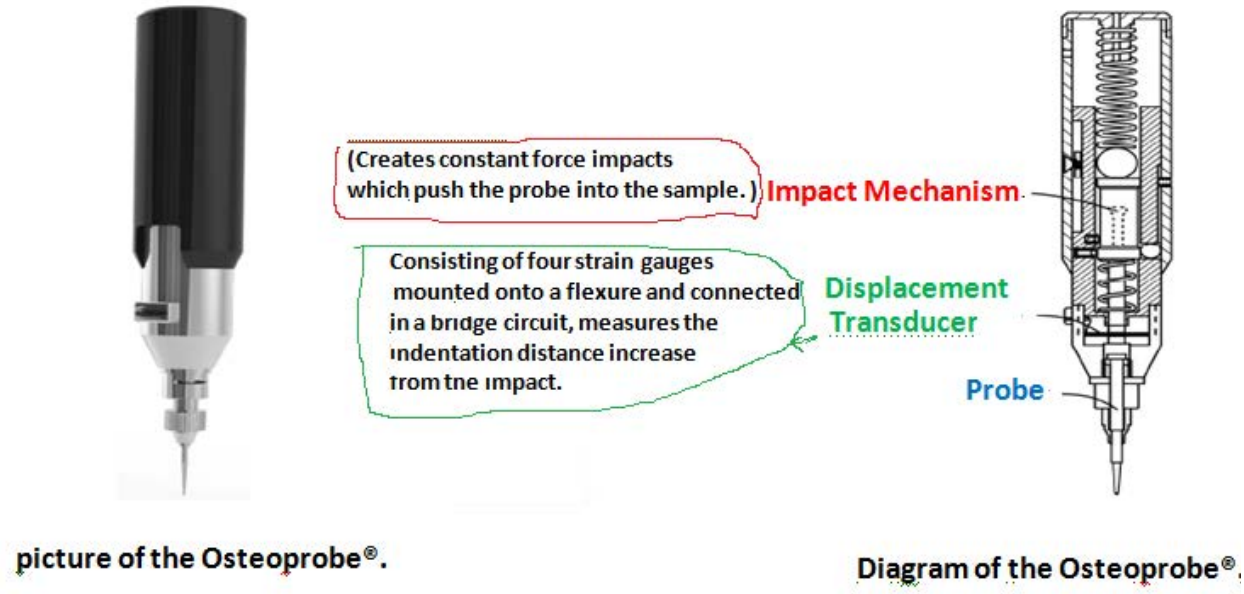


Figure 21

Figure 21- Diagram and picture of the Osteoprobe®. Osteoprobe® main components are an impact mechanism, a displacement transducer, and a probe

The operations are: (1) pre-load, (2) triggering, (3) impact, and (4) unloading. These operations are labeled in a force vs. time graph to clearly show the forces on the sample during a test cycle Figure 22 [102]. During the loading phase, typically lasting around 1 s, the probe of the instrument is manually pushed into the bone up to a maximum

loading force of 10 N. Once the loading force reaches 10 N, an indentation is triggered. The indentation impacts the probe into the bone with a peak force of 40 N (30 N due to the impact alone). After the impact, the operator removes the instrument during the unloading phase. The measured parameter is the **indentation distance increase** from the impact. The short duration of the impact (of order 1 ms) means that the time for the measurement is short relative to the times over which a living patient or horse moves. Thus the measurement is relatively insensitive to movement of the patient.

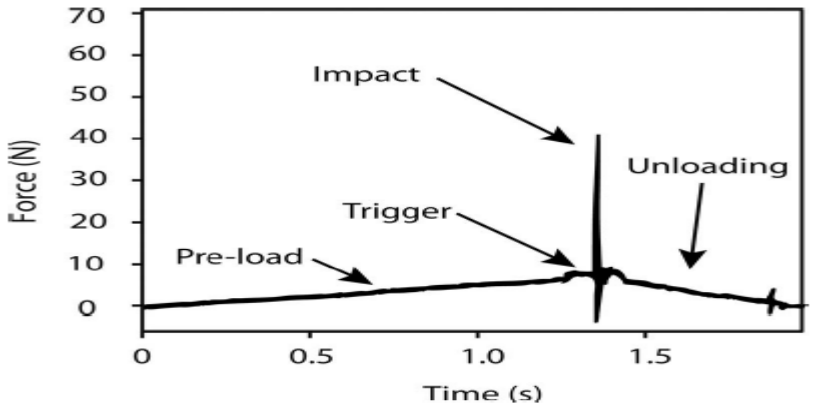


Figure 22

Figure- 22 . Force-Time plot during indentation on bone.

Operation technique for testing a sample is shown in Figure 23 [102].

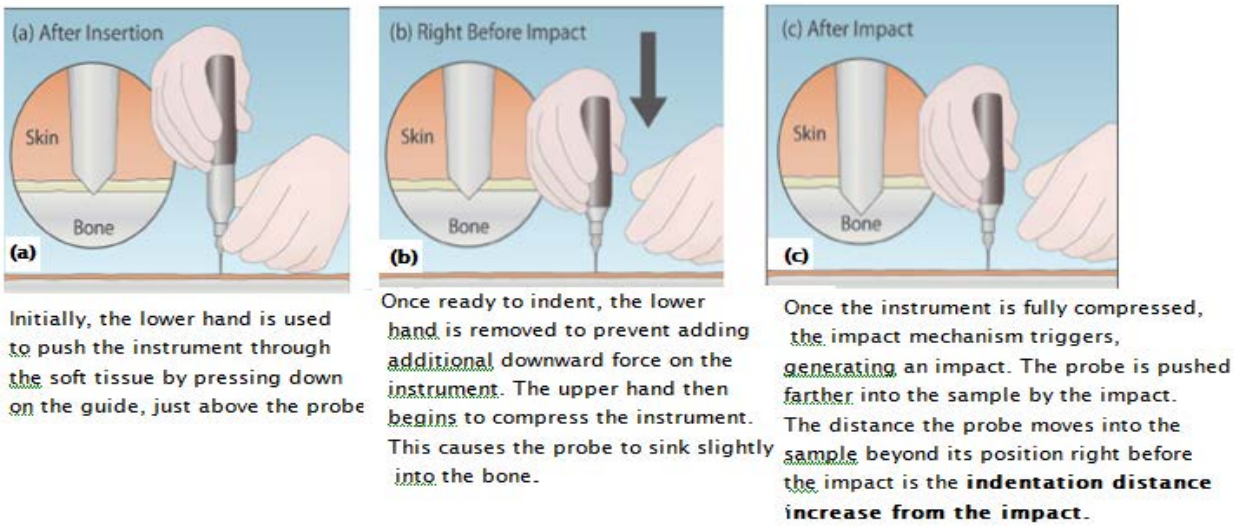


Figure 23

Figure 23- Basic operation of the Osteoprobe. The BMS is defined as 100 times the ratio of the average indentation distance increase from the impact into a calibration phantom [Poly (methyl-methacrylate), PMMA, plastic] over the indentation distance increase from the impact into bone.[102].

There are some instrumental variables in regard of Osteoprobe:

1-Angular dependence: It should be held perpendicular to the test specimen. The operator should always keep an eye on the Osteoprobe during testing. If during compressing the spring the operators observe, for instance the computer screen, they typically do not maintain a perpendicular relationship between the sample and the instrument.

2- Speed dependence: compression duration alterations throughout the indentation have influence on the measurement of values.

3- Probe sharpness dependence

The tip of probe is 90 degree conical and radius of sharpened tip is equal or less than 10 micrometer. When tip radius being more than 10

micrometer on the probe, unnormalized BMS suddenly slope upwards. Probe tip radius more than10 micrometer traverse with difficulty through skin and soft tissue. Thus tips should be sharpened equal or less than 10 micrometer.

4- Sample mass dependence

This concern is notable for small animal bones with little mass. Additionally, the indentations have been optimized for human bone testing.

5- Thermal dependence

Instrument warming prior to procedure is not necessary .Thus environment temperature change has no significant effect on OsteoProbe® measurements.

Hypothesis & Objectives

The Hypotheses of the current research are:

A.-Values of impact microindentation are independent of age

B.-Gender influences impact microindentation values

C-Inter Observer Coefficient variance is less than 5%.

Objectives are:

A-Establish the reference values for BMSi in normal Population from Barcelona, Spain.

B-To assess the influence of age, sex and BMI in BMSi.

C-To assess the Inter observer Coefficient variance for IMI in Hospital del Mar.

Methods

Material: Databases of 98 healthy control individuals (age 18 and older) obtained from 1500 individuals of Microindentation databases from a specialized osteoporosis clinic of the department of internal medicine in a tertiary medical setting of Hospital del Mar- Barcelona, Spain. These databases are comprised of 68 females and 21 males of Spanish Catalan origin, healthy volunteers. BMSi values obtained from the anterior aspect of mid-shaft of cortical tibia bone by Osteoprobe® (Active Life Scientific, Santa Barbara, CA, USA) and, by microindentation procedure that had been performed by different investigators at hospital del Mar between 2010 and 2018. In this experiment it must be pointed out that some investigators (EEP=3, EDTP=3, RST=2, GCM=3, ADP=2) had few samples that is not practical from statistical analysis perspective, Thus these investigators were grouped into another group and called **"others**".

Study Population: Criteria for selecting healthy control samples from databases of internal medicine department specialized osteoporosis clinic in order to prepare dataset for each sample, are summarized in the Table 2 .

Table 2

Table-2 Criteria for selection of healthy samples
1-Age 18 and older
2-No history of fragility fracture and also traumatic tibia fracture
3- No history of diseases or conditions with bone effects e.g. DM, RA, CKD, CD, …
4- No history of medications with bone effects e.g. Glucocorticoids, Anti-malignant drugs, …
5- No history of primary and secondary bone diseases as well as metastatic bone diseases
6- No history of congenital or acquired lower limb deformity most notably of tibia bone
7- No history of osteoporosis and use of anti-osteoporotic medications
N.B. Each individual should have all above criteria to be included in healthy samples group.

After selection of healthy control individuals, we extracted the following information from each healthy volunteer recorded file for dataset preparation

Table 3:

Table 3

Table-3 Dataset information about healthy individuals extracted from recorded file in Hospital del Mar	
individual personal Information	1- ID Number 2- Age and categorized according to decade of age intervals 3-Gender and categorized according to genders 4-height 5-weight
Investigator	1-Name of investigator 2-Date of microindentation procedure 3-Number of microindentation procedures by each investigator 4-Samples categorized according to genders for each investigator
DXA-BMD values	LS T-Score FN T-Score TH T-score
BMI	1-BMI Calculated with according to height and weight 2- BMI Categorized according to BMI values.(Thin≤20 ,Normal=20-25, 25>Overweight<30, Obese≥30, Morbid Obesity≥35)
BMSi value	

After refining the information data from the samples files. We categorized individuals to males and females and the following comparisons were performed:

Samples	89
Male	21
Female	68

Figure 24

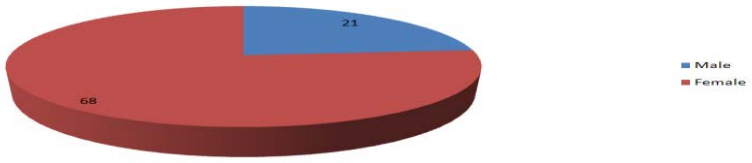


Figure 25

There were nine investigators that had performed BMSi procedure between 2010-2018.Samples of each investigator specified and categorized in male and female and following information were obtained.

Investigat	No. of sam	Male	Female
XNS	18	3	15
LMS	8	0	8
LVM	35	8	27
RGF	14	5	9
EEP	3	0	3
EDTP	3	0	3
RST	2	0	2
GCM	3	2	1
ADP	2	0	2
	89	21	68

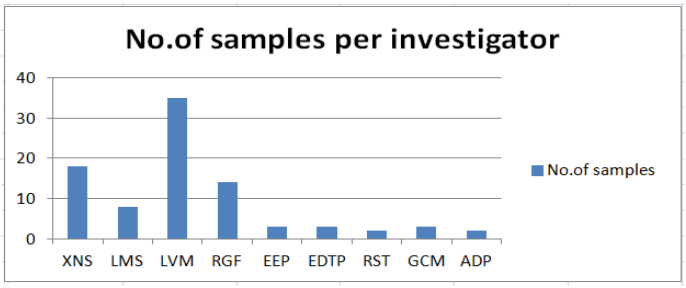


Figure 26

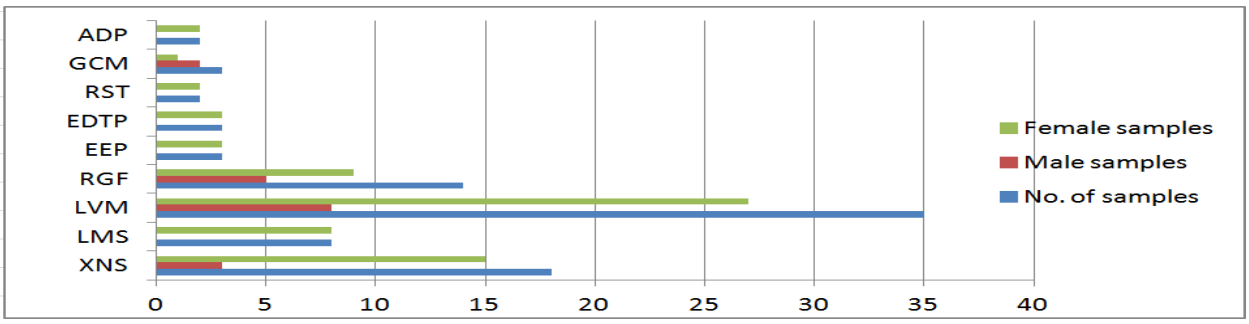


Figure 27

Individuals in each group of male and female categorized by age decade and number of individuals in each decade was determined. Most samples were between ages 20-60. There were few cases after 60 perhaps predictable since most people after age 60 are suffering from a disease like hypertension, diabetes, etc.

Age range	Male	Female
20-30	7	13
30-40	3	13
40-50	0	14
50-60	3	16
60-70	3	7
70-80	1	6
80-	1	1

Figure 28

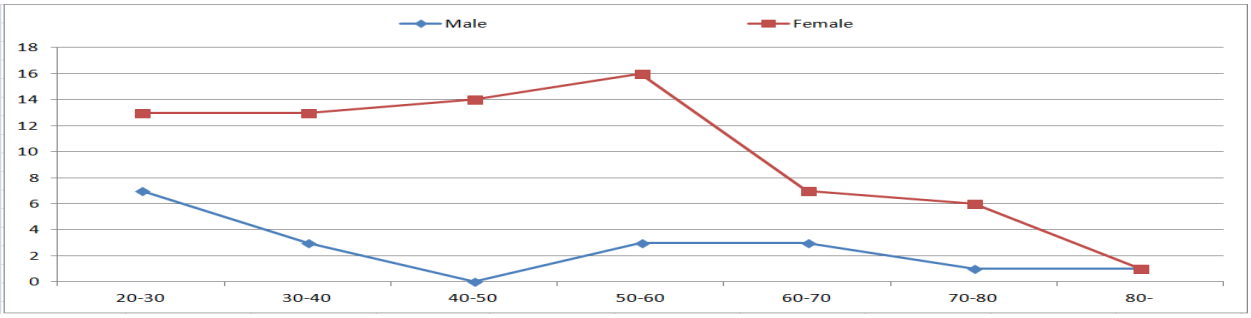


Figure 29

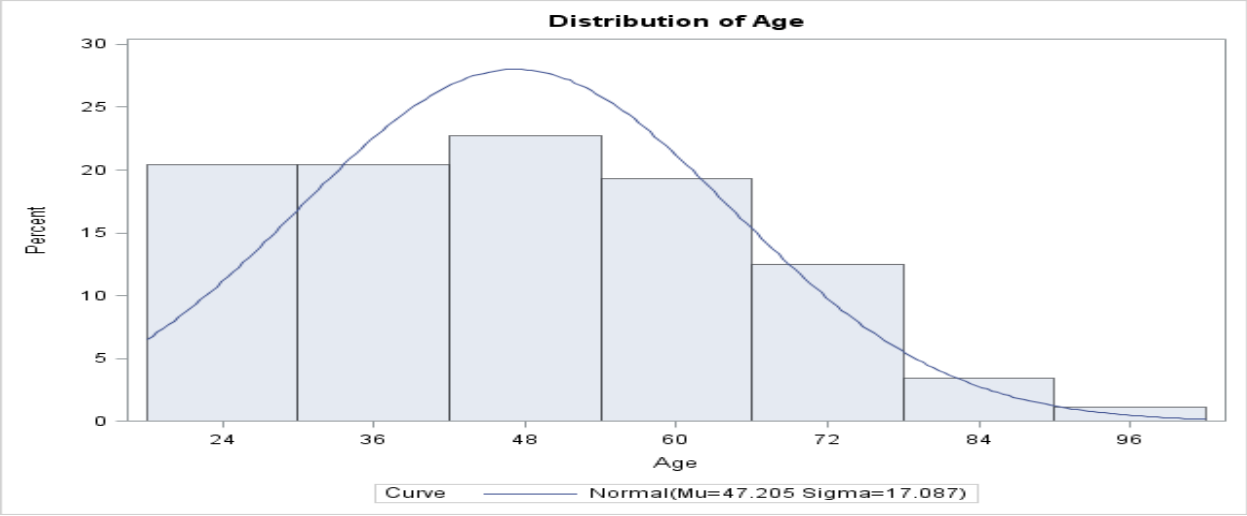


Figure 30

Individuals were categorized according to BMI values. In male group there were four cases thin and one obese but most of the volunteers were normal or overweight. In female group there were nine thin samples, seven obese, 10 samples over weight but most samples (33) were normal weight. All these data are shown in the following graphs.

BMI	Male	Female
>20	4	9
20-25	7	33
25-30	7	10
30-35	1	5
<35	0	2

Figure 31

Age distribution by gender

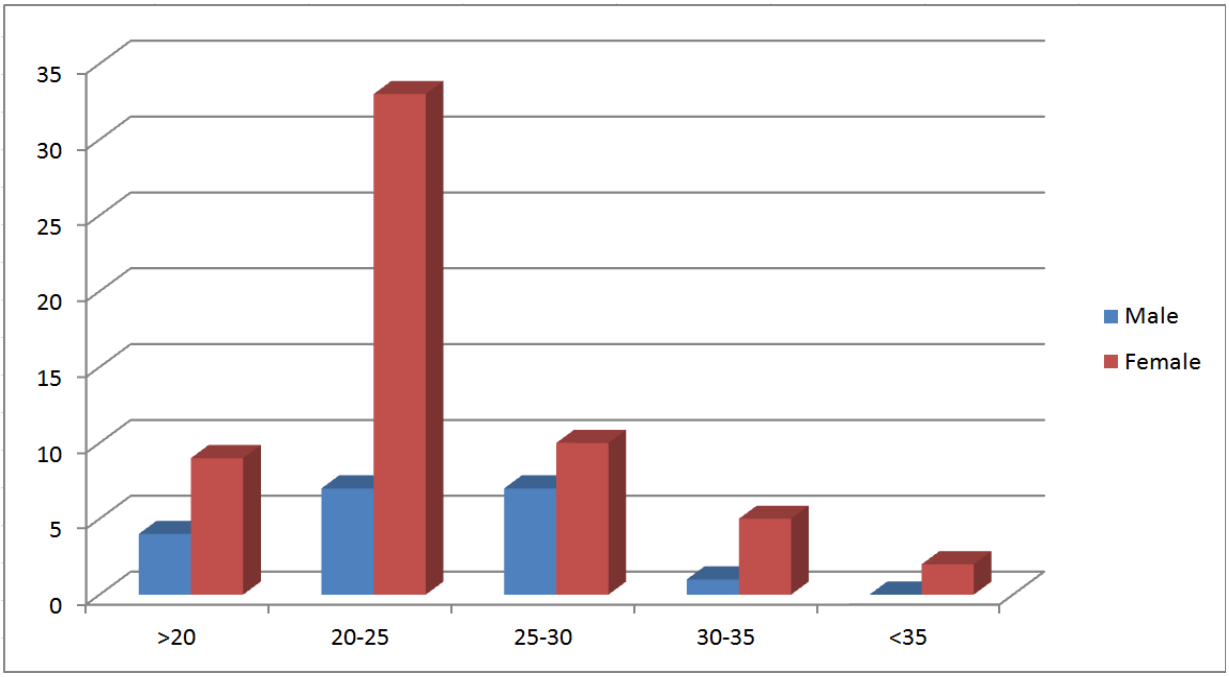


Figure 32

Histogram of age and gender in the healthy volunteers population

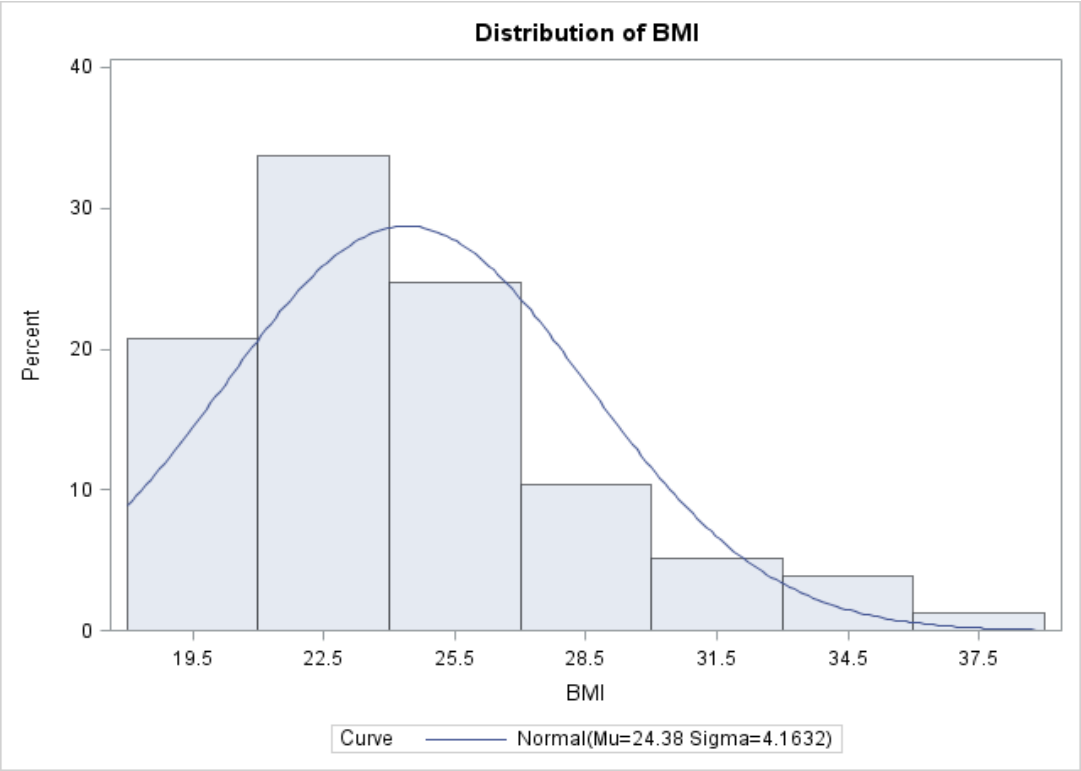


Figure 33

BMSi values of individuals categorized from 70 to 90 in both male and female groups by strata of interval 5.

BMSi	Male	Female
>70	0	2
70-75	1	8
75-80	2	17
80-85	4	18
85-90	8	18
90-95	2	4
<95	4	1

Figure 34

Male and female graph of frequency in BMSi in the studied population

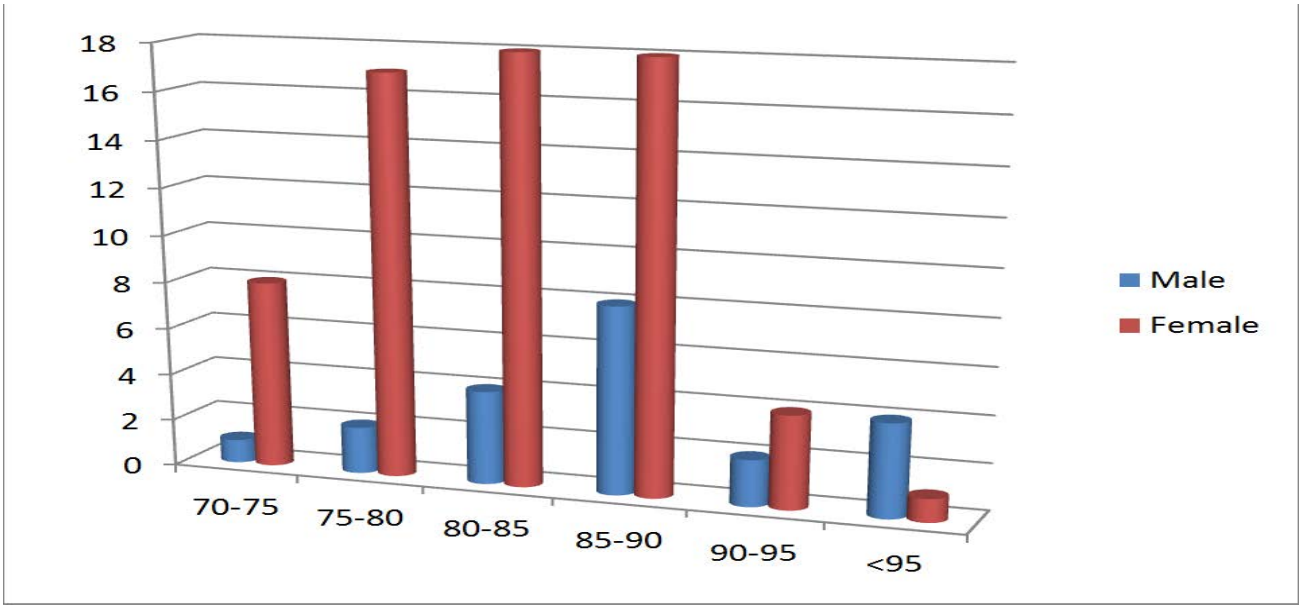


Figure 35

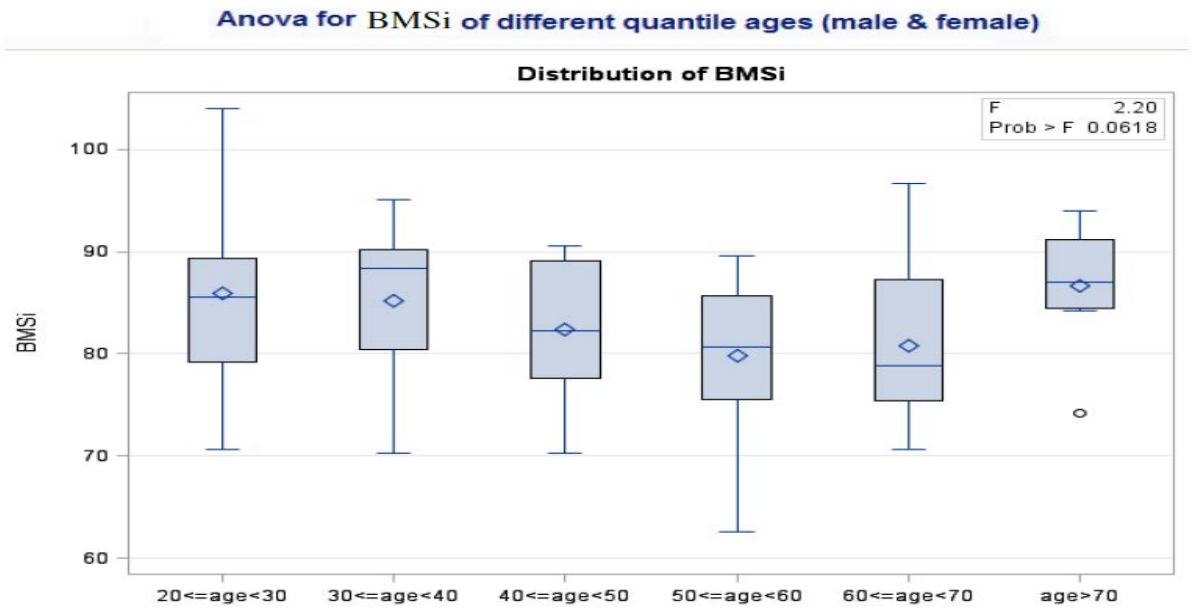
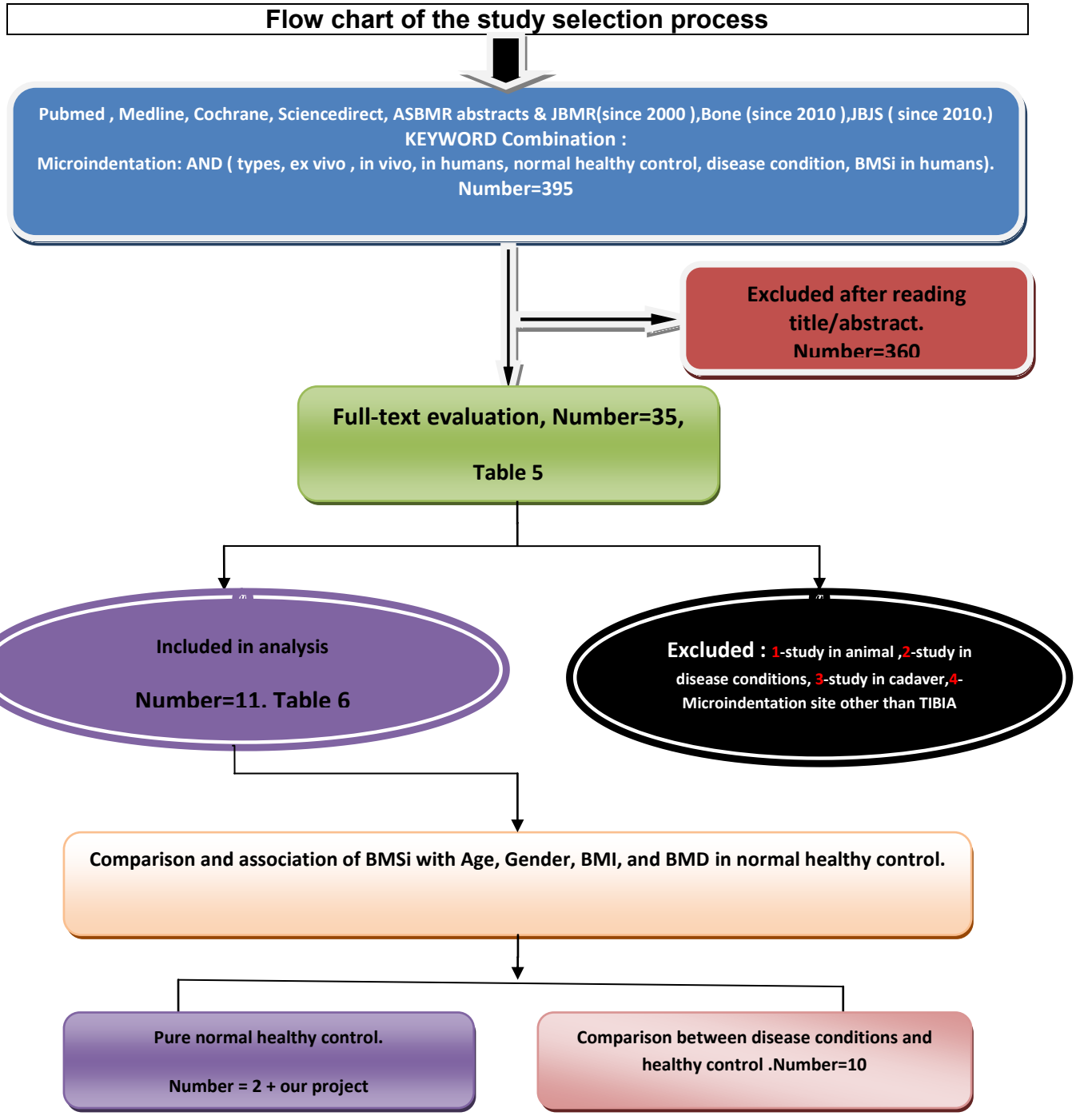


Figure 36

For comparison our study findings of BMSi values in normal healthy populations with other studies that have been performed so far, the systematic literature search was performed and systemic reviews for bone Microindentation was done as shown in following flow chart:



Impact Microindentation technique:

For comprehensive details concerning this matter readers refer to paper that has been published recently [79].

1-Patient "set-up" and "Scrubbing up":

A-Proper Position of patient at bed and patient's leg position:

The best position of the patient is supine and non-dominant leg is suitable for procedure unless there are contraindications Table 4 [79], like skin infection, in these cases the opposite leg should be utilized. The best position of the leg is external rotation till the flat surface of the medial tibia diaphysis horizontally becomes easily accessible for procedure.

Table 4

Table-4.	
Skin	Skin lesions at the indentation site i.e edema ,cellulitis,pyoderma
Bone	1-Previous tibial fracture 2-Primary bone diseases at the microindentation site e.g. PBD. Gaucher 3-Primary or Metastatic bone disease at the microindentation site 4-Osteomyelitis at the microindentation site
Systemic	1-febrile conditions 2- Systemic infectious processes 3-Obesity of severe type 4-allergy to local anesthetics
Miscellaneous	No fitness for microindentation with according to operator opinion

B-Identification of indentation sites, pre-procedure preparation and asepsis, and local anesthesia:

Identify mid distance between the medial border of the tibia plateau and the medial malleolus by a measuring tape. Preparation of procedure site with disinfectants:

- 2 per cent chlorhexidine (effective for more than 4 hours, potent against Gram- positive and -negative organisms, some viruses, less effective against the tuberculosis bacillus);
- 7.5 per cent povidone-iodine (duration of effect shorter, highly bactericidal, fungicidal and viricidal; some effect against spores and good anti-tubercle effect];
- Alcohols (highly effective against all but spores and inexpensive). Allergic reactions are recognized to both chlorhexidine and iodine solutions [103].

Patients at risk for these types of infections include those who are elderly, immunosuppressed, or diabetic; those who suffer from peripheral vascular disease; or those with a combination of these factors [104]. Local anesthetic drugs used to provide analgesia as a sole agent. Lidocaine 2%, mepivacain 2% or equivalent, with or without adrenaline, can be used. The hands are then washed systematically with disinfectants. Once operator gloved, the procedure begins.

2-Procedure techniques: (For better and optimal procedure, operator should be unable to see the computer's monitor)

C-Instrument set up:

On a stable surface put the BMSi-100 reference material cube and hold within standard holder then Insert a sterile probe into the Osteoprobe. At mid diaphysis of the tibia on the pre-identified site Make a hole through the skin and traverse through the underlying soft tissue and periosteum up to cortical bone. Adjust the angle of probe to be at vertical position on the tibia surface without losing contact of probe on the bone surface with up to 10° variation, Figure 37 [96,104].

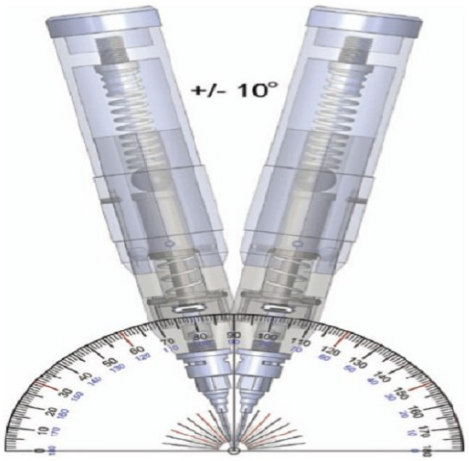


Figure 37

Figure 37-Acceptable angular range of holding the instrument in regard of sample during microindentation procedure. If the instrument holds within range of _+/-10 degree _the unnormalized values of BMSi will be within a few % of BMSi value measured just vertical to the sample.

D-Procedure and outputs collection:

- Usually 8-10 measurements are required during procedure.
- Acceptable procedure should have at least 5 valid measurements

- After every indentation for 2 to 3 seconds the body of the device slowly should be pulled down.
- First indentation because of inadequate probe penetration through periosteum, measurement of first indentation should be disregarded .
- New location of indentation should be at least 2mm apart from previous indentation and probe's angle readjustment is mandatory.

E- RPI outputs and results:

-Invalid results should be removed

-By eight times indentation in the BMSi-100 Reference Material ,the normalization phase starts

-Bone material strength index (BMSi) as the result of indentation will be displayed on the monitor. Review of published literature for comparison of our results is summarized in the following Table 5.

Table 5

Table 5									
Clinical investigations with Microindentation in different disease conditions									
Year of data Publication & Place of Investigation	Authors	Device used for BMSi	No. of samples	No. of male/female samples	Type of samples	BMSi measurement site	Patients BMSi	Controls BMSi	Results of study
2009/ UCSF-USA	Christina Salas et al. [105]	Osteoprobe II™	12	Not specified	fresh- -frozen human cadavers	Distal radius	Not specified	Not specified	Osteoprobe II™ BDI moderately correlate with clinical DXA measurement for bone density with clinical DXA measurement for bone density
2010/ Barcelona-Spain	Adolfo Diez Perez et al. [134]	Biodent®(RPI)	35(27 women with fragility Fx and 8 controls)	27 F /0 M	Living human	Mid-shaft anterior tibia	Not specified	Not specified	RPI by inducing microscopic fracture, directly measures bone mechanical properties at the tissue level.
2013/ Barcelona Spain	Roberto C. Guerri-Fernandez et al. [84]	Biodent® (RPI)	70 (6 AFF, 38 typical osteoporotic fractures , 6 long term bisphosphonate and 20	70 F	Living humans	Mid-shaft anterior tibia	Not specified	Not specified	After adjusting by age, significant differences in BMSI parameters(IDI) were

			controls)						seen between controls and typical and atypical fractures
2013/ USA & Spain	Connor Randall et al. [106]	Biodent® & Osteoprobe®	2	2 F/ 0 M	human cadavers	Mid diaphysis of the tibia	66.2	89.8	Ostoprobe® is an easy-to-use instrument, does not require extensive training and a very simple instrument to operate.
2015/ Gothenburg-Sweden	Daniel Sundh et al. [107]	Osteoprobe®	202	202 F/0 M	Living human	Mid diaphysis of the tibia	72-79	No control in this study	Fat mass was independently and inversely associated with BMSi .
2015/ Leiden Netherlands	Frank Malgo et al [108]	Osteoprobe®	90	53 F/37M	Living human	Mid diaphysis of the tibia	79.9-83.9	Not specified	BMS in patients with fragility fractures is decreased independently of BMD
2015/ Barcelona Spain	Leonardo Mellibovsky et al. [109]	Osteoprobe®	52	25 F/27 M	Living human	Mid diaphysis of the tibia	70-81.6	No control in this study	RPI is sensitive enough to reflect changes in cortical bone indentation after treatment

									with osteoporosis therapies in patients newly exposed to glucocorticoids.
2015/ Oslo-Norway and Barcelona-Spain	Daysi Duarte Sosa et al. [110]	Osteoprobe®	88	88 F(42 Norwegian and 46 Spanish)/ 0 M	Living human	Mid diaphysis of the tibia	No patients in this study	Norwegian BMSi 77±7.1, Spanish BMSi 80.7±7.8	Ethnic differences in bone material properties may partly explain the higher propensity for fracture in Norwegian women.
2015/ Sweden and Barcelona-Spain	R.Rudang et al. [111]	Osteoprobe®	211	211 F/0 M	Living human	Mid diaphysis of the tibia	Mean BMSi value 75.6±7.6 with a range from 52.9 to 93.3	No control in this study	No any difference in BMSi between fractured and non-fractured subjects, but a weak association between BMSi and aBMD
2016/ Southampton-UK	Thomas Jenkins [112]	Biodent®	62 (56 Femoral neck samples were collected at surgery	38 F/24 M	Femoral neck samples were collected at	Femoral neck	Not specified	Not specified	RPI at femoral neck discriminated fracture cases from controls independent

			after low-trauma fracture + 16 Cadaveric control samples without bone disease)		surgery after low-trauma fracture + Cadaveric control samples without bone disease)				of BMD and traditional risk factors but dependent on location.
2016/Norway	Kristin Matre Aasarod et al. [113]	Osteoprobe®	58	29F/29M	Living human	Mid diaphysis of the tibia	Not specified	Not specified	No significant differences between patients and controls for BMSi in total group as well as for the male and female groups
2016/ CUCPS-USA	Jeaaica R. Furst et al. [114]	Osteoprobe®	35	35F/0M	Living human	Mid diaphysis of the tibia	63.69±1.9	70.12±1.9	Bone material properties are impaired in postmenopausal women with T2DM as determined by RPI.
2016/ Barcelona-	Roberto Guerri-	Osteoprobe®	85(50 Infected with	26F (15 pts+11	Living human	Mid diaphysis	84.5 (83 to 87)	90 (88.5 to	HIV infection is associated

Spain	Fernandez et al. (129)		HIV+ 35 Controls)	controls)/59 M (35 pts+24 controls)		of the tibia		93)	with bone damage
2017/ Leiden- The Netherlands	F. Malgo et al [115]	Osteoprobe®	132	85F/47M	Living human	Mid diaphysis of the tibia	79.7±0.6 in females, 80.0±0.8 in males	In this study there was no healthy control samples	BMSi as measured by IMI on the tibia is associated with increased bone fragility at all relevant skeletal sites.
2017/ Leiden – The Netherland	F Malgo et al. [116]	Osteoprobe®	92(48 Acormegaly patients+ 44 Controls)	44F (22 pts+22 controls)/48 M (26 pts+22controls)	Living human	Mid diaphysis of the tibia	79.4±0.7	83.2±0.7	BMSi was significantly lower in acromegaly patients than that in controls
2017/ Barcelona Spain	Sabina Herrera et al. [117]	Osteoprobe®	45(16 GD1 patients+ 29 Controls)	32F (9 pts+23 controls)/13 M (7 pts+6 controls)	Living human	Mid diaphysis of the tibia	72.74±	81.76±	BMSi in GD1 patients eas significantly lower than controls.
2017/ Norway	Daysi Duarte Sosa et al. [118]	Osteoprobe®	132 (66 osteoporosis and 66 control)	132F/0 M	Living human	Mid diaphysis of the tibia	71.5±	76.4±6,2	Low BMSi constitutes a risk factor for all osteoporotic fractures and vertebral fracture severity in women with osteoporosis

									,independent of BMD,age and bone turnover .
2017/ Barcelona-Spain	Xavier Nogues et al. [119]	Osteoprobe®	39(21 cases with Fx and 18 cases without Fx)	39 F/ 0M	Living human	Mid diaphysis of the tibia	73.76±6.49(in cases with new Fx during oral BP therapy) 84.64±6.26(in cases without new Fx during oral BP therapy)	In this study there was no healthy control samples	Cases with Fx during oral BP therapy is associated with low BMSi in comparison to no Fx cases
2017/ Barcelona-Spain	Maria Jose Perez-Saez et al. [120]	Osteoprobe®	131(38 cases with KT and 93 controls)	97F (23 pts with KT+74 controls)/37 M (17 pts with KT+20 controls)	Living human	Mid diaphysis of the tibia	79.1±7.7	82.9±7.8	BMSi was lower in KTR
2017/ Barcelona-Spain	Roberto Guerri-Fernandez et al. [121]	Osteoprobe®	63 HIV patient on different group of medications (TDF-FTC vs ABC-3TC)	16F/47M	Living human	Mid diaphysis of the tibia	81.02 vs 82.68 (TDF-FTC vs ABC-3TC)	In this study there was no healthy control samples	Long term anti-HIV treatment leads to impaired bone health.
2017/ Leiden – The Netherlands	Frank Malgo et al. [122]	Osteoprobe®	20(9 pts with Paget's disease of tibia + 11 subjects without tibia pathology:4	Not specified	Living human	Mid diaphysis of the tibia	74.7±1.7 pagetic tibia vs 78.7±1.3 contralateral non-affected	82.2±1.3 Dominant leg vs 81.4±1.3 non-dominant leg	In paget's disease mean BMSi was lower that of the contralateral non-affected

			Osteoporosis +5 Osteopenia+ 2 normal)				tibia		tibia, but inpatients without paget's disease there was no differences in mean BMSi between both legs.
2017/ Barcelona-Spain	Sabina Herrera et al [123]	Osteoprobe®	32(3Cases of Camurati-Engelmann disease+29 controls)	26F(2 pts +24 controls)/6M(1 pt + 5 controls)	Living human	Mid diaphysis of the tibia	76.9	81.8	BMSi value were low or very low in comparison to normal controls
2017/ USA	Tamara D Rozental [124]	Osteoprobe®	192(99 Pts with Fx + 93 controls)	192 F/0M	Living human	Mid diaphysis of the tibia	74.6±8.5	77.4±8.8	BMSi was 4%lower in pts with Fx compared to control group
2018/ Gothenburg-Sweden	Lisa Johansson et al. [125]	Osteoprobe®	1027 osteoporotic women (750 with no VFs+ 277 with VF)	1027F/0M	Living human	Mid diaphysis of the tibia	77.9±7.4(cases without VF)+ 76.9±7.3(cases with VF)	In this study there was no healthy control samples	Prevalence of VF was not associated with BMSi or cortical porosity indicating an inferior role of cortical bone quality in the pathogenesis of VF.
2018/ Barcelona-Spain	Roberto Guerri-Fernande	Osteoprobe®	40 HIV pts	7 F/33 M	Living human	Mid diaphysis of the tibia	86.07 At baseline vs 89.04	no healthy control	BMSi values were significantly

	z et al. [126]						after 48 weeks of TDF-based ART treatment	samples	higher after 48 weeks of TDF-based ART treatment vs baseline value .
2018/ HMS-USA	Lamya Karim et al. [127]	Osteoprobe® And Biodent®	20 human tibia and femur pairs from female donors	20 F/0 M	human cadavers	Mid diaphysis of the tibia	no pts samples	83.06±7.44 (68.44-93.63)	BMSi was independent of cortical thickness, and were not associated with age.
2018/Gothenburg-Sweden	Daniel Sundh et al., [128]	Osteoprobe®	20	20F/0M	Living human	Mid diaphysis of the tibia	no pts samples	76.6±5.5	a 3-month high-impact jumping exercise program was able to substantially increase BMSi in postmenopausal women,

After refining the papers with according to exclusion criteria of the flow chart, the Results of systematic review of papers for Microindentation can be seen in the following Table 6.

Table 6

Table 6												
Papers **Samples** **Specifications**	Hospital del Mar 2010-2018 Barcelona Spain	Tamara D Rozenta etal/ BMSi as measured by IMI in postM women with distal radius and hip fractures/ 2017 [124]	S.Herrera et al /Discrepency between bone density and BMSi in three siblings with Camurati-Engelmann disease/ 2017 [123]	Frank Malgo et al, Impact Microindentation: Consistency of Serial Measurements and Alterations in Patients With Paget's Disease of the Tibia/2017 [122]	Pérez-Sáez MJ et al, Bone Density, Microarchitecture, and Tissue Quality Long term After Kidney Transplant/ 2017 [120]	Daysi Durate Sosa et al,Reduced BMS is associated with increased risk and severity of osteoporotic fractures.An Impact Microindentation study/2017 [118]	S.Herrera et al /Assessment of bone health in patients with type 1 Gaucher Disease using impact microindentation/ 2017 [117]	F Malgo et al.BMSI as measured by impact microindentation is altered in patients with acromegaly / 2017 [116]	F Malgo et al. Bone material strength index as measured by impact microindentation is low in patients with fractures irrespective of fracture site/ 2017 [115]	Roberto Guerri-Fernandez et al. HIV Infection Is Associated With Worse Bone Material Properties, Independently of Bone Mineral Density/ 2016 [129]	Jessica R. Furst et al. Advanced Glycation Endproducts and Bone Material Strength in Type 2 Diabetes/ 2016 [114]	Daniel Sundh,et al. High Impact Mechanical Loading Increases Bone Material Strength in Postmenopausal Women — a 3-Month Intervention Study /2018 [128]
City Country	Barcelona Spain	Boston USA	Barcelona Spain	Leiden ,The Netherlands	Barcelona Spain	Oslo Norway	Barcelona Spain	Leiden ,The Netherlands	Leiden ,The Netherlands	Barcelona Spain	New York/ USA	Gothenburg/ Sweden
Number of healthy Samples	89	93	29	2	94	66	29	44	31	35	19	20
Age **(year)**	47.20±17.08	67.3±7.6	NS	61.9	50.2±16	66.5±7.9	48.72±15.8	60.5±8.5	57.5±9.9	33.9	65.6±1.2	55.5±2.3
Male/	20/69		5/24	7/4	20/74		3/23	22/22	11/20	24/11	0/19	20/0

Female												
Height	164.71±9.64	163±7	NS	NS	NS	165.7±5.8	NS	NS	NS	NS	158±2	166.7±5.7
Weight	66.26±11.91	70.9±17.1	NS	NS	NS	67.8±10.1	NS	NS	NS	70	7.5.8±3	64.5±7.5
BMI	24.37±4.16	26.8±6.5	NS	27.3±1.7	24.8±4	24.6±3.9	NS	26.6±4.3	25.1±4.7	22.9	30.5±1.3	23.3±3.3
LS-BMD	0.98±0.13	-0.64±1.46	NS	NS	NS	0.3±1.0	NS	-0.3±1.2	NS	-0.65	-0.86±0.29	0.94±0.09
TH-BMD	0.90±0.13	-0.62±0.90	NS	NS	NS	0.2±0.7	NS	NS	NS	-0.6	-0.5±0.1	0.86±0.09
FN-BMD	0.78±0.12	-1.16±0.91	NS	NS	NS	0.3±0.8	NS	-0.8±0.8	NS	-0.65	-1.3±0.1	0.72±0.08
BMSi	83.010±7.877	77.4±8.8	81.8	82.1±1.3	82.9±7.8	76.4±6.2	81.76±1.44	83.2±0.7	76.6±4.9	90	70.12±1.9	76.6±5.5
Site of Microindentation	Mid diaphysis of the tibia	Mid diaphysis of the tibia	Mid diaphysis of the tibia	Mid diaphysis of the tibia	Mid diaphysis of the tibia	Mid diaphysis of the tibia	Mid diaphysis of the tibia	Mid diaphysis of the tibia	Mid diaphysis of the tibia	Mid diaphysis of the tibia	Mid diaphysis of the tibia	Mid diaphysis of the tibia
Devise	Osteoprobe®	Osteoprobe®	Osteoprobe®	Osteoprobe®	Osteoprobe®	Osteoprobe®	Osteoprobe®	Osteoprobe®	Osteoprobe®	Osteoprobe®	Osteoprobe®	Osteoprobe®
CV	<5%	NS	NS	2.2%	NS	9.1%	NS	2.2%	2.2%	3%	8,7%	7%

Statistical analysis

Based on the database of a tertiary medical settings of hospital del Mar-Barcelona, Spain i.e. "a specialized osteoporosis clinic of department of internal medicine" dataset of normal healthy samples were prepared. Our destinations were to: 1-extract information in order to collect the dataset of normal healthy populations from this sizeable database and thus to determine reference normative data of BMSi values in normal and healthy samples irrespective of genders as well as BMSi values in both male and female genders respectively and also 2- to determine the associations of BMSi values with age, gender, BMI, BMD, height, weight. Descriptive statistics including distribution values of BMSi were estimated for the healthy control group after checking for normality and 3- BMSi values per different investigators regardless of genders as well as in males and females genders separately. The statistical analysis were adjusted according to different conditions i.e. Age, Gender, BMI, BMD, Height , Weight.

By using the UNIVARIATE Procedure and with according to statistical analysis, BMSi value distribution curve (regardless of gender) was performed. Comparing means of BMSi between males and females was performed and analyzed by using Student's t-test.

By using the ANOVA procedure BMSi per different investigators irrespective of genders as well as males and females samples were performed and analyzed. By using the Pearson's correlation coefficients, Correlation between BMSi, BMI, and BMD Regardless of genders as well as males and females were performed and analyzed.

By using ANOVA for BMSi with Different Interval Ages for The Control healthy Group were performed and analyzed.

By using ANOVA for comparing means of BMSi in different BMI intervals for the Control healthy Group were performed and analyzed.

By using a nonparametric regression approach based on Regression Splines to find existing associations between covariates (Age, Gender, BMI, BMD, Weight, and Height) and BMSi were performed and analyzed.

All the statistical analyses were performed by using SAS version 9.2 (Statistical Analysis System, SAS Institute Inc., Cary, NC, USA) and R VERSION 3.3.

Results

A total 89 healthy individuals data were extracted with according to selection criteria and categorized per investigators and genders.

Frequency table for each investigator

The FREQ Procedure

USER				
USER	Frequency	Percent	Cumulative Frequency	Cumulative Percent
ADP	2	2.27	2	2.27
EDTP	3	3.41	5	5.68
EEP	3	3.41	8	9.09
GCM	3	3.41	11	12.50
LMS	8	9.09	19	21.59
LVM	35	39.77	54	61.36
RGF	14	15.91	68	77.27
RST	2	2.27	70	79.55
XNS	18	20.45	88	100.00
Frequency Missing = 2				

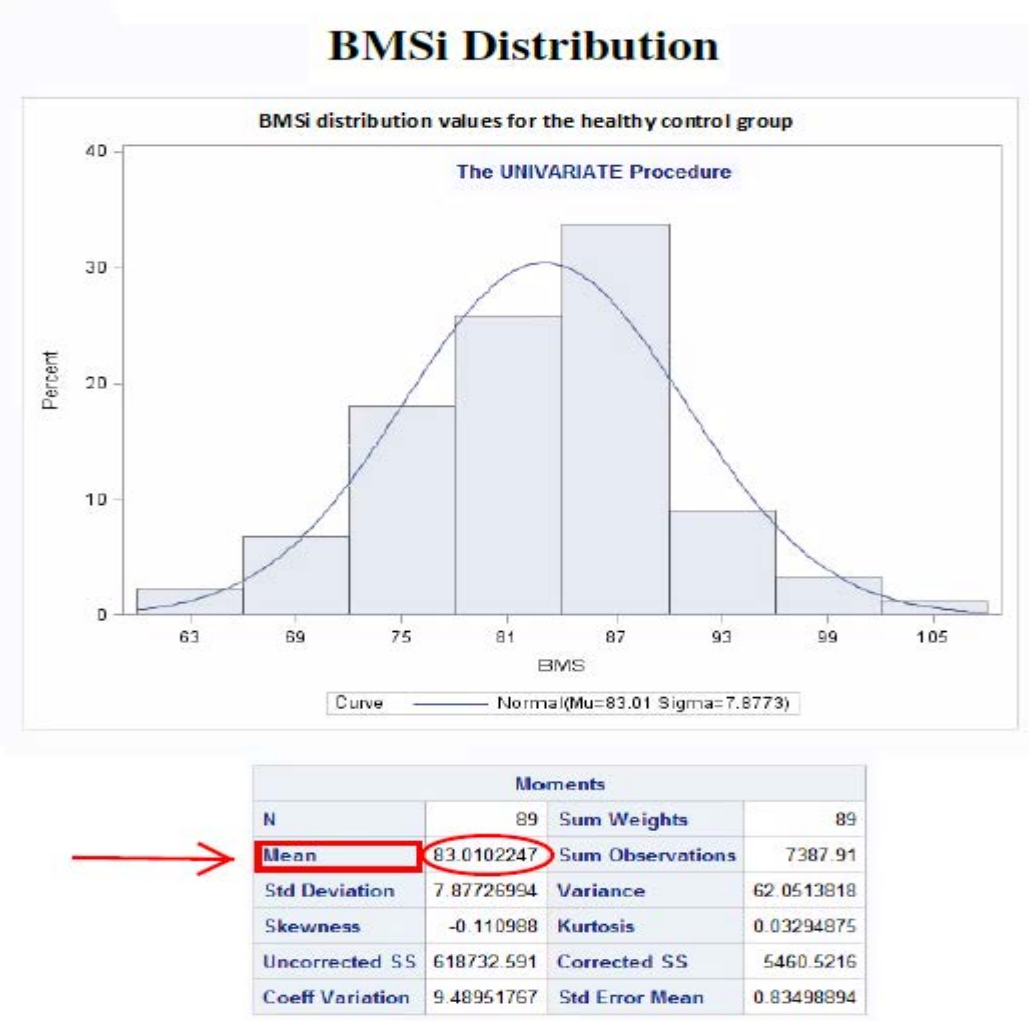
Figure 38

The gender distribution of the group was as follows

GENDER				
GENDER	Frequency	Percent	Cumulative Frequency	Cumulative Percent
Female = 1	69	77.53	69	77.53
Male = 2	20	22.47	89	100.00
Frequency Missing = 1				

Figure 39

In this section all the statistical analyses were performed using SAS version 9.2 (Statistical Analysis System, SAS Institute Inc., Cary, NC, USA) and R VERSION 3.3. Descriptive statistics including distribution values of BMSi were estimated for the healthy control group after checking for normality. With according to statistical analysis by using the UNIVARIATE Procedure, BMSi value distribution curve (regardless of genders) is symmetric and is not skewed with Mean=83.0102247 and SD=7.87726994.



Moments			
N	89	Sum Weights	89
Mean	83.0102247	Sum Observations	7387.91
Std Deviation	7.87726994	Variance	62.0513818
Skewness	-0.110988	Kurtosis	0.03294875
Uncorrected SS	618732.591	Corrected SS	5460.5216
Coeff Variation	9.48951767	Std Error Mean	0.83498894

Figure 40

Above Picture and table represents the BMSi distribution values for the healthy control group regardless of gender.

As previously mentioned there were a number of investigators that collected normal data:

Investigat	No. of sam	Male	Female
XNS	18	3	15
LMS	8	0	8
LVM	35	8	27
RGF	14	5	9
EEP	3	0	3
EDTP	3	0	3
RST	2	0	2
GCM	3	2	1
ADP	2	0	2
	89	21	68

Figure 41

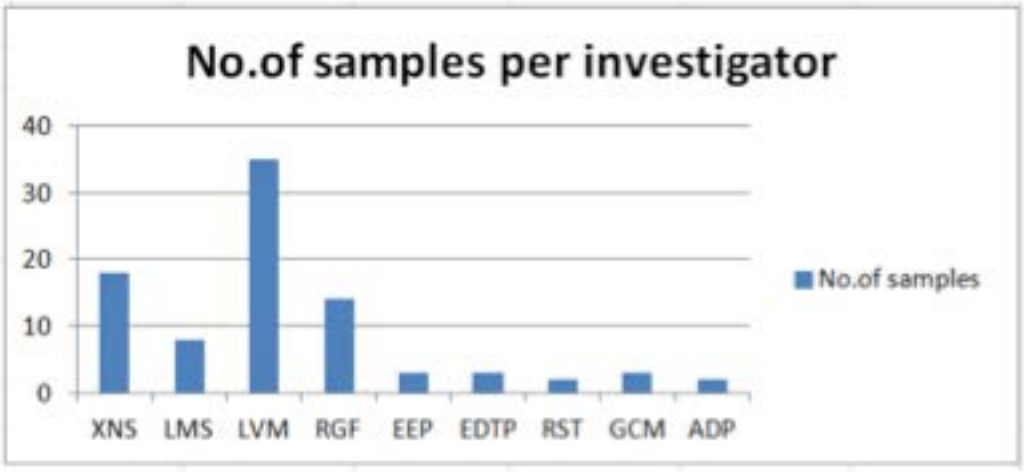


Figure 42

ANOVA for BMSi / different investigators in healthy group regardless of genders

The ANOVA procedure shows that the means of BMSi for different investigators (groups) are different. P-value is 0.0064, which is less than the 0.05 significance level. Based on the given sample, only for investigators LVM and others the means of BMSi are significantly different. The interpretation of this finding is most probably that most samples of LVM are females (females=27 vs. Males=8) and according to our findings in this

study, BMSi values are different between the two genders) It must be pointed out that some investigators (EEP=3, EDTP=3, RST=2, GCM=3, ADP=2) had few samples that is not practical from statistical analysis perspective. Thus these investigators were grouped into another group called "others" [EEP=3, EDTP=3, RST=2, GCM=3, ADP=2]. BMSi values distributions were estimated in each gender group. In Male group Mean of BMSi value is equal to 87.8089 with SD=7.9994 and in Female group Mean is equal to 81.6952 with SD=6.7557 respectively.

gender_dummy	N	Mean	Std Dev	Std Err	Minimum	Maximum
Female	61	81.6952	6.7557	0.8650	65.3000	91.6000
Male	18	87.8089	7.9994	1.8855	74.4300	104.0
Diff (1-2)		-6.1136	7.0491	1.8908		

Figure 43

The following graphs represent the sampling distributions of BMSi for males and females. It is clear that there is a significantly difference between means of BMSi for each group. As plot shows the applied t-test is correct since the distributions of samples in each group are close to standard normal distribution demonstrated by according to quintile plots.

T-test for comparing means of BMSi between males and females in the healthy control group

The values of BMSi in both genders were:

gender_dummy	Method	Mean	95% CL Mean		Std Dev	95% CL Std Dev	
Female		81.6952	79.9650	83.4255	6.7557	5.7336	8.2246
Male		87.8089	83.8309	91.7869	7.9994	6.0026	11.9922
Diff (1-2)	Pooled	-6.1136	-9.8787	-2.3485	7.0491	6.0902	8.3694
Diff (1-2)	Satterthwaite	-6.1136	-10.3895	-1.8378			

Figure 44

And the histogram of the distribution is:

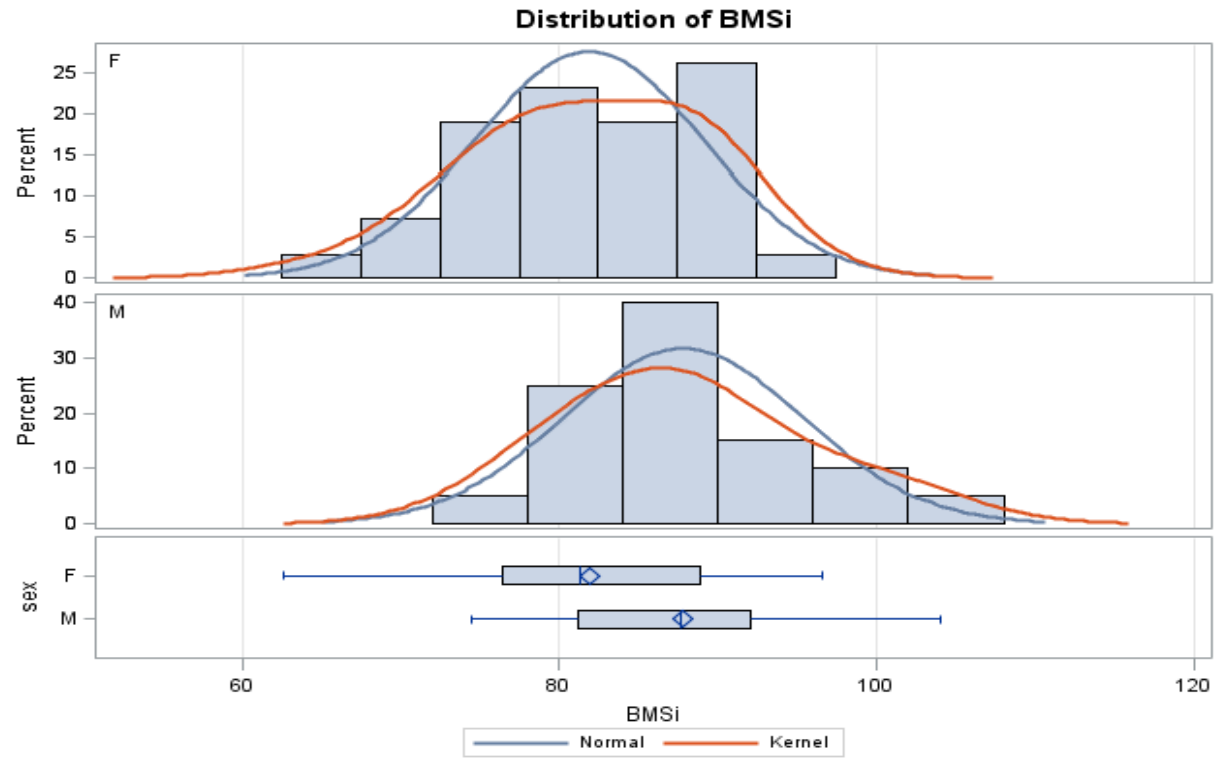


Figure 45

And the plot distribution of the individual measurements is displayed as follow, categorized in quantiles

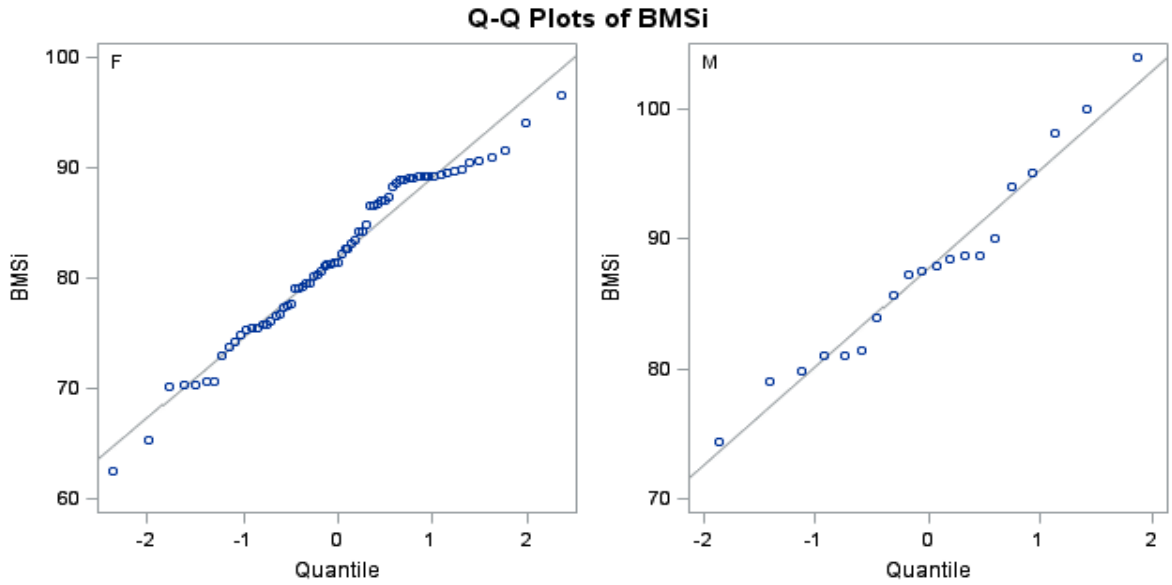


Figure 46

(N.B. Quantiles: Divisions of a probability distribution or frequency distribution into equal, ordered subgroups, for example quartiles or percentiles.)

ANOVA for BMSi per different investigators and Female samples (Gender=1)

gender_dummy	N	Mean	Std Dev	Std Err	Minimum	Maximum
Female	61	81.6952	6.7557	0.8650	65.3000	91.6000

Figure 47

Based on the given sample (69 observations) the ANOVA procedure represented, there is no significant difference (p-value=0.0718) between means of BMSi for each investigator in female patients.

ANOVA for BMSi/different investigators and Male samples (Gender=2)

gender_dummy	N	Mean	Std Dev	Std Err	Minimum	Maximum
Male	18	87.8089	7.9994	1.8855	74.4300	104.0

Figure 48

Based on the given sample (20 observations) the ANOVA procedure represented, there is no significant difference (p-value=0.0871) between means of BMSi for each investigator in male patients.

T-Test for Means of BMSi between Males and Females in the healthy control group

The following table represents the results of the independent t-test for testing means of BMSi between males and females in the healthy control group, there are 69 and 20 observations without prevalent fractures for females and males respectively. Also because based on the given sample the variances of BMSi for males and females are the same (p-value 0.7495), the pooled t-test was used.

The result of this t-test represents that there is a statistically significant difference between means of BMSi for each group (p-value =0.0020).

T-test for comparing means of BMSi between males and females in the healthy control group :

Two sample t-test for means of BMSi between Males and Females

The T-test Procedure

Variable: BMSi (BMSi)

sex	N	Mean	Std Dev	Std Err	Minimum	Maximum
F	69	81.9070	7.2319	0.8706	62.6000	96.6100
M	20	87.8330	7.5709	1.6929	74.4300	104.0
Diff (1-2)		-5.9260	7.3073	1.8557		

sex	Method	Mean	95% CL Mean		Std Dev	95% CL Std Dev	
F		81.9070	80.1697	83.6442	7.2319	6.1943	8.6902
M		87.8330	84.2897	91.3763	7.5709	5.7576	11.0579
Diff (1-2)	Pooled	-5.9260	-9.6145	-2.2376	7.3073	6.3642	8.5810
Diff (1-2)	Satterthwaite	-5.9260	-9.8149	-2.0371			

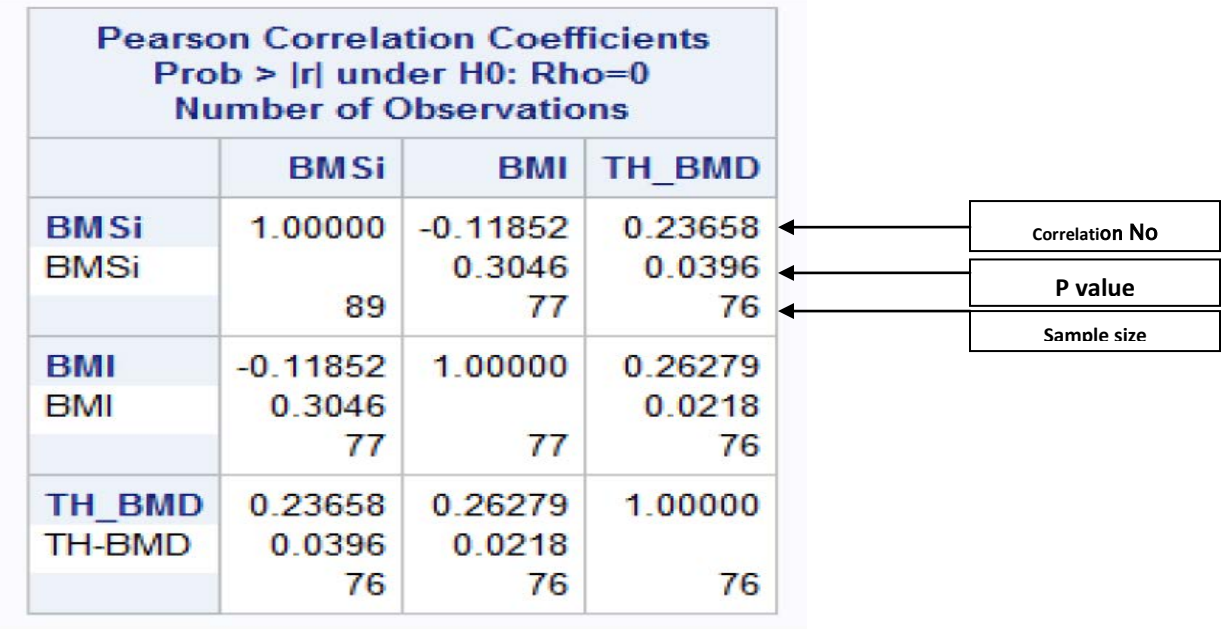
Method	Variances	DF	t Value	Pr > |t|
Pooled	Equal	87	-3.19	0.0020
Satterthwaite	Unequal	29.797	-3.11	0.0041

Equality of Variances				
Method	Num DF	Den DF	F Value	Pr > F
Folded F	19	68	1.10	0.7495

Figure 49

Correlation between BMSi, BMI, TH-BMD Regardless of gender

Based on the given sample (89 observation), for BMSi the Pearson's correlation coefficient shows a statistically significant level (p-value =0.0396) of positive correlation (r=0.23685) with TH-BMD. BMSi does not have statistically significant correlation with BMI as demonstrated bellow (highlighted in the first row):



Pearson Correlation Coefficients Prob > |r| under H0: Rho=0 Number of Observations			
	BMSi	BMI	TH_BMD
BMSi	1.00000	-0.11852	0.23658
BMSi		0.3046	0.0396
	89	77	76
BMI	-0.11852	1.00000	0.26279
BMI	0.3046		0.0218
	77	77	76
TH_BMD	0.23658	0.26279	1.00000
TH-BMD	0.0396	0.0218	
	76	76	76

Figure 50

The results of the similar procedure for each gender are as follows:

For females (69 observations) there are no statistically significant correlations between BMSi and BMI, TH-BMD, FN-BMD and LS-BMD, all p-values are greater than 0.05

Correlation between BMSi and BMI and TH_BMD, FN_BMD, LS_BMD (females)

Pearson Correlation Coefficients
Prob > |r| under H0: Rho=0
Number of Observations

	BMSi	BMI	TH_BMD	FN_BMD	LS_BMD
BMSi	1.00000	-0.07356	0.11676	0.13792	0.01583
BMSi		0.5831	0.3871	0.3062	0.9070
	69	58	57	57	57

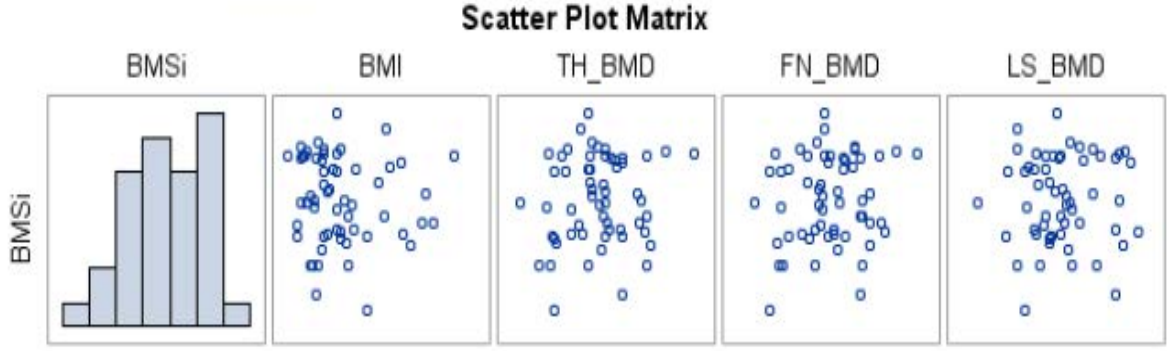


Figure 51

For males (19 observation) there are no statistically significant correlations between BMSi and BMI, TH-BMD, FN-BMD and LS-BMD, all p-values are greater than 0.05.

Correlation between BMSi and BMIand TH_BMD FN_BMD LS_BMD (males)

Pearson Correlation Coefficients Prob > |r| under H0: Rho=0 Number of Observations					
	BMSi	BMI	TH_BMD	FN_BMD	LS_BMD
BMSi	1.00000	-0.42594	0.07009	0.02709	-0.04701
BMSi		0.0690	0.7756	0.9123	0.8484
	20	19	19	19	19

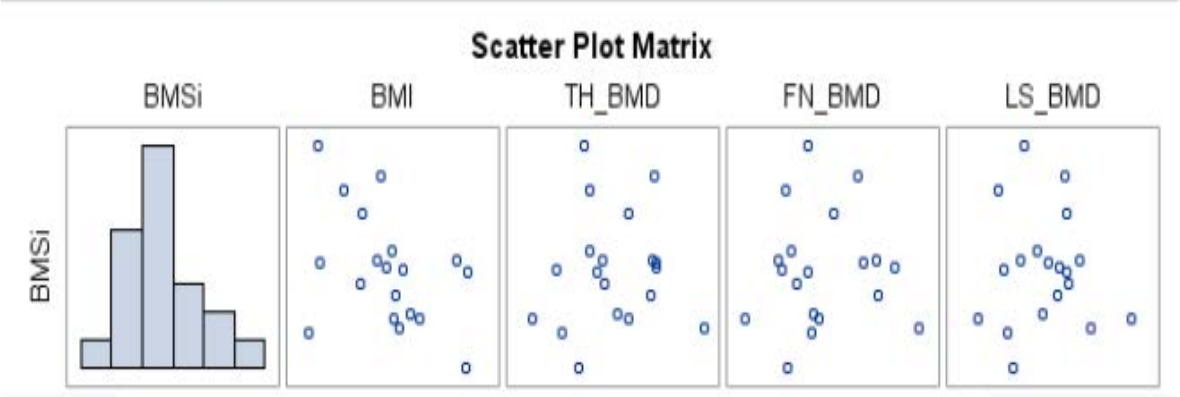


Figure 52

ANOVA for BMSi with Different Interval Ages for The Control healthy Group

The following diagram shows that based on the given sample of healthy group for different interval ages, there is no significant difference between means of BMSi for each interval (p-value=0.0618).

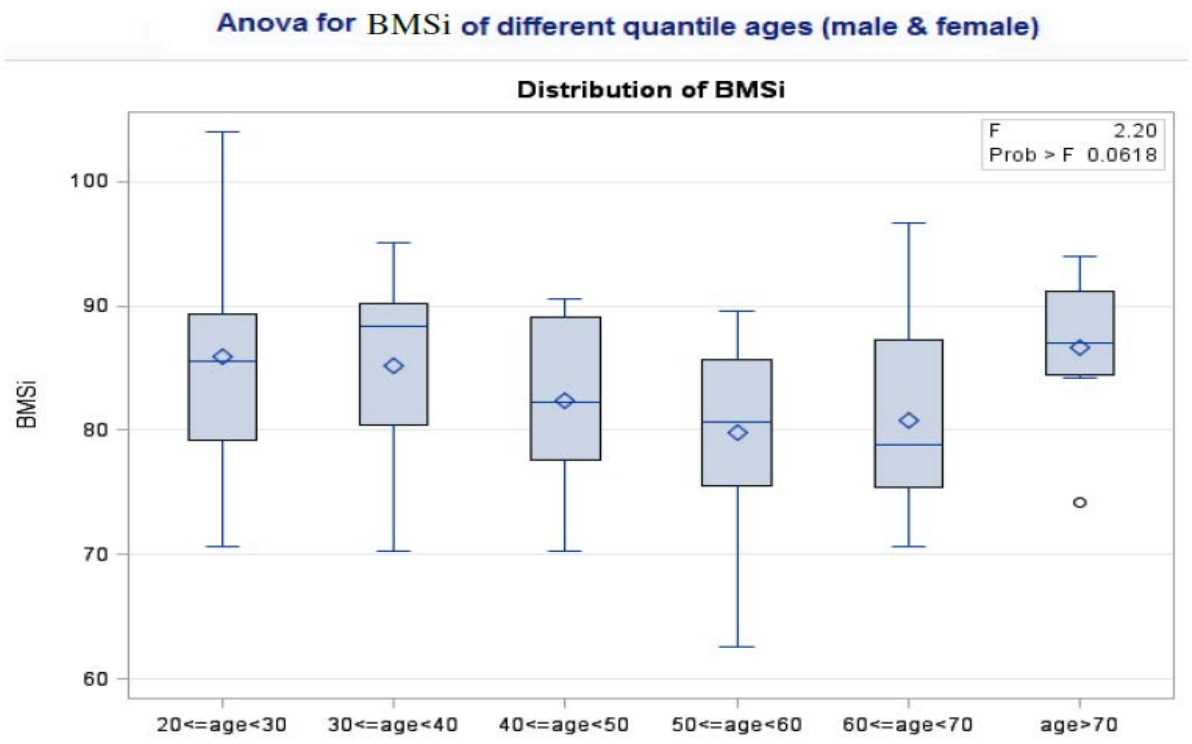


Figure 53

BMSi in different interval ages of both genders

The following diagram shows that based on the given sample (19 observations without prevalent fractures) for different interval ages, there is no significant difference between means of BMSi for each interval (p-value=0.2755) for male gender.

In the diagram is displayed the distribution of age in males group

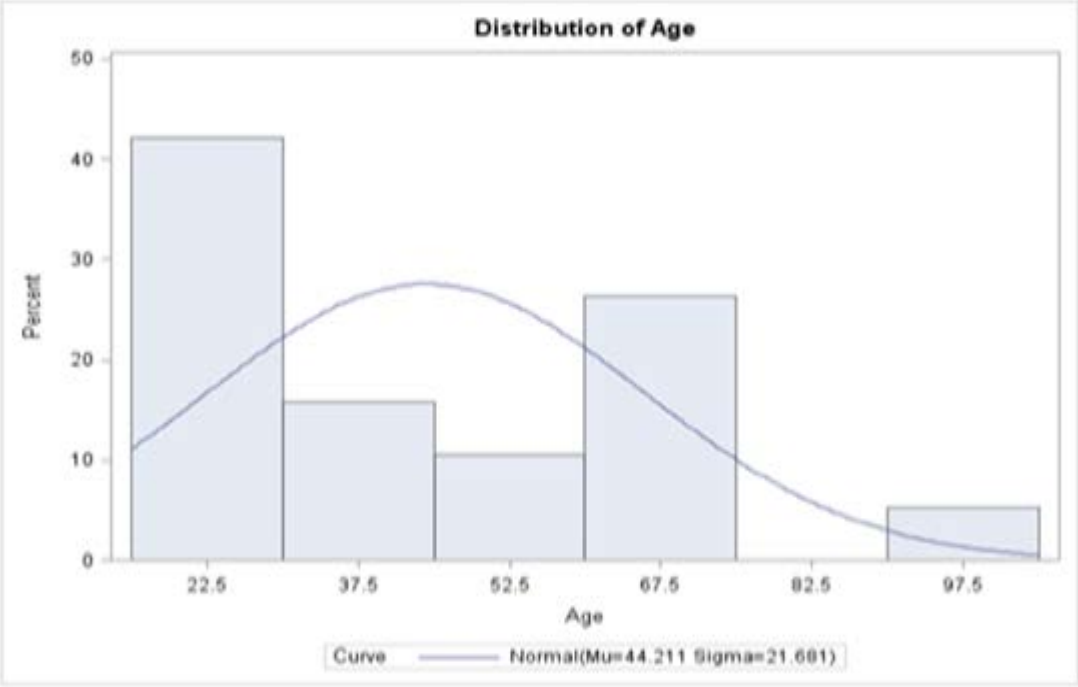


Figure 54

The following diagram shows that based on the given sample (69 observations without prevalent fractures) for different interval ages, there are also, like in men, no significant difference between means of BMSi for each interval (p-value=0.4319) for females.

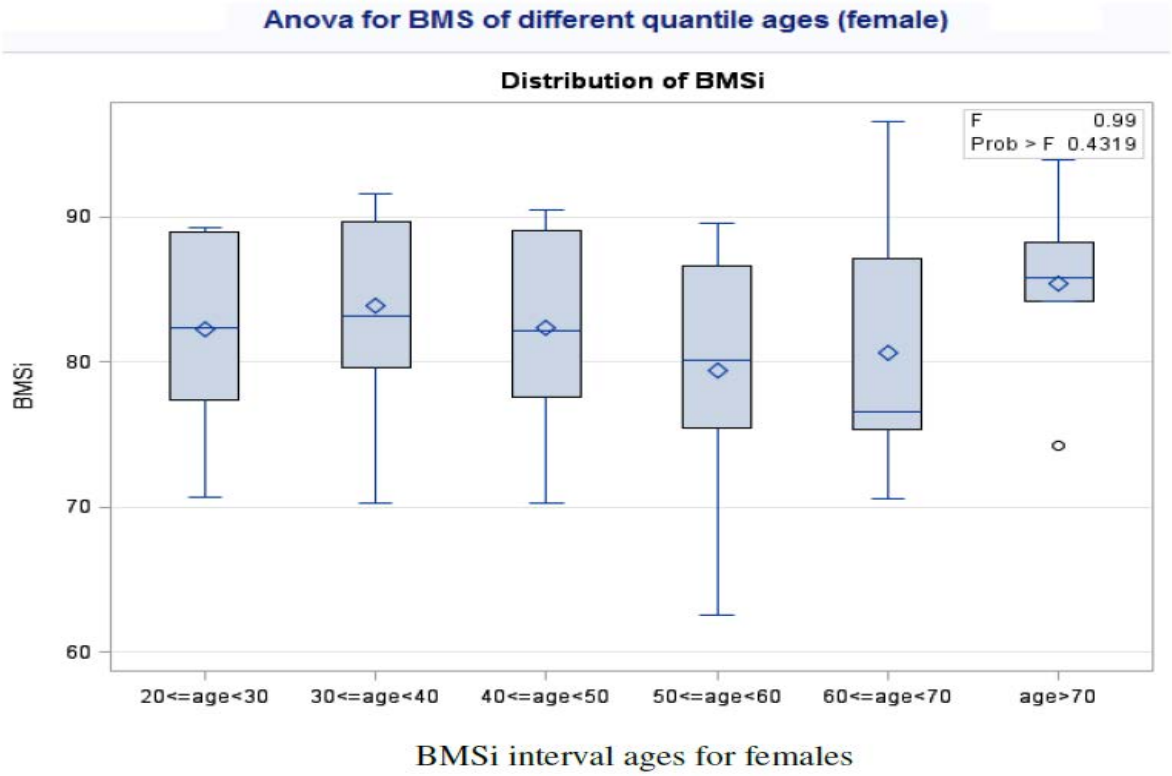


Figure 55

The following diagram shows the distribution of age in females group:

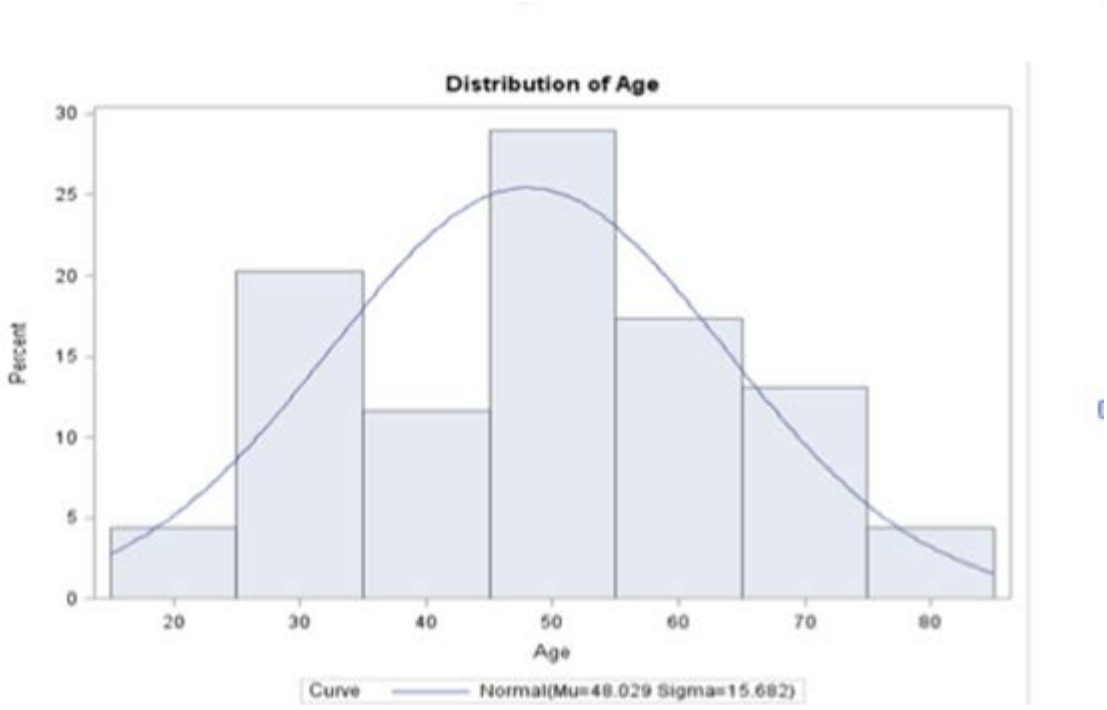


Figure 56

ANOVA for comparing means of BMSi in different BMI intervals

The following table represents the results of ANOVA for testing means of BMSi in different BMI categories in the given sample .This test represents that for a given data set there is no significantly difference between means of different BMI categories (p-value=0.3879).

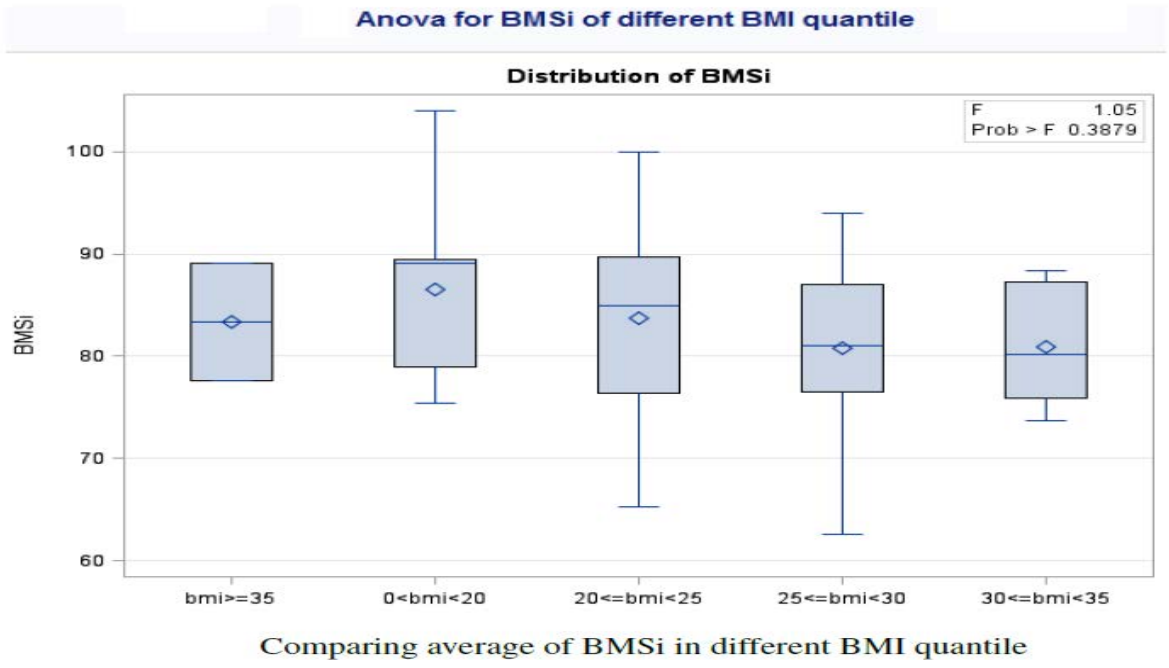


Figure 57

And the histogram of distribution of BMI in both females and males is as follows:

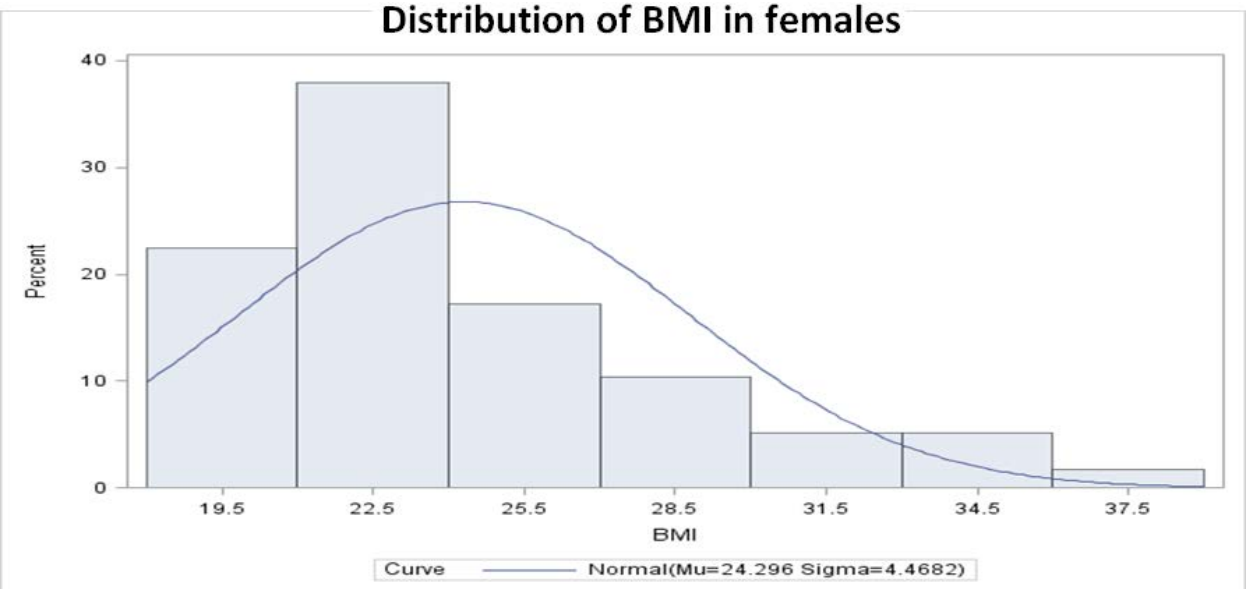


Figure 58

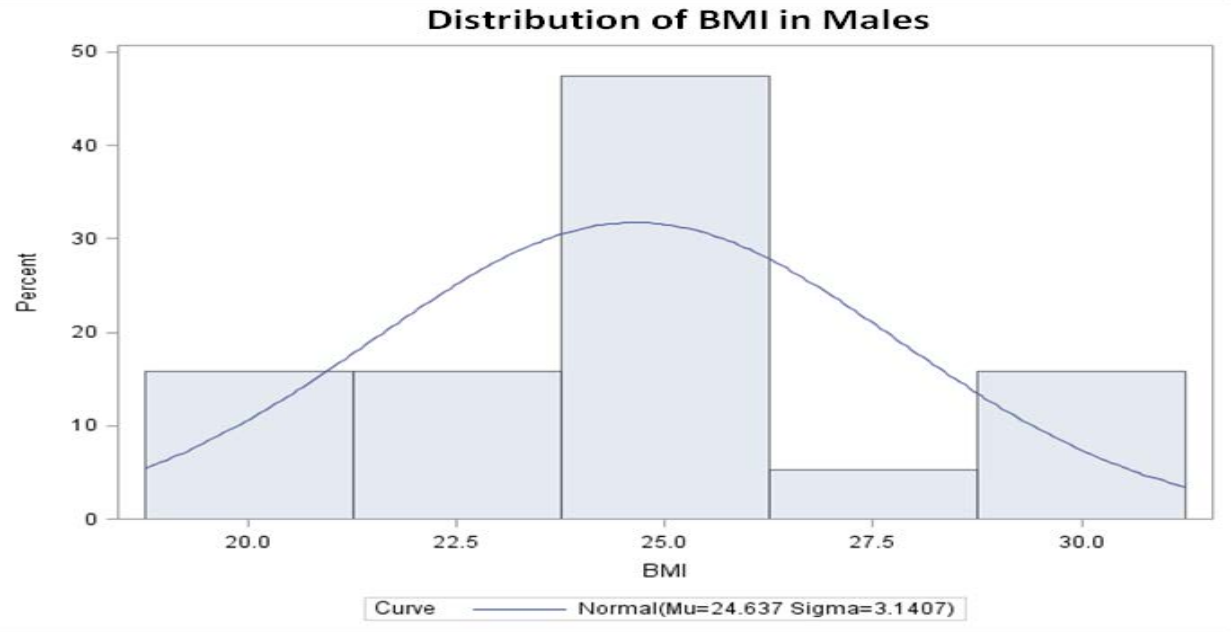


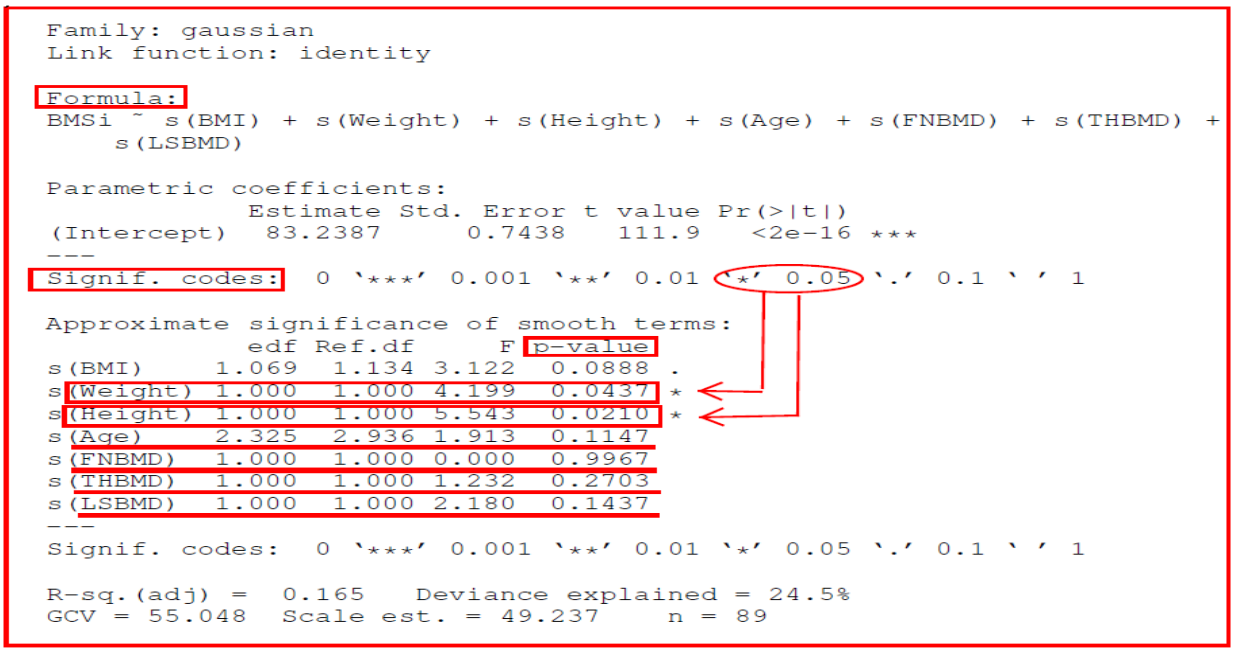
Figure 59

Association between variables and BMSi

In this section in order to explore existing associations between covariates and BMSi, a nonparametric regression approach [288] based on Regression Splines is proposed. In other words the model has the following form adjusting with variables irrespective to genders:

BMSii = f(BMIi) + f(Weighti) + f(Heighti)+f(Agei) + f(FN - BMDi) + f(TH - BMDi) + f(LS - BMDi) + εi, :

Where function f() denotes nonparametric terms. The rationale behind this approach is that, nonparametric regression can grasp any kind of association between covariates (features) and the target variable, in other words it does not assume any functional form like linear or logarithmic as a prior knowledge. The result of nonparametric regression is as follow (R output):



```
Family: gaussian
Link function: identity

Formula:
BMSi ~ s(BMI) + s(Weight) + s(Height) + s(Age) + s(FNBMD) + s(THBMD) +
    s(LSBMD)

Parametric coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  83.2387     0.7438   111.9   <2e-16 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Approximate significance of smooth terms:
            edf Ref.df     F p-value
s(BMI)    1.069  1.134 3.122  0.0888 .
s(Weight) 1.000  1.000 4.199  0.0437 *
s(Height) 1.000  1.000 5.543  0.0210 *
s(Age)    2.325  2.936 1.913  0.1147
s(FNBMD)  1.000  1.000 0.000  0.9967
s(THBMD)  1.000  1.000 1.232  0.2703
s(LSBMD)  1.000  1.000 2.180  0.1437
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

R-sq.(adj) =  0.165   Deviance explained = 24.5%
GCV = 55.048  Scale est. = 49.237     n = 89
```

Figure 60

edf = expected degree of freedom Ref.df= residual degree of freedom F=F test Value

As these results show, only "Weight" and "Height" has some kind of association with "BMSi" and the rest of covariates does not have any Associations irrespective to Genders

After adjusting for gender & other variables, the model has the following formula:

BMSi ~ Gender + s(BMI) + s(Weight) + s(Height) + s(Age) + s(FNBMD)
+s(THBMD) + s(LSBMD) :

Where function f() denotes nonparametric terms.

The rationale behind this approach is that, nonparametric regression can grasp any kind of association between covariates (features) and the target variable, in other words it does not assume any functional form like linear or logarithmic as a prior knowledge. The result of nonparametric regression is as follow(R output):

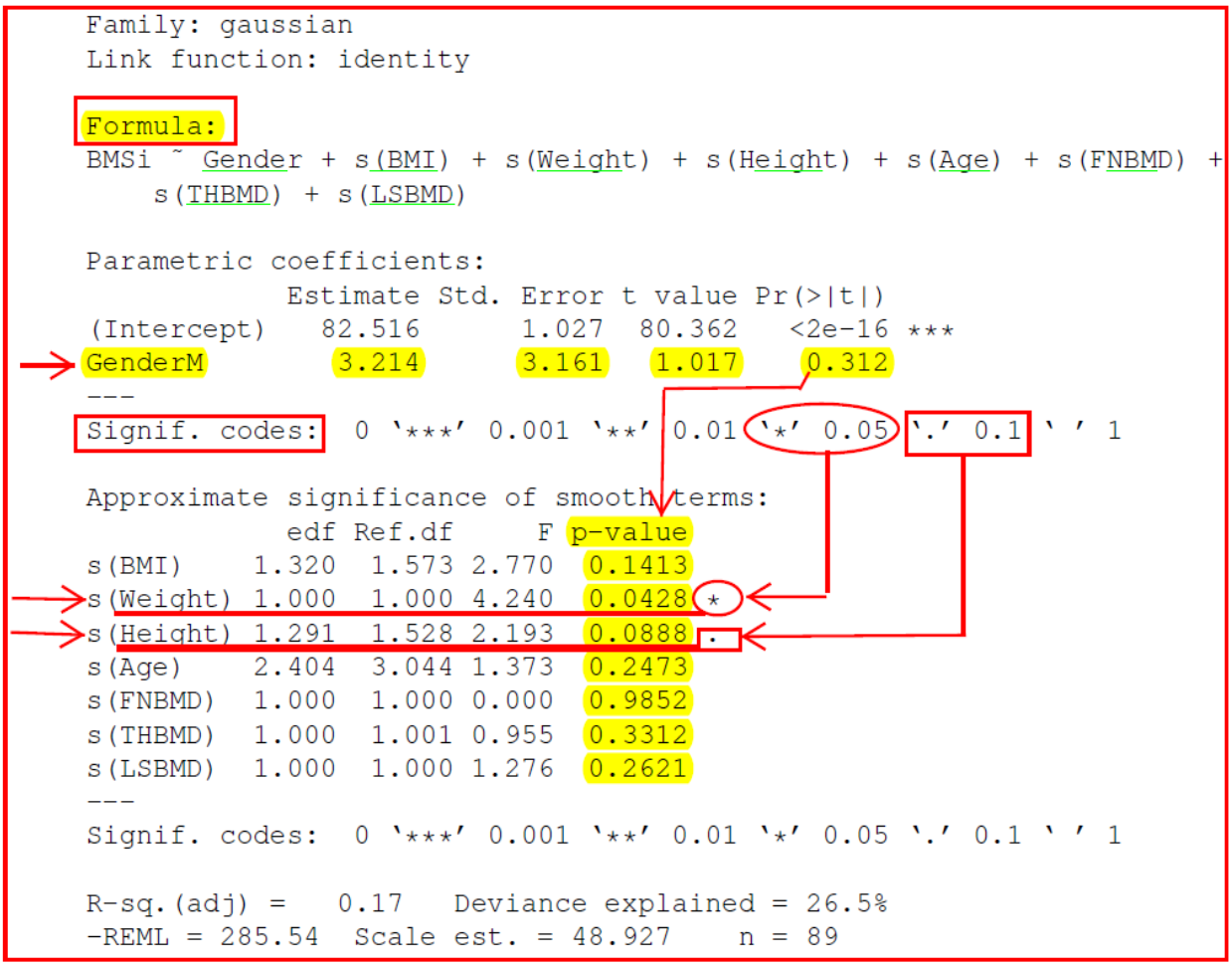


Figure 61

edf = expected degree of freedom Ref.df= residual degree of freedom F=F test Value

There are associations between BMSi & Height and Weight irrespective of Genders due to fact Dummy variable i.e. Gender is not statistically significant.

After adjusting for gender & other variable excluding BMI, the model has the following form:

BMSi ~ Gender + s(Weight) + s(Height) + s(Age) + s(FNBMD) + s(THBMD)

+ s(LSBMD)

And after the exclusion of BMI

Computing the association between BMSi and other variables (excluding BMI):

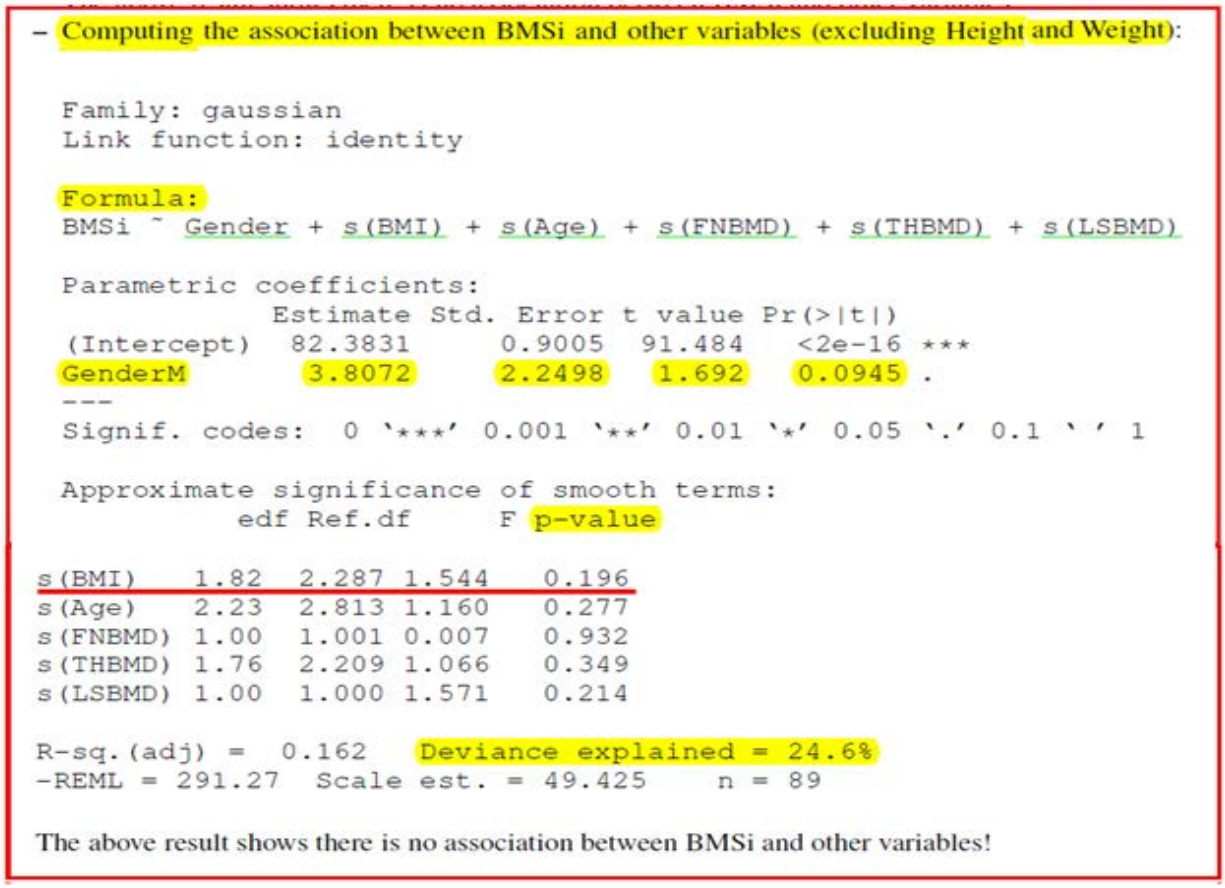
```
Family: gaussian
Link function: identity

Formula:
BMSi ~ Gender + s(Weight) + s(Height) + s(Age) + s(FNBMD) + s(THBMD) +
    s(LSBMD)

Parametric coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)   82.521      1.035  79.719   <2e-16 ***
GenderM        3.193      3.165   1.009    0.316
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Approximate significance of smooth terms:
            edf Ref.df     F p-value
s(Weight) 1.000  1.000 2.478   0.119
s(Height) 1.001  1.003 1.104   0.296
s(Age)    2.195  2.787 0.974   0.339
s(FNBMD)  1.000  1.000 0.088   0.767
s(THBMD)  1.670  2.087 0.859   0.422
s(LSBMD)  1.000  1.000 1.541   0.218

R-sq.(adj) =  0.146   Deviance explained = 23.2%
-REML = 289.47  Scale est. = 50.341     n = 89
```

The above result shows there is no association between BMSi and other variables!

Figure 62

After adjusting for genders & other variable excluding Height & Weight, the model has the following form:

BMSi ~ Gender + s(BMI) + s(Age) + s(FNBMD) + s(THBMD) + s(LSBMD)



– Computing the association between BMSi and other variables (excluding Height and Weight):

```
Family: gaussian
Link function: identity

Formula:
BMSi ~ Gender + s(BMI) + s(Age) + s(FNBMD) + s(THBMD) + s(LSBMD)

Parametric coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept)  82.3831     0.9005  91.484   <2e-16 ***
GenderM       3.8072     2.2498   1.692   0.0945 .
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Approximate significance of smooth terms:
           edf Ref.df     F p-value
s(BMI)    1.82  2.287 1.544   0.196
s(Age)    2.23  2.813 1.160   0.277
s(FNBMD)  1.00  1.001 0.007   0.932
s(THBMD)  1.76  2.209 1.066   0.349
s(LSBMD)  1.00  1.000 1.571   0.214

R-sq.(adj) =  0.162   Deviance explained = 24.6%
-REML = 291.27  Scale est. = 49.425     n = 89
```

The above result shows there is no association between BMSi and other variables!

Figure 63

The conclusion of the three nonparametric regressions is that, all three variables BMI, Height and Weight must be included in the experiments since as the above results show the deviance explained for the model which have all mentioned variables is 26:5% which is more than the corresponding deviance explained of the rest models. It is true that BMI is made of Height and Weight but in statistical reasoning, increasing dimension of the problem (increasing number of features) by creating new features based on available features improves the predictive ability of the estimated model.

The following plots show the effects of each feature on "BMSi". As the plots relating to "Weight" and "Height" show the association with "BMSi" are negative and positive respectively

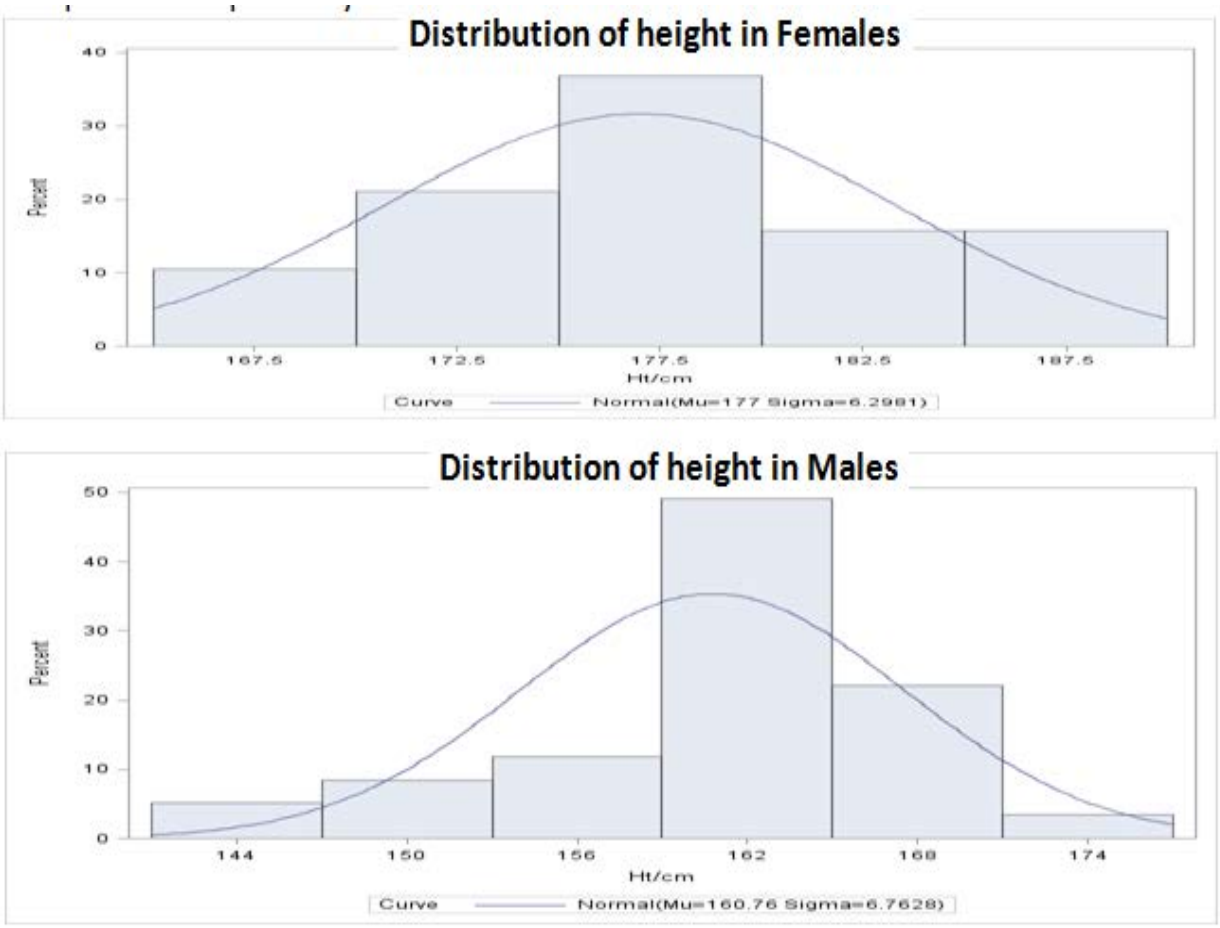


Figure 64

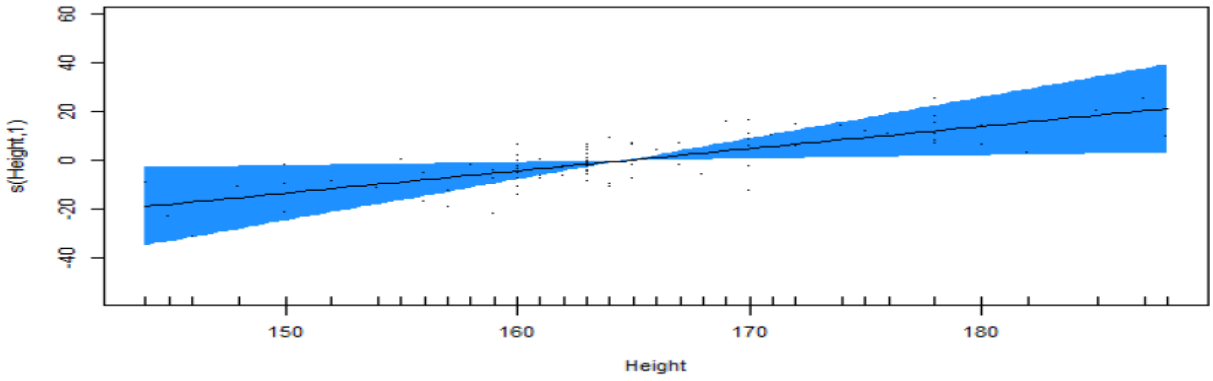


Figure 65

As the plot shows the association of the height and BMSi is positive.

It does mean that the taller is a person the more value of BMSi is expected irrespective of gender (p-Value=0.0210).

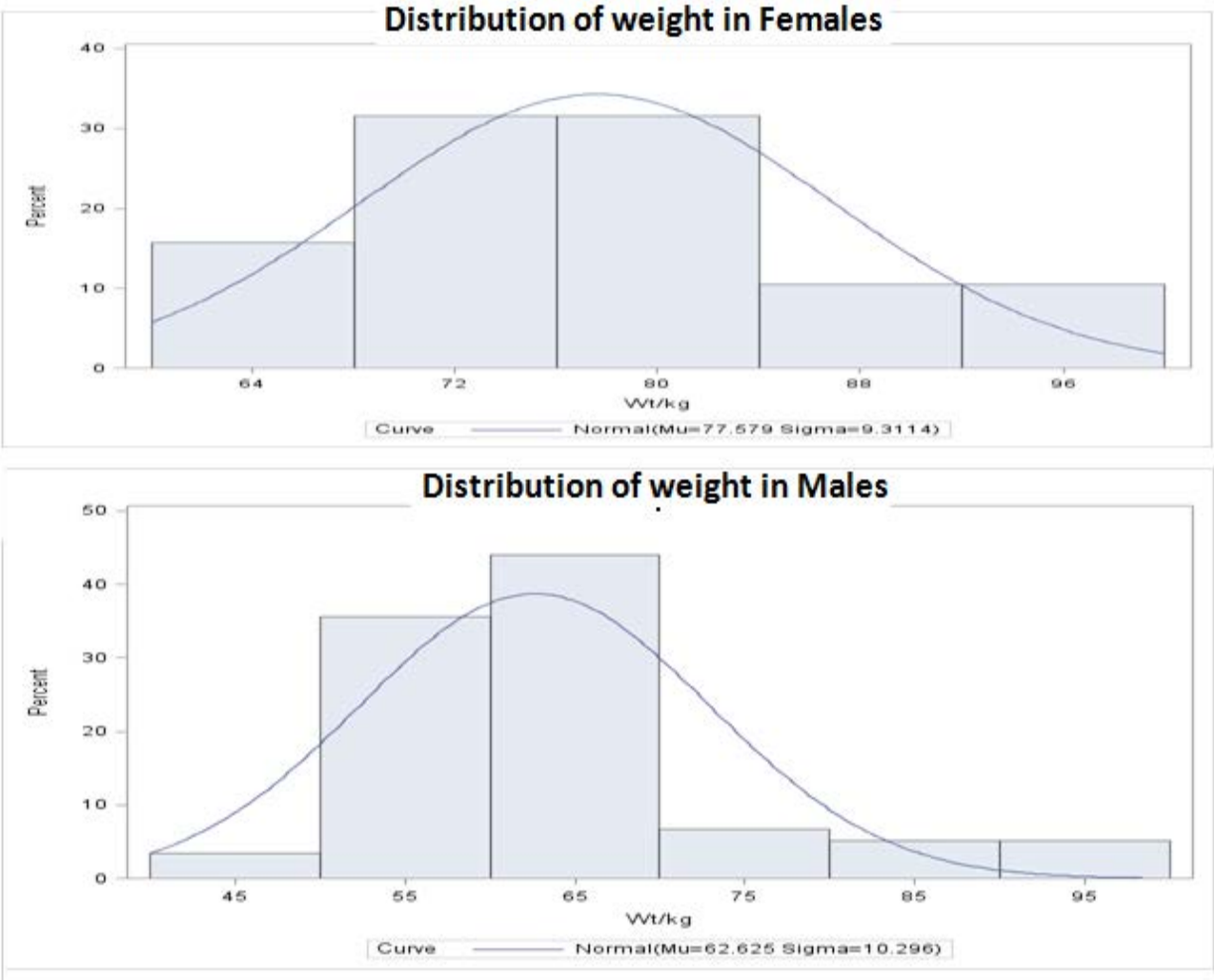


Figure 66

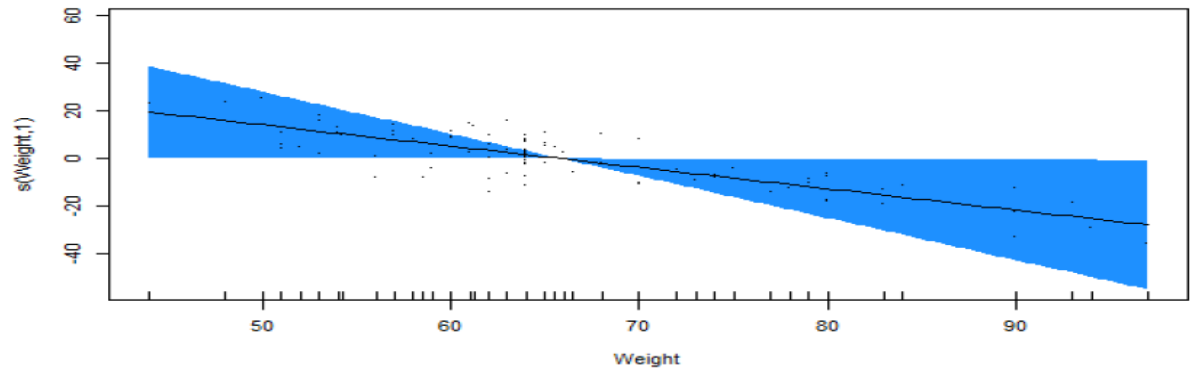


Figure 67

As the plot shows the association of the weight and BMSi is negative, it does mean that the heavier is a person the less value of BMSi is expected irrespective of gender (p-Value=0.0437).

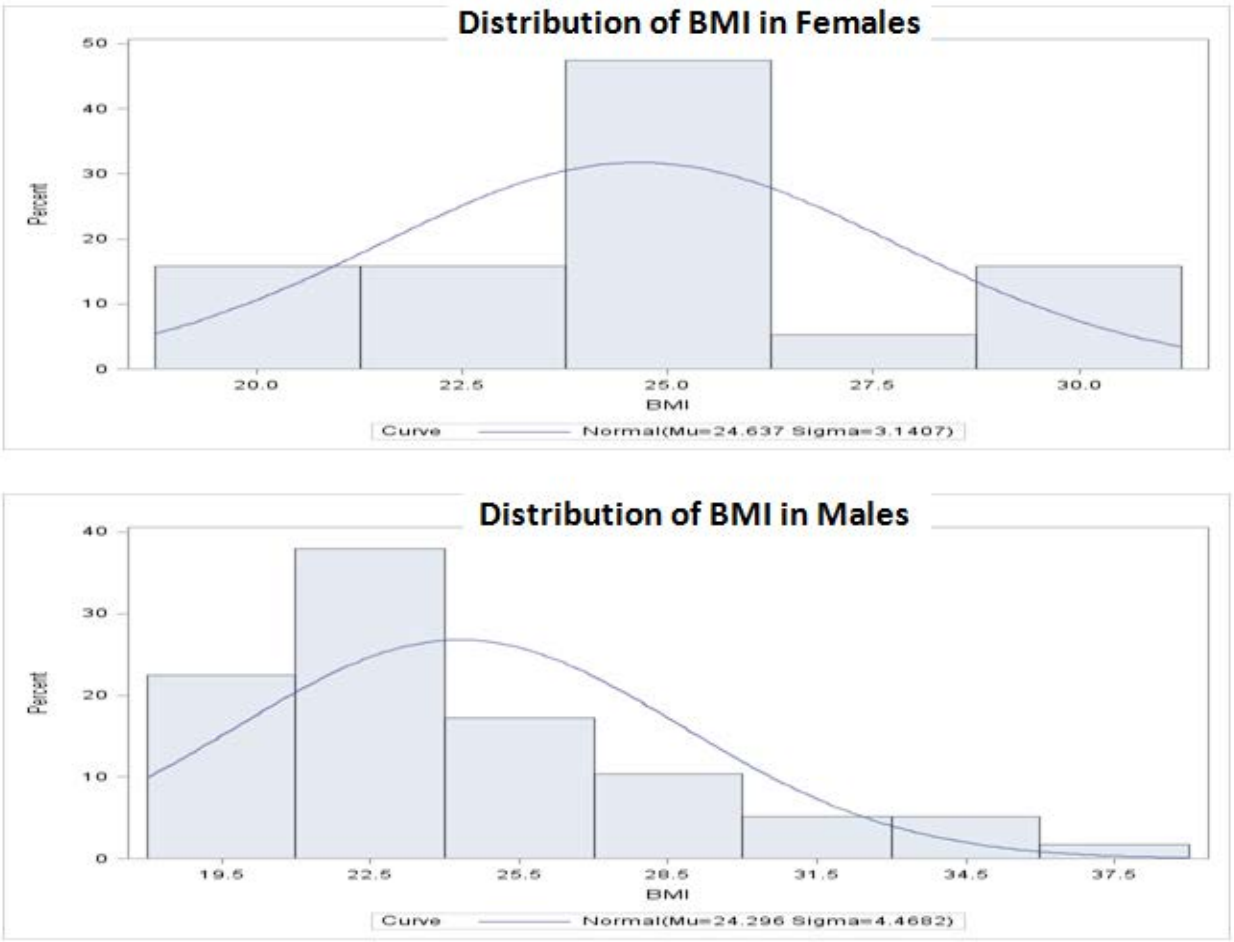


Figure 68

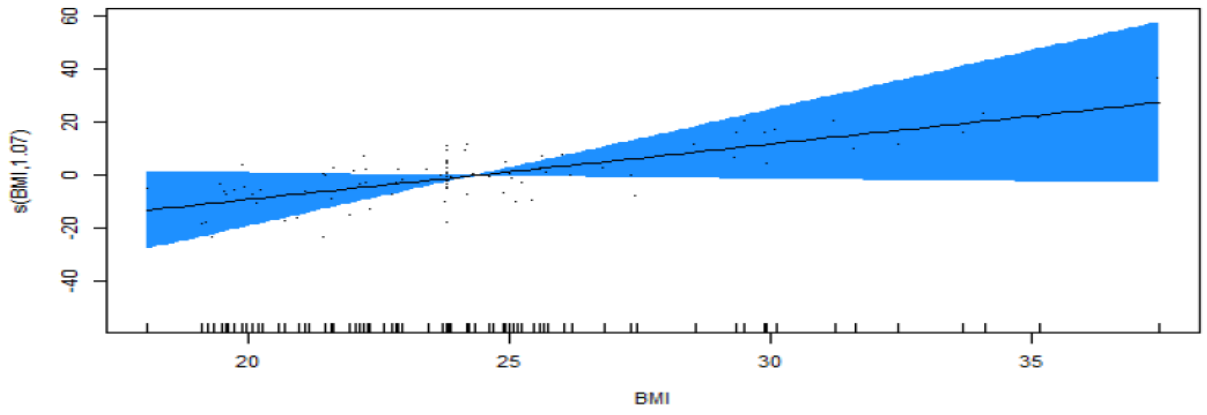


Figure 69

As the confidence bands (blue region in the picture) show there is no association between BMI and BMSi due to zero is included in confidence band over the range of BMI irrespective to genders(p-Value=0.888) and after adjusting to genders(p-Value=0.1413).

Figure 70

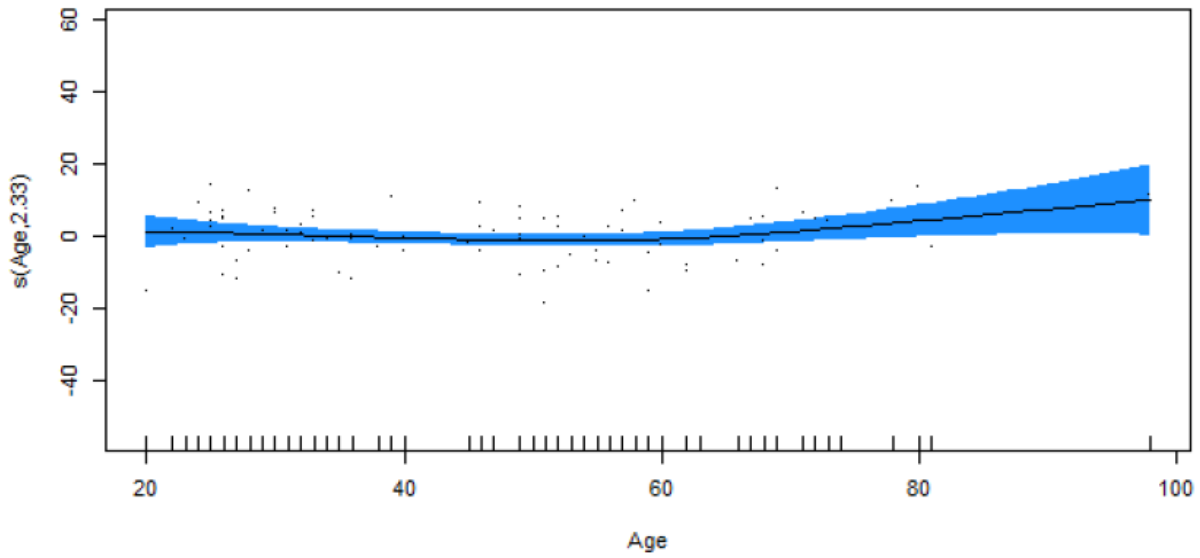


Figure 71

As the confidence bands (blue region in the picture) show there is no association between Age and BMSi due to zero is included in confidence band over the range of Age, irrespective to gender (p-Value=0.1147) and after adjusting to gender (p-Value=0.2473).

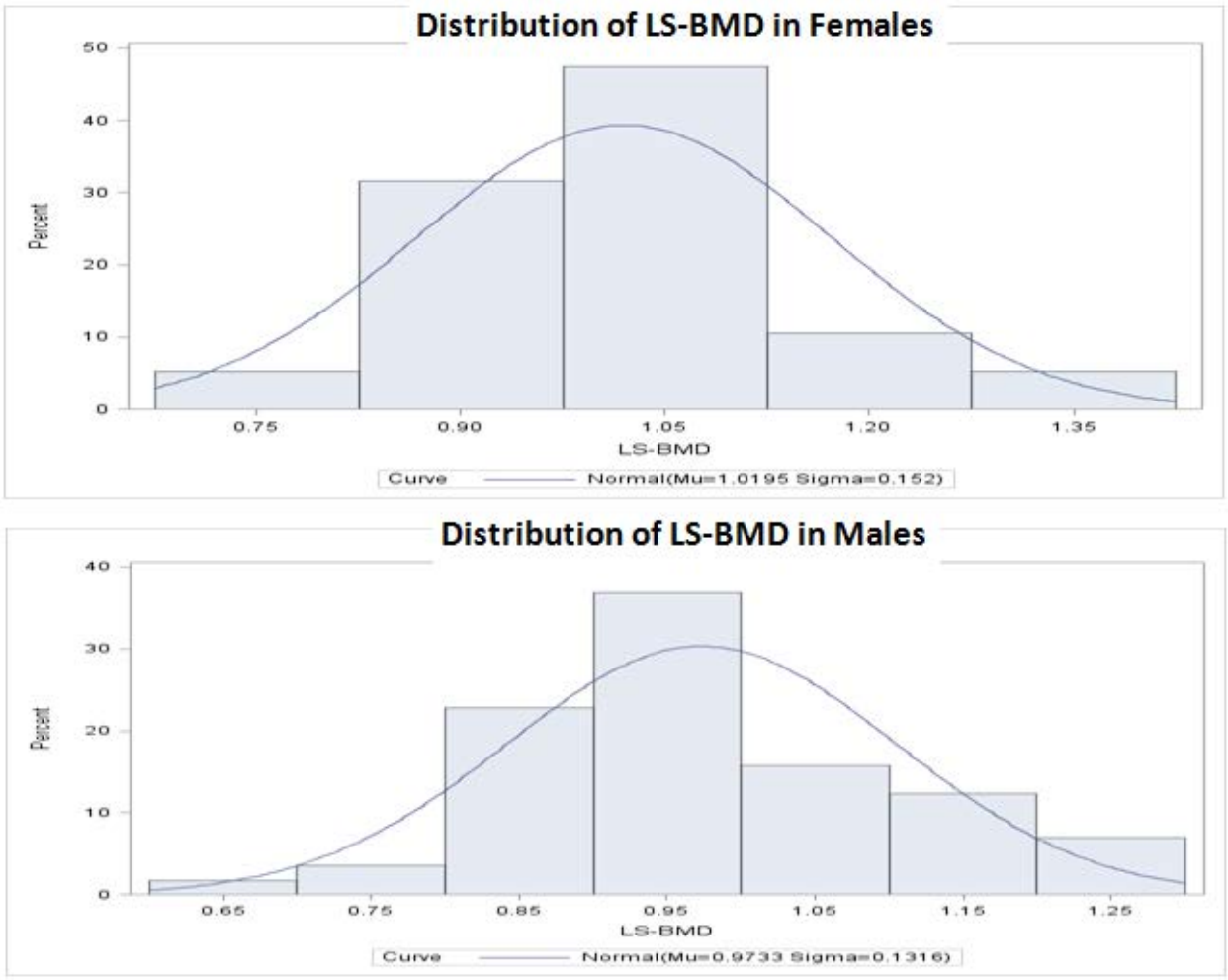


Figure 72

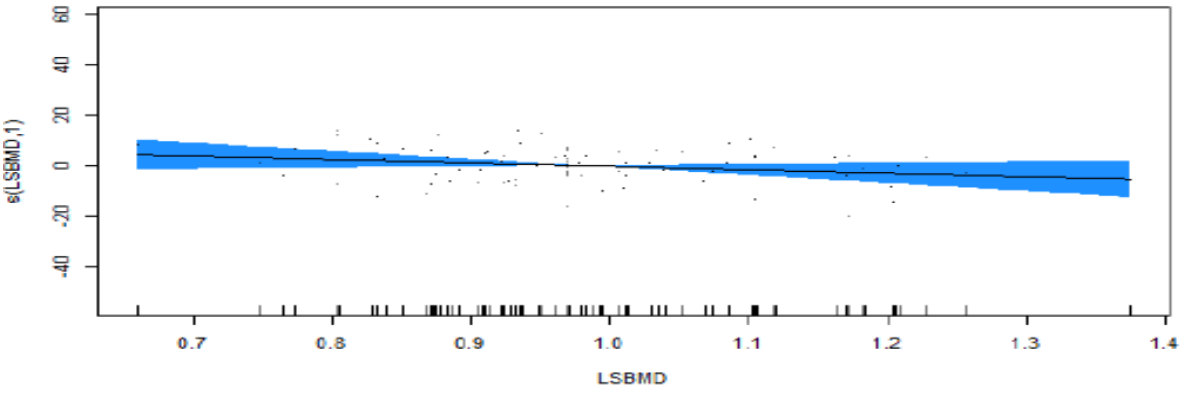


Figure 73

As the confidence bands (blue region in the picture) show there is no association between LS-BMD and BMSi due to zero is included in confidence band over the range of LS-BMD, irrespective to genders(p-Value=0.1437) and after adjusting to genders (p-Value=0.2621).

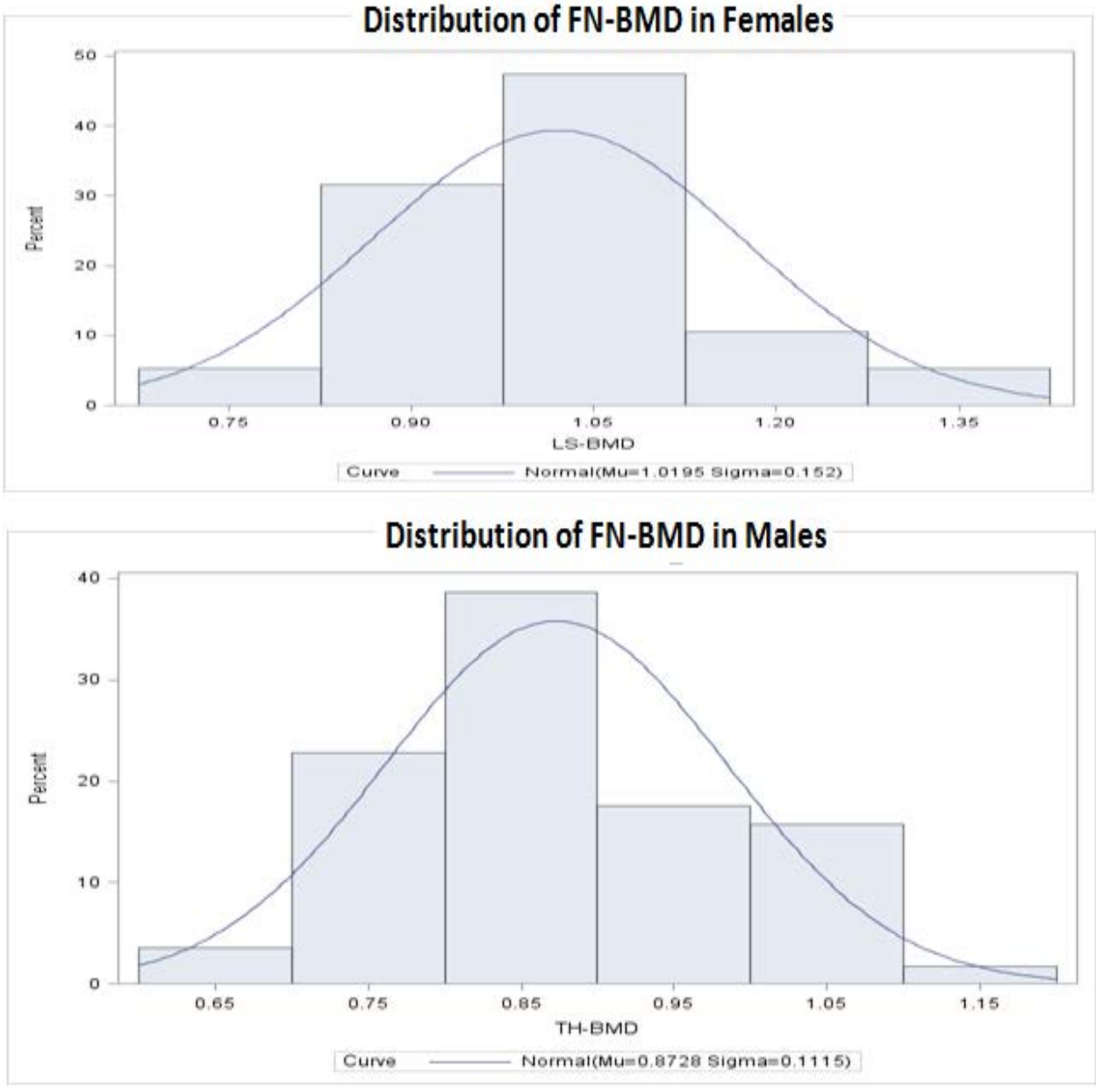


Figure 74

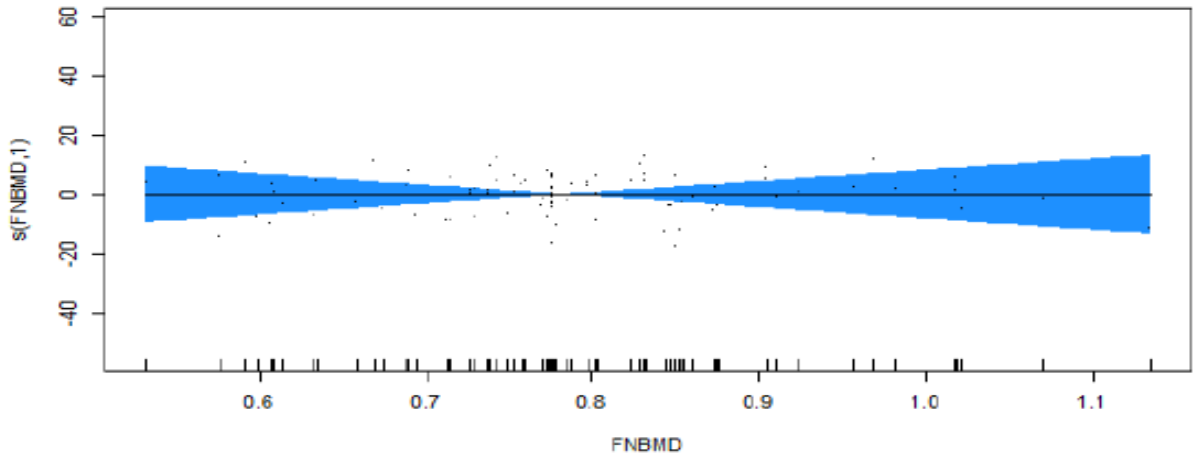
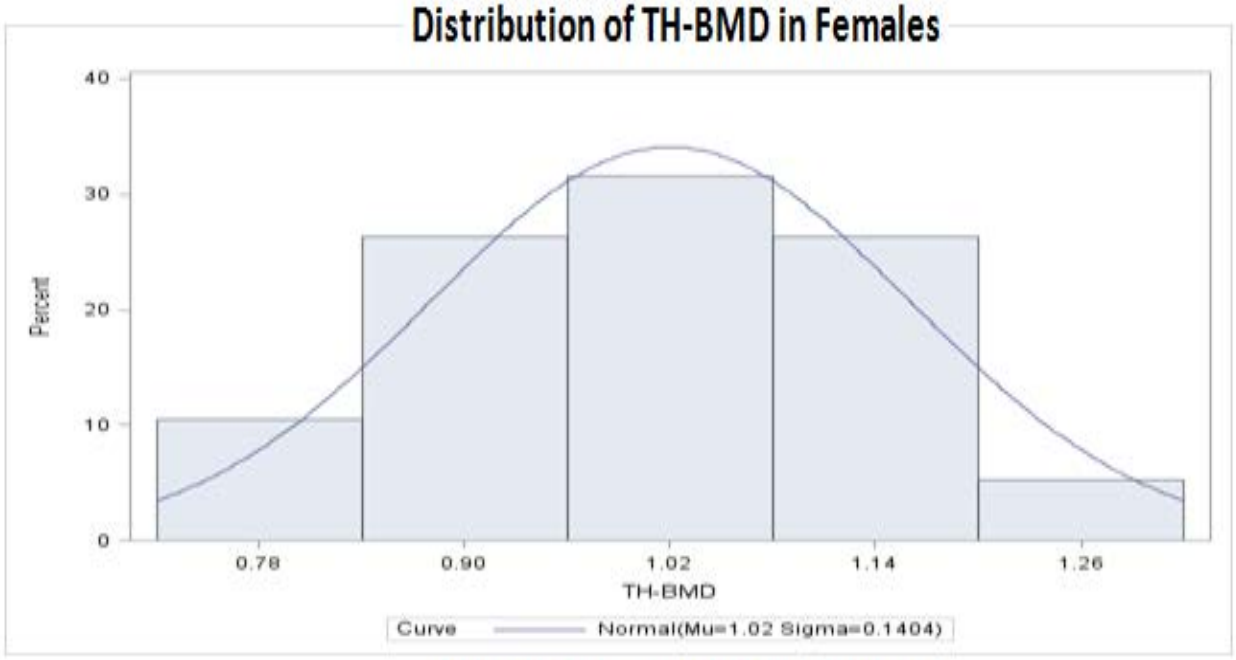


Figure 75

As the confidence bands (blue region in the picture) show there is no association between FN-BMD and BMSi due to zero is included in confidence band over the range of FN-BMD, irrespective to gender (p-Value=0.9967) and after adjusting to gender (p-Value=0.9852).


Distribution of TH-BMD in Females
Percent
40
30
20
10
0
0.78
0.90
1.02
1.14
1.26
TH-BMD
Curve
Normal(Mu=1.02 Sigma=0.1404)

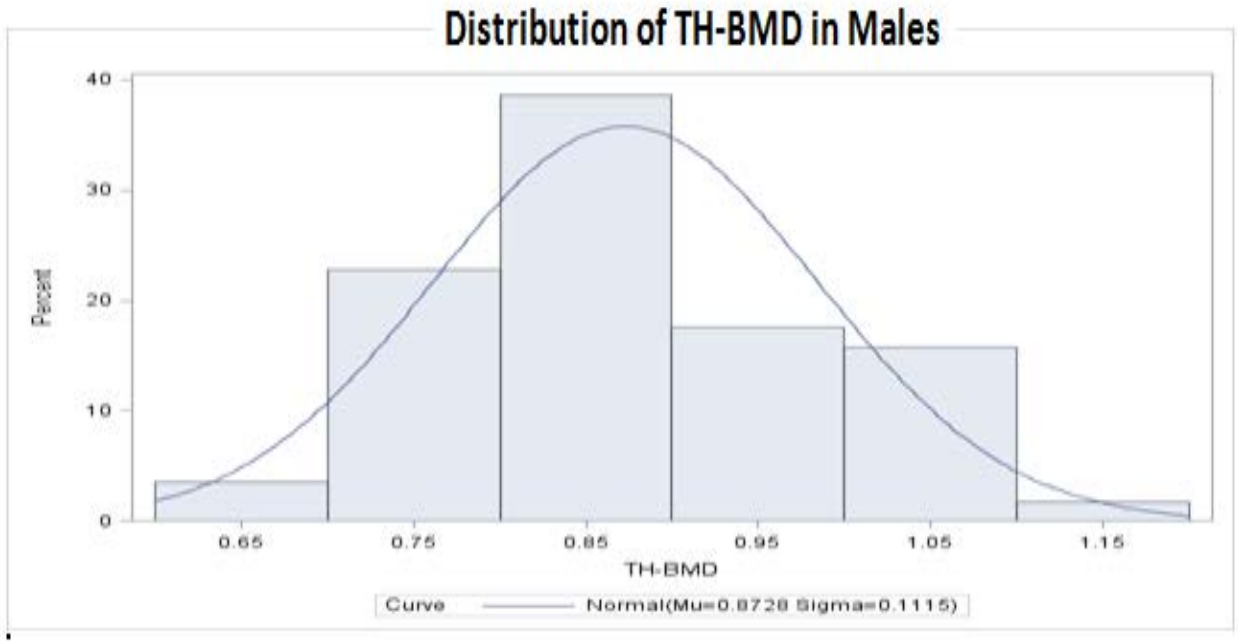

Distribution of TH-BMD in Males
Percent
40
30
20
10
0
0.65
0.75
0.85
0.95
1.05
1.15
TH-BMD
Curve
Normal(Mu=0.8728 Sigma=0.1115)

Figure 76

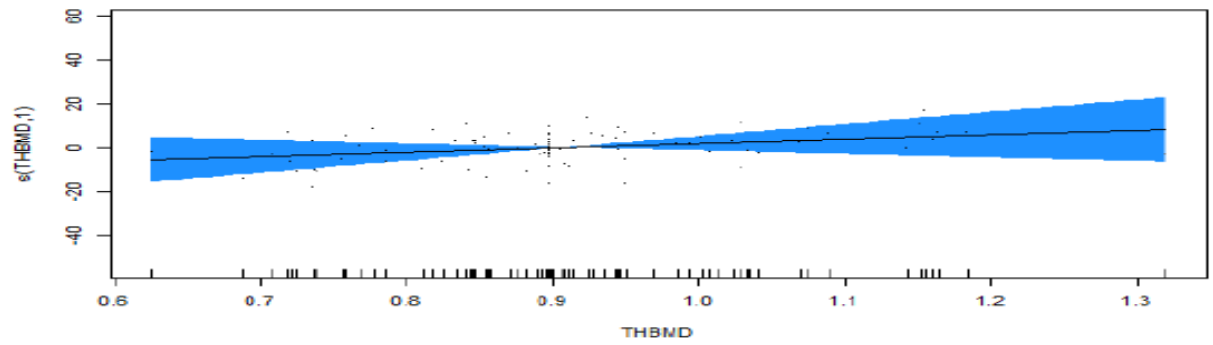


Figure 77

As the confidence bands (blue region in the picture) show there is no association between TH-BMD and BMSi due to zero is included in confidence band over the range of TH-BMD, irrespective to gender (p-Value=0.2703) and after adjusting to gender (p-Value=0.3312).

Note:

One question may arises why we used BMI as well as "Height and Weight" in the formula for calculation of association between BMSi and variables?

As we know BMI derived from Height and Weight from this formula BMI=Wt (Kg):Ht² (m), in some cases with overweight and obesity i.e. BMI >25 or >30 respectively , they are osteopenic or osteoporotic by DXA-BMD due to Sarcopenia. We assumed that perhaps Wt and Ht may have directly association with BMSi. For this reason we performed this calculation despite these assumption were not included in our hypothesis.

Finally our results and findings pertaining to BMSi are seen in the Table 7and Comparisons of our findings with other published papers are summarized in Table 8:

Table 7

Table 7. Summary of BMSi results					
Results					
BMSi	**Irrespective of Genders (M&F=89)**	**Males (M=21, 24%)**	**Females (F=68, 76%)**		**Comments**
BMSi in different sex (gender)	83.010±7.877	87.8089±7.9994	81.6952±6.7557		Means of BMSi is Significantly Different Between Male and Female. The pooled t-test (P-value =0.0020).
BMSi in interval Age	ANOVA for BMSi with Different Interval Ages for The Control healthy Group revealed: There is no significant difference between means of BMSi for each interval (p-value=0.0618).				
BMSi in interval BMI	ANOVA for testing means of BMSi in different BMI categories of the samples reveals there is no significantly difference between means of different BMI categories (p-value=0.3879).				
CV*	**<5%**				
Association between **BMSi as "Reference Normative Values** in normal population" and other Co variables					
	In both genders (89)	Male(21) 24%	Female(68) 76%	Association	Comments
Genders(M/F)	**83.010±7.877**	**87.8089±7.9994**	**81.6952±6.7557**	**Significantly Difference between means of BMSi of Male and Female**	**the pooled t-test (P-value =0.0020)**

Age		**47.20±17.08**	**44.21±21.68**	**48.02±15.68**	**NSSC****	**P =0.2473**
BMI		**24.37±4.16**	**24.63±3.14**	**24.29±4.46**	**NSSC****	**P>0.05**
Height		**164.71±9.64**	**177±6.29**	**160.76 ±6.76**	**Positive association**	**nonparametric regression@ (P-value <0.1)**
Weight		**66.26±11.91**	**77.57±9.31**	**62.62 ±10.29**	**Negative association**	**nonparametric regression@ (P-value <0.05)**
LS-BMD		**0.98±0.13**	**1.019±0.151**	**0.97 ±0.13**	**NSSC****	**P>0.05**
FN-BMD		**0.78±0.12**	**0.87±0.12**	**0.75±0.10**	**NSSC****	**P>0.05**
TH-BMD		**0.90±0.13**	**1.02±0.14**	**0.87 ±0.11**	**NSSC****	**P>0.05**

**NSSC= No Statistically Significant Correlations, *CV= Inter Observer Coefficient Variance

@Non-parametric methods : Statistical techniques of estimation and inference that are based on a function of the sample observations, the probability distribution of which does not depend on a complete specification of the probability distribution of the population from which the sample was drawn. Consequently, the techniques are valid under relatively general assumptions about the underlying population. Often, such methods involve only the ranks of the observations rather than the observations themselves. Examples are Wilcoxon's signed rank test and Friedman's two-way analysis of variance. In many cases, these tests are only marginally less powerful than their analogues, which assume a particular population distribution (usually a normal distribution) even when that assumption is true, Also known as Distribution-free methods.

Table 8

Table 8. Comparison of our results with other studies from different groups										
Authors	Year and Place	Mean of BMSi	Number of Samples	Type of statistical system	Type of analysis	Variables association with BMSi in normal controls	BMSi & Age association in normal controls	BMSi & Gender association in normal controls	BMSi & BMD association in normal controls	BMSi & BMI association in normal controls
Our Project/ ADP	2018 Spain Barcelona	83.010±7.877	89	SAS ,R	ANOVA, student t test, non-parametric regression	Performed & Determined	No Association	BMSi dependent on gender type	No Association	No Association
Tamara D Rozenta et al. (124)	2017 USA Boston	77.4±8.8	93	SAS	ANOVA	Not Performed & Not Determined	Not Performed & Not Determined	Not Performed & Not Determined	Not Performed & Not Determined	Not Performed & Not Determined
S.Herrera et al (123)	2017 Spain Barcelona	81.8	29	Graph Pad Prism	Student's *t*-test /Mann–Whitney test	Not Performed & Not Determined	Not Performed & Not Determined	Not Performed & Not Determined	Not Performed & Not Determined	Not Performed & Not Determined
Frank Malgo et al, (115)	2017 The Netherlands Leiden	82.1±1.3	2	Graph Pad Prism	Student's *t*-test /Mann–Whitney test	Not Performed & Not Determined	Not Performed & Not Determined	Not Performed & Not Determined	Not Performed & Not Determined	Not Performed & Not Determined
Pérez-Sáez MJ	2017 Spain	82.9±7.8	94	Not specifi	Logistic regression	Not	Not	Not	Not	Not

et al (120)	Barcelona			ed		Performed & Not Determined	Performed & Not Determined	Performed & Not Determined	Performed & Not Determined	Performed & Not Determined
Daysi Duarte Sosa et al. (110)	2017 Norway Oslo	76.4±6,2	66	SPSS for Mac	Student's *t*-test /Mann–Whitney test	Not Performed & Not Determined	Not Performed & Not Determined	Not Performed & Not Determined	Not Performed & Not Determined	Not Performed & Not Determined
S.Herrera et al (117)	2017 Spain Barcelona	81.76±1.44	29	SPSS for Windows	ANOVA	Not Performed & Not Determined	Not Performed & Not Determined	Not Performed & Not Determined	Not Performed & Not Determined	Not Performed & Not Determined
Frank Malgo et al, (116)	2017 The Netherlands Leiden	83.2±0.7	44	SPSS for Windows	2-sample *t* tests or chi-square tests.Pearson/ Spearman correlation coefficients	Not Performed & Not Determined	Not Performed & Not Determined	Not Performed & Not Determined	Not Performed & Not Determined	Not Performed & Not Determined
Frank Malgo et al, (122)	2017 The Netherlands Leiden	76.6 ± 4.9	31	SPSS for Windows	Student's t test, ANOVA, and Chi square test or a Mann-Whitney U test or	Only in patients group	BMSi was inversely and significantly related with age (r = −0.485,	There was no difference in BMSi values between women and men	There was no correlation between BMSi with BMD in	No Association

					Kruskall-Wallis test		p < 0.001) in patients group	(79.7 ± 0.6 vs. 80.00.8; p = 0.789). in patient group	patients group.	
Roberto Guerri-Fernandez et al. (129)	2016 Spain Barcelona	90 (88.5-93)	35	Stata/IC 13.1.	2-sample t tests and x2 test	Not Performed & Not Determined	Not Performed & Not Determined	Not Performed & Not Determined	Not Performed & Not Determined	Not Performed & Not Determined
Jessica R. Furst et al. (114)	2017 USA New York	70.12 _ 1.9	19	Not specified	two-sample *t* tests and x^2 tests	Not Performed & Not Determined	Not Performed & Not Determined	Not Performed & Not Determined	Not Performed & Not Determined	Not Performed & Not Determined
Daniel Sundh,et al. (128)	2018 Sweden Gothenburg	76.6±5.5	20	SPSS version 23	Paired sample T-test	Not Performed & Not Determined	Not Performed & Not Determined	Not Performed & Not Determined	Not Performed & Not Determined	Not Performed & Not Determined

Discussion

Our aims of this study were 1) to determine BMSi as Reference Normative Values in normal population and 2) determination of relationships of Age, Gender, BMI, and BMD with BMSi in normal population and 3) to determine interobserver Covariance of Microindentation in hospital del Mar, Barcelona-Spain.

1-According to our hypothesis we determined the "Reference Normative Values in normal population with Mean=83.01 ±7.87 (±SD) regardless of gender Figure 40 so that in male group mean of BMSi value is equal to 87.80 ±7.99 Figure 43 and in female group mean of BMSi value is equal to 81.69 ±6.75 Figure 43 respectively." With according to T-Test for Means of BMSi between males and females in the healthy control group Figure 49, the result of this T-Test represents that there is a significantly difference between means of BMSi for each group (p-value =0.002) Figure 49. The importance of BMSi Reference Normative Values in normal population can be explained by this fact that for routine application of Microindentation in daily practice of medicine a "Reference Normative Values in normal population" is mandatory in order to clinical decision making in regard of patient management. As we know approximately half of cases of osteoporotic fractures are not detected by DXA-BMD Imaging method that is currently gold standard for diagnosis of osteoporosis and high-risk patients in clinical practice. Therefore the clinical need to have a complementary tool i.e. Microindentation with its "Reference Normative

Values in normal population" for disclosing these large percentages of high-risk patients at the bedside or on the ambulatory basis seems to be mandatory and a logical effort. For comparison our study findings of BMSi in normal healthy populations with other studies that have been performed so far, the systematic literature search was performed and systemic reviews for bone Microindentation was done as shown in Table 5 . In virtually all studies performed so far, BMSi measurement assigned to the bone material properties or strength in disease conditions like Diabetes and compared with non-disease condition and also in comparison with BMD on occasions. Experimental studies ex vivo on cadaveric samples and lab animals have shown the bone strength for prediction of fragility fractures but there is no pure study that only has been focused on the normal population (free of any disease condition and on no any medications) in order to measure the BMSi value range regardless of genders and also in males and female respectively. There are few and perhaps two studies that were performed on normal healthy samples, Daniel Sundh, et al. in 2018 from Sweden [128] that compared jumping effect of exercise on the BMSi and Lamya Karim et al published a paper in 2018 from the USA [127] that performed on the cadaveric samples of normal donors and all samples were from female gender. There is also a paper published in Osteoporos Int by F. Malgo et al from the Netherland in 2017 [115] that noted. There was no difference in BMSi values between women and men (79.7 ± 0.6 vs. 80.00.8; p = 0.789). The important note about this paper is that this study was carried out on the osteoporotic samples with and without fractures and not on the healthy normal peoples

Table 6. BMSi as "Reference Normative Values in normal population"

from our study in comparison to other reported papers are summarized in Table 8. Reviewing papers about the difference between BMSi in females and males, we have limited studies and information about the influences of gender on the human cortical bone tissue properties. In 1996 Norman et al published a paper in J. Biomech., about Resistance to crack growth in human cortical bone is greater in shear than in tension. In this study that is in regard of human cortical bone fracture toughness revealed that tissue toughness of cortical bone gradually decreases between 55 to 89 years of age and there is no significant differences between men and women bone toughness [130].

From biomechanics and pathomechanic perspectives it is sufficed to say that susceptibility of age-related fractures is more pronounced in females than males. Bone tissue properties i.e. strength and fracture toughness, as well as structural characteristics i.e. bone size and bone shape are major factors involving in fragility fractures. With according to facts that revealed, bone structural differences quantitavely are more effective than tissue properties differences in regard of higher incidence fractures in women [131].

2-Our second goal was to determine relationships of Age, Gender, BMI, and BMD with BMSi in normal population. And our hypothesis was that BMSi values are independent of age, according to our findings regardless of gender and, also, there is no significant difference between each interval age group in males and females Figure 53. Therefore this hypothesis confirmed that there is no significant difference between means of BMSi for each interval age (p-value=0.0618) Figure 53 and thus there is no

association between BMSi values and age and BMSi is independent of age. In the reviewing of the literature only in 2017 one paper by F. Malgo et al [115], from Leiden,

The Netherlands was noted that there was no correlation between BMSi with age. Again important note about this paper is that this study was performed in peoples with osteoporosis associated with and without fragility fractures-Table 5.

One of the main health concerns in postmenopausal women and both aging genders are bone fragility fractures. The main load bearing tissue is cortical bone. Cortical bone is under influences of age and gender and the cortical bone tissue mechanical competence changes pathologically are interesting in regard of timely diagnosis and proper management of osteoporosis. Obviously with according to evidence, deterioration of cortical bone strength in human takes place 2-5% per decade. And in a paper, a study on bone obtained from donors ranging from 20 to 98 years old reported that the amount of this deterioration in femur and tibia were compared. According to this paper the amount of decrease in the tensile strength of cortical bone in tibia is 1.2% and 2.1% in femur respectively, also in this study yield strength of the femur (tension) decreases 2.2% per decade, while yield strength of the tibia decreases 0.5% per decade [132]. In regard of relation between BMSi and gender, according to our findings there is a significantly difference between means of BMSi for each group Figure 44.

Distribution of BMSi in each gender as seen in graphs as well as plot shows Figure 45 the applied t-test is correct since the distributions of samples in each group are closed to standard normal distribution

demonstrated by according to quintile plots Figure 46. Comparison of our finding about relation between BMSi and gender, and other reported papers can be seen at Table 8. With according to this Table 8 that can be seen, in only one paper by Frank Malgo et al, 2017 from Leiden, [115], the relation of BMSi and gender was studied and reported that there was no difference in BMSi values between women and men (79.7 ± 0.6 vs. 80.00.8;p value = 0.789). The important note about this paper is that this study was performed in osteoporotic patients with and without fractures and not in normal healthy peoples Table 6. The interpretation of difference of BMSi in male and female are multi factorial but one of main and critical regulators of bone growth in both genders i.e. male and female, are sex hormones at the time of increasing and decreasing mass of bone. A minimum of 50% of peak bone mass during adulthood is acquired during puberty when there is rapid growth of bone. Both estrogens i.e. the primary sex hormones of female as well as androgens i.e. primary sex hormones of males have positive impacts on remodeling of bone and bone mass maintenance in normal and healthy adult bone (133).

Correlation between BMSi, BMI, BMD Regardless of genders:

In regard of association between BMSi and BMI, as our finding shows, based on the samples for BMSi the Pearson's correlation coefficient represents BMSi do not have statistically significant correlation with BMI. In comparison to other reported papers as seen Table 8, there is only one paper by Frank Malgo et al, in 2017 [115], that reported, there is no association between BMSi with BMD. As noted before this study was

performed on the osteoporotic samples with and without fragility fractures and not on the normal and healthy populations. In regard of association between BMSi and BMD, Based on the given sample, for BMSi the Pearson's correlation coefficient discloses that BMSi does not have statistically significant correlation with BMD. As shown in Table 8 there is only one reported paper by Frank Malgo et al, in 2017 [115], that noted, there was no correlation between BMSi with BMD in patients group. According to a nonparametric regression approach based on Regression Splines in order to find existing associations between covariates (BMI, Weight, Height, Age, FN – BMD, TH – BMD,LS - BMD) and BMSi Figure 60. As results show, only "Weight" and "Height" has some kind of association with "BMSi" and the rest of covariates does not have any associations Figure 61. The plots show the effects of each feature on "BMSi". As the plots relating to "Weight" and "Height" show, the association with "BMSi" is negative and positive respectively. It does mean that the taller is a person the more value of BMSi is expected Figure 65 and that the heavier is a person the less value of BMSi is expected Figure 67.

3-our third objective was to determine interobserver Covariance of Microindentation in hospital del Mar, Barcelona-Spain. Our finding as shown in table 7, the Interobserver Covariance was <5%, and comparison with other studies performed so far can be seen in table 5**.** According to our finding as ANOVA for BMSi/different investigators and Male & Female samples revealed that there is NO significant difference (p-value=0.0871 for men and p-value=0.0718 for women) between means of BMSi for each investigator in male and female patients. In our study only for two

investigators the means of BMSi were significantly different. The interpretation of this finding was most probably that most samples of one investigator were females (females=27 vs. Males=8, and with according to our finding in this study, BMSi values are different between two genders) and another had few samples that were not practical from statistical analysis perspective .

As we explained throughout the Microindentation [RPI Technology] Section, there are two major types of Microindentation [CMI i.e. BioDent® vs. IMI i.e. OsteoProbe®] that so far has been utilized for stiffness evaluation of cortical bone and our project has been focused only on IMI for determination of "Normative Reference Values in normal and healthy population" in order to applying it for patient management decision making clinically. In CMI, there are some parameters that produced during CMI procedure namely IDI, CID, TID that interpretation of these parameters are problematic in contrast to IMI that during the procedure we have an output called- Bone Material Strength Index (BMSi).

In regard of IMI clinical application, currently there are few studies one from USA [127] and two reports from Europe [115,107] that were performed only on the normal healthy population. But there are several reports and studies that have focused on the differences between BMSi values in disease and non-disease conditions Table 5 . As a rule of thumb, in all these studies it is accepted that higher values of BMSi derived from IMI is indication of better mechanical properties of bone.

In regard of BMSi values ranges in different reported papers, there are several factors that should keep in mind about the variability around this range and for precise disclosure of these factors we need to have larger

samples from different regions of the world that can explain and detect the influences of age, gender, ethnicity, nutrition, climate, cultural issues in regard of daily living standards on the BMSi.

Our participants were collected from inhabitants of northeast part of Spain i.e. Catalonia. They have sun most time throughout the year; they consume popular and familiar Mediterranean diet and have a very relaxed lifestyle in comparison to northern part of Europe for example. So in my opinion it is necessary and mandatory to have a large collaborative multinational investigation about the influences of aforementioned factors on the normal range of BMSi.

Our experience with microindentation has a number of limitations because of: 1- BMSi estimation did not performed at the same time by standard mechanical testing technique and microindentation on the same bone for the comparison and it may be not feasible because of critical bone damaging by conventional standard mechanical testing; 2- limitation of samples size in 5th decade and older at the age group because there are few persons over 55 year in a purely healthy condition. And this fact is more correct in age over 70.

Conclusions

In this project our aims and destination of investigation was about to determine the normal values of BMSi in normal healthy living humans with the following conclusions:

1-Normal BMSi value regardless of gender is: Mean=83.01 and SD=7.87.

2-Normal BMSi value in Male gender is: 87.80 ± SD=7.99 and in Female gender is: 81.69 ± SD=6.75.

3-In regard of Age and BMSi value: BMSi values irrespective of gender are independent of age both in male and in female individuals.

4-In regard of gender and BMSi value: BMSi values regardless of gender are statistically significant to the gender type (Male or Female), It does mean that BMSi is gender dependent.

5-Inter Observer Coefficient variance of Microindentation in hospital del Mar is < 5%.

Future investigation

The microindentation in clinics is a novel technique and, therefore a large number of future research projects are opened by these and previous results

1. International development

These results will be the Spanish contribution to an ongoing project for establishing international normative reference values of impact microindentation in cooperation with the University of Oslo (Prof. Erik F Eriksen), Leiden (Prof. Natasha Applebaum-Distra), Harvard (Prof. Mary L Bouxsein and Rochester (Mayo Clinic, Prof. Sundeep Kohsla).

2. Clinical development

There are several questions that should be answered by this technique of microindentation in future and by researchers in bone field:

• Where is the best site for MI (RPI) in human bones and is this site a real surrogate of all bones for estimating BMSi?

• Is MI capable of distinguishing between normal and diseased cortical bone in preclinical state? ” Window of opportunity” concept in osteoporosis?

• What are false negatives and false positives regarding the MI results?

• What are mechanical bone properties alterations detectable by MI?

• Is MI sufficient for detecting bone mechanical properties alterations?

• What are the characteristics of pathomechanics of human bone in regard of MI (cRPI vs. IMI)?

• What are mechanical properties alterations of cortical bone with aging and in genders?

• What are MI characteristic in the elderly?

• What are characteristics of MI in different metabolic, inflammatory induced and metastatic bone diseases?

• Is it feasible to consider MI as a component of "Periodic Health Examination" in future for preventing fragility fracture?

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Last words:

“Anyone who stops learning is old, weather at twenty or eighty.

Anyone who keeps learning stays young.

The greatest thing in life is to keep your mind young.” [135].

Henry Ford (1863-1947)

Resum de la tesi

Antecedents:

No s'han establert valors de referència normals de BMSi per IMI en homes i dones majors de 18 anys.

Hipòtesi per provar:

A-Per determinar valors normals de BMSi en poblacions sanes normals d'ambdós sexes és a dir, home i dona.

B- Els valors BMSi són independents de l'edat

C- La variància del coeficient d'observador intermedi és inferior al 5%.

Objectius:

A- Establir els valors de referència de BMSi en la població normal de Barcelona, Espanya.

B- Avaluar la influència de l'edat, el sexe i l'IMC a BMSi.

C-Avaluar la variància del Coeficient Inter-observador d'IMI a l'Hospital del Mar.

Mètodes:

La base de dades de 1.500 persones (pacients i control sense malaltia) es van recollir del sistema de base de dades del departament de medicina interna de l'Hospital Del Mar entre 2008 -2018 de diferents grups d'investigadors en aquest període de temps. La informació rellevant derivada d'aquestes bases de dades es va sotmetre a avaluació especialitzada en bioestadística i anàlisi estadística. S'ha analitzat la variabilitat entre observadors i intra-observadors per tal de validar la precisió i la reproductibilitat de l'IMI. Aquest projecte se centra en

part en la variabilitat de diferents investigadors que van aconseguir resultats amb impacte Microindentació per mesurar BMSi en ossos humans.

Resultats:

1-El rang normal del BMSi independentment del sexe és mitjana = 83.01 i SD = 7.87.

2- En la població normal de 18 anys i més, el valor de BMSi en els barons és de 87,80 ± 7,99 i en les dones: 81,69 ± 6,75 (mitjana + SD).

3- Els valors de BMSi en la cohort global, tant en homes com dones, són independents de l'edat.

4- Els valors de BMSi són significativament més alts en homes que en dones. Significa que el BMSi és dependent del gènere.

5- El Coeficient d'Observació Inter de Variabilitat és <5%

Conclusions:

En aquest projecte BMSi, independentment del gènere, es de mitjana 83.01 i SD = 7.87, i el rang normal derivat de BMSi 87.80 ± SD = 7.99 en home i BMSi 81.69 ± SD = 6.75 en dones de la població normal de 18 anys o més. Els nostres resultats també mostren que el valor de BMSi es independent de la edat però es significativament depenent del gènere.