

Tuberculosis origin

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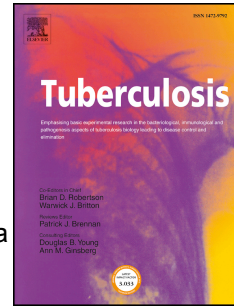
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Tuberculosis origin: the Neolithic scenario

Israel Hershkovitz^{at}, Helen D. Donoghue^{bt}, David E. Minnikin^c, Hila May^a, Oona Y-C. Lee^c,
Michal Feldman^a, Ehud Galili^d, Mark Spigelman^{ae}, Bruce M. Rothschild^f, Gila Kahila Bar-
Gal^g

^aDepartment of Anatomy and Anthropology, Sackler Faculty of Medicine, Tel-Aviv
University, Tel-Aviv, Israel

^bCentres for Clinical Microbiology and the History of Medicine, University College London,
London, UK

^cInstitute of Microbiology and Infection, School of Biosciences, University of Birmingham,
Edgbaston, Birmingham, UK

^dIsrael Antiquities Authority, Jerusalem, and Zinman Institute of Archaeology, Haifa
University, Israel

^eKuvin Center for the Study of Infectious and Tropical Diseases, Hebrew University-
Hadassah Medical School, Jerusalem, Israel

^fBiodiversity Institute and Departments of Anthropology and Geology, University of Kansas,
Lawrence KS 66045, USA,

^gThe Koret School of Veterinary Medicine, The Hebrew University of Jerusalem, Rehovot,
Israel

Email addresses:

anatom2@post.tau.ac.il; h.donoghue@ucl.ac.uk; d.e.minnikin@bham.ac.uk;

hilamay@gmail.com; leeoy@bham.ac.uk; michalfe@gmail.com;

udi@israntique.org.ilspigelman@btinternet.com; bmr@ku.edu; gila.kahila@mail.huji.ac.il;

***Corresponding author:** Professor Israel Hershkovitz, Department of Anatomy and
Anthropology, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv 69978, Israel
Tel: 972-3-6409495, Fax: 972-3-6408287, e-mail: anatom2@post.tau.ac.il

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30 † These authors share senior authorship

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33 **Summary**

34 This paper follows the dramatic changes in scientific research during the last 20 years
35 regarding the relationship between the *Mycobacterium tuberculosis* complex and its hosts –
36 bovids and/or humans. Once the *M. tuberculosis* and *M. bovis* genomes were sequenced, it
37 became obvious that the old story of *M bovis* evolving into the human pathogen should be
38 reversed, as *M. tuberculosis* is more ancestral than *M. bovis*. Nevertheless, the timescale and
39 geographical origin remained an enigma.

40 In the current study human and cattle bone samples were examined for evidence of
41 tuberculosis from the site of Atlit-Yam in the Eastern Mediterranean, dating from 9250-8160
42 (calibrated) years ago. Strict precautions were used to prevent contamination in the DNA
43 analysis, and independent centers used to confirm authenticity of findings. DNA from five *M*
44 *tuberculosis* genetic loci was detected and had characteristics consistent with extant genetic
45 lineages. High performance liquid chromatography was used as an independent method of
46 verification and it directly detected mycolic acid lipid biomarkers, specific for the *M.*
47 *tuberculosis* complex. These, together with pathological changes detected in some of the bones,
48 confirm the presence of the disease in the Levantine populations during the Pre-pottery
49 Neolithic C period, more than 8,000 years ago.

50 **Key words:**

51 Ancient DNA; Neolithic; origin of tuberculosis; paleopathology;

52

53 1. Introduction

54 Human tuberculosis (TB) persists as a global epidemic with disproportionate effects on low-
55 income populations. Modern genetic data supported by the archaeological evidence indicate
56 that the *Mycobacterium tuberculosis* complex (MTBC) may have co-existed with humans for at
57 least 15,000 years since the Neolithic.^{1,2} The disease reached near-epidemic proportions in the
58 rapidly urbanizing and industrializing societies of Europe and North America in the 18th and
59 19th centuries.³

60 Despite extensive research over period of more than 100 years, the timing, cause and
61 geographical origin of TB in humans is still under debate. Until the end of the previous
62 century, it was commonly believed that animals, especially bovine, transmitted the ancestral
63 *Mycobacterium* to humans – divergent evolution. As infection with tuberculosis spreads in two
64 major ways, by the respiratory route directly from another infected person (e.g., *M.*
65 *tuberculosis*) or by the gastrointestinal route mainly by drinking milk infected or milk
66 products with the bovine tubercle bacillus (*Mycobacterium bovis*),⁴ the notion that newly
67 domesticated cattle, sheep or goats in the Eastern Mediterranean region during the
68 agricultural revolution (ca. 8,300-5,500 BC), is the source of the disease in humans, became
69 common.⁵ Even when this idea of zoonotic transmission of *M. bovis* to Early Neolithic farmers
70 was widespread, we pointed out the following criticisms:⁶ 1) It was unclear when and how
71 the *M. bovis* spread among domesticated cattle; 2) The oldest known human skeletal evidence
72 of TB from the Mediterranean region, other than those of the Pre-Pottery Neolithic (PPN) C
73 site of Ain Gaazal,⁷ were all roughly dated to a much later period – the fourth millennium BC
74 or later.^{6,8} Also, this later date was reflected by pathological and molecular findings reported
75 for Egyptian mummies (some dating back to the XXIst Dynasty) and skeletons (the oldest
76 dated to 3300 BC) that were reported to have tuberculosis pathology;^{9,10} 3) The spread of TB
77 from cattle to human occurs largely by drinking infected milk, yet milk consumption did not
78 start until the “Secondary Products Revolution” in the fifth-fourth millennium BC.¹¹
79 Furthermore, according to Keusch et al.,¹² by two years of age virtually all Neolithic children

80 were lactase-deficient, i.e., they lacked the ability to metabolize milk. Biological tolerance of
81 adult populations to bovine milk and milk products only began in the Neolithic period.¹³ In
82 this case only infants would have consumed milk and thus contracted bovine TB; 4) When
83 considering TB infection, herd size is of greater relevance than human population size.¹⁴ With
84 few exceptions, the harsh unpredictable Mediterranean environmental conditions, including
85 large arid zones and hilly topography, are suitable for goats but not for raising large herds of
86 cattle. Based on the above arguments, at that time we rejected the 'domesticated-bovine-
87 hypothesis' for TB and concluded that the appearance of human TB was probably associated
88 with the beginning of urbanization in the Fertile Crescent region during the fifth-fourth
89 millennium BC, during the Chalcolithic-Early Bronze Age c. 3.500 BC.

90 In the last decade of the 20th century it was shown that the identification of *M. tuberculosis*
91 DNA in ancient bones is possible.¹⁵ Less than 10 years later, the plethora of molecular studies
92 of the MTBC – both ancient and modern – showed that there is no direct evolutionary
93 relationship between *M. bovis* and *M. tuberculosis* but these were divergent evolutionary
94 lineages, with *M. tuberculosis* being more ancestral.¹⁶ Genetic analysis of the pathogen from a
95 Pleistocene bison bone (17,000 years) showing tubercular-like infection indicated greater
96 similarity to *Mycobacterium tuberculosis* and *M. africanum* rather than to *M. bovis*.¹⁷
97 Furthermore, the overwhelming majority of studies that have examined MTB complex aDNA
98 by spoligotyping^{17,18} demonstrate that the organisms are not *M. bovis*. The sole exception to
99 date is the detection of *M. bovis* in a group of Iron Age semi-nomadic pastoralists from Siberia
100 dating from the 4th century BC to 4th century AD.¹⁹ Further genetic studies, based on
101 coalescence analysis have even suggested the possibility of human to bovine transmission of
102 TB, whereby the most ancestral human MTB may have infected livestock and through a
103 parallel evolutionary process established tuberculosis in cattle (*M. bovis*) and goats
104 (*Mycobacterium caprae*).²⁰ Nonetheless, this and other DNA studies adhered to two basic
105 notions: the first that the origin of the disease in humans is within the Fertile Crescent; the
106 second that the transition from human to domesticated animal hosts is linked to the
107 development of agriculture some 13,000 years ago.^{1,16,20}

108 As TB is still one of the leading infectious diseases worldwide, with an estimated 1.4 million
109 deaths in 2011²¹ the questions of the time and conditions surrounding the emergence of *M.*
110 *tuberculosis* are important. The primary aim of the current research was to present both the
111 published and later findings from the Pre-pottery Neolithic C site of Atlit-Yam in an attempt
112 to answer those questions.

113 *1.1 Background on the site and its inhabitants*

114 Atlit-Yam is one of the major submerged sites discovered and studied during the 1980s and
115 1990s. Hershkovitz et al. (2008)²² gives the full bibliography that describes the site, its
116 structures and occupation. The site is located 300 to 500 m offshore and 8-12 m below sea
117 level in the North Bay of Atlit, 10 km south of Haifa (34°56' E, 32°42.5' N). Stone foundations
118 of several rectangular structures, paved floors, long straight walls, hearths, round megalithic
119 structures, storage and production installations, and water wells have been discovered, all
120 embedded in dark clay. The structures and installations are sparsely scattered over the site
121 with wide-open spaces between them. The site was dated to the end of the Pre-Pottery
122 Neolithic period (PPNC). Radiocarbon dates on charcoal and waterlogged plant remains
123 range from 8180 to 7250 years BP (9250-8160 BP calibrated). The rich, well-preserved finds of
124 Atlit-Yam include botanical and faunal remains, stone, flint and bone tool assemblages, and
125 human bones. The site is one of the earliest prehistoric Mediterranean fishing villages ever
126 excavated. Human bones were revealed in ninety-one different locations at the site, of which
127 forty-six were recognized as graves dug into the clay. Most burials (70%) were located in
128 specific areas, adjacent to walls or installations. No grave showed evidence of stone
129 construction, or surface marking. Burials were mainly primary, containing mostly (75%)
130 single interments, situated around the rectangular structures and rarely in within them. In
131 some cases, grave goods were added to the graves. Secondary burials were rare. Grave goods
132 were found in fifteen burials.

133 The health status of the Atlit-Yam population was relatively good, as attested by the life
134 span of the population. The pathologies identified are mainly associated with infectious
135 diseases, such as ear infections due to diving (auditory exostosis), spondylolysis due to

136 intensive rowing activities, anemia due to the marshy environment and probably tuberculosis
137 following cattle domestication.²² Dental wear associated with weaving fishing nets and dental
138 diseases was also identified.

139

140 **2. Materials and Methods**

141

142 The remains of 64 individuals from Atlit-Yam were examined for TB lesions. All human
143 bones are housed at Tel Aviv University. Identification of TB was based on both
144 morphological (macro and micro) and molecular analyses. All cases with bony lesions
145 indicative of TB were sampled for MTBC aDNA, either directly from the lesion itself or
146 from a bony area with a rich blood supply.

147

148 *2.1. Morphological analysis*

149 Osseous criteria for TB: As many infectious diseases tend to produce similar bone
150 changes, osseous criteria alone are not sufficient to reach a definite diagnosis of TB.

151 *2.1.1. Osseous criteria for the presence of TB in infants, children and adolescents*

152 All skeletons were inspected for the following gross osseous changes, all of which are
153 indicative for potential presence of tuberculosis in sub adult and children: (a) convoluted
154 engraving on the inner aspect of the cranial bones, a phenomenon termed '*Serpens Endocrania*
155 *Symmetrica*' (SES);²³ (b) periosteal reactive bone of tubular bones characterized by destruction
156 of the cortex and formation of an expanded shell of periosteal reactive bone;²⁴ (c) growth
157 deficit and/or intrauterine growth retardation; (d) deformity of long bones (due to foci
158 destroying a growth plate);²⁵ (e) presence of multiple lesions throughout the skeleton.

159 *2.1.2. Osseous criteria for the presence of TB in adults*

160 Osseous changes, indicative for potential presence of tuberculosis in adults are: (a) presence
161 of SES;²³ (b) presence of hypertrophic osteoarthropathy;²³ (c) local destruction and cavitation
162 in cancellous bone; (d) local changes in the epiphyses of long bones, mainly undermining and
163 resorptive grooving along the line of the synovial attachments; (e) bony ankylosis;²⁵ (f)

164 cavitation and or collapse (wedge-shape vertebra) of vertebral body; (g) destruction of hip
165 and/or knee joints; (h) proliferative bone reaction on the ribs.²⁴

166 2.2. *Histological sections*

167 Fragments of affected bones were used for histological sections. The bones were cleaned
168 with water (ultrasonic bath) and immersed in alcohol (90%). The bones were then
169 embedded in methymethacrylate. The tissue block was cut into 150 µm thick sections
170 using a slow-speed diamond saw (Isomet: Buehler). The sections were ground and
171 polished (Phoenix Beta: Buehler) to a final thickness of 15-30 µm and surface stained with
172 H&E.

173 2.3. *Molecular analysis-Human bones*

174 All molecular work was conducted in dedicated aDNA laboratories, taking strict precautions
175 against contamination. DNA was extracted from two Atlit-Yam samples, an adult female and
176 an infant, using guanidine thiocyanate lysis buffer and silica-based purification. The extracted
177 DNA was amplified via PCR and characterized using deletion analysis, spoligotyping and
178 sequencing.²² The presence or absence of the *M. tuberculosis*-specific deletion (TbD1) was
179 determined by targeted PCR²² and by spoligotyping pattern.¹⁸ Negative PCR findings are not
180 proof of absence, due to the damage and breakdown of aDNA over time and the localization
181 of pathogen molecular markers within the host. However, a positive result does confirm TB,
182 especially in combination with typical TB-associated morphology, histology and
183 biochemistry.

184 2.4. *Molecular analysis-cattle bones*

185 Samples were taken from five cattle bones with no visible pathological changes and
186 were processed as described above.

187 2.5. *Lipid biomarkers*

188 Extraction, derivatisation and high performance liquid chromatography (HPLC) analysis of
189 mycobacterial cell wall mycolic acids was carried out on samples from both the infant and
190 adult. For examination of lipid biomarkers an established protocol was carried out.²²

191

192 3. Results

193 3.1. Paleopathology

194 The skeletal remains of well-preserved individuals from the site of Atlit-Yam were
195 examined for lesions consistent with a possible diagnosis of tuberculosis. Among the 64
196 specimens studied, three specimens showed bone pathology suggestive of tuberculosis: a – an
197 adult woman buried together with an infant (Fig. 1); these skeletons were later sampled for
198 molecular examination (see below); b – an adult male. The infant, though small in size, was
199 estimated (on a very fragmented skeleton), to be less than 1 year old based on crown
200 development and long bone dimensions. The infant shows SES on the inner aspect of the
201 cranial bones (Fig. 2c) and hypertrophic osteoarthropathy (HOA) lesions – a periosteal
202 reaction of tubular bones characterised by the formation of an expanded shell of periosteal
203 reactive bone on the long bones (Fig. 2a,b). Both lesions are indicative of tuberculosis. The
204 woman, estimated to be around 25 years old based on teeth attrition, epiphyseal ring
205 ankylosis and separated symphysis pubis, had a periosteal reaction affecting the distal
206 diaphysis of one tibia, a bony change associated with HOA. The adult male exhibited a
207 destruction of the anterior vertebral body of a thoracic vertebra (Fig. 3), known as Pott's
208 disease and characteristic of TB.²⁶ No proliferative bone reaction was observed on the ribs.
209 The histological analysis (Fig. 2b) clearly shows that the new bone formation rests on the
210 original bone surface without infiltrating or destroying it. This indicates that the
211 inflammatory process originates in the periosteum and/or the surrounding soft tissue, and
212 not in the medullary cavity, as the consistency of the compact bone is undisturbed.

213 3.2. Molecular analysis

214 Ancient DNA analysis was conducted on the ribs and several limb bones of the woman and
215 from the long bones of the infant. *Mycobacterium tuberculosis* (MTB) complex DNA was
216 detected in the bones of both the woman and infant.²² Multi-copy IS6110 and IS1081
217 amplicons were obtained and sequenced from the rib of the woman and the infant long bone.
218 The results were replicated in two laboratories: at UCL an IS6110 123bp product from the
219 woman (right rib) and a 92 bp nested IS6110 product from the infant were obtained,

220 sequenced and found to be identical to contemporary *M. tuberculosis* sequences.²²
221 Additionally, a 104 bp sequence of the IS1081 gene fragment obtained from the infant long
222 bones was found to be identical to contemporary *M. tuberculosis* sequences.²² The
223 amplification and direct sequences of the IS6110 gene region were successfully replicated at
224 the Hebrew University of Jerusalem.

225 A TbD1 flanking PCR, based on a single site on the DNA strand, was successfully
226 amplified for the infant sample and a complete DNA sequence for the 128bp amplicon with
227 the outer primers was obtained²² identical to that in the *M. tuberculosis* reference sequence.
228 Nested PCR was also successful. Spoligotyping was successfully performed on both adult
229 and infant specimens. There were several faint or dubious positives, and it was noted that
230 spacers 33, 35, 37-43 were present and that spacers 2, 8, 21, 34 and 36 were either absent or
231 only faintly positive on three or more occasions. However, a consensus spoligotype, based on
232 any positive result, contained no missing spacer regions.

233 None of the 5 bones of cow analyzed for MTB aDNA yielded positive results.

234 3.3. Lipid biomarkers

235 Long-chain fatty acids were extracted as pentafluorobenzyl (PFB) esters, and fractions
236 corresponding to PFB mycolates were obtained.²² After treatment with pyrenebutyric acid
237 (PBA) these fractions produced PBA-PFB mycolates, which, after reverse phase HPLC, gave
238 profiles closely similar to standard *M. tuberculosis*.²² Further normal and reverse phase HPLC
239 gave detailed profiles for each sample, reinforcing the identity with *M. tuberculosis*.

240

241 4. Discussion

242 The current study sought answers to three basic questions regarding TB, namely when,
243 where and how did *M. tuberculosis* first infect humans and cause disease? The morphological
244 (macro and micro) examination, molecular investigations and lipid analysis have shown
245 clearly that people at the Atlit-Yam site dated to the Pre-pottery Neolithic C period (6,200-
246 5,500 BC) were infected by *M. tuberculosis* and that it was of a TbD1-deleted lineage. Further
247 support for this finding is from a contemporaneous PPNC site of Ain Gaazal, in Jordan,

248 where vertebrae with osseous lesions typical of those caused by the TB bacillus were found.⁷
249 Not surprisingly, TB appeared several hundred years later in the early Neolithic populations
250 of central Europe, ca. 5400-4800 BC.² There are archaeological and genetic studies²⁷ indicating
251 that early farmers from the Near East started migrating into Europe during the 6th millennium
252 BC. Did they (or their cattle) carry the TB bacillus with them? The genetic evidence for Near-
253 Eastern origins of European cattle²⁸ appears to be significant. Interestingly, sub-typing the
254 aDNA of the bacillus found in the Neolithic European site of Derenburg revealed that, in
255 contrast to modern European *M. tuberculosis* lineages, four MTBC strains still harbored the
256 TbD1 region.² In the world today, such TbD1-intact strains are found mainly in the Far East
257 and Pacific Rim. Also at Derenburg, one strain was found to belong to the RD9-deleted MTBC
258 lineage that includes *M. africanum* and *M. bovis*.

259 Current data suggest that the MTBC is as old as 40,000 years.²⁰ However, it is notable that
260 there are no documented cases of TB among human populations prior to the PPNC period. Of
261 more than a thousand Natufian and Pre-Pottery Neolithic A and B skeletons excavated in the
262 eastern Mediterranean region, none demonstrated osseous lesions associated, directly or
263 indirectly, with TB. This contrasts with the evidence for the rise of infectious diseases among
264 early farmers compared to their preceding hunter/gatherers.²⁹ Furthermore, there are global
265 data to suggest that the transition to farming and animal husbandry not only subjected
266 humans to new pathogens but also increased the risk of infectious diseases due to living
267 conditions and diet.³⁰ It therefore seems the presence of cattle was pertinent for TB after all.
268 Atlit-Yam is the only Neolithic site where cattle bones dominate the zooarcheologic record
269 and where cattle were a major component of the diet. In the absence of detectable *M. bovis*,
270 the cattle may be important by supporting a larger and denser human population, thus
271 indirectly encouraging the conditions for the long-term maintenance and transmission of *M.*
272 *tuberculosis*.

273 Finally we conclude that the infant had disseminated primary tuberculosis: the only DNA
274 sequences for single copy sites were obtained from the infant material, which suggests a
275 higher bacterial load during life. In infants less than a year old the present risk of developing

276 active disease on infection with *M. tuberculosis* is high due to the inadequacy of their immune
277 system. The size of the infant's bones, and the extent of the bony changes, suggest a case of
278 acquired neonatal tuberculosis, where an adult suffering from contagious pulmonary
279 tuberculosis infects an infant shortly after birth. Childhood tuberculosis is closely linked with
280 adult disease, and is usually a sentinel event in the community, demonstrating recent
281 transmission. In the absence of any effective treatment, advanced tuberculosis carried
282 significant mortality for both mother and child, so it is unsurprising for a presumed mother
283 and child to succumb and be buried together. We believe that these are the earliest confirmed
284 cases of the disease. Based on the spoligotype and TbD1 deletion, the genetic lineage
285 resembles the Principal Genetic Group PPG1b. The relationship between genetic variants of
286 *M. tuberculosis*, geographical location and the presentation of disease is poorly understood at
287 present. Our study, we believe, provides a marker in real-time to indicate how this major
288 pathogen has changed its relationship with its human host.

289

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293

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295 Not required

296

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301

302

303 **Author contributions**

304 I.H. and E.G. conducted the archaeological excavation; I.H., H.M. and M.F. assessed the
305 palaeopathology; H.D.D. did the DNA molecular analysis in London and shares senior
306 authorship with I.H.; D.E.M. and O.Y-C.L. analyzed lipid biomarkers; M.S. coordinated
307 the project; B. M. R. was a leading researcher in the TB project; G.K.B. performed PCR and
308 spoligotyping in Israel and initialized the second stage of the TB aDNA study at Atlit-
309 Yam.

310 All authors discussed the results and commented on the manuscript.

311

312 **Competing interests**

313 The authors declare no conflict of interest

314

315

316

317

318

319

320 **Figure legends**

321 **Figure 1:** The mother and the child from Atlit Yam. Both were confirmed positive for TB by
322 both morphological and aDNA analysis

323

324 **Figure 2:** Evidence for TB on the infant long bones: new bone formation on the shaft of a long
325 bone - HOA (a), radiating appearance of the appositional bone on the infant long bone (b),
326 grooves (SES) on the inner table of the calvaria (c).

327

328 **Figure 3:** Beveled thoracic vertebra of an adult person suggestive of TB

329

330

331

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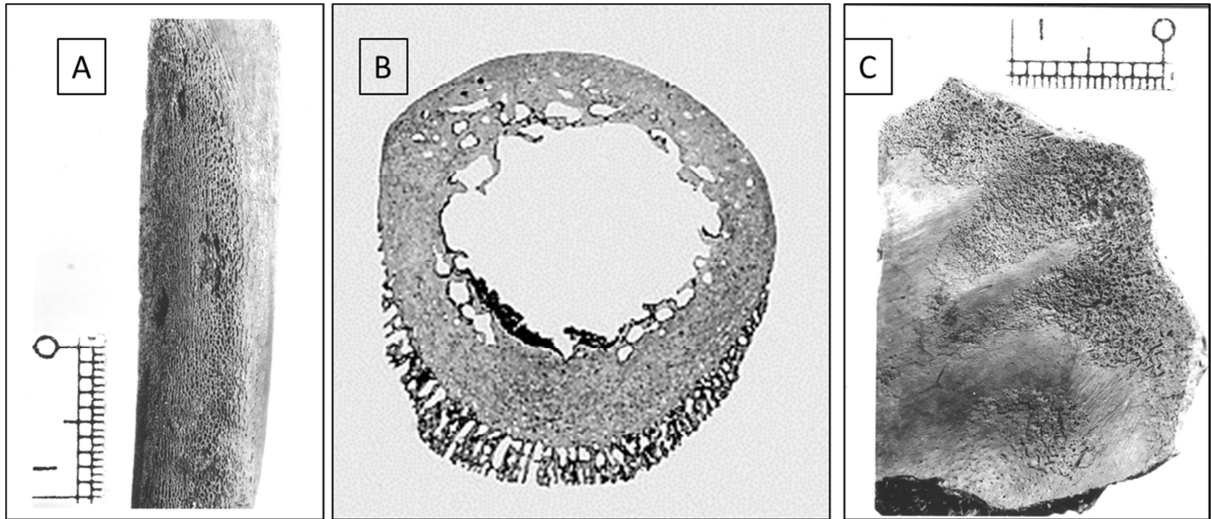
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