ESTIMATION OF AGE AT DEATH BY COUNTING NUMBER OF OSTEONS FROM THE STERNAL RIB

Dissertation submitted in partial fulfillment of the requirement Degree

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CERTIFICATE

This is to certify that the work embodied in this dissertation entitled **"ESTIMATION OF AGE AT DEATH BY COUNTING NUMBER OF OSTEONS FROM THE STERNAL RIB"** has been carried out by Dr.S.Saravanan M.B.B.S. a Post Graduate student under my supervision and guidance for his study leading to Branch XIV M.D. in Forensic Medicine during the period of May 2010 to April 2013.

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DECLARATION

I, Dr. S. Saravanan, solemnly declare that the dissertation titled "**ESTIMATION OF AGE AT DEATH BY COUNTING NUMBER OF OSTEONS FROM THE STERNAL RIB"** is the Bonafide work done by me at Institute of Forensic Medicine, Madras Medical College, Chennai – 3 under the expert guidance and supervision of Capt. Dr. B.SanthaKumar, M.Sc. (Forensic Science), M.D.(Forensic Medicine) Dip N. B. (Forensic Medicine), Director and Professor of Institute of Forensic Medicine, Madras Medical College, Chennai. This dissertation is submitted to The Tamilnadu Dr.M.G.R Medical University, Chennai towards partial fulfillment of requirement for the award of M.D., Degree (Branch XIV) in Forensic Medicine.

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ABBREVIATIONS

avg_osteons - average number of Osteons

CA - Cortical Area

OPD - Osteon Population Density

OS - Osteon Size

Osteon_Avg – Average number of Osteons

Osteon_Gr - Osteon Group

SD – Standard Deviation

SE - Standard Error

TABLE OF CONTENT

ESTIMATION OF AGE OF OSSIFICATION OF HYOID BONE BY RADIOLOGICAL AND HISTOPATHOLOGICAL EXAMINATION OF AUTOPSY SPECIMEN

Identification is essential in living persons, recently dead persons, decomposed bodies, mutilated bodies and skeletal remains. The main part of corpus identity of the dead body.

The three primary characteristics of identification of a person are *Sex, Age and Stature*. Visual identification becomes difficult and impossible in cases of explosions, fires, advanced decomposition, mutilation, earthquakes, aircraft accidents, mass disasters and other terrorist activities. The aim of the study To estimate the age of fusion of greater horn of the hyoid bone with its body using radiological and histological examination. To find out the sexual variations in the fusion of greater horn of the hyoid bone with its body. Hyoid bones collected from the cadaver and examined radiologically and histologically. In this study the incidence of fusion of the body and the greater cornu of the hyoid bone increases with age and maximum number of hyoid bones with bilateral complete fusion are observed in the age group of 51 – 55 years

INTRODUCTION

General:

Prior to opine about the cause of death, time since death and sometimes manner of death, it is the first and foremost task for the autopsy surgeon, to establish the identity of the corpse. Identification is difficult, when the body is decomposed. Furthermore, it is more complicated one, in case of mummified or skeletonized bodies. When the skeletonized body is subjected for autopsy, the first step is what forensic anthropologists say "doing the big four"—identifying age, sex, stature and race[21].

Identity or identification is defined as the recognition of the individuality of a person, either live or dead. It is vital among both the cases. The results of trials, in court of law, often depend upon the exact identity of the deceased. Other important situation for identity is mass disasters in natural calamities like earth quake, bomb blast or conflagration of a crowded building[4]. Where ever the skeletal remains have been recovered, they should be submitted for experts' opinion[16]. The most elemental is to distinguish human from non-human remains.

Experts who can make contributions towards solving identity include pathologists, physicians, anatomists, physical anthropologist and dentists. For the living, the identity is much easier than for the dead one.

For the living, the following features are useful for the identity: Photography, Handwriting, Speech, Finger Prints etc. To identify the dead one, we have to look the following demographic features: Age, Sex, Stature, Race and Religion[6], Communal Characters, Stature, Hair, Dentition and dental records, Tattoo marks, Moles, Scars, Finger, Foot and Lip Prints, DNA Profile and Brain Finger Printing[4]. Dental records are used by the developed countries for identity.

Personal identification is the traditional goal in the recovery of human skeletal remains. These identification processes may be carried out through several levels viz. anatomical, biological and circumstantial.

Age as identification:

Assessment of individual biological characteristics like age, ancestry, gender and stature constitutes the basic of personal identification. In which, the assessment of age is very much essential to establish personal identity. It is a primary characteristic in the identification, and its estimation is of considerable importance. The skeleton and teeth are the principle sources of information towards the post – mortem age estimation, because of resistance of degradation and age related changes. Identification is classified into Complete and Incomplete or Partial and Legal Identity[6]. Identification by skeletal remains is also called as Presumptive identification. If parts of the body, complete individual bones or fragments of bones are recovered, efforts

must be taken to establish the appropriate individuality[14]. It is not only possible to differentiate between human and non-human bone fragments **(Schranz, 1954)**, but it is also possible to differentiate the age of the individual bone[27]. The skeleton is hoped, to give an exact estimation of age, but environmental, nutritional, and pathological conditions often cause changes in the skeleton, which may mask the true age of the individual. If analyzers have got the full skeleton without absence of any bone, they might establish the complete identity positively. Skeletal remains are usually examined by an anthropologist – hopefully, a forensic anthropologist.

Krogman (1960) reviewed the reliability of the identification of human skeletal remains[6]. Description of morphological characteristics, dentition, osteon pattern and ossification of bones are useful for determining the age[4].The estimation of age relies up on the assessment of the physiological age of the bones, as opposed to the chronological age of the concerned individual. The physiological age is based on the relative growth patterns.

In younger individual, the estimation of age wholly depends up on the developmental changes observed. In younger, the age, which assessed will be précised one. But in older ones, aging pattern is accomplished via the destructive changes, so that determined age is in less accuracy.

AIMS AND OBJECTIVES OF THE STUDY

1. To arrive at the regression formula for assessing the age, based on number of osteons in the sternal rib.

2. To estimate the age at the time of death by using the regression formula.

REVIEW OF LITERATURE

Methods of estimation of age:

If any corpse is submitted for autopsy, whether it is fresh or decomposed, mutilated or not and complete or partial, it is the duty of autopsy surgeon to estimate the age at death. Numerous methods have been developed to determine age at death, depending upon the available state of the body. Experts may attempt to determine the age, with stature, histological methods and many more. It also depends upon the suspected dead one would be adult or not. The age assessment should be based on numerous indicators. A holistic analysis of all the possible age-related changes is the best for an overall estimate. Some of them include:

1) General development, in case of children
2) Secondary sexual characters
3) Dental Eruption and Occlusion
4) Cortical Bone Histology
5) Cranial Suture Closures
6) Ossification of bones
7) Pubic Symphysial Face Morphology
8) Age-Related Degenerative Conditions
9) Phase Changes in the Sternal Rib

1. General development:

Gestational age of the fetus is calculated from the maturation of chorionic villi, length and weight of the fetus, head and chest circumferences, femur length, scalp hairs, nails, position of testes as in case of male fetus, foot length and appearance of ossification centers.

In childhood, age determination may be made from anthropometric measurements, closure of anterior and posterior fontanelle, appearance and fusion of ossification centers and eruption of teeth.

2. Secondary sexual characters:

While using secondary sexual characters, to assess the age in both gender, mustache, axillary, and pubic hair are used. In males, position of testes will be used for age estimation in intra uterine life. In females, menstruation, breasts changes and external genitalia changes are useful. Secondary sexual characters are used to estimate age from 5^{th} year to 21^{st} year age[37].

Sometimes the professionals who investigate may use numerous scientific formulae to arrive the conclusions to assess the age at death. Age estimation are the most difficult to make; the sad reality is the positive and personal identification is often neither possible nor

pragmatic in poor nations and areas suffering from recent conflicts, in which less or no infrastructure[14]. However, this determination is becoming easier now that microscopic analyses are being performed[5].

3. Dental Eruption and Occlusion:

Smith (1991) and **Scheuer and Black (2000)** used dental eruption methods for age determination. While using the dentition, age estimations are based on the eruption of the deciduous and permanent teeth. This method is more useful up to about 15 years of age only. Since the third permanent molar erupts after 15 years of age, if it erupts at all, this method is not a highly reliable indicator. Ubelaker's illustrations about eruptive phases of the teeth are pointing out the standard deviations. Occlusal wear is also one of the indicators of age. But this is highly inaccurate.

While dentition is used for age estimation, the following characters are noted, viz. teeth development and eruption. The alveolar cavities are formed at or around the third or fourth month of intrauterine life. Both the upper and lower jaw bones contain rudiments of all the temporary and first permanent molars at birth. In age estimation, teeth are useful according to the state of development i.e. temporary or permanent teeth, and secondary changes. Gustafson’s method is used for age estimation by analyzing the secondary changes of teeth like

Attrition, Paradentosis, Secondary dentin, Cementum apposition, Root resorption and Transparency of root.

The method to estimate age of dead infant is Boyde's method. In this method, daily incremental line in the enamel of teeth is used[17].

4. Cortical Bone Histology:

The first age estimating equation, based on the histological observations in bone sample was derived by **Balthazard and Lebron (1911)**. Alqvist and Damsen have given a method that can be used to the femur histology[48]. However, **Kerley (1965)** was the first one, who published the technique of prediction of human skeletal age[20]. He used histology of bone for age estimation, based on counting the number of osteons in bone bits taken from mid-shaft region of long bone sections. In his analysis, the samples were taken from the outer one third of the cortex of femur. Ordinary light microscope was used. In 100 X field, the variables were counted in four peripheral fields. A percentage assessment was estimated. These percentages were applied into regression formula or pre-determined age profile chart. Kerley concluded that, a reliability of almost 90% with standard deviation of +/- 5 years, with the good correlation from the fibula, then the femur and the tibia. He analyzed, the variables like number of osteons, number of

osteon fragments, and percentage of lamellar bone the percentage of circumferential lamellae cortex of bone.

Singh and Gunberg (1970) also analyzed the bone microscopy for various indicators including osteons and related components[28]. In these indicators, the number of osteon and osteon fragments are increasing with age. In the histological method of age estimation, it is also observed that size and shape of osteons increase with age[3]. In contrast, the non haversian canals and percentage of circumferential lamellar bones are decreasing with age and they will disappear completely after fifty years of age.

With the similar type of study, **Rai et al** had derived a regression equation for estimation of age[2]. The equation is Age = number of osteons + 8.3.This method is useful in sub adult and adult population.

Apart from the manual measurements of histomorphometric changes of the bone, automated measurements through entire cross section of femoral diaphysis would enable the estimation of age[7].

Other histological methods used for age estimation in adults are osteon remodeling **(Ortner DJ 1975)**, histomorphometry of femur **(Watnabe Y et al, 1998)** and double lamellae in trabecular osteons in ilium **(Boel L W, et al, 2007)**.

C. D. L. Thomas et al, studied femur for estimation of age, by using morphology and histology. He concluded that use of automated global measurements in cortical bone leads to poor estimation of age[49].

5. Cranial Suture Closures:

This method was based upon the degree of closure, union or ossification of the cranial sutures in stipulated ages. Till recently, these methods have been considered inaccurate. But **Meindel and Lovejoy (1985)** have identified, the closure of parietal ectocranial suture is significant indicators of age above 40 years.

And **Mann et al (1987)** have stated that the four maxillary sutures and their patterns of closure are reliable age estimators.

6. Ossification of bones:

Krogman and Iscan (1986) used epiphyseal closure and ossification method for age assessment. Cartilaginous bridges between the growing bones form the endochondral bones of postcranium by union and ossification. This process is used to follow a pattern of growth algorithm. It is used to assess age at death on the union/non-union basis. **McKern and Stewart** have defined five grades of epiphyseal union: unobservable (0), beginning (1), active (2), recent (3), and complete (4). These observations can give a possible accuracy

estimation of age. Ossification centers were used by **Francis et al (1935), Noback (1954) Scheuer and Black (2000) and Scheuer (2002)** for age assessment. Ossifications of bones are used to estimate age between 5 and 21 years of age[17].

7. Pubic Symphyseal Face Morphology:

In the young age, the pubic symphyseal face is categorized by an undulating surface of typical non-fused epiphyseal plate. From age 18 onwards, this surface undergoes continuous progressive metamorphosis. With this character, **Suchey and Brooks** have studied age estimation with the male pubic symphysis.

8. Age-Related Degenerative Conditions:

The osteophytic growth forms on the outer margins of the vertebral body, over a period of time. Steward had computed an age evolution histogram over 21 years based on the percentage of extra lipping as a function of age for the dorsal and lumbar vertebrae. Vertebral column was also studied for assessment of age by **Albert and Maples (1995)** and **Scheuer and Black (2000);**

9. Phase Changes in the Sternal Ribs:

Iscan and Loth have established a method of age estimation based on observable changes at the sternal end of the fourth rib. They

are parallel to those that occur on the pubic symphyseal face. They are of exact morphological nature and occur on the costochondral joint between rib and sternum. The variables are as follows: an upsurge in the depth of the articular depression and the degenerative fragmentation, thinning and augmented porosity at the edges of the articular surface over the period of time[22.]

Some medico legal importance of estimated age:

1. From birth to 1 year of life - Infanticide. In India, no separate law is available for infanticide, it is considered as child murder.

2. 5 years of age - Custody of the individual, who has not completed the age of 5, shall rest with the mother.

3. 7 – 12 years of age – (a) Child below 7 years of age is exempted from criminal liability, since the child is not having criminal intent (82 IPC); (b) Child above7 years below 12 years may or may not held guilt depending on presence of or absence of maturity (83 IPC); (c) Kidnapping or abducting child below 10 years is punishable (369 IPC);

4. 14 years of age - Child below 14 years can not be employed in factory.

5. 15 years of age – (a) Child above 15 years can be employed in factory, while production of fitness certificate; (b) Police can not compel the individual below 15 years of age other than their residence (160 IPC)

6. 16 years of age – (a) Kidnapping a male child below 16 years of age (361 IPC) and maiming for employing for begging (363 A IPC) are punishable; (b) Consenting age for sexual intercourse by a female;

7. 17 years of age - Candidate should attain 17 years of age for MBBS admission.

8. 18 years of age – (a) Qualifying age for marriage for the bride; (b) Kidnapping a female child below 18 years of age (361 IPC) and maiming for employing for begging (363 A IPC) are punishable; (c) By completion of age 18 one can become ‘major’; (d) Below 18 years can not valid consent to suffer any harm; (e) Abetment of suicide of one below 18 years of age is punishable; (f) Above 18 years of age can exercise vote, can authorize organ removal and can make a ‘valid will’.

9. 20 years of age - To any individual below 20 years of age, selling, distributing, exhibiting or circulating obscene objects is punishable (293 IPC).

10. 21 years of age - (a) Below 21 years, not attaining majority while in the guardianship of court of law; (b) Qualifying age of marriage for bridegroom.

11. 25 years of age - (a) Minimum age for contesting in MLA and MP elections (b) Maximum age for entering in to Government service.

12. 35 years of age – (a) Minimum age for appointment as President, Vice- President or Governor in India; (b) unless, the pregnant mother is above 35 years of age, no prenatal diagnostics tests is used.

12. 55-60 years of age - Age of retirement in State Government or some Autonomous bodies.

14. 60 years of age - Age of retirement in central government in India.

15. 70 years of age - Age of retirement for medical teacher in Private Institutions.

Anatomy:

Bones are the hard connective tissues used for the purpose of appearance, movement, support and protective mechanisms of the human or animal body. Long bone, short bone, irregular bone, flat bone and pneumatic bone are the types of bone. Long bones are longer in one dimension. For the other dimension, they are shorter. Examples are femur, tibia, fibula and rib. The parts of the long bone are epiphysis, metaphysis and diaphysis (shaft). In short bones, both the length and the breadth are almost equal. Examples are metacarpal and metatarsal bones. Flat bones are plate like bones. Examples are temporal bone etc. Irregular bones are shapeless. Pneumatic bones contained air sinuses. Examples are paranasal bones.

Morbid anatomy of the ribs:

There are 12 pairs of elastic arches or ribs in the human skeleton. They are curved long bones. They are separated from each other by the intercostal spaces. They increase in length from first to seventh ribs. The greatest breadth is at their anterior end. First to seventh ribs or True ribs attach through their own cartilages to the sternum[8]. So they are called as **Sternal Ribs**. And all the pairs of ribs articulate with the vertebral column posteriorly[3]. The third to tenth ribs conform to common features. A typical rib has a shaft, anterior end and posterior end. At

anterior end, costal cartilage is attached. Shaft is convex one and its upper border is rounded and its lower border is grooved. Posterior end has head as two facets, neck and tubercle[1].

Embryology of the ribs:

Sclerotomes derived from the somites produce mesenchymal cells. Mesenchymal cells, around the developing spinal cord and notochord form vertebrae and mesenchymal costal process. These mesenchymal costal processes of developing thoracic vertebrae, give rise to the bony portions of the ribs by independent growth[10]. Costal cartilages are formed by sclerotome cells that migrate in to the adjacent lateral plate mesoderm[9]. As the rib grows, it is described as the costal arch. At first, each arch is completely chondrified[1]. A rib begins growing endochondrally at their primary centers of ossification. During, the second intrauterine month at seventh week intrauterine life **(Scheuer and Black, 2004)** one primary ossification centre appears close to the posterior angle of the shaft, and then it ossifies both dorsally and ventrally. At birth, the ribs have got the basic morphology like in adults. During puberty, one secondary ossification centre for the head and two secondary ossification centers for the tubercle appear[11]. Thus, both the chondrification and ossification form the complete ribs. There are two types of ossification namely enchondral ossification, which the most common one and intramembranous ossification.

Ossification:

As we know already, process of formation of bone is ossification. Even though, the bones are developed through two distinct processes, the histologic characters are same.

(a) Enchondral ossification:

In human body, enchondral ossification begins in the second trimester and continues up to early adulthood. In human, many bones are produced through enchondral ossification. Endochrondral ossification is responsible for formation of short and long bones and takes place in hyaline cartilage, which already resembles a small version of the fully grown bone. This process starts with structure known as bone collar. The bone collar is produced by the process of intramembranous ossification within the local perichondrium. During the process, the blood vessels penetrate through the bone collar and transfer the osteoprogenitor cells in this region. The bone collar or cartilage model proceeds to develop by both the appositional and interstitial means. The bone collar is merely bone tissues appearing a shallow bone cylinder surrounding the mid portion of the cartilage.

Then the chondrocytes divide, enlarge, mature and cartilage begins to calcify. Now the perichondrium ultimately becomes the periosteum. While the calcification proceeds, the diffusion of nutritional

particles and gaseous materials through the calcified matrix decreases. The characteristics of the step include cell enlargement, matrix calcification and cell death. Following death of chondrocytes, the small pieces of calcified matrix serves as framework for deposition of bony materials.

The result of these processes is an area of three dimensional structures, which is formed by the remnants of the calcified cartilage matrix**.** Subsequently, the inner perichondrial exhibit their osteogenic potentials. A thin periosteal collar of bone forms around mid-shaft region of bone. Then osteoprogenitor cells, osteoblasts, osteocytes and osteoclasts develop. Mesenchymal tissues, blood vessels and osteoblasts form primary ossification centre at the diaphyseal region of the bone and secondary ossification centre on the epiphyseal region of the bone[12]. Secondary ossification centers form at the swellings in the extremities and together with the primary ossification centers form cavities. These cavities gradually fill with bone marrow. The epiphyseal cartilage is responsible for the growth in length and when the epiphysis closes there will be the cessation of the bone growth.

Epiphyseal cartilage can be divided in to seven zones:

1. Resting zone
2. Zone of proliferation
3. Hypertrophic zone

4. Zone of calcification
5. Zone of retrogression
6. Ossification zone
7. Resorption zone

Resting zone is otherwise known as the quiescent zone. It is mainly composed of hyaline cartilage. It is present nearest to the end of the bone. As ossification approaches, this initial short zone progressively shortens and generally slows down the growth in all the directions.

The next zone, the zone of proliferation is an active zone and involves the cells of the resting zone and producing daughter cells. It aligns themselves in distinct columns parallel to the long axis of the bone. Each row of cells grows by the addition of the more cells and so forth. This mechanism allows the cartilage to increase in length.

The third zone involves the maturation of the cells, in which large lacunae can be found with thin adjoining septae.

The next zone is involving the process of calcification of the matrix surrounding the enlarged lacunae.

Following calcification, there is zone of ossification. Here, the osteoblasts differentiate from the mesenchymal cells and gather the

exposed plates of calcified cartilage. At this stage, osteoblasts lay down the bone, and enchondral bone tissue appears.

The last stage is known as the resorption zone. Here, the resorption of the bone in the center of the diaphysis takes place and results in increase in size of the marrow cavity.

(b) Intramembranous ossification:

In this method, bone development is not preceded through cartilage model. But it develops from mesenchyme itself. It takes place within the condensation of the mesenchymal tissues. The mesenchymal cells directly differentiate into osteoblasts. Then, osteocytes and osteoclasts are developed. The clavicle, flat bones of cranial vault, mandible and maxilla are examples of intramembranous ossification[12]. This process also contribute thickening of long bones[13]. It is also responsible of the growth of the short bone.

The first evidence of this process is taking place in the eight week of the intrauterine life. The mesenchymal tissue consists of the primary connective tissue cells. They differentiate into osteoblasts. The osteoblast begins to lie down on the bone matrix and gradually separate from each other during the process. Once the bone matrix has surrounded by the osteoblasts, then it undergoes the process of calcification. These osteoblasts are now known as osetocytes or mature

osteoblasts. They are located in the forming lacunae. Subsequently, the primary cells in the surrounding membrane give rise to the osteoprogenitor cells. They, in turn, develop into osteoblasts. During the initial process of growth, these osteoblasts emerge on the surface of the developed bone. Through this process of apposition, the bone is able to become thicker and more.

Histology of ribs:

Ribs, like all the bones, are made up of both cortex and trabeculae. Trabecular bone has a much more surface area than cortical bone and is highly vascular. The bone marrow lies within the trabecular bone and in which, the red blood cell production occurs. All the bones, including ribs are specialized form of connective tissues having cells, fibers and extracellular calcified **matrix**. There are three types of cells viz. **osteocytes, osteoblasts** and **osteoclasts.**

Bone matrix:

Bone matrix contains both inorganic and organic matters. Calcium and Phosphorus are the most abundant inorganic matters. Sodium, potassium, magnesium, citrate and bicarbonate are present in small amount. Apart from these elements, both hydroxyapatite calcium phosphate crystals and noncrystalline calcium phosphate are also

present. A layer of water and ions forms around the crystals. This layer facilitates ion exchange between the body fluids and crystals.

Organic matter are type I collagen fibres and ground substance, like proteoglycan aggregates and specific structural glycoprotiens. These glycoproteins are responsible for calcification of bone matrix. Because of high collagen content, the decalcified bone matrix intensely binds with the stains for collagens fibres. Even after decalcification, the bone preserves its shape, because of the association of collagen fibres with minerals. But it becomes fragile and crumbling easily when handled.

The type of cells is as following:

(i) Osteoblasts:

Osteoblasts synthesize organic components of bony matrix and they are responsible for deposition of inorganic components of bone and subsequent calcification of the matrix. They are also responsible for collagen and non-collagenous protein synthesis and for the orderly arrangement of proteins. They are derived from the osteoprogenitor cells. They are located side by side, on the surface of the bone. And they have varied shapes like oval, triangular or cuboidal shaped epithelium. They have only one large nucleus and only one prominent nucleolus. The nucleus is ovoid in shape and euchromatic. The cytoplasm is basophilic. It has well developed Golgi apparatus. The cytoplasmic

processes of the osteoblasts extend through the osteoid. By means of gap junction, they have got connected to the adjacent cell processes. They communicate each other through these cytoplasmic processes. They deposit osteoid on preexisting mineralized surface. Bone mineralization occurs only when there are adequate supplies of phosphate and calcium in extracellular fluid. These mineral supplies depend upon the availability of 1, 25 di hydroxy vitamin D_3 and parathyroid hormone. This indicates the vital role of hormones in bone formation.

(ii) Osteocytes:

Osteocytes are mature bone cells. They are long living cells. When osteoblasts are being surrounded by the newly formed matrix, it became osteocytes. They are present numerously in young bones. The numbers gradually decrease with advancing age[3].They lie in the **lacunae**. Lacunae are nothing, but a space produced between the lamellae, during the changing process of osteoblasts to osteocytes. Each lacuna contains only one osteocyte. Osteocytes are flat and almond shaped cells with cytoplasmic processes. They have eosinophilic or lightly basophilic cytoplasm. They play a role in removal or deposition of calcium and bone matrix, when needed. They are responsible for integrity of canaliculi and lacunae. They keep open the channels responsible for diffusion of nutrition.

(iii) Osteoclasts:

Osteoclasts are bone removing cells. They are very large, branched, multinucleated, giant cells. They are 20 - 100 micrometer or more in diameter. Since, they are derived from the fusion of bone marrow derived mono nucleated cells, they are multinucleated. Their bodies contain 5 - 50 nuclei or sometime more. They are motile cells. They are found in the place, where the bone is undergoing resorption. They are responsible for resorption of mineralized bone, calcified cartilage and dentine. They are seen in the grooves known as Howship's lacunae. These lacunae are formed by the erosive activity of the near lying osteoclasts. Under the electron microscope, they have seen with numerous cytoplasmic processes and microvilli on the outer border of cell. The cytoplasm showed numerous mitochondria and lysosomes. These microvilli and cytoplasmic processes facing the bone matrix and known as the 'ruffled border'. A zone of cytoplasm can be observed enclosing the ruffled border and is responsible for adhesion by the osteoclasts with the bone matrix. Following that, there is creation of microenvironment, in which the resorption process can occur. They are also involved in remodeling of bone[13].

(iv) Osteoprogenitor cells:

Osteoprogenitor cells are otherwise known as resting cells. They have fibroblasts like appearance, present in the inner most cellular layer

of periosteum, Volkmann canals, Haversian canals and marrow cavities. They are spindle like shaped. These are mesenchymal originated cells. They may proliferate or convert themselves into either osteoclasts or osteoblasts. There are two types of osteoprogenitor cells. One is pre-osteoblast and the other one is pre-osteoclast. Apart from transformation into osteoblasts and osteoclasts, they supply and maintain the nutrition of the embedded osteocytes.

Internal and external surfaces of bone are covered by layers of connective tissues and bone forming cells. And they are called by endosteum and periosteum respectively.

The endosteum is thinner than the periosteum. It lines, all the internal cavities of bone. It is composed of both the single layer of flattened osteoprogenitor cells and little amount of connective tissues.

The periosteum, the thicker, consists of both the outer layer of collagen fibres and inner layer of osteoprogenitor cells.

Compact bone:

In the cortex of the bone there are two types of areas. In outer area, there are dense areas without cavities known as **compact bone.** In inner area, there are areas with interconnecting cavities known as

spongy (**cancellous**) **bone**[12]. However, both the types have same histological appearance under microscope.

(a) **Cortical or compact bone** is hard densely organized and gives the rib its overall shape and structure. It is vascular in nature. It has uniform smooth structure. It is situated in cylindrical outer part or shaft region of the long bone. In compact bone, the collagen fibres are arranged in thin layers of bone. Most of the compact bone consists of osteons. When we examine a section of compact bone, we find that this type of bone is made up of lamellae, and is pervaded by lacunae, and by canaliculi.

(b) **Cancellous bone:**

Within the medullary cavity there lies cancellous or spongy or trabecular bone. It is not dense like compact bone. It is situated inner to the compact bone and adjacent to the bone marrow cavity. It contains numerous interconnecting spaces. If we examine, the ends of the bone, we can see the meshwork of tiny rods or plates of bone called **trabeculae** containing numerous spaces appearing like that of a sponge. So it is also called as spongy bone[18].

Primary bone:

In microscopic examination of the bone, there are two varieties. They are **primary, immature** or **woven bone** and **secondary, mature** or **lamellar bone.**

Primary bone tissues are usually temporary in the body. These are newly formed bones. These newly formed bones do not have lamellae. In these varieties, the collagen fibres are found randomly interlacing each other. These are woven bone. Except near the cranial sutures, tendon insertions and tooth sockets, they are replaced by lamellar bone or secondary bone tissues, in future, in adults[19].

Secondary bone:

In contrast, secondary bone tissues are usually found in adults. It characteristically shows the lamellae. If the bone is made up of layers or **lamellae**, then it is called as **lamellar bone**. Lamellae are of two types. They are interstitial lamellae and circumferential lamellae.

(a) **Interstitial lamellae** are present in the angular intervals, between the adjacent osteons. These are remnants of osteons.

(b) **Circumferential lamellae** are arranged near and parallel to the surface of compact bone[19]. Lamella is thin plate of bone consisting of collagen fibres and mineral salts that are

deposited in a gelatinous ground substance[4]. Lamellae are 3 – 7 micrometer thick[13]. They are parallel to each other in periphery of the bone. They are mostly arranged as concentric rings around a blood vessel. The outer circumferential lamellae are deep to the periosteum. The inner circumferential lamellae surround the marrow cavity.

Osteons:

The word **osteon** comes from the Greek word meaning bone. Osteons usually lie parallel with each other and, in elongate bones such as those of appendicular skeleton with the long axis of the bone[1].The term is defined as elongated cylinder like structure with a canal like space inside the cylinder. Osteons are concentric lamellae surrounding the canals with blood vessels, nerves, osteocytes and some cells, and loose connective tissues (Fig.1). They are otherwise known as **Haversian systems**. These are the basic functional units of bone tissues. Each Haversian system starts as a broader channel at the outer part of the bone[18]. Osteons run predominantly in longitudinal direction in long axis of shaft of bone. They are not always parallel. They may in spiral course, may branch, or may join with one another. In cross section, they may appear oval, ellipsoidal or circular. Generally osteon has smaller diameter in the regions where the bone frequently experiencing more stress.

The number of lamellae per osteon is not equal in all. The average number is six[19]. Osteons are readily distinguished from their counterparts and from the interstitial lamellae by a **cement line.** This cement line contains little or no collagen fibres. It composed of mainly inorganic matrix. It is strongly basophilic, because, it has high content of glycoprotiens and proteoglycans[1].Osteons can be used to determine the sex of an individual also[47]. They are present in many bones of most mammals and some bird species.

Osteoclasts drill the compact bone by enzymatic activity along the long axis of the bone to produce a hollow. The osteoclasts resorb the primary bone tissue. Soon the osteoblasts fill the inner circumference and neurovascular channels and lymph vessels fill outer circumference of the hollow. The osteoblasts secrete thin layer of cement called reversal line. Osteoblasts surrounded by the bone matrix are called as mature osteoblasts or osteocytes. Volkmann's canals between the osteons are responsible for nutrition, innervation and lymphatic supply.

When osteon has been examined under polarized light, it shows two bright band that cross each other. This is called birefringence[19].

Primary osteons:

Primary osteons or **atypical Haversian system** are described as those osteon which have formed first during bone formation, and they do not have clear lamellar structure, but consists of woven bone.

Secondary osteons:

Secondary osteons or **typical Haversian systems are** described as those which have replaced or superimposed the previously existing osteons[3].

Types of secondary osteons:

There were four types of secondary osteons yet identified. They are Type I, Type II, drifting and zonal varieties. Even though, these are identified as different types, all are the product of the same actions of the basic functional unit**.**

(i)Type I osteons:

Type I osteons are the most common. They have seen in human adult**.** Type I osteons are generally known as secondary osteons. They contain of circular concentric layers of lamellar bone surrounding a Haversian canal.

Under the microscope, in cross section, these osteons appeared as discrete circular bundles of concentric bone that are bounded by a scalloped reversal or cement line**.** Type I osteons result from normal intracortical remodeling

(ii)Type II osteons:

Type II osteons are also known as embedded osteons. They embedded fully in large type I osteon. They are not crossing the reversal line. They result from remodeling along a complete length of Haversian canal inside the already existing osteons. They are found in the radial remodeling of the pre formed haversian systems. In cross section under microscope, type II osteons appeared as osteon within osteon and exhibit two scalloped cement lines one within the other. And they could be linked to dietary stress. Because of presence of type II osteon can be used as evidence for disturbed normal intracortical osteon production. Some research has suggested that the density of type II osteons is positively correlated with age.

(iii)Drifting osteons:

A drifting osteon is classified as the osteon which runs both longitudinally and transversely through the entire cortex. An osteon can "drift" into one or several directions leaving tail of lamellae behind the advancing canal of havers[53]. That is, it will be in resorption state in one end and formation of bone other end. Drifting or waltzing osteons appeared similar to type I osteons except they appeared like elongated rather than circular in microscopic view. They also have eccentricHaversian canals. Drifting osteons are the most common type of osteons found in sub adults.

(iv)Zonal osteons:

Similar to type II osteons, zonal osteons are also the result of a disturbed normal intracortical osteon production. They appeared during the infilling stage of Type I osteon. Under microscope, they appeared like type II osteons. They looked like small osteon within an osteon. But, they differed containing one or supplementary smooth arrest lines in addition to parallel contours of concentric lamellae. There was a disturbance in radial closure during the formation of a new type I osteon. Zonal osteons have been associated with disease and aging. Zonal osteons have also been found to increase with age at a rate of approximately 4% per decade in individuals between 20 and 80 years of age

Osteon fragments:

They are older osteons and that have been partially obliterated during the formation of a newer osteon. The relative area of interstitial bone is inversely proportional to age decreasing over time as it is progressively replaced by osteons**.**

Osteon Population Density:

Osteon population density (OPD) is a measurement of the total number of osteons (whole and fragmentary) per mm^2. As an individual

advancing age, osteon population density increases on the cortical surface. Numerous studies have demonstrated that OPD is positively associated with age. Because of this relationship, OPD has been employed in the determination of age at death by histological method.

Haversian canal:

Haversian canal or **central canal** or **canal of Havers**is the space in which the osteon contains blood vessels and nerves[12].

Bone growth and remodeling:

Any hard tissues like bone grow by apposition. Apposition is deposition of new one over the old one. By apposition, long bone grows in both length and thickness. Around the shaft of cartilaginous model, periosteum deposits layer of bone. This is called as periosteal collar. This collar extends gradually through the entire length of shaft or diaphysis.

If this depositing process of the layers of the bone continues the periosteal bone becomes thicker and thicker. As bone enlarges, the osteoclasts come in to the internal surface of the shaft, remove the bone from this aspect and reconstruct it. This removal and reconstruction is to avoid becoming the bone further thicker. It is in constant state of turnover. It is called as remodeling. They also remove the bone lying in

the trabeculae in the centre of the bone. In this way, the marrow cavity is formed. This marrow cavity extends through the entire length of the diaphysis, but not to the epiphysis. Remodeling is also considered as the process by which, inert bone is transformed in to metabolically active one. As an average, spongy bone undergoes remodeling every two years. This remodeling process needs interactive cellular activity. And it is regulated by both metabolic and biochemical factors. Because of remodeling bone maintains its shape and integrity during growth. The reason for remodeling is get adaptation to the mechanical stress through the life of the individual. The associated process of bone deposition and resorption helps for maintaining the shape of growing bone.

During the early deposition of the lamellar bone, minute blood vessels, are incorporated in to the already depositing lamellar bone. These vessels are vulnerable to become surrounded by the concentric lamellae. When this happens, the canals are known as primary osteon. If the vessels are without any lamellae, surrounding the periphery of the canal, they are simply termed as non-haversian canal. These primary canals are distinguishable from the haversian canals by the lack of small amounts of concentric lamellae. The central canal of the primary osteon is approximately 100 micrometer and lacks the characteristic cement line as observed in the secondary osteon.

Before **Kerley's** analysis, the non-haversian canals were usually not recognized as separate type. It was realized that, these canals are also the part of the Haversian system. The process of the remodeling involves in the formation of the primary osteon. Usually, the primary osteon is relatively small and can have up to three rings of lamellae. They are seldom large structures. Primary osteons commonly organize in distinct rows or layers and are more widespread through a young growing skeleton.

Factors that affect bone formation:

There are number of factors that affect the bone remodeling and formation. They are either intrinsic or extrinsic. And additionally, they have impact on the age estimation process also.

Intrinsic factors:

The age estimating methods using osteon are usually got affected by intrinsic factors like chronic conditions[23]. They are nutrition and their deficiencies, and pathological conditions.

(a) Nutrition and their deficiencies:

Calcium, Vitamin C, Vitamin D and Iron are the most important for bone development and growth. The deficiencies of above nutritional values, definitely affect the bone's integrity and strength. Calcium is the

main and most important constituent of the bone. Its deficiency produces rickets in pediatric age group and osteomalaica in adult population. They are leading into under calcification of newly formed bones.

Scurvy, due to deficiency of vitamin C, is characterized by weakened connective tissues, reduced osteoid content and hemorrhagic disorders. In adults, there is subperiosteal hemorrhage due to thinning of cortex of the bone and reduction in the bone formation with normal resorption activity.

Rickets, due to deficiency of vitamin D, resulted in deformation of bone, because of poor mineralization of the newly formed bone. In children, calcium is also the cause of rickets.

The influence of nutrition and their deficiency definitely played role in histologically assessed aging methods as per the study by Stout and Paine (1992). The study showed that the results of the study were under estimated age, when the histological methods are applied to the individual who were affected by the nutritional deficient diseases.

(b) Systemic and musculoskeletal conditions:

Osteoporosis is the disease caused by the increased resorption of bone. It is because of the imbalance between the bone formation and

resorption. The ultimate result is loss of bone mass. It leads to increased risks of fractures. Mostly they are seen in females and older people. Sometimes, young people also got affected by idiopathic osteoporosis. The causes of osteoporosis are menopause, estrogen deficiency, chronic steroid medication, smoking and drug induced. Mostly it affects the femoral neck and vertebrae.

Paget's disease has unknown cause. It affects the turnover of the bone. The resultant bone will be disorganized one. There will be rapid resorption of old bone and rapid deposition of new bone leads to increased surface of the bone. It is more prevalent in people above the age of forty.

Hyperparathyroidism may increase the number of osteons. So, in case, if the individual is suffering from hyperparathyroidism, there will be over estimation of the age.

Differences present in the microstructure of bone histology were not been considered by the earlier analysts while preparing regression formulae for age assessment. So, there were numerous results of under estimation or over assessment of age up to 70%, as occurred in previous studies[26]. There is definite presence of differences in age estimating regression formulae in various populations. Why because, there is presence of differences in the osteon population density[25].

Extrinsic factors:

Variations in the extrinsic factors like gender and population have also influences among the age estimation using bone histology.

(a) Gender:

The elderly male and female gender usually shows variable rates of bone remodeling. But, it is not clear that whether the differences in bone remodellng are present in younger counterpart or not. According to Thompson et al, females have larger haversian canals and males have more number of haversian canals in mid – diaphyseal femur[52]. And the differences are because of distribution of samples. But, there is non-availability of studies pertaining to the significant effect of the differences over the estimated age. Thompson and Ericksen have divided the regression equation separately to incorporate sex specific. Followed by they have proved that gender oriented formulae were more useful than the combined formulae.

There are differences in size of osteons between males and females. In 1990, Burr et al had concluded that size of osteons was seems to increase with age in females and decrease with age in males. The causative factors explained for the variations are skeletal maturation and time of cessation of growth.

Another important factor plays role in gender difference is micro damage. Micro damage is the consequence of constant strain on the musculoskeletal system throughout the life time. Norman and Wang proved in their study that, micro damage is more prevalent in females and found in mid diaphyseal region of the weight bearing bones like femur and tibia. Micro damage is age specific, in such a way that, it will stimulate the upsurge of the remodeling of bone. As we know early, it will influence the microscopic bone structures.

There are differences in the degree of mineralization of bones in both genders. Goldman et al have studied the effect of mineralization of bones. He found that, while increasing age, the degree of mineralization is decreased[55]. Males have higher range of mineralization in the age group of 45 – 65 years when compared to the same age group of females. They believed that, the gender variations in the bone mineralization are vital factors to understand the biochemical adaptation of bone and its age.

In a study of Dutch population, Maat et al concluded that, there are no significant gender differences in the percentage of unremodelled bone.

(b) Population variations:

When **Kerley** derived regression formulae to assess the age of death, he did not consider about the differences in various group of

population. The successors also applied the same ideas and did not take efforts to analyze the differences in the age estimating formula in various population categories. The well-known reason for not considering the different types of population is the deficiency of varied samples and resources. Thompson was the first who indicated the need of adequate amount of samples from known age and population. And **Ericksen** used the variety of population in USA. The study by Cho et al concluded that there is a definite presence of differences in the OPD, OA and the relative cortical area of the bone, while considering the study in various population groups[56].

Lately in **2006, Qiu et al** studied the differences between the osteocyte and lacunar densities in the iliac bone of the pelvis in black and white American women.

Similar studies:

Jowsey (1960) had studied the histology of bone by using femur and its relationship with age[56]. He indicated that, bones of young individuals are showing high bone turn over and more number of osteons.

Kerley (1965 and 1969) carried out first and several effective studies on bone histology and age related changes in the bone[29]. He used 126 ground samples of long bone using femur, tibia and fibula. Under 100X magnification, he studied four variables viz. total number of osteons, total number of old Haversian fragments, percentage of circumferential lamellar bone and total number of non haversian canals. He concluded that, osteons and fragments of osteons are increasing from birth till adulthood.

Ericksen MF et al (1976 and 1991) estimated age at death by using histology of anterior cortex of the femur. The samples were obtained from anatomy dissecting room in United States and cemeteries from Dominican Republic. They were 328 already documented individuals. The samples were 1.0 cm wide and taken from the mid – shaft region of the femur opposite to the linea aspera. To provide permanent record and to define the field, five 0.886 mm^2fields were identified at the periosteal edge and photographed. The variables like

secondary osteons, type II osteons numbers, osteon fragments, resorption spaces non-haversian canals were counted as /mm^2. A 100 space grid was used to count average percent of unremodelled, osteonal and fragmental bone. Step wise regression analysis done for each and every variables and for both gender separately and combined. Most formulae have SE of estimate of about 10 years. Coefficient determination range was from 0.48 to 0.72[46].

Thompson (1979) was the first one who established the age estimation equation by histology of bone by not taking full thickness of the bone, but with the portion of the cross section, for the study. This technique was useful as one could preserve the remaining portions for further analysis and repeatability. His study contained 116 samples (64 males and 52 females) with mean age were 71. 4 years. He used portion of bone with only 0.4 cm diameter sampled from the anterior mid diaphysis of femur, medial mid diaphysis of tibia and mid-shaft of humerus. The required samples were removed from the main bone with the help of high speed dremel drill. 19 variables were used for the study purpose. Phase contrast microscope was used for examination of histology. All the variables were analyzed by linear regression analysis methods. And the results were consistent with age estimated with most accuracy. The study also indicated that the cortical thickness and bone marrow density showed degradation after the age of fifty[52].

Stout S D et al (1982) studied the effects of field size in age estimation while using Kerley's histological method of bone. The field size, at which the bone read, affects the results, using Kerley's histological method for age determination. In this study, they have suggested field size as close to 2.06 mm^2 as possible to be used when using Kerley's method[35].

Stout S D et al (1989, '92, '94, '96 and '98) had modified Kerley's method to assess age at death. He used percent osteonal bone instead of actual osteonalbone. This is to avoid the discomfort of differentiating the actual osteon counts from the fragmentary osteons. The study was using radius, tibia and fibula. A significant age estimation is obtained when the entire cross section f he bone was used. When actual osteonal count used, significant correlation of age at death, was obtained for all the three bones. But when percent osteonal count was used significant correlation found with radius only. Repeated measures revealed that, percent osteonal counts and actual counts differ among the all used bones with in single individual.

Yoshino et al (1994) preceded the study to determine the age at death by using humerus sample. Only males of 40 members were included in the age group of 23 years to 80 years. The bone bits were taken from the shaft of the humerus near the surgical neck. The variables observed are number of secondary osteons, number of double

zoned osteons, number of type II osteons, number of low density osteons, number of osteon fragments, resorption space number, total osteon area, total haversian canal area, average osteon area, and average haversian canal area. The data were subjected for both stepwise and regression analyses. The equations derived were most promising with standard error of 5.1 years[54].

Ritz S et al (1994) analyzed bone with age dependent accumulation of D - aspartic acid in bone to determine age at death. D - aspartic acid is content of bone osteocalcin. It is in turn an aging peptide of organic bone matrix. D – aspartic acid content of purified osteocalcin represents good relationship with age at death. In this study 53 specimens from skull bones were analyzed. The amount of racemization of D – aspartic acid was determined. This study is looking as precise and good reproducibility than other analysis using histology of bone to age assessment[42].

Aykroyd R G et al (1997) studied the accuracy of post mortem determination of age. It is always posing problem in the respect of high variability among the physiological indicators. Confounding this variability is systematic tendency for age estimation irrespective of the indicators employed. It may be too high for younger and too low for older individuals. In this paper, we have come to know that at least a portion of this error is unavoidable consequence in the statistical

procedures. And magnitude of the error is inversely proportional to how well the age indicator is correlated with age[45]. Chatterji S et al have analyzed the changes in structure of human bone with age[30].

Watnabe et al (1998) studied the femur to estimate of age at death, in Japan. They designed the study by using histomorphometry. In this analysis, femurs from cadavers of 72 males in the age group of 43 days to 92 years and 26 females from 2 years to 88 years were sampled. The sections were not decalcified. The area, the length, the width and the perimeter of the complete osteons and the haversian canals were measured. The type II osteon number, fragment number and are of the triangle were also analyzed. These parameters were examined by an image analyzer. The parameters of the osteon showed high correlation with age where as those the Haversian canal showed low correlation coefficient with age[34].

Balwant et al (2005-07 and 2005-09) in India studied about the osteon as an age determinant. The histological methods were done on 107 subjects of both sexes (Male 55 and Female 52) in the compact bone of the mandible to analyze the changes in the number of osteons. In majority of the cases, it was noted that osteon number were increased with age (12 years to 57 years). The study population included from 55 males and 52 females of known age, brought for autopsy, at Rohtak. Bone samples with any bone anomalies were not included for the study.

In this study bone samples were prepared by calcified method. The bone samples were treated in boiling soap solution (Nirma Soap) for first five hours. This will facilitate the removal of organic material. After through washing, they were suspended into the chloroform for next 50 hours in order to remove fat materials. Each bone was cut by the help of Jeweller's saw into numerous sections. Sections prepared were manual hand grinded till become transparent. These transparent sections were fixed on microscope slides with the help of D.P.X. Mountant. In each slide, the number of osteons, consisting of complete Haversian system was counted in four fields. Then the average number of osteons was taken for preparing regression formulae.

It was observed that, 12 osteons at 20 years; 15 osteons at 23 years; 30 osteons at 38 years; 40 osteons at 48 years; 50 osteons at age of 58 years respectively were present. It has shown that the number of osteons is increasing according to the age. Then he derived the regression equation with statistical analysis.

Paine R R et al (2006) studied about the dietary health, which is affecting the histological age assessment, in which age was estimated using secondary osteons from the rib. In this study, 26 samples were collected from cadavers of known age and known cause of death, which may either due to malnutrition or pellagra. The number of intact and fragmentary secondary osteons for the entire cross–section of the rib

was counted. For each case, rib osteon population density values were calculated. This study suggested that, measurements based on healthy population may not be useful in the analysis of individual with poor health and diet[33].

Maat et al (2006), in the recent study, used the sample population like, Caucasian, Dutch, West Europeans and White peoples, in which 86 were males and 76 were females. The study group comprised the age group of 15 years and 96 years. He used transmission light microscopic with 10 X ocular lens and 10 X objective lens.

Sarajli C N et al (2006) used the rib phase analysis method of Iscan et al to estimate age of exhumed skeletal remains. Previously, several researchers have used this analysis, in other populations. They have proposed their modifications in their age estimating formula. The author studied about the applicability of the formula in Bosnian populations, by using 4^{th} sternal rib but sometimes 3^{rd}, 5^{th} or 6^{th} rib. Finally adjusted age ranges were developed[44].

Kim Y S et al (2007) were assessed the histomorphological features of the fourth rib. And, they developed age estimating formula for Koreans. Totally 64 rib samples were used. Among them, 36 were males and 28 were females. By manual grinding method, two thin sections per sample, each measuring less than 100 micron thick were

prepared. Multiple variants were used for analysis of covariance. The results showed significant differences in variables between sexes. OPD, average osteon area were correlated with unknown sex. Relative cortical area was not related significantly to the age[41].

Chan A H et al (2007) studied femur histology and its impact on age estimation. Here, secondary osteon lamellae and Haversian canal ratio and thickness of cortex were quantified. To assess the age, Thomson's All Males Left Femur regression formula was utilized. Results of the study showed that significant variation in the assessed ages derived from the posterior aspect of mid diaphysis of femur. To conclude, the cortex of the femur has the potential role to create significant differences amongst age estimation[43].

Boel et al (2007) conducted a study for age estimation in bone histology by counting number of double lamellae in trabecular osteons. This study also provides information about age associated changes in the body. In this study, ilium bones were collected from 25 male cadavers. The samples were cut and studied by light microscopy. New trabecular bone was formed in disk shaped osteons with a clear double lamellar structure. In each sample, they counted the number of double lamellae in complete osteons[32].

Keough N et al (2009) studied whether factors like chronic disease, nutrition, population group and sex have been influencing the rate of bone turn over and in turn the histological method used for determination of age at death. The study purpose was to evaluate the repeatability and accuracy of histomorphometric variables used for age estimation. In the study, the dissection hall cadavers of known age and sex of 146 sample comprising 105 males and 41 females have been taken. From the front of mid-shaft of femur, exactly opposite to the lineaaspera, sample of size 0.2 x 1 cm removed and studied the histology. The variables like total osteon number, percentage unremodelled bone, total number of non haversian canals and average percentage of osteon fragments have shown acceptable correlation with age but minimal or no correlation had established with total number measureable osteons, average number of lamellae per osteon and resorption spaces. To conclude, a data bank should be complied in order to estimate the relationship between the age and above mentioned variables[39].

Han S H et al (2009) studied anterior cortex of the femur in Korean adults to develop the age estimating formulae. 72 samples from the femur of Korean cadavers were used. By manual grinding method, thin sections of bone measuring less than 100 micron thick were sliced, undecalcified and stained by Villaneuva bone stain. Analysis showed that there was no significant in age adjusted histomorphological

variables between genders. OPD, average osteon area and width of the most anterior cortex were produced a high regression coefficient. The average Haversian canal area was not significantly related to age.

Di Gangi E A et al (2009) were developed a new method to estimate the age at death from first rib, as modification of Kunos et al. From the original study of **Kunos et al** several variables were extracted. They were utilized in this new study. Ribs from 470 male cadavers of known age were used. Data were collected from three places of the first rib like costal face, rib head and tubercle facet. Ages at the time of death range from 12 to 90 years. The lowest correlation was between the costal face and tubercle facet[40]. During slide preparation from the shaft of long bone like femur for estimation of the age by histological method, micro cracks were observed. They are number and lengths and orientation with osteons were analyzed. 13 male cadavers were used to take sample from mid shaft of bone. They have stained with basic fusichin.

Streeter M (2010) estimated age at death based on sub adult rib cortex. Histomorphometric age at death estimation methods were developed for use in adults, which was based on age associated accumulation of osteons. Until the latter half of second decade, there was a poor correlation between osteon numbers and the age. In this study, rib cortex of 72 sub adults in the age group of 2 – 21 years was

analyzed. This was a qualitative method which was based on the systemic changes in the rib cortical morphology. This was used to classify ribs into one of the four age phases[37].

Cucina A et al (2010) studied fourth human rib, using histomorphological changes to estimate age at the time of death in Maya population. In the study, 36 individuals of known age were estimated the age at death from the fourth rib. Thin sections from the rib were taken and examined in polarized light microscopy. The following variables viz. osteon size (OS), osteon population density (OPD) and cortical area (CA) were recorded. Using all the above variables in combined form, seven algorithms were analyzed. On an average, the OPD based formulae deviate 8.7 years from the known age. The OS and the CA based formulae deviate between 12.8 and 10.7 years. To be concluded, for different ethnic group, algorithms should be formulated using OPD, since they have shown lesser deviation from known age[38].

Cannet C et al (2011) estimated age based on histomorphometric techniques in paraffin embedded ribs. In this analysis, 80 left side fourth ribs were collected. Picrosirius dye provided the staining of the decalcified paraffin embedded ribs. The total bone cortical area, haversian canals, osteon areas of intact and remodeled secondary osteons and the area of the non – haversian canals were evaluated. It was noted that, morphometric measurements in the internal cortex of the rib

showed less variability than in the external cortex. It was analyzed that, osteon population density was sufficient to discriminate between three age groups 20 – 39, 40 – 59 and group more than 60 of age[36].

Principles of block selection and fixation of bone:

The preparation of old and dried bone previous to making thin sections used for analyzing bone micro-anatomy is an important first step. To maintain the strength of the bone sample during entire procedure is vital for documenting accurately the histology of bone [22]. But new recently defleshed bone and wet heat treated bones with small cut sections, i.e. rib, clavicle and metacarpal usually not require an embedding step in the bone sampling for sectioning.

The cutting of thin sections of bone, teeth, teratomas containing bone tissue, and calcified tissues are impossible. So, these types of tissues must be treated in order to remove the calcium like hard substances. These removal methods are known as decalcification in which by the use of acids to dissolve the bone salts. Fixation is accomplished by selecting exact blocks of 2 to 4 mm thickness by the help of fine hacksaw or jig saw. The longer time is required in formalin so that the nucleic acids will become resistant hydrolytic action of decalcifying acids.

There are two types of bone sample preparation viz. decalcified bone and undecalcified (mineralized) bone.

To study bone histology, undecalcified bone samples are very much useful than the conventional decalcified bone sections in cases of hyperparathyroidism, Vitamin D, osteoporosis and osteomalacia. There is no need of staining methods like decalcified bone, while using mineralized bone.

Techniques for the cutting of sections of bone:

There are three groups of methods for bone cutting viz. mechanical cutting, without or with adhesive tape and grinding.

Grinding method:

This is the oldest method, preferably followed by the analysts of dental tissues. Many successive scientists have modified the grinding methods. **Saxby, in 1959** used the abrasive lathe wheel for initial grinding. Then Friend and Smith have adopted fossil grinder to obtain blocks less than 50 micrometer thin. In which we have used frost technique to obtain bone blocks. It is the simplest of all the methods. There is need of minimum and cheaper equipment and little time only. All that required are small Jeweler's jaw, waterproof adhesive paper and sheet of plate glass[31].

Mechanical cutting:

There are sophisticated machineries available for bone cutting by the use of high speed diamond impregnated cutting edge. With it one can cut bone blocks about 6 to 12 micrometer with ease and rapidity.

Adhesive tape method:

The main problem in cutting mineralized bone is compression and fracture. To overcome this, firm adherence of strips of Sellotape over the bone to be cut should be made.

MATERIALS AND METHODS\

The bone samples, used in this study composed of sections which have been taken from the shaft region near the anterior ends of fourth sternal ribs of 41 South Indian Population.

After getting proper permission, concurrence and clearance well in advance, from the Institute Ethical Committee, the samples were selected randomly from the autopsied bodies with known age group ranging from 20 years to 50 years of age, at the Institute of Forensic Medicine, Madras Medical College, Chennai – 3, Tamil Nadu, South India. The materials are mostly from the male bodies and few from the female bodies. Since the autopsies for female body usually less in number, our samples also have similar less number of female samples.

Inclusion and exclusion criteria:

In this study, the inclusion criterion was the given age ranging from 20 years and to below 50 years of age. The exclusion criterion was the age group of below 20 years and above to 50 years. The sample materials were taken from the already known age group. Samples with suspicious pathological condition were positively omitted from the study.

As mentioned earlier, the age ranged between 20 years and 50 years with a mean age of 34 years. The range of age for male gender was between 22 and 48 with mean age of 28.22 years. The range of age for female gender was between 20 and 42 with mean age of 29.66 years. There was a good representation of all the categories of age.

Procedures followed for taking samples:

Bone slides were prepared by the method proposed by **Maat et al (2003)**[15]. After completion of autopsies of known age group individual, the portion of bone was removed from the shaft of the fourth sternal rib near their anterior ends because near the anterior end, the bone has maximum breadth. And the sternal ends of fourth rib change as when the people get older. The bone was cut across the longitudinal axis of the bone. The portion chosen has very much resistant to the decomposition changes.

Step 1:

The part of fourth rib, about 4 cm long, was removed from the thoracic cage, 5 cm lateral from the costochondral junction, by the help of hack saw.

Step 2:

Then, with fine edged permanent markers, two parallel circumferential lines 0.2 to 0.3 cm apart across the shaft of the rib bone

was made to mark the cutting line. With the help of fine toothed hack saw slice (Fig.2)the bone was cut to make a sample of 0.2 - 0.3 cm thick (Fig. 3 & 4). While we were cutting the bone block, there should be continuous poring of water over the cutting bone sample for the following purposes. Poring of water would reduce the heat generation while bone was cut and in turn, to make the process easier, smooth and to minimize the frequent changing of cutting blade.

Step 3:

Even though, with careful cutting of the bone, there was chance to breakage of the rib, while the half way of cutting. This was because of rough handling, old blade and shorter the thickness less than 0.2 cm of the block. This unwanted breakage of the block, while cutting the bone could be overcome by increasing the thickness of the block about up to 0.4 to 0.5 cm. But thicker the block of bone would result in longer the grinding.

Step 4:

There were two types of water proof abrasive papers bought grit no. 320 and grit no. 400 (Fig. 5 & 6). Both were used one after another, while grinding the samples. The grit no. 320 was used first. Then, the grit no. 400 used for fine grinding. A sheet of water proof abrasive paper 31 x 23 cm was cut into half section and made in to two equal pieces of 23 x 15.5 cm. A plate glass 30.4 x 30 cm was used. It was

painted with the Fevicol on one side. The abrasive paper was painted with the Fevicol on the non-abrasive or smooth surface of the abrasive paper. And both the Fevicol painted surfaces were pasted with each other.

Step 5:

A middle area of the abrasive paper moistened with water. The bone to be ground was placed on the water spread central area of the abrasive paper. With the tip of the index finger at minimal pressure, the block was started to grind in rotating motion. In order to make smooth, flat and even surfaces, the block should be ground equally on the both upper and lower surfaces of the block or medial and lateral surface of the rib. Care was to be ensured not to topple the section. Grinding was started in the central area of the sheet and gradually moved to the periphery. Water should be poured frequently to avoid friction and in turn damage or breakage of the block. Block should be ground till the cut bone became transparent in naked eyes. During grinding, first we used the abrasive paper with grit no. 320.And then we used grit no. 400 for finer grinding.

Step 6:

With the help of non-toothed forceps, the section was cleaned with Xylene or Xylol to remove the dust particles. The glass microscope slide 7.4 x 2.5 cm was cleaned with 90% alcohol. Xylol cleaned bone

section should be placed on the cleaned slide in such a way that the long axis of the bone section should be parallel to the long axis of the slide (Fig. 7 & 8). This type of fixation is for the convenience of the authors while counting the number of osteons. The bone section was mounted with DPX Mountant fluid and glass cover slip 2.5 x 2.5 cm.

Step 7:

With pointed tip permanent marker, details about the bone sample was written in the glass slide. Now the slide (Fig. 9) was ready for analysis.

Histological analysis of bone slides:

The bone section in the slide was placed in the well illuminated ordinary transmission light microscope. With 10 X objective and 10 X ocular lens, the section of the bone and consequently, the osteons were focused. After focusing the osteons, the entire cross section of the bone was scanned completely. This would enable to study the distribution of the osteons in full thickness of the compact bone of the rib. Then, the number of osteons per single field (Fig. 10, 11 &12) was counted. The counting field was selected in such a way that the number of osteons should be relatively maximum in number. Two fields were selected randomly to count the number of osteons. Then, the average number of osteons was taken to analytical purpose. The count included all he positively identified osteons.

OBSERVATIONS AND RESULTS

Statistical analysis was done in SPSS (Software Package for Social Science) for the study of regression analysis. Regression analysis offers the mathematical formula used to predict one variable. Observed data has been documented in the scatterplot diagram. We can get visually analyzable relationship between the variables.

In this study, age was plotted in the x – axis and the number of osteons were in the y – axis. A correlation coefficient (r) is derived. It summarizes the significance in the relationship of two variables. The following observations were obtained.

In the statistical studies, if 'r' value ranging from 0.75 to 0.99 means, the study was considered as having high correlation value. And if from 0.5 to 0.74, having moderate and if from 0.25 to 0.49, having low correlation values.

The limitation of the 'r' value is the dependency of the sample size. So, the best way to determine the relationship is calculation of r^2. " r^2" is the coefficient determination. If the "r^2"is closer to 1.0, then the study is for positive relationship and if away from 1.0, negative for relationship.

In this study simple linear regression analysis was done.

Table.1 - Over all Mean, Median and S.D. for both the genders.

Mean	28.63
Median	26.00
Std. Deviation	7.172

In this study, over all mean was 28.63, median 26.00 and S.D. was 7.17

Table.2- Mean Median and Standard Deviation for male samples

Sl.No.	**Mean**	**Median**	**S.D.**
1	28.46	26	7.01

This table indicates the average mean of the male samples and it was 26. The S.D. in this sector was 7.01. This value is because of less number of samples.

Table.3 - Mean Median and Standard Deviation for female samples

Sl.No.	**Mean**	**Median**	**S.D.**
1	29.6	30.0	8.7

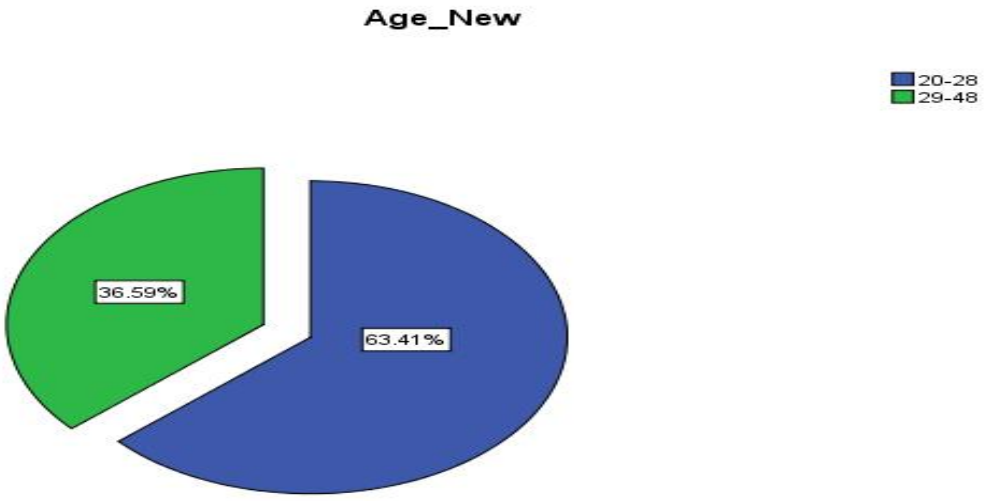
This table indicates the average mean of the male samples and it was 29.6. The S.D. in this sector was 8.7. This value is because of less number of samples.

Table.4 - Frequency of distribution of osteons

Sl. No.	Distribution of age	Frequency of distribution of Osteons	Percent
1	20 -28	26	63.4
2	28-48	15	36.6
3	Total	41	100.0

This table indicates the out of 41 samples, the age group of 20 – 48 years, distribution of osteons more (63.4 %) in the 20 – 28 years of age and less (36.6%) in the age group of 28 – 48 years of age.

Pie Chart 1.



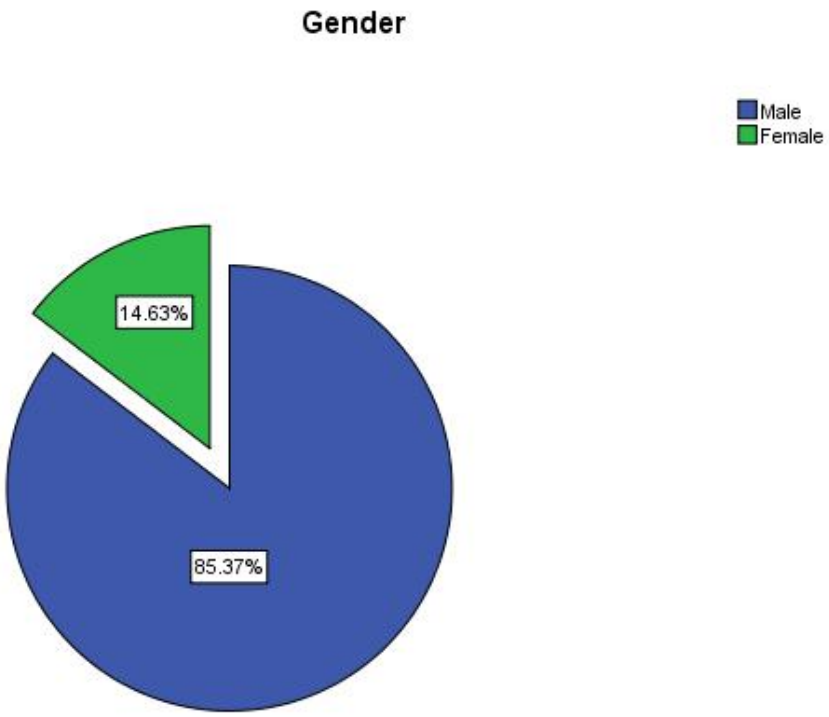
For easy analysis of study sample, the age group was separated into two types comprising age group of 20 – 28 years (63.41%) and 29 – 48 years (36.59%).

Table.5 - Frequency of gender distribution of osteons

Sl.No.	Characteristics	Male	Female
1	Total samples	35	6
2	Distribution of osteons (%)	85.4	16.6

Since the study group comprised lesser number of female gender, distribution of osteons are also lesser 16.6%.

Pie Chart 2



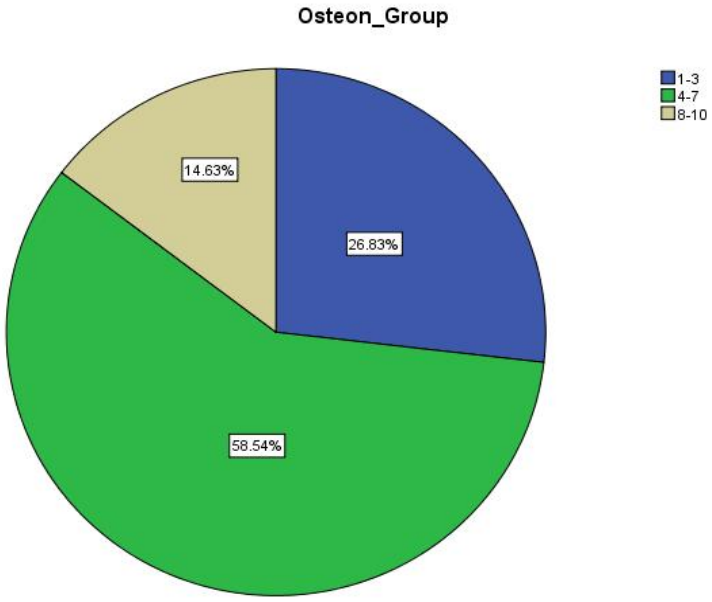
The study comprised 85.37% of males and 14.63% of females.

Table.6- Frequency of distribution of osteon group

Sl. No.	Osteon group	Number of cases	Percent (%)	Valid Percent	Cumulative Percent
1.	1 – 3	11	26.8	26.8	26.8
2.	4 -7	24	58.5	58.5	58.5
3.	8 -10	6	14.6	14.6	14.6
4.	Total	41	100.0	100.0	100.0

This table indicates, out of 41 cases, 24 cases (58.5%) have 4 – 7 osteons per field. 26.8% cases (11 samples) have only 1 – 3 osteons. Maximum number of osteons per field is 8 – 10 and only 6 cases (14.6 %) have 8 – 10 osteons per field.

Pie chart 3



Osteon_Group

This pie chart shows the distribution of osteon groups comprising 1 – 3 osteons in 26.83% of cases, 4 – 7 osteons in 58.54% of cases and 8- 10 osteons in 14.63% of cases.

Table.7Osteon_Group * Gender Crosstabulation

			Gender		
			Male	**Female**	**Total**
Osteon_Gr	1-3	Count	11	0	11
		% within Osteon_Group	100.0%	.0%	100.0%
	4-7	Count	18	6	24
		% within Osteon_Group	75.0%	25.0%	100.0%
	8-10	Count	6	0	6
		% within Osteon_Group	100.0%	.0%	100.0%
	Total	Count	35	6	41
		% within Osteon_Group	85.4%	14.6%	100.0%

This chart shows the distribution of osteon groups comprising 1 – 3 osteons in 26.83% of cases, 4 – 7 osteons in 58.54% of cases and 8- 10 osteons in 14.63% of cases.

Table.8- ANOVA**

	Sum of Squares	Df	Mean Square	F	Sig.
Regression	31833.259	2	15916.629	161.622	.000
Residual	3840.741	39	98.481		
Total	35674.000	41			

The independent variable is Osteon_Avg.

**The equation was estimated without the constant term.

From this study, p-value is measured

as 0.001. It indicates satisfactorily significant in age estimating formula.

Table 9.Quadratic

Model Summary*

R	R Square	Adjusted R Square	Std. Error of the Estimate
.945	.892	.887	9.924

The independent variable is Osteon_Avg.

* The equation was estimated without the constant term.

The $r^2$is 0.89, that is 89% of the variance in age estimation from the sample can be explained by counting the number of osteons.

Table.10 – Coefficients

	Unstandardized Coefficients		Standardized Coefficients		
	B	**Std. Error**	**Beta**	**t**	**Sig.**
Osteon_Avg	11.086	.975	2.067	11.366	.000
Osteon_Avg^2	-.933	.136	-1.247	-6.857	.000

Regression equation: $Y= ax^2+bx+error$

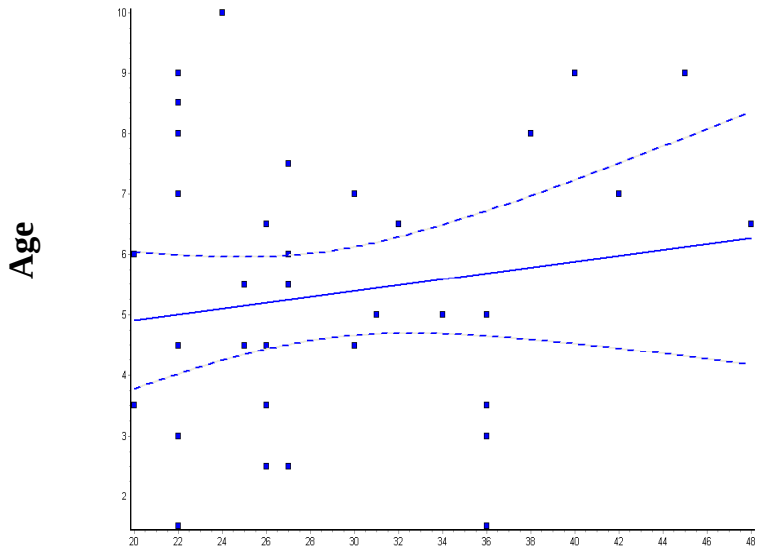
(Y= Age; A and B = No of osteon's average; X= 2.067; X^2 = -1.247)

Graph 1 showing distribution of osteons for both genders:

(Total number of samples, n = 41)

____ Linear Regression

------ 95% Confidence Level



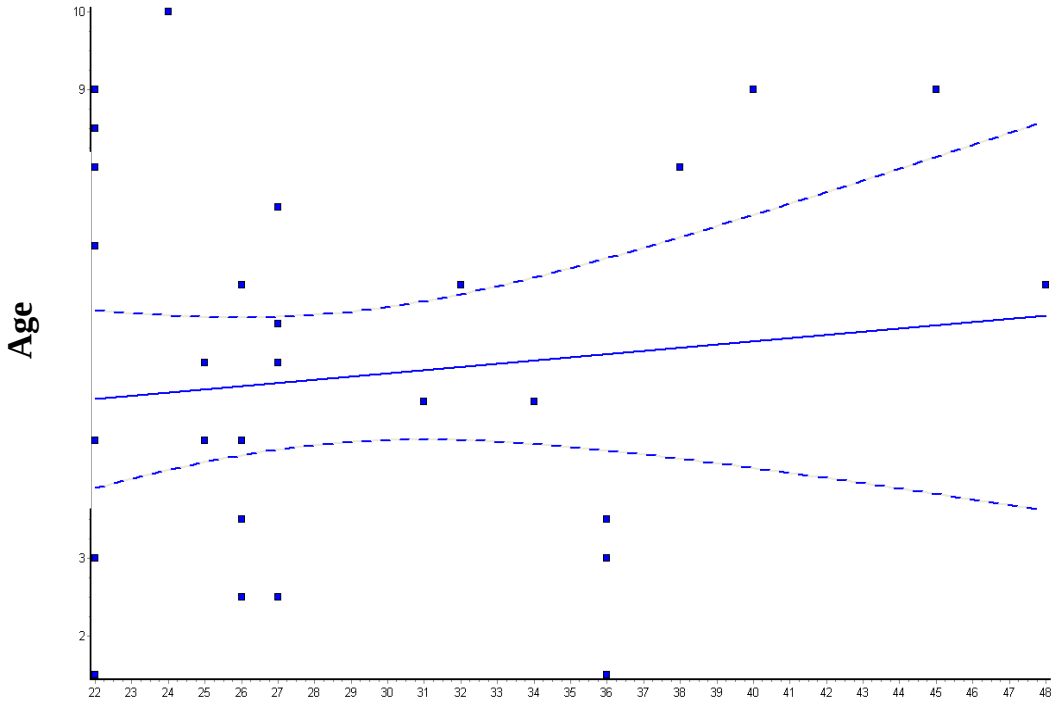
This linear regression graph showing that the number of osteons is increasing viz. 20 years – 4.8 osteons; 22 years – 5 osteons; 32 years – 5.5 osteons; 42 years – 6 osteons and 48 years – 6.5 osteons.

Graph 2 showing distribution of osteons for male gender

(Number of male samples, n = 35)

____ Linear Regression

------ 95% Confidence Level



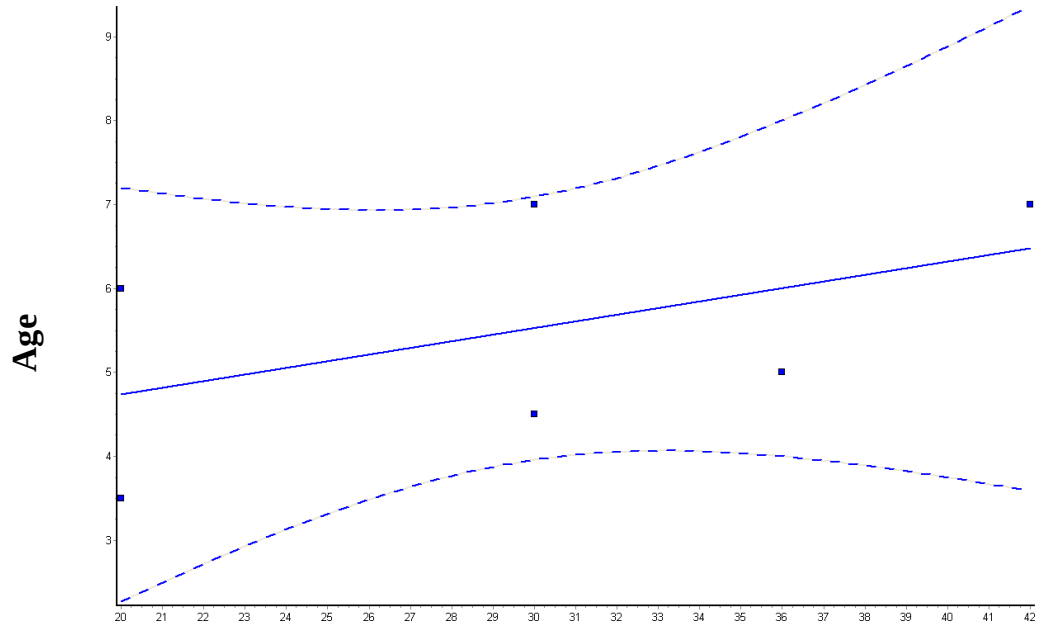
This linear regression graph showing that the number of osteons is increasing viz. 22 years – 5 osteons; 32 years – 5.5 osteons and 42 years – 6 osteons.

Graph 3 showing distribution of osteons for female gender:

(Number of female samples, n = 6)

____ Linear Regression

------ 95% Confidence Level



Osteons

This linear regression graph showing that the number of osteons is increasing, viz. 20 years – 3.5 osteons; 30 years – 4.5 osteons and 36 years – 6 osteons.

DISCUSSION

After the complete analysis, the study showed that the number of osteons is in increasing trend as the age advances. It is supporting the other studies using the microscopic analysis of rib[51] to estimate age. The number of osteons is increasing such a way that, the number of osteons is increasing viz. 20 years – 4.8 osteons; 22 years – 5 osteons; 32 years – 5.5 osteons; 42 years – 6 osteons and 48 years – 6.5 osteons.

The equation for detecting age at death, derived is,

Regression equation: "Age = $Y= ax^2+bx+error$" in which Y= Age; A and B = No of osteon's average; X= 2.067; X^2 = -1.247.

In this study, the 'r^2'value is estimated as 0.892. And so, the study concluded that, the study is positively correlated the similar previous studies and as the age advances, the number of osteons is also increased.

This study indicates, the distribution of osteons within a given cross-section of rib do not follow a consistently dispersed pattern, despite the fact that, remodeling can occur on any bone surface not covered by cartilage or osteoid. This study correlates well with the study done by **Martin et al., 1998** and **Pankovich et al., 1974.**These estimates have proven most accurate when analyzing individuals between the third and fifth decades of life.

This method continues to be useful until the entire cortical surface has been filled with osteons signaling asymptote during the fifth or sixth decade of life. At this point any new osteons will be completely obliterating any evidence of previous osteon fragments.

This study also indicates OPD is significantly correlated well with age of the individual. This increasing trend is proved in both the genders.

Age dependent variables that have been associated with increasing age include cortical area and osteon density. Some studies have suggested using mean osteon size as an age dependent variable though it remains controversial.

The association of OPD to aging is well documented and several age estimation formulas have been proposed based on remodeling theory.

The number of samples should be increased. And, the selection of bone samples should be concentrated from the 20 – 60 years of age group.

For developing the study further, more than one variable should be taken instead of using only one like osteon number.

For the best results, osteon counting method should be used with phase analysis method of rib.

Use of ribs in age estimating studies:

At first, it is easily accessible and removable bone from the body in the post mortem examination , unlike the other long bones like femur, tibia etc. Removal of just one rib causes almost no mutilation or disfigurement of the body.

Unlike other long bones, which are usually weight bearing bones, ribs are less vulnerable to mechanical stress and in turn stress induced aging changes.

Here, there is absence of sampling error, which occurs in using wedges of bone sample taken from the thick bone like tibia or femur. Absence of sampling error gives high positive correlation values.

Next, the entire diameter of the rib is smaller (1.2 x 1.0 cm). So, it is completely well fit against the microscope glass slide which is measured 7.4 x 2.5 cm. And so, cutting, taking sample from the rib for cross section and counting of osteons are simple, easy and less time consuming procedures. It is very much important because of the less probability for inter observer variations in osteon counting.

Another added advantage of using sternal end fourth rib, is the possibility of age estimation by assessing observable phase changes like, increase in the depth of articular depression and increased porosity at the margins of articular surface in the gross appearance itself[50].

Limitations of the study:

Like other studies, this study also had some typical and peculiar difficulties in each and every stage of the study. They may affect or may not affect the results.

1. While taking bone samples from the rib cage, there may be chance to get unevenly broken in the half the way with partial thickness of the rib. This may have been overcome by selecting the adequate thickness of the bone to cut.

2. While grinding the bone sample, if it had got ground thinner than the adequate, there were chances to get broken of the sample.

3. If the bone had not ground adequately, difficulties may be encountered not only in counting the osteons and also in visualizing the osteon itself.

4. There was possibility of getting pricked by the uneven fractured edges, in the grinding finger, when the bone sample is becoming thin and in turn occupational hazards. To overcome this, the analyzer may use double gloves when grinding. Once again, grinding the bone sample by using of double gloves may lead to lesser grip in grinding.

CONCLUSION

This study illustrates the estimation of age at death by counting number of osteons in the rib from the age of 20 to 48 years. It was proved that the number of osteons is increasing when the individual gets aging as per all the previous studies (Kerley 1965; Alqvist and Damsten 1968 and Waatnabe et al 1998).

In this study, the age of the individual is able to estimate maximum up to 48 years of age. Here, we could not able to determine whether the graph linear would go on increasing continuously or may decrease after some age. If we increase our samples to the maximum extent of life, it would be able to predict the pattern of number of osteon.

The available r^2 value in this study was 0.892, which is well correlated with the similar previous studies.

But, because of the lesser availability of female samples (n = 6), the differences between the osteon count in both the genders could not be predictable.

Because of less destructive nature of this method, for better results, the study may be continued with multiple variables instead of single one. It is also recommended that, usage of gross change in rib phase analysis in association with histological analysis, for best yields.

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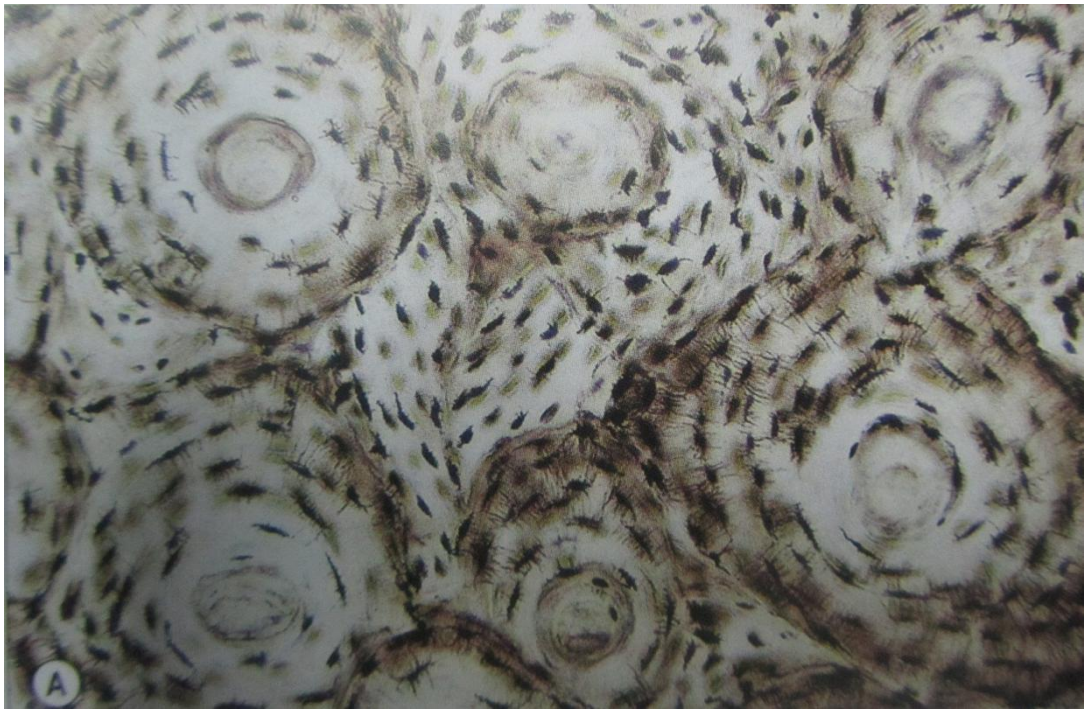

Fig.1 - Osteons in dry ground transverse section of bone. (Photograph from Gray's Anatomy of Clinical Practice, Fortieth Edition).

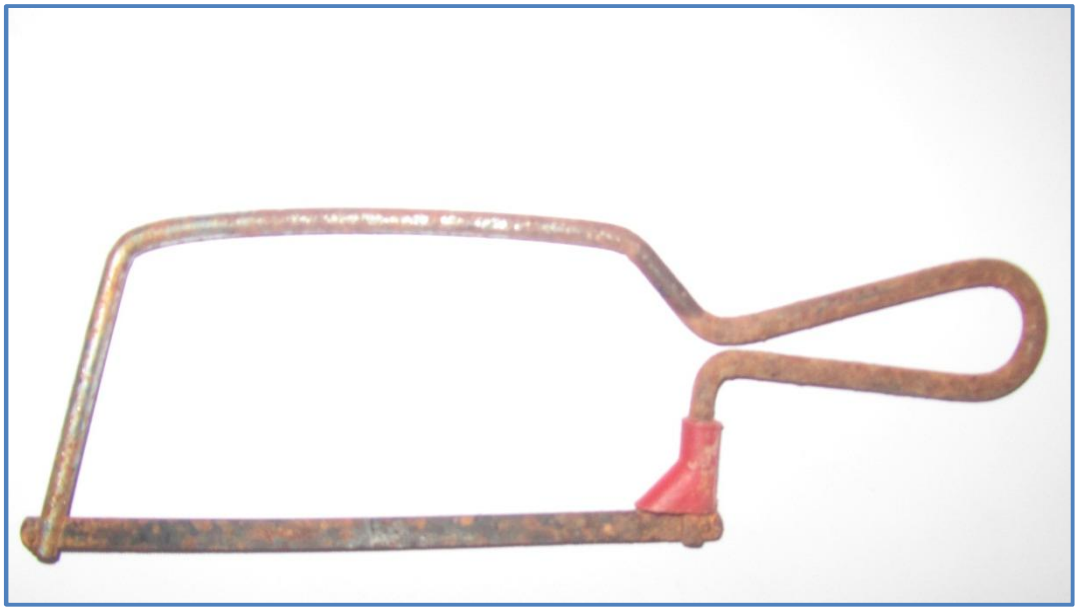

Fig.2 - Fine toothed hack saw for rib cutting.

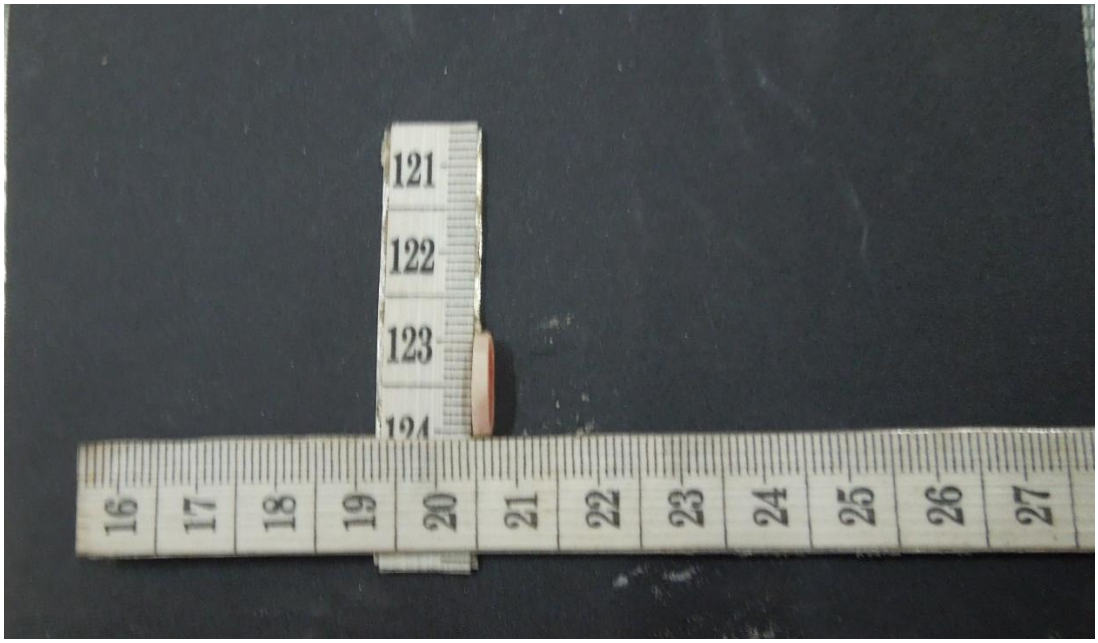

Fig.3 – Slice of the rib on the grinding surface of the abrasive paper.

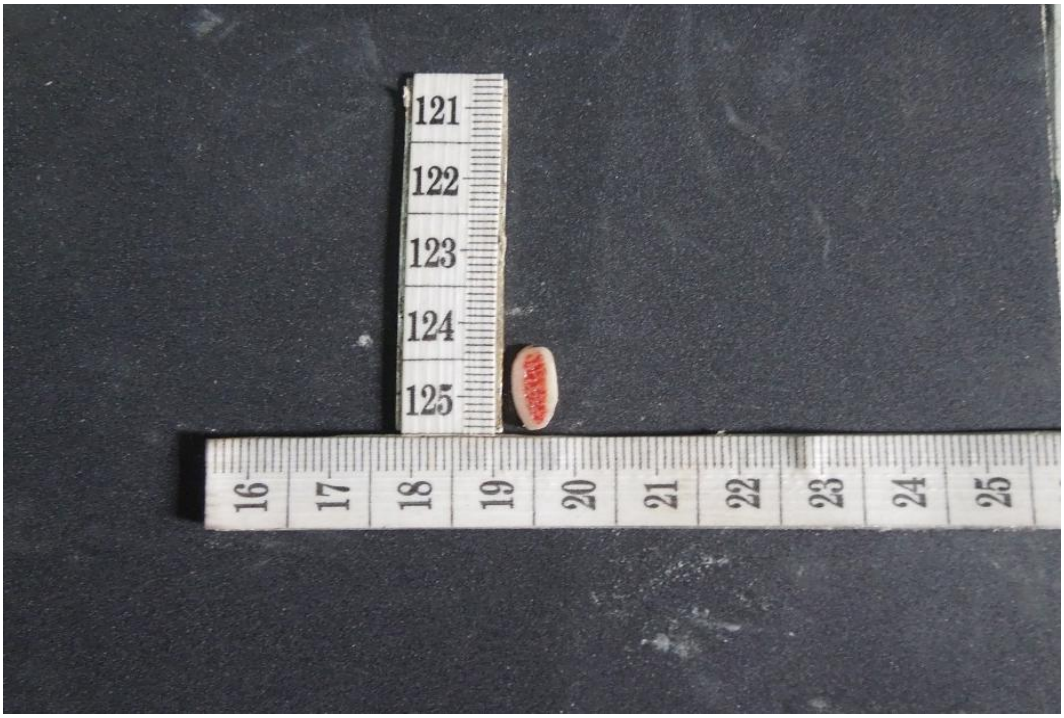

Fig.4 – Cross sectional view of the sliced rib on the grinding surface of the abrasive paper.

Fig.5 - Water proof Carborundum abrasive paper grit no. 320.

Fig.6 - Water proof Carborundum abrasive paper grit no. 400.

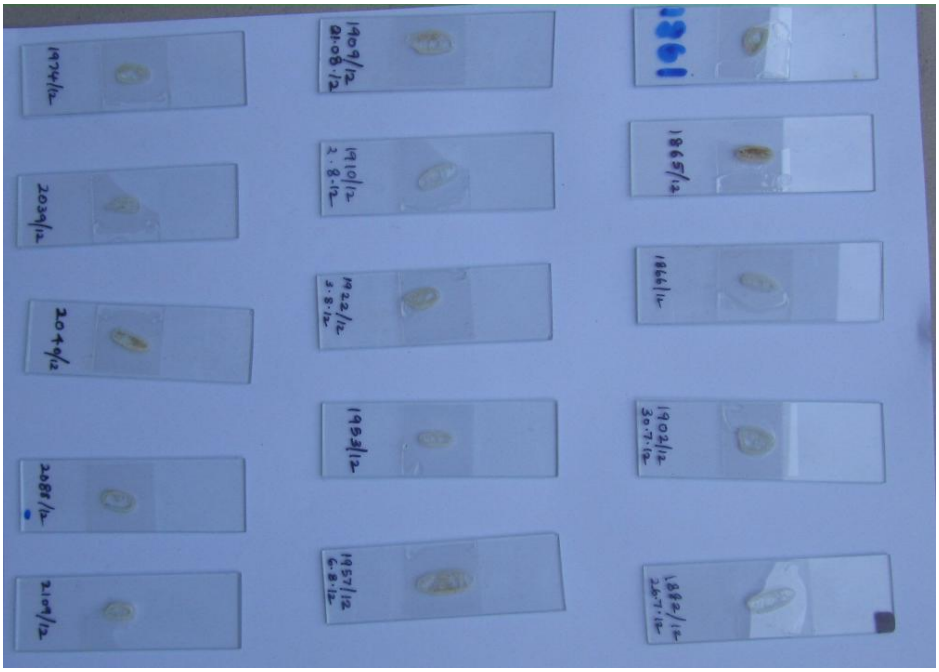

Fig.7 - Samples of bone slices in glass slides.

Fig.8- Closure view of slice of bone in glass slide.

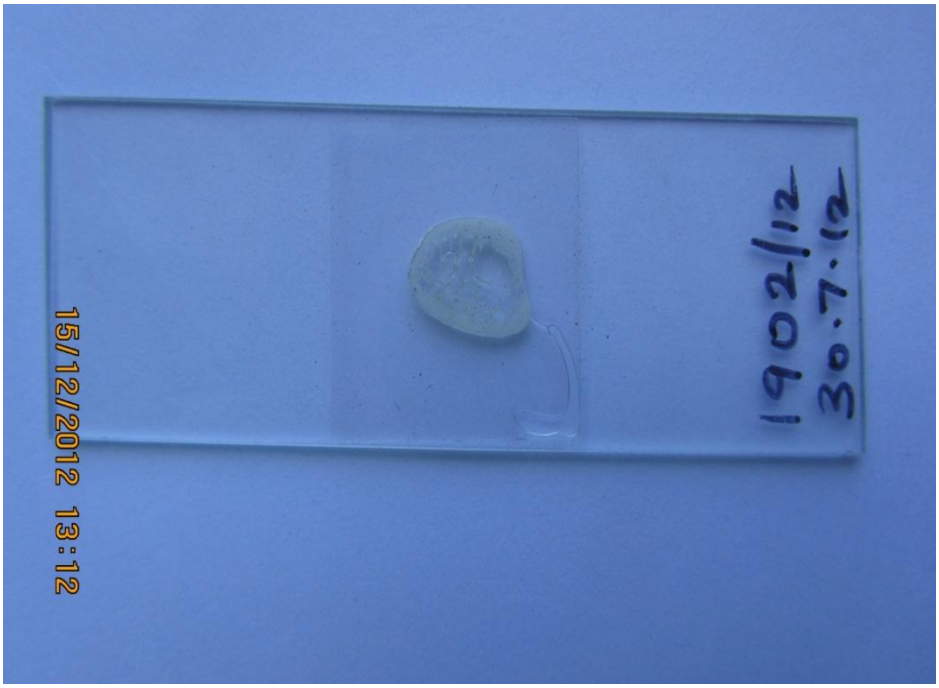

Fig.9 - Slice of bone in glass slide with details of the bone sample.

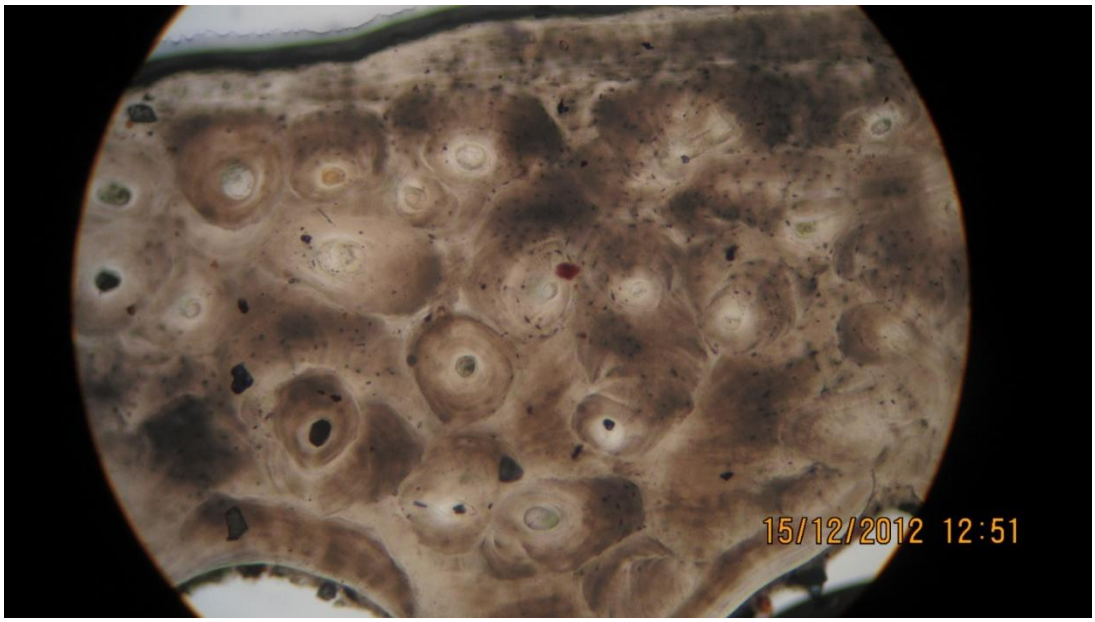

Fig.10 - Osteons in microscopic cross section of rib. Under 10 X.

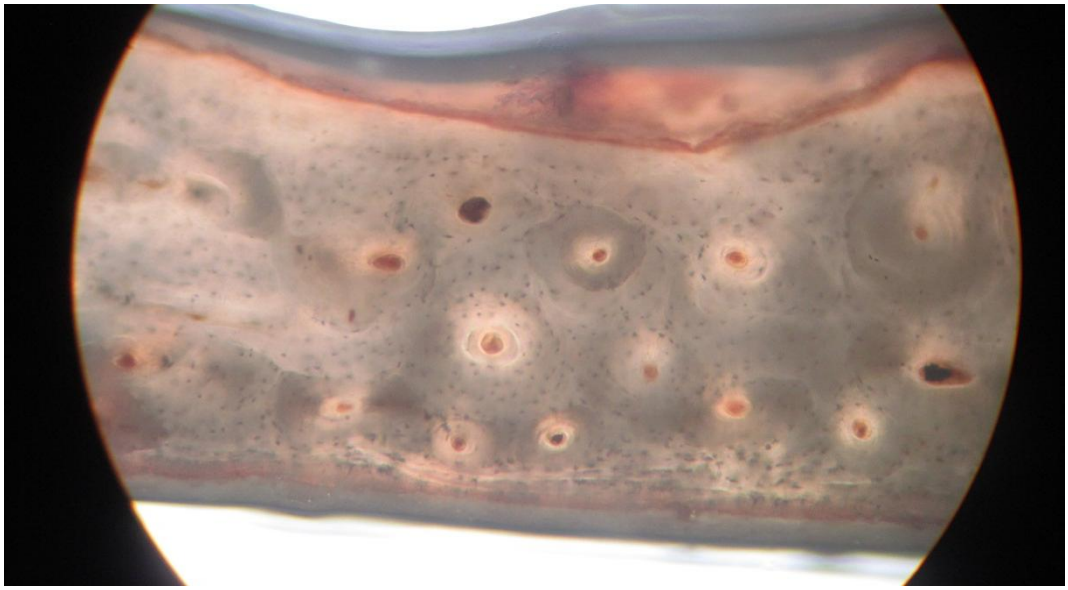

Fig.11- Osteons in microscopic cross section of rib. Under 10 X.

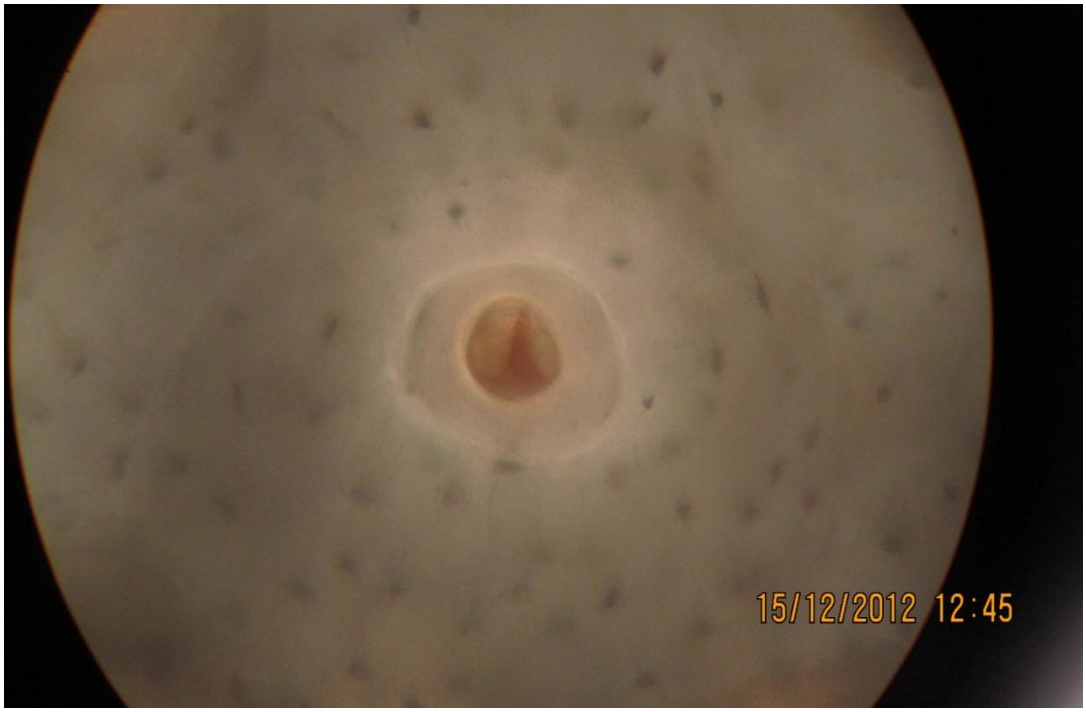

Fig.12 - Closure view of single osteon. Under 100 X.

MASTER CHART

S.No	PM NO	Age	Gender	Cause of Death	No.of osteons in field1	No.of osteons in field 2	Average no. of osteons
1	1536/12	20	F	Poisoning	3	4	3.5
2	1861/12	20	F	Fall	6	6	6
3	1512/12	22	M	RTA	4	5	4.5
4	1577/12	22	M	RTA	4	2	3
5	2040/12	22	M	RTA	1	2	1.5
6	1585/12	22	M	RTA	2	1	1.5
7	2088/12	22	M	RTA	8	10	9
8	2171/12	22	M	TTA	8	8	8
9	1737/12	22	M	RTA	7	7	7
10	1656/12	22	M	TTA	6	8	7
11	1731/12	22	M	RTA	9	8	8.5
12	2416/12	22	M	RTA	5	4	4.5
13	1866/12	24	M	RTA	10	10	10
14	1462/12	25	M	RTA	5	4	4.5
15	1684/12	25	M	RTA	5	4	4.5
16	1537/12	25	M	Fall	6	5	5.5
17	1865/12	26	M	RTA	2	3	2.5
18	1882/12	26	M	Poisoning	3	4	3.5
19	1579/12	26	M	Fall	4	5	4.5
20	2310/12	26	M	RTA	2	3	2.5
21	2335/12	26	M	RTA	7	6	6.5
22	1902/12	27	M	RTA	5	6	5.5
23	2110/12	27	M	TTA	3	2	2.5
24	2227/12	27	M	RTA	5	7	6
25	2417/12	27	M	RTA	6	9	7.5
26	2419/12	27	M	Fall	5	6	5.5
27	1505/12	30	F	Poisoning	8	6	7
28	1627/12	30	F	Fall	4	5	4.5
29	1957/12	31	M	Hanging	5	5	5
30	1922/12	32	M	Hanging	7	6	6.5
31	1805/12	34	M	Hanging	5	5	5
32	1828/12	36	M	TTA	3	4	3.5
33	1953/12	36	M	TTA	3	3	3
34	2109/12	36	M	RTA	2	1	1.5
35	2316/12	36	M	RTA	3	3	3
36	2422/12	36	F	RTA	5	5	5
37	1910/12	38	M	Suspicious	7	9	8
38	2429/12	40	M	Fall	8	10	9
39	2039/12	42	F	RTA	6	8	7
40	1909/12	45	M	Poisoning	10	8	9
41	2719/12	48	M	RTA	7	6	6.5