UNIVERSITY OF BELGRADE
School of Medicine

Ksenija M. Đukić

Bone macromorphology at muscle attachment sites: its relationship with the microarchitecture of the underlying bone and possible implications for the reconstruction of habitual physical activities of past populations

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Makromorfološki izgled kosti na mestu mišićnih pripoja: odnos makromorfologije i mikroarhitekture kosti na mestu pripoja i moguće implikacije na rekonstrukciju svakodnevih fizičkih aktivnost drevnih populacija

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Doctoral Advisor:

Prof. Dr. Marija Djurić, University of Belgrade – School of Medicine, Institute of Anatomy, Laboratory for Anthropology, Dr Subotica 4, 11000 Belgrade, Serbia

Reviewing Committee:

1. Vladimir Bumbaširević, MD PhD, Full Professor of Histology, School of Medicine, University of Belgrade.

2. Milan Milisavljević, MD PhD, Full Professor of Anatomy, School of Medicine, University of Belgrade.

3. Vujadin Ivanišević – PhD, Principal Research Fellow, Institute of Archaeology, Belgrade.

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Author

ABSTRACT

Bone macromorphology at muscle attachment sites: its relationship with the microarchitecture of the underlying bone and possible implications for the reconstruction of habitual physical activities of past populations

Background: The term "enthesis" is usually used to mark the sites of muscle attachments, as well as attachments of ligaments and joint capsules to bones. In the last three decades, studies concerning entheses in human skeletal remains have attempted to reconstruct the habitual physical activities of past populations. The evaluation of entheseal morphological appearance was suggested for the identification of gross workload patterns, which could be used in the interpretation of labour division on a gender, age or social basis in ancient populations.

There have been four major research streams in literature with regard to entheses and entheseal changes (EC). The first group of studies mainly focused on the analysis of the histological structure of the attachment site. The second group of studies focused of visual scoring methods based on the macromorphological features of the attachment surface with the aim of evaluating the degree of muscle use. The third group included studies dealing with the relationship between EC and the biomechanical properties of long bones, while a fourth group of studies was aimed at reconstructing the habitual physical activities of past populations, using visual scoring methods.

Regardless of the fact that habitual physical activities or stress patterns of past populations are frequently reconstructed based on EC, there is no direct experimental evidence for the relationship between muscular activity and particular macroscopic entheseal scores.

Although entheses were investigated from macroscopic, histological and biomechanical aspects, it is surprising that microarchitectural studies of the underlying bone are still lacking, despite the well-known potential of bone microarchitecture to reflect mechanical loading. It is widely accepted that the bone adapts its structure to changes in its mechanical environment, and a number of studies have reported the relationship between bone microarchitecture and loading patterns in different skeletal sites. However, despite numerous studies reporting that bone morphology is affected by mechanical loads and that bone has a self-optimising capability, there is no

comprehensive study dealing with this issue in the region of the entheses. Specifically, it is not known if different macromorphological expressions of muscle attachment sites correlate with the microstructural characteristics of the underlining bone.

The present study analysed the macromorphological and microstructural characteristics of the bone at the muscle attachment sites, hypothesising that mechanical loading influences the microarchitecture and macromorphology of the bone at the entheses. Previous studies mostly focused only on the macroscopic appearance of entheses and it is unknown whether different morphological expressions of muscle attachment sites correlate with the microstructural characteristics of the bone. Therefore, the reliability of interpretation of widely used macroscopic scores of EC is questionable. A microstructural evaluation of entheseal morphology will ensure a more reliable methodology for the interpretation of labour division on a gender, age or social basis of archaeological populations. Also, when analysing the differences in the macroscopic morphological appearance of EC between the medieval Avarian population of horse riders and the agricultural population of medieval Vinča and *Sirmium*, the criteria for the identification of horse riders in human skeletal remains was investigated.

The microstructural assessment of the entheses may contribute to the understanding of the biomechanical relevance of entheseal gross morphology, further clarifying the role of EC morphology in the interpretation of the habitual physical activities of past populations.

Hypotheses: Our hypotheses were that the bone morphological changes of muscle attachment sites follow the overall bone adaptation to different mechanical loadings; also that macroscopic changes of the bone on the muscle attachment site correlate with microarchitectural changes in the underlying bone; and that the different morphological appearances of the muscle attachment sites in human skeletal remains are the consequence of different muscle activities and, therefore, could be helpful in the reconstruction of the habitual physical activities of past populations.

Material and method: The skeletal material used in this study derives from four medieval cemeteries: Pionirska Ulica and Čik in Bečej, Vinča and *Sirmium*. The published archaeological reports indicate that the sites of Pionirska Ulica and Čik are medieval Avarian populations of horse riders while the medieval cemeteries of Vinča and *Sirmium* consist of an agricultural population. The anthropological and macro-structural analysis of entheses in human skeletal remains was conducted in the Laboratory for Anthropology, Institute of Anatomy, School of Medicine, University of Belgrade. Anthropological analyses included the estimation of sex and age at the moment of death, palaeopathological examination, as well as the assessment of dental status. Palaeopathological analyses included macroscopic observation and radiographic analysis.

For the purpose of macrostructural analysis of EC, 19 muscle attachments on the lower and upper limbs were evaluated using the visual three-stage scoring system, proposed by Villotte (2006, 2010).

The microstructural analyses of entheses were carried out in the Laboratory for Anthropology, Institute of Anatomy, School of Medicine, University of Belgrade and at University Medical Center Hamburg-Eppendorf.

The bone samples were harvested using a slow rotating medical saw. The specimens were scanned using micro-computed tomography (Scanco μCT 40, Scanco Medical, Switzerland and SkyScan μCT 1172, Bruker, Belgium) with an isotropic resolution of 10 μm. The microarchitecture of the cortical and trabecular bone was evaluated automatically using a micro-CT evaluation program with direct 3D morphometry. In order to evaluate the normality of data distribution, a Kolmogorov-Smirnov test was performed. The Pearson's Chi-Square test was used for comparing non-parametric features between different groups. The relationship between the obtained data and age was tested by Pearson correlation or Spearman correlation, depending on data distribution. The relationship between the macroscopic stages ("stage" and "site" selected as factors) and the microscopic parameters (selected as "dependent variable") was assessed using analysis of variance (ANOVA) for parametric data, while in the case of non-Gaussian, the distribution of data was assessed using the Kruskal–Wallis test. Bivariate correlation analysis was performed to determine the association between various cortical and trabecular microarchitectural parameters in each of the macroscopic stages.

The statistical significance was set at a level of 0.05. All statistical analyses were performed using SPSS for Windows, version 15.

Results and conclusions: The results suggest that with horse riders the physical activities of the upper limbs were more or less consistent, i.e. performed from younger to older age, while the activity of the lower limbs are related to the intensive use of these muscles in younger individuals. Among the agricultural population, the results show that the entheseal changes are more pronounced in the older age categories. Sexual dimorphism of EC scores in the group of horse riders was noted on the lower limbs only, while in the group of agriculturals it was noted on both the lower and upper limbs. All attachment sites which demonstrated significant differences, showed greater markers of stress in males. A statistically significant difference of the entheseal morphological appearance between the riders' and the agricultural population was found in the upper limbs only in the subscapularis muscle, indicating a greater exposure to stress for the riders' population group, while in the lower limbs only the muscles which are specific for horse riders were singled out. In a further study, the possible successive nature of the widely used three-stage scoring system of entheseal macroscopic changes was investigated, by comparing EC scores with the microarchitectural features at the musculoskeletal attachment sites. Overall, comparing EC scores with the microarchitectural features at the musculoskeletal attachment sites showed a lack of consistent correlation between the established stages of the macroscopic scoring system and the microarchitecture at the entheses, suggesting that the macroscopic entheseal stages might not represent distinct successive phases in bone adaptation to mechanical loading. Microscopic analyses of the differences between the "proliferative" and "resorptive" phases of entheseal changes demonstrated that from the initial flat surfaces at the point of the muscle attachment site, two directions of EC can be developed.

However, the bony part of the entheses has not received due consideration in literature. In particular, in order to profoundly comprehend the bone's microstructural adaptation to mechanical loading by the muscle, the stress transfer between muscle and bone has to be understood. Further studies are needed to demonstrate how the stress transfer process is influenced by the bone microstructure of the entheses.

Keywords: enthesis, entheseal changes, micro CT, horse riders, everyday activities, past population.

RESEARCH FIELD: MEDICINE – Skeletal Biology.

REZIME

Makromorfološki izgled kosti na mestu mišićnih pripoja: odnos makromorfologije i mikroarhitekture kosti na mestu pripoja i moguće implikacije na rekonstrukciju svakodnevih fizičkih aktivnost drevnih populacija

Uvod: termin enteze se koristi da označi mesto na kome se za kost pripajaju mišić, ligament ili zglobna kapsula. U studijama koje se bave proučavanjem skeletnih ostataka humanog porekla istraživanja enteza se uglavnom sprovode sa ciljem da se rekonstruiše svakodnevna fizička aktivnost drevnih populacija. Proučavanje morfološkog izgleda enteza je predlagano kao metod pomoću koga možemo da pretpostavimo pojedine obrasce fizičke aktivnosti koji u kasnijoj interpretaciji mogu da nam sugerišu kakva je bila podela rada u odnosu na pol, godine starosti ili socijalni status pojedinca u jednoj populaciji.

U stručnoj literaturi postoje četiri osnovne grupe istraživanja koja se bave proučavanjem enteza i entezalnim promenama. Prvu grupu čine studije koje se uglavnom fokusiraju na istraživanje histološke strukture tkiva na mestu pripoja. U drugu grupu se ubrajaju studije koje imaju za cilj da uspostave vizuelni skoring sistem koji je baziran na promenama u morfološkom izgledu koštanog dela enteze. Treća grupa su studije koje istražuju vezu između morfoloških promena enteze i biomehaničkih karakteristika dugih kostiju, dok četvrtu grupu čine studije koje na osnovu promena u morfološkom izgledu kosti na mestu mišićnog pripoja pokušavaju da rekonstruišu obrasce ponašanja koji su dominirali u svakodnevnim fizičkim aktivnostima arheoloških populacija.

Uprkos činjenici da je svakodnevna fizička aktivnost drevnih populacija često rekonstruisana na osnovu promena morfološkog izgleda enteza, ne postoje direktni eksperimentalni dokazi koji bi potvrdili povezanost između određene fizičke aktivnosti i specifične morfološke promene na mestu pripoja mišića.

Iako su enteze do sada bile istraživane sa aspekta histologije, biomehanike, kao i sa stanovišta morfoloških promena koje se javljaju na mestu pripoja, iznenađuje činjenica da istraživanja mikroarhitektonskih promena koje se događaju unutar kosti, još uvek nedostaju uprkos dobro poznatoj činjenici da mehanička sila koja deluje na kost utiče i na promene koje se događaju u mikroarhitekturi kosti.

U stručnoj literaturi široko je prihvaćena činjenica da se kost strukturno prilagođava promenama u njenom biomehaničkom okruženju. Međutim, uprkos brojnim studijama koje sugerišu da se morfologija kosti menja u skladu sa mehaničkom silom koja deluje na nju, kao i da kost ima sposobnost remodelovanja, ne postoji sveobuhvatna studija koja se bavi ovom problematikom na mestu enteza.

Naročito, za sada ostaje nepoznato da li različit morfološki izgled na mestu mišićnog pripoja korelira sa mikrostrukturnim promenama koje se događaju u kosti na tom mestu.

Hipoteze: Naše hipoteze su bile da morfološke promene kostiju na mestu pripoja mišića prate sveobuhvatan obrazac adaptacije kosti na različitu mehaničku silu; zatim, da makromorfološke promene na mestu pripoja mišića prate mikroarhitekturne promene u kosti na mestu pripoja; kao i da različit morfološki izgled mesta pripoja mišića je direktna posledica različite mišićne aktivnosti, i zbog toga nam može pomoći u rekonstrukciji svakodnevnih fizičkih aktivnosti arheoloških populacija.

Meterijal i metod: Skeletni materijal koji je korišćen u ovoj studiji potiče sa četiri srednjovekovne nekropole: Pionirska ulica i Čik u Bečeju, *Sirmium* u Sremskoj mitrovici i Vinča nadomak Beograda. Na osnovu objavljenih arheoloških podataka Pionirska ulica i Čik su srednjovekovne avarske nekropole konjanika, dok je na srednjovekovnim nekropolama u Sirmijumu i Vinči sahranjena uglanom zemljoradnička populacija. Antropološka analiza skeltnih ostataka, kao i analiza markera stresa obavljena je u Laboratoriji za antropologiju Instituta za anatomiju Medicinskog fakulteta u Beogradu. Antropološka analiza podrazumevala je odredjivanje pola i starosti individua u momentu smrti, palopatološku analizu, kao i analizu dentalnog statusa individue. Paleopatološka analiza podrazumevala je makroskopsku i radiološku analizu potencijalnih patoloških promena. Kada govorimo o makroskopskoj analizi markera stresa posmatrali smo 19 mišićnih pripoja na gornjim i donjim ekstremitetima u skladu sa preporukama Villotta (2006, 2010). Mikrostrukturalna analiza enteza obavljena u Laboratoriji za antropologiju Instituta za anatomiju Medicinskog fakulteta u Beogradu, i na Institutu za osteologiju i biomehaniku u Hamburgu. Sečenje i priprema koštanih uzoraka obavljena je spororotirajućom medicinskom testerom. Uzorci su skenirani primenom mikrokompjuterizovane tomografije (micro-CT: Scanco Medical µCT 40, Switzerland i SkyScan µCT 1172, Bruker, Belgium) sa izotropnom rezolucijom od 10 µm. Mikroarhitektonske karakteristike kortikalne i trabekularne kosti evaluirane su korišćenjem automatskog mikro-CT programa za evaluaciju i 3D rekonstrukciju. Za statističku obradu podataka u slučaju procene normalnosti raspodele podataka primenjen je Kolmogorov-Smirnov test. U zavisnosti od dobijenog rezultata, primenjeni su parametarski, odnosno neparametarski statistički testovi. Pirsonov hi-kvadrat test korišćen je za poređenje neparametarskih podataka između različitih grupa. Odnos između dobijenih parametarskih podataka i godina individua testiran je korišćenjem Pirsonovom ili Spirmanovom korelacijom u zavisnosti od raspodele dobijenih podataka. Odnos između makroskopskih faza (faza ili lokacija označena kao zavisna varijabla) i mikroarhitektonskih karakteristika (nezavisna varijabla) kostiju testiran je primenom analize varijanse (ANOVA) za parametarske podatke ili primenom Kruskal–Wallis testa za neparametarske podatke. Dvosmerna analiza kovarijanse primenjena je prilikom računanja povezanosti između različitih kortikalnih i trabekularnih mikroarhitektonskih parametara u svakoj makroskopskoj fazi.

Statističke analize su sprovedene u programu SPSS, verzija 15, za Windows operativni sistem. Sve analize su sprovedene na nivou značajnosti od 0,05.

Rezultati i zaključci: Sa pretpostavkom da mehanička sila koju mišić ili tetiva proizvode prouzrokuju promene na makro i mikro planu u zoni enteze ova studija je analizirala makro-morfološke i mikro-arhitektonske karakteristike kosti na mestu pripoja mišića. Naši rezultati sugerišu da je fizička aktivnost kod konjanika, u slučaju gornjih ekstremiteta, bila uglavnom konzistentna tj. kada jedna aktivnost počne da se koristi u mlađem starosnom dobu nastavlja se sa istom i u starijim starosnim dobima na veoma sličan način. Međutim, kada govorimo o mišićnim pripojima na donjim ekstremitetima uočava se intenzivnija upotreba u mlađim u odnosu na starija starosna doba. Kod pripadnika zemljoradničke populacije entezalne promene su daleko izraženije kod starijih individua. Kada govorimo o podeli rada koja je bazirana na polnim razlikama, naši rezultati sugerišu da su enezalne promene na donjim ekstremitetima konjanika specifičnije za muski pol, dok su u grupi zemljoradnika muškarci imali izraženije mišićne pripoje i kod gornjih i kod donjih ekstremiteta. Značajna statistička razlika između morfološkog izgleda mišićnih pripoja kod konjanika i zemljoradnika sugeriše da su konjanici u odnosu na zemljoradnike imali izraženije mišićne pripoje gornjih ekstremiteta, dok je u slučaju donjih ekstremiteta signifikantna razlika uočena u slučaju mišića koji imponuju jahanju. Dalje, poredeći entezalne promene i mikroarhitekturu na mestu mišićnog pripoja istraživali smo moguću sukcesivnu prirodu široko primenjivanog trostepenog skoring sistema. Ustanovili smo da je korelacija između stepena izraženosti mišićih pripoja i mikrostrukturnih promena na mestu pripoja izostala, kao i da procena makromorfoloskog izgleda u skladu sa preporučenom metodologijom najverovatnije ne prati sukcesivne faze u procesu adaptacije kosti na dejstvo mehaničke sile. Analiza mikrostrukture u okviru “proliferative” i “resorptivne” faze entezalnih promena pokazala je da možemo razlikovati dva razvojna smera entezalnih promena.

Iako se enteze do sada zauzele značajno mesto u antropološkoj i medicinskoj literaturi, ipak još uvek ostaje nekoloko nerešenih pitanja. Posebno je važno napomenuti da je povezanost imeđu morfoloških promena na mestu pripoja mišića koje nastaju kao posledica mišićne aktivnosti i mikrostrukturne promene koje se na mestu pripoja događaju kao odgovor na dejstvo mehaničke sile, još uvek nejasna.

Ključne reči: mišićni pripoj, enteze, entezalne promene, mikro-CT, fizička aktivnost, konjanici

NAUČNA OBLAST: MEDICINA – Biologija skeleta.

TABLE OF CONTENTS

1. INTRODUCTION

The term "enthesis" is usually used to mark the sites of attachment of muscles, ligaments and joint capsules on bones, although some authors consider "enthesis" only as the site of muscle attachment (1). It is also called an insertion site, osteotendinous or osteoligamentous junction (2). Benjamin and McGonagle (2001) established the term 'enthesis organ' to define a collection of structures adjacent to, and associated with, the enthesis itself, that help to reduce stress concentration at the attachment site (3). They also introduced the term 'functional enthesis' to describe the wrap-around regions of tendons or ligaments (4). Functionally, entheses provide strong and stable anchorages that promote musculoskeletal movement with concomitant joint integrity. However, they must serve as more than simple anchors because, in linking soft to hard tissue, entheses also need to minimise the risk of damage in the face of high levels of mechanical loading, by allowing the smooth transfer of force between soft and hard tissue (2, 5, 6).

In medicine, entheses are of particular concern to orthopaedic surgeons because of the need to re-attach a tendon or a ligament to a bone. Injury and damage to connective tissues, specifically to tendons and ligaments are common conditions in orthopaedics and often require operative intervention. It is uniquely challenging to recreate the natural smooth transfer of load from a tendon/ligament to a bone that simulates the healthy, original attachment site (7). A variety of surgical techniques have been pioneered that attempt to do so, but most simply involve stapling the tendon/ligament to the bone (2).These procedures are often associated with high failure rates and, consequently, require revision procedures. The management of tendinous injuries and the reconstruction of the insertion site is becoming a popular topic in the field of orthopaedic medicine.

Skeletal attachment sites have also long been of interest to bio-anthropologists and archaeologists in relation to physical activity. It has been widely accepted in anthropological literature that habitual physical activities of past populations can be reconstructed based on musculoskeletal markers in human skeletal remains, i.e., particular morphological features of muscles attachment sites (8-13). Osteological studies are based exclusively on the fact that it is sometimes possible to 'read' dried bones in a macerated skeleton with the assumption that greater physical activity (e.g. when comparing males to females) is reflected by different enthesis markings. The constant stressing of a muscle during daily repetitive tasks of various types, gives bio-anthropologists and archaeologist skeletal marks which reflect habitual activity patterns and this has contributed to the understanding of a wide range of issues related to ancient populations. The evaluation of the so called "Musculoskeletal Stress Markers" (MSM) was suggested for the identification of gross workload patterns, which could be used in the interpretation of division of labour on a gender or social basis (14, 15). For instance, studying health changes at the Dickson Mounds population, Goodman et al. (1984) suggested an increase in physical stress with the adoption of agriculture and a sex-dependent pattern of physical stress. Similarly, through analysis of MSMs of the upper limb, it was reported that physical stress increased with the adoption of agriculture, as the mean MSM scores were higher in the Neolithic populations compared to the Natufian in Levant (9). Based on social character and sexual dimorphism, Havelkova et al. (2011) investigated the prevalence of enthesopathies among individuals living in different everyday environments in the early medieval Great Moravian population. Authors found significant differences in the occurrence of enthesopathies between the population who lived in the castle zone and those who lived in the hinterland (12). In their most recent paper, Villotte and Knüsel (2014) presented the results of an analysis of enthesopathies of the elbow in three time-successive population samples which comprised the prehistoric, pre-industrial historic, and modern European eras. The authors postulated the existence of a persistent sexual division of labour in these European populations involving one or several strenuous activities linked to unilateral limb use (13). Regardless of the fact that habitual physical activities or stress patterns of past populations are frequently reconstructed based on musculoskeletal markers (MSM), there is no direct experimental evidence for the relationship between muscular activity and particular macroscopic entheseal scores, although a number of studies have reported the relationship between bone micro architecture and loading patterns in various skeletal sites, excluding entheses (16-19). Nevertheless, an experimental study on an animal model showed no significant effect of exercise treatment on enthesis morphology (20). Niinimaki (21) analysed the effects of physical activity on bone structural adaptations by studying the relationship between MSM and the cross-sectional geometry of humeral diaphysis (average bending rigidity). She distinguished that covariance between the polar second moment of area and MSM exists at cross-sectional locations under muscle insertions as well as at more distant locations. These results demonstrate both the direct and general effects of muscular loadings applied to diaphyses. Furthermore, Hirschberg (2005) provided a profound study investigating the biomechanical basis for the development of a tubercle or a pit at the position of the muscle attachment site.

1.1 Historical Note on Terminology

The earliest origin of the word ‘enthesis’ is unclear. However, Khan (2002) considers that the word is derived from the adjective *enthetic*. According to the Greek roots of this adjective, this means ‘fit for implanting’. Also, he states that the term *enthetic* was initially used in the l9th century to refer to diseases which had been inoculated or entrenched into a human body from some external source (e. g. infections). During the following century, the use of the adjective reduced, so the noun *enthesis* was used as a synonym of artificial materials used to repair a defect (2). Given that the term *enthesis* was initially used to determine some phenomenon other than a muscle attachment site, it is clear that an *enthesis* has much more recently come to mean the site where a tendon or ligament attaches to a bone. Such a meaning originated in rheumatology, but the usage is now common and has spread to many other branches of medicine and anthropology. One of the very first descriptions of musculoskeletal attachment sites was given by Dolgo-Saburoff (1929) (2). He described the anatomy of a cat’s patellar ligament and its multilayered appearance (2). In 1959, G. La Cava used the term "enthesis" in order to create the notion of "enthesitis". His term "enthesitis" was designed to denote inflammation of tendon attachments onto bones. Subsequently, in the 1970s, authors proposed using the word "enthesis" to designate the area where a tendon, a capsule or a ligament attaches to a bone and "enthesopathy" to indicate any pathological changes of this structure (22).

In bio-anthropology, as researchers have begun a more intensive engagement in the study of these types of morphological features, a variety of terms have been proposed and used. In 1986, Dutour suggested the term enthesopathies, Robb (1998), muscle markings, while muscle crests was proposed by Angel et al. (1987). However, the most well known and widely used terminology was provided by Hawkey and Merbs’ publication (1995), in which they proposed the term Musculoskeletal Stress Markers (MSM) (23-26). Terms such as “Evidence for Occupation”, “Skeletal Markers of Occupational Stress” and “Activity-Induced Stress Markers” were also in use until the mid-nineties (22). Although it is inherently imprecise and in some ways misleading, the use of the terminologically referent “MSM” has increased in popularity over time. However, the most unsuitable aspect of the MSM terminology is that it presupposes the involvement of a primary etiological agent. The terminological problem has become increasingly common with the more comprehensive reassessment of entheseal changes. Thus, it is suggested that, while simple, popular, and easily remembered, the “MSM” terminology be replaced with something that is both less biased and more accurately descriptive. Research into several terms was carried out using ScienceDirect (from September 25, 2009) (22). However, among researchers the prevailing opinion is that for terminology which could be used for both pathological and non-pathological cases, "entheseal change" or "entheseal changes" appear to be the most neutral. The term entheseal changes (EC) does not actually imply a causal agent (stress, for instance), a specific nature (e.g. degenerative) or a specific aspect (entheseal new bone formation) (22). Also, researchers have unanimously agreed (International Journal of Osteoarchaeology, special issue for 2013) that entheseal changes abbreviated as EC instead of musculoskeletal markers abbreviated as MSM should be used, as this change in terminology is accompanied by a changed perception of the causal factors behind entheseal changes.

1.2 Anatomical background

1.2.1 A brief overview of entheseal types

According to the characteristics of the tissue at the point where tendons and ligaments meet bone, entheses may be classified into two main groups: fibrous and fibrocartilaginous entheses (5, 27). Although the division between fibrous and fibrocartilaginous entheses seems clear enough, some additional considerations regarding their apparent distinctiveness have been made. Consequently, Villotte et al. (2013) strongly suggest that changes in the two main types of enthesis do not indicate the same phenomena, and it is to be expected that biological anthropologists will no longer combine them in a single study (28). It should be noted that some attachments are actually ‘mixed’ (28). For instance, the majority of the entheses of the masticatory muscles are of this type. Thus, the insertion of the m. masseter is partly periosteal, partly osseous and partly fibrocartilaginous (28, 29). In a fibrocartilaginous enthesis, the periphery may have little or no fibrocartilage (5, 27). Also, fibrocartilage may exist in a small amount at a fibrous enthesis, particularly on the metaphysic, an example of which is the m. pectoralis major insertion on the humerus (5, 27). Finally, the example of the fibrocartilaginous attachment site of the m. iliopsoas located on the lesser trochanter shows that the most distal part of this insertion, at the junction between the lesser trochanter and the femoral shaft, may correspond to the variety of fibrous insertions of the m. iliacus and, as a consequence, this region is highly variable (28). However, the distinction between enthesis into fibrous and fibrocartilaginous entheses is widely accepted and well established in the literature.

1.2.1.1 Fibrous enthesis

In contrast to fibrocartilaginous attachments, anatomical and histological descriptions for fibrous entheses are particularly rare. They attach soft tissues to bone directly or via a mediating layer of periosteum (5) (Fig. 1). Therefore, fibrous entheses may be subdivided into two categories: periosteal and bony where there is no intervening layer of cartilage between the tendon and bone surface (5, 27). In periosteal fibrous entheses the tendon attaches to the periosteum, which consequently indirectly attaches the tendon to the bone. In bony fibrous entheses the periosteum is absent and the tendon inserts directly into the bone itself. In some ways it could be suitable to classify these fibrous attachments as ‘indirect’(periosteal) and ‘direct’ (bony), respectively (5). The division of fibrous entheses into these two subcategories based on the tissue type in the interspaces seems to remain inconsistent. Evidently, a periosteal fibrous enthesis can become a bony one with age, as the periosteum disappears from the attachment site with skeletal maturity (30). However, some fibrous entheses continue to be periosteal throughout life (5). Anchorage at a point of fibrous entheses is achieved through collagen fibres, the so called “Sharpey’s fibres” comprising periosteum, tendon or ligament, which are embedded into the bone (31). At fibrous entheses, blood vessels from the tendon or the ligament may anastomose with those of the bone (28). In medical and bio-anthropological literature, even though they are associated with some of the largest and most powerful muscles in the human body (the deltoid muscle or some large

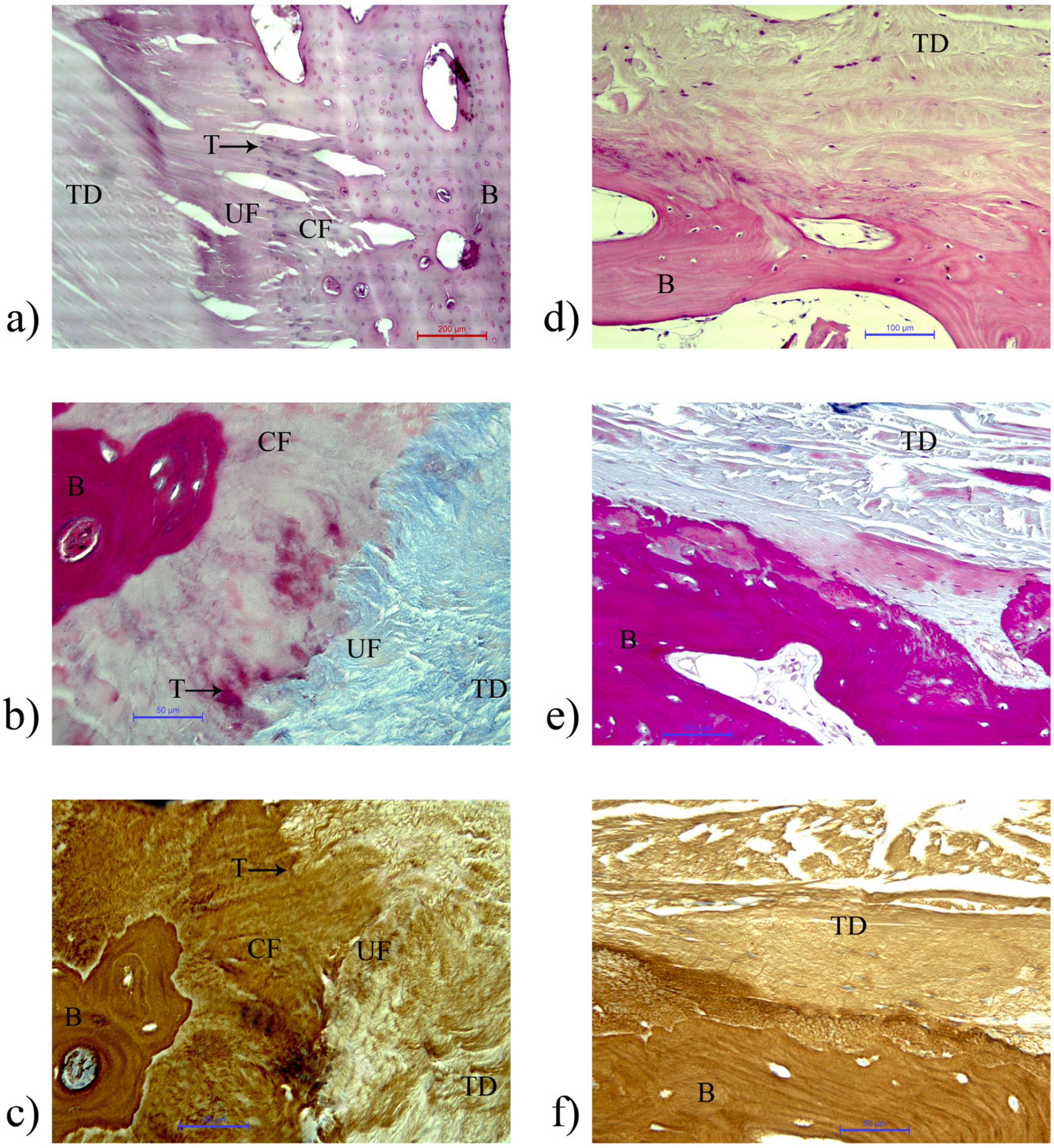


Figure 1. Histological picture of fibrous (attachment site of *m. adductor magnus*) and fibrocartilaginous (attachment site of *m. iliopsoas*) enthesis: a) Hematoxilin impregnation, scale bare 200 µm (B-bone; CF-fibrocartilage; T-tidemark; UF-uncalcified fibrocartilage; TD-tendon), b) Masson's trichrome impregnation, scale bare 50 µm (B-bone; CF-fibrocartilage; T-tidemark; UF-uncalcified fibrocartilage; TD-tendon), c) Silver impregnation, scale bare 50 µm (B-bone; CF-fibrocartilage; T-tidemark; UF-uncalcified fibrocartilage; TD-tendon), d) Hematoxilin impregnation, scale bare 100 µm (B-bone; TD-tendon), e) Masson's trichrome impregnation, scale bare 100 µm (B-bone; TD-tendon), f) Silver impregnation, scale bare 50 µm (B-bone; TD-tendon).

muscles that are attached to the linea aspera of the femur), relatively little attention has been paid to fibrous entheses (5, 27, 28). This partly reflects a clinical bias toward fibrocartilaginous entheses which represent tissue more vulnerable to overuse injuries. Also, the attraction of working with the richer variety of tissues that fibrocartilaginous entheses can offer shouldn't be neglected (5). One should, however, note that when a tendon is surgically reattached to the bone, the newly formed enthesis is initially fibrous even though fibrocartilage may finally be re-formed (5, 32, 33).

The enthesis is usually fibrous at the diaphyses of long bones and on the vertebral column, generally at those points where there are substantial quantities of compact cortical bone (5, 28). Areas of fibrous entheses are often far more extensive than fibrocartilaginous attachments and the boundaries of fibrous attachment sites are less well defined. Where periosteal fibrous entheses predominate, markings on bones are mostly smooth (29). Bony fibrous attachments are those which are associated with raised ridges, or a definite roughening of the bone (e.g. the deltoid tuberosity on the humerus or the inferior temporal line) (5) (Fig. 2).

Figure 2. The deltoid tuberosity on the humerus.

1.2.1.2 Fibrocartilaginous entheses

A fibrocartilaginous enthesis shows a gradual shift from tendinous to bony tissue and is organised histologically in four zones with different cellular and extracellular properties: dense fibrous connective tissue, uncalcified fibrocartilage, calcified fibrocartilage and bone (5) (Fig. 1). At the point of fibrocartilaginous attachment sites there is no periosteum, although, for instance in rats, some tendons that develop to have fibrocartilaginous entheses in adulthood attach to the perichondrium in early development (34). Fibrocartilaginous entheses occur close to the joints of the long bones, but also on short bones and some parts of vertebrae (28). The zones of uncalcified fibrocartilage and calcified fibrocartilage are avascular and are separated from each other by a basophilic line called the tidemark which represents a calcification front (5). The tidemark is practically the mechanical boundary between soft and hard tissues and the widespread view of its significance is largely based on observations of comparable tidemark zones in articular cartilage (5). The tidemark is relatively rectilinear, suggesting that the mineralisation process generally produces a flat surface and is not crossed by blood vessels (28). This is clearly important for minimising the risk of damage to the soft tissues at any enthesis where tendons change their insertional angle with joint movement. In contrast to the tidemark, the junction between the calcified fibrocartilage and subchondral bone is highly irregular (5). The collagen fibres of the tendon continue across the tidemark, as they change from uncalcified fibrocartilage to calcified fibrocartilage. The fibres generally cross the tidemark at right angles. If the enthesis is immediately adjacent to an articular cartilage, the tidemark is continuous across both (27). In some cases the tidemark may be duplicated. This has been interpreted in articular cartilage as a consequence of 'start-stop' phases of calcification (5).

It is not complicated to decide whether a particular type of bony imprint strongly suggests a fibrocartilaginous enthesis. As the tidemark represents the point at which soft tissues are removed during maceration and is relatively straight with the fibrocartilage zones avascular, the site of attachment in a healthy enthesis should be smooth, well circumscribed and devoid of vascular foramina (e.g. as in the markings left by the supraspinatus, popliteus and Achilles tendons) (27) (Fig. 3).

Figure 3. Attachment site of musculus supraspinatus.

1.2.2 The concept of an 'enthesis organ'

Numerous authors have discussed the boundaries of the concept of enthesis (for fibrocartilaginous attachments), and all agree that it cannot be reduced to the attachment of a tendon or ligament. Benjamin et al. (2002, 2004) formalised this idea defining the concept of 'enthesis organ' with the aim to denote a collection of structures adjacent to the attachment site itself, which are functionally associated with the enthesis and which also play an important part in reducing stress concentration at the soft hard tissue interface (4, 5). The idea that there is more to an enthesis than just the attachment site itself appears to have been first considered by Niepel and Sit'aj (1979) (35), however, it's full attention in literature was obtained over a quarter of a century later.

Many entheses have bursae and fat near the insertion site and both of these serve to promote frictionless movement. Collectively, the fibrocartilages, bursa, fat pad and the enthesis itself constitute the enthesis organ. The archetypal enthesis organ is that of the Achilles tendon, where intermittent contact between tendon and bone immediately proximal to the enthesis leads to the formation of fibrocartilages on the deep surface of the tendon and on the opposing calcaneal tuberosity, but similar functional modifications are widespread throughout the skeleton (36). The concept of an enthesis organ is of general significance in understanding attachment sites and may explain the diverse pathological changes, including synovitis, bursitis, and extracapsular changes, seen adjacent to tendon/ligament entheses in spondylarthropathies (4).

1.3 Entheseal changes through life: the effects of sex, age and hormones

Although in anthropological and archaeological literature skeletal changes associated with different life styles in past populations are considered to be an important source of knowledge, there are several limitations to studies of entheseal changes as occupational stress markers. One should not ignore the fact that the position of musculoskeletal attachment sites is a multi-factorial zone where conditions are modifiable depending on various circumstances. Throughout literature scholars have mainly analysed the influence of different confounding factors, such as age, sex and body size, on different morphological expressions of muscle attachments sites (26, 37-39). During aging, bones gradually become thinner and weaker both in men and women. Bone resorption on the endocortical, intracortical, and trabecular surfaces reduces the amount of bone, while simultaneously periosteal bone formation partly offsets the removal of bone on the inner surface. The absolute and relative movement of the periosteal and endosteal surfaces produced by bone formation and bone resorption during growth and aging determine the size, mass, geometry and architecture of the skeleton, as well as its strength (40). The net loss of bone changes through life because of numerous factors such as periosteal apposition, subendocortical resorption, cortical porosity, and trabecular volumetric bone mineral density (41). For instance, the net loss of bone is lower in men than in women because periostal apposition is greater in men (42). Also, the process of bone turnover is increased in menopausal women as well as during pregnancy, when sex hormone levels are significantly changed (43-45). Changes in the qualitative patterns of the bones during aging have a great influence on the bone transformation at the point of an entheses. Therefore, one should keep in mind that even though numerous authors reported more pronounced EC in the elderly, this cannot be exclusively caused by long-term use of muscle.

Other hormonal disorders could affect bone turnover, such as changes in the levels of parathormone (PTH). For instance, in this milder form of the primary hyperparathyroidism, bone density is typically reduced with a proclivity for the greatest reduction at the 1/3 radius, a site of cortical bone. The lumbar spine, a site comprised predominantly of cancellous bone, tends to be preserved and similar to age-matched control subjects (46).

1.4 Enthesopathies

Entheses are vulnerable to overuse injuries and these present as a number of poorly understood, pathological changes that are collectively referred to as enthesopathies. Any such injury can have a significant impact on the lifestyle of the general population and on the ability of athletes to pursue their sport.

Enthesopathies consist both of degenerative-reparative phenomena (fibro-ostosis) and inflammatory reactions (fibro-ostitis) (47). Both processes can lead to hyperostosis with spur formation, as well as to osseous resorption. The corresponding osseous changes in the spine are referred to as syndesmophytes and parasyndesmophytes. Fibro-ostosis is a traumatic or degenerative enthesopathy (47). It also occurs with sclerosis and acromegaly. An especially extensive manifestation is diffuse idiopathic skeletal hyperostosis (DISH). Pathologic findings, clinical presentation, and imaging features depend on the duration, location, and extent of the change. Repetitive trauma also causes changes on entheseal sites including 'inflammatory' tissue reactions with lymphocytic infiltration, edema, and resorptive changes in the bone. This can progress to calcifications and cystic bone defects. The typical location of these types of enthesopathies are calcaneus (posterior and plantar calcaneal spur), greater trochanler, ischial tuberusity, olecranon, and iliac crest, whilst other locations are less common (47). In the second type of enthesopathy –fibro-ostitis, two forms are distinguished: productive fibro-ostitis (new bone formation), and rarefying fibro-ostitis (inflammatory insertion erosions). Fibro-ostitis is a sign of inflammatory joint disease, especially common in psoriatic arthritis, Reiter syndrome and ankylosing spondylitis (47).

In physical anthropology, enthesopathies are often considered as ‘‘musculoskeletal stress markers’’, and are assumed to reflect the activity of the attaching musculature (11, 12, 48). However, as enthesopathy surely includes a pathologically altered condition, can we still consider them as ‘‘musculoskeletal stress markers’’? Even if it is assumed that some enthesopathies could be caused by extreme or repeated muscles use, the further development of this state can be conditioned by various circumstances. For instance, the trauma of the bone of one side limb (e.g. femur) will probably cause a forced use of the other limb. Therefore, scholars should evaluate all issues related with one individual skeleton before an analysis of the morphological appearance of the EC.

1.5 Brief methodological introduction

Understanding the particulars of people's daily lives is a key goal of bio-anthropological and archaeological research. As part of this, the reconstruction of habitual physical activities through entheseal changes (EC) has been widely undertaken in the last 20 years or so. In order to investigate the physical activities of a past population, EC studies have been the most commonly used method. Most EC studies have been performed on archaeological skeletal samples (23, 26, 49-51) as well as those undertaken on skeletons of individuals with known occupational backgrounds, but most have focused on developing and testing methods (10, 48). The studies based on samples with a known occupational history are of vital importance in determining whether the interpretations of EC are valid. The studies aimed at searching for the most effective methodological approach were mainly focused on visual scoring methods based on the macromorphological features of the attachment surface (1, 24, 26, 48). However, Villote (11) noted that all these scoring methods are prone to methodological shortcomings due to the neglect of novel data regarding histological types and the organisation of entheses. As such, according to the characteristics of the tissue at the bone-tendon interface, tendon entheses may be classified into two groups: fibrous and fibrocartilaginous entheses (5). Taking into account these facts, Villotte recommended a visual scoring system for both types of entheses, fibrous and fibrocartilaginous (10, 11, 52).

Such studies frequently considered several morphological grades (phases, stages) of muscle attachment sites, suggesting that macroscopically more pronounced muscular insertions (higher scores, stages) reflect increased activity of the associated muscles (1, 10, 24, 26, 48, 52). However, macroscopic scoring systems were not validated against absolute muscle activities, and there is no direct data indicating that different macroscopic "stages" (scores) are really the successive/temporal stages in the process of activity related enthesis development.

Previous anthropological studies of ECs focused only on the macroscopic appearance of entheses and, hitherto, it has been unclear whether different morphological expressions of muscle attachment sites correlate with the microstructural characteristics of the bone. Consequently, it would appear important to note whether the interpretation of widely used macroscopic scores of EC can be deemed reliable. Therefore, one of the goals of this current thesis is to analyse bone microstructural characteristics at the muscle attachment sites and correlate these features with EC macroscopic scores. It was speculated that a microstructural assessment of entheses may contribute to understanding the biomechanical relevance of entheseal gross morphology and further clarify the role of the ECs' morphology in the interpretation of habitual physical activities of past populations.

The investigation of macromorphological and microstructural patterns of entheses in this study was based on research hypotheses that the bone morphological changes of muscle attachment sites follow the overall bone adaptation to different mechanical loadings. Also, macroscopic changes of the bone on the muscle attachment site correlate with microarchitectural changes in the underlying bone. It was assumed that the different morphological appearances of the muscle attachment sites in human skeletal remains are the consequence of different muscle activities and, therefore, could be helpful in the reconstruction of the habitual physical activities of past populations.

2. RESEARCH GOALS

In order to investigate bone macromorphology at muscle attachment sites and its relationship with the microarchitecture of the underlying bone the specific aims of the current thesis are:

1) To observe whether the macroscopic morphological appearance of EC is related to the age and sex of individuals in the investigated archaeological population.

2) To investigate whether different habitual activities influence the morphology of the muscle attachment sites by comparing the macroscopic morphological appearance of EC of the medieval Avarian population of horse riders and the agricultural population of medieval Vinca.

3) To examine if the macroscopic changes of the bone on the muscle attachment site correlate with microarchitectural changes in the underlying bone through analysis of the structure of four selected entheseal sites on lower extremities.

4) To investigate the possible successive nature of the widely used three-stage scoring system of entheseal macroscopic changes, by comparing EC scores with the microarchitectural features at the musculoskeletal attachment sites.

5) To investigate whether different attachment sites demonstrate different patterns of morphological changes at a macroscopic and microscopic level.

3. MATERIAL AND METHODS

3.1 Study sample

The skeletal material used in this study derives from four medieval cemeteries: Pionirska Ulica and Čik in Bečej, Vinča near Belgrade and *Sirmium* (site No 85) in Sremska Mitrovica (Fig. 4). The published archaeological reports indicate that the sites of Pionirska Ulica and Čik are Medieval Avarian populations of horse riders (53, 54) while the medieval cemeteries of Vinča and *Sirmium* (site No 85) consist of an agricultural population (55)[1].

Figure 4. Location of the archaeological sites.

[1] Information related to the medieval necropolis of Vinča was obtained from a personal comunication with Prof. Dr Nenad Tasic, Department of Archaeology, Faculty of Philosophy at the University of Belgrade.

3.1.1 Pionirska Ulica sample

Pionirska Ulica is an archaeological site located on the southern periphery of the city of Bečej, within the city zone. Archaeological excavations were conducted on three occasions 1979, 1988 and 2003. Excavations were led by the Bečej City Museum. The necropolis of Pionirska Ulica is dated between the 6th and 8th century (53).

3.1.2 Čik sample

The archaeological site of Čik lies between two roads in the area of Bačko Petrovo Selo, on a hill beside a stream that bears the same name. The village of Bačko Petrovo Selo is situated at the point where the Čik rivulet flows into the Tisza River, in the far eastern part of Bačka, northern Serbia. This area represents an excellent example of a place that served as a settlement during several prehistoric and historic periods (54). Systematic archaeological excavations were conducted from 1968 to 1972 by the Provincial Cultural Heritage Preservation Institute in Novi Sad as well as the Department of Archaeology, Faculty of Philosophy (University of Belgrade) (54). The majority of this multifaceted site represents an Avar necropolis from a settlement which is dated between the 6th and 7th century (567-670 AD). During excavations in this locality, 134 graves (118 Avar and 16 Sarmatian) were explored, under the supervision of Professor Jovan Kovačević (54). Although there were 16 Sarmatian graves in all (11.94% of the total number of explored graves on the site) in the Čik necropolis (54), for the purposes of this study only those graves identified as Avarian were considered.

3.1.3 Vinča sample

The archaeological site of Vinča is located in the village of Vinča, just a few miles from Belgrade, on the coastline of the Danube. This archaeological site is famous as being one of the largest prehistoric Neolithic settlements in Europe. The medieval necropolis of Vinča was first discovered in 1906 above the prehistoric settlement. The first systematic archaeological excavations were conducted in 1911 (56). Investigations have continued, with interruptions, until the present day. The human skeletal remains used in this study derive from the medieval necropolis excavated between 2011 and 2014. According to archaeological findings, this necropolis was dated to the period between the 11th and 14th centuries[1].

3.1.4 Sirmium sample (Site No. 85)

Site No. 85 is located at the corner of Vuk Karadžić and Saint Sava Street in the area of a demolished prison in the centre of Sremska Mitrovica, ancient *Sirmium* (Serbia). Systematic archaeological excavations have been conducted since 2002, by the Institute of Archaeology in Belgrade. On these occasions a part of the *Sirmium* imperial complex was explored, along with a Gepidian cultural layer from the 5th century and parts of two medieval necropolises with skeletal burials between the 10th and 12th century and between the 13th and 16th century (55).

The human osteological material used in this study is a part of a medieval necropolis from the 13th to 16th century and was excavated in 2015 (systematic archaeological excavations have been conducted by the Institute of Archaeology, Belgrade). In this territory, during the 13th century, a new medieval settlement of artisans and traders was formed and *Sirmium* was given a new name after the monastery dedicated to the town patron St. Demetrius – *Civitas Sancti Demetrii* (55).

3.1.5 Sample selection

During macromorphological and microstructural analyses, individuals showing pathological changes that could affect the results, such as DISH, osteoarthritis or other arthropathies involving vertebrae and the major joints of lower extremities were excluded. Additionally, no skeletal signs of hyperparathyroidism were found in the included individuals.

To observe whether the macroscopic morphological appearance of EC is related to the age and sex of the individuals in the investigated archaeological population, as well as to investigate whether different habitual activities influence the morphology of the muscle attachment sites of the medieval Avarian population of horse riders and the agricultural populations of medieval Vinča and *Sirmium*, only adult individuals were selected. In further analyses the functional sample size was 82 individuals: 33 females and 49 males.

With the aim to avoid the possible effects of hormonal status on bone structure during menopause and pregnancy (28, 43-45), only adult male skeletons were selected for microstructural analyses. Also, to avoid age and sex as confounders, women, children, adolescents and elderly individuals were excluded, as were individuals showing pathological changes.

In order to compare the relationship between the macroscopic scores of the entheses and their cortical and trabecular microstructural design and to investigate whether different attachment sites demonstrate different patterns of morphological changes at a microscopic level, four insertions on the femur and tibia were evaluated using the EC score visual reference system proposed by Villotte (2006) (10) for the fibrous and Villotte (2013) (52) for the fibrocartilaginous entheses (Fig. 5). The bone samples were taken from 24 male individuals with different macroscopic expression scores of EC: 8 specimens with entheses scored as macroscopic stage A, 8 with stage B, and 8 with stage C. Also, given that the morphological expression of enthesis in stages B and C involves changes which mainly show signs of bone formation (stage B(a) and stage C(a)) as well as bone resorption (stage B(b); stage C(b)), in this part of the study only stages B(a) and C(a) were chosen, as they were expected to show the most straightforward relationship with microarchitecture. It was assumed that such sample homogeneity might provide a better and clearer standpoint for micro-CT analyses.

The morphological expression of enthesis in stages B and C involved changes mainly showing either signs of "bony formation" (stage B(a) and stage C(a)) or "bony resorption" (stage B(b); stage C(b)). The micro-CT analyses of the gluteus maximus enthesis were performed with the aim of investigating the relationship between phases of bone formation and bone resorption and the microarchitectural design of entheses. According to the type of tissue at the attachment site of the gluteus maximus muscle, it belongs to the group of fibrous entheses (Fig. 6). This means that in the interspace between the tendon and the bone fibrous tissue dominates.

Bone specimens from 16 femurs were included. For the purpose of micro-CT analyses of the gluteus maximus only adult males individuals were selected. A macromorphological analysis of those specimens was carried out using the EC score visual reference system proposed by Villotte for the fibrocartilaginous entheses (28).

It should be noted that, regardless of the possible influence of genetic and body mass as confounding factors, osseous modifications of fibrous types of entheses appears to be somewhat more complex than fibrocartilaginous when analysing entheseal structure as indicators of stress applied to the bone (11).

Furthermore, an additional classification of the macroscopic entheseal morphology into two stages was used, i.e., the lesions scored as present/absent, according to recent recommendations (57). In this simplified classification, stage A represents an absence of any changes, while the combination of stages B and C, present another category where any type of change is present.

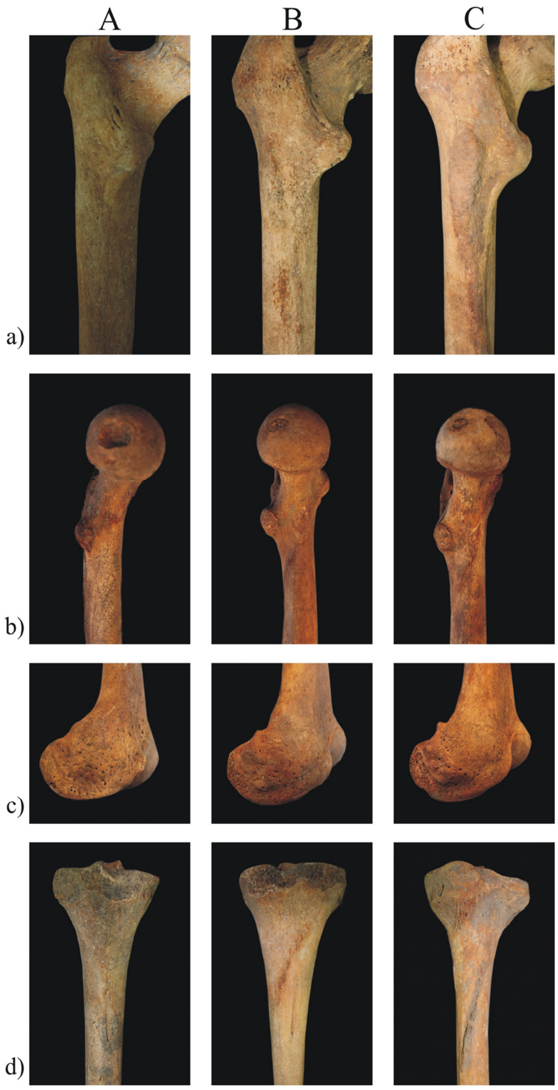


Figure 5. Investigated muscle attachment sites: macroscopic entheseal stages:

A - stage A (see classification in material and method section).

B - stage B (see classification in material and method section).

C - stage C (see classification in material and method section).

a - insertion of gluteus maximus on gluteal tuberosity

b - insertion of iliopsoas muscle on lesser trochanter

c - insertion of adductor magnus muscle on tuberculum adductorium

d - origin of soleus muscle on soleal line.

a)
b)
c)

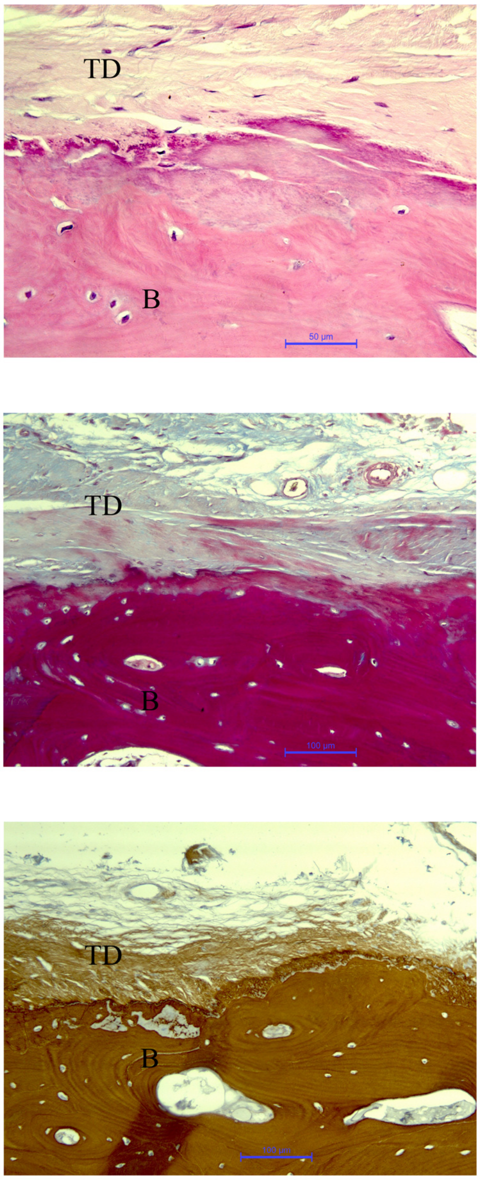


Figure 6. Histological picture of attachment site of *m. gluteus maximus*: **a)** Hematoxilin impregnation, scale bare 50 µm (B-bone; TD-tendon), **b)** Masson's trichrome impregnation, scale bare 100 µm (B-bone; TD-tendon), **c)** Silver impregnation, scale bare 100 µm (B-bone; TD-tendon).

3.2 Anthropological and paleopathological analysis

The anthropological and paleopathological analysis of human skeletal remains was conducted in the Laboratory for Anthropology, Institute of Anatomy, School of Medicine, University of Belgrade.

After archaeological excavation, the bones were washed and cleaned using moderately warm water and small dental instruments.

For the purpose of this study, the anthropological analyses included an estimation of sex and age at the moment of death and a paleopathological examination.

Since hip bones present the most reliable indicators of sex in the human skeleton (58), in this study sex determination was primarily based on the dimorphic features of the os coxae (59). An additional method for sex assessment in this study was based on cranial morphology and standard anthropological criteria were followed (59).

The age assessment of adults was based on the morphological appearance of the pubic simphysis in accordance with the recommendations of the Suchey-Brooks (60) age scheme. However, the final estimation also included Iscan's methods (61, 62) based on the appearance of the sternal end of the ribs, the cranial suture closure (63), dental wear (64) and degenerative changes of the skeletons.

Finally, individual skeletons were distributed into seven age categories (65):

Infans I – 0-7 (years)

Infans II – 8-14 (years)

Juvenilis – 15-22 (years)

Adults – 23-39 (years)

Maturus – 40-59 (years)

Senilis I – 60-70 (years)

Senilis II – over 70 years

Occlusal dental wear was scored by dividing cases into four categories, following the recommendations of Gustafson (1950) (64):

1 – no attrition,

2 – attrition within enamel,

3 – attrition reaching dentin, and

4 – attrition reaching pulp.

Age at death estimation of non-adults followed standard osteological procedures based on the epiphyseal union method (66), diaphyseal length measurement (67, 68) and dental age estimation, based on macroscopic analyses of tooth eruption (69), as well as dental age estimation based on radiographic methods (70, 71).

The skeletons were analysed for signs of bone disease, using diagnostic paleopathological procedures comprising gross examination and digital radiography. Gross examination was based on the macroscopic observation of each bone, including detailed written and photographic descriptions, while radiographs were taken in all cases where pathological changes were observed or suspected.

3.3 Macrostructural analysis of entheses

For the purpose of the macrostructural analysis of EC 19 muscle attachments (Table 1) on the upper and lower limbs, evaluations using the visual three stage scoring system proposed by Villotte (10, 52) were employed.

On the upper limbs the following muscles were analysed: on the humerus, the m. supscapularis, mm. supraspinatus and infraspinatus, m. pectoralis major, m. deltoideus, m. flexor carpi radialis, m. palmaris longus, m. flexor carpi ulnaris, m. flexor digitorum superficialis, m. extensor digitorum, m. extensor digiti minimi, m. extensor carpi ulnaris and m. supinator; on the radius, the m. biceps brachii and m. pronator teres; and on the ulna, the m. triceps brahii. On the lower limbs the following muscles were analysed: on the pelvic bones, the m. biceps femoris (Caput longum), m. semimembranosus and m. semitendinosus; on the femur, the m. gluteus minimus, m. gluteus medius, m. gluteus maximus, m. iliopsoas, m. adductor brevis, m. adductor longus and m. adductor magnus; on the tibia m. soleus; on the patella, the m. quadriceps femoris and on the calcaneus, the m. triceps surae.

3.3.1 Macrostructural analysis of fibrocartilaginous entheses

For the fibrocartilaginous entheses the following EC expression scores were assigned, taking into account changes on the inner part of the attachment site as well as on the outer part of the entheses. On the inner part, three scores were defined: 0 – absent, attachment surface is regular without foramina or cysts; 1 – minor changes, this phase is divided into two sub-phases (a – the entire surface presents a slight irregularity, and b – less than half of the surface is affected by another type of change, such as bone production or erosion, etc); 2 – more than half of the surface is affected by major changes.

On the outer part also, three scores are defined: 0 – absent, attachment site characterised with a regular margin; 1 – minor changes with a salient or irregular margin, and 2 – major changes, involving entheses with present enthesophytes. The final stages for the fibrocartilaginous entheses were obtained using the sum of the values for the inner and the outer part, representing the following stages: stage A = 0; stage B = 1 - 2; and stage C = 3 - 4 (52).

3.3.2 Macrostructural analysis of fibrous entheses

For the fibrous entheses, the following EC expression scores were assigned: A – surface is globally regular; B – (a) presence of significant irregularity in most of the insertion zone, absence of enthesophytes and (b) presence of lacuna of cortical bone with length less than 20 mm; C – (a) presence of a significant irregularity in most of the insertion zone, presence of one large or several smaller reliefs distinguished from the insertion zone and (b) presence of lacuna of cortical bone with length greater than or equal to 20 mm (10).

3.4 Microstructural analyses of entheses

3.4.1 Preparation of specimens

After archaeological excavation, the bones were washed and cleaned using moderately warm water and small dental instruments. The bone samples were harvested using a slow rotating medical saw. The specimen size was adjusted to a size of 1 cm^2.

3.4.2 Micro-CT scanning procedure

The microstructural analysis of entheses was carried out in the Laboratory for Anthropology, Institute of Anatomy, School of Medicine, University of Belgrade and at the University Medical Centar Hamburg-Eppendorf.

The specimens were scanned using micro-computed tomography (Scanco μCT 40, Scanco Medical, Switzerland and SkyScan μCT 1172, Bruker, Belgium).

Each bone specimen was attached to a sample holder of the micro-CT with a consistent proximal-distal orientation and was scanned in dry conditions. The trabecular and cortical bone of the whole specimen was scanned using micro-computed tomography. Movement artifacts during scanning were prevented by fixing the specimens with foam inside the specimen holder. The micro-CT was operated at 55 kVp and 144 μA, with an isotropic resolution of 10 μm, 2048×2048 pixels per slice and a 200 ms integration time per projection.

3.4.3 Micro-CT evaluation procedure

The segmentation procedure comprised a manual marking of the contours of the region of interest (ROI) on various slices, and used the Morph function of the micro-CT program to interpolate the contours of all slices, and in such a way the volume of interest (VOI) was produced. In further analyses, the microarchitecture of the cortical and trabecular bone was evaluated automatically using the micro-CT evaluation program V6.5-1 with direct 3D morphometry.

3.4.3.1 Trabecular microarchitectural parameters

The microarchitectural parameters of cancellous bone included:

- *trabecular bone volume fraction* (BV/TV, %) which represents the ratio of the bone volume (without voids) to the total volume of the region of interest (bone plus voids),
- *trabecular number* (Tb.N, 1/mm) is a measure of the average number of trabeculae per unit length,
- *trabecular thickness* (Tb.Th, mm) represents the mean thickness of trabeculae, assessed using direct 3D methods,
- *trabecular separation* (Tb.Sp, mm) is the mean distance between trabeculae, assessed using direct 3D methods,

- *structure model index* (SMI) is an indicator of the shape of trabeculae; SMI will be close to 0 if parallel plates predominate and closer to 3 if cylindrical rods predominate, while negative values indicate a dominance of concave trabecular surfaces. It is worth noting that plate-like trabeculae develop in high stress regions while rod like trabeculae develop in low stress areas (72).
- *connectivity density* (Conn.D, 1/mm^3) is a measure of the degree of connectivity of trabeculae normalised by tissue volume,
- *degree of anisotropy* (DA) describes the orientation of trabeculae, where lower values reflect an isotropic and higher values depict an anisotropic orientation of trabeculae (16, 73).

3.4.3.2 Cortical microarchitectural parameters

The following microarchitectural parameters were determined for cortical bone:

- *cortical bone volume per tissue volume* (Ct.BV/TV, %) represents the ratio of the bone volume (without voids) to the total volume of the region of interest (bone plus voids),
- *cortical porosity* (Ct.Po, %), in a given cortical region represents the volume of pores (Po.V, mm^3) divided by the total volume of the cortical bone compartment (Ct.V, mm^3),
- *cortical pore diameter* (Po.Dm, mm) represents the mean diameter of the cortical pores, assessed using direct 3D methods,
- *cortical pore separation* (Po.Sp, mm) is the mean distance between the cortical pores, assessed using direct 3D methods,
- *mean cortical thickness* (Ct.Th, mm), which represents the average cortical thickness at the enthesis,
- *minimum cortical thickness* along enthesis (min Ct.Th, mm),
- *maximum cortical thickness* along each enthesis (max Ct.Th, mm).

3.5 Statistical analyses

The Kolmogorov-Smirnov test was used to assess the normality of the data distribution of macromorphological and microstructural parameters.

To observe whether the macroscopic morphological appearance of EC is related to age and sex, as well as whether different habitual activities influence the morphology of the muscle attachment sites by comparing macroscopic morphological appearance of EC between the two investigated populations, the data acquired is categorical and ordinal. Accordingly, non-parametric tests were predominantly used for the statistical calculations. In order to compare the relationship between the different macroscopic appearances of enthesis and age groups, the Kruskal–Wallis test was performed. To investigate different macroscopic appearances of enthesis among all three age groups, the Mann–Whitney *U* test was applied. For post-hoc comparisons, the Bonferroni correction was applied. To analyse differences between EC scores and sexes, the Chi-squared test was used. The Wilcoxon signed-rank test was applied to evaluate the relationship between the different macroscopic stages of the upper and lower limbs and the different sides. The Chi-squared test was used for the overall evaluation of the differences between the population groups.

The relationship between the macroscopic stages ("stage" and "site" selected as factors) and the microscopic parameters (selected as "dependent variable") was assessed using analysis of variance (ANOVA). For post-hoc inter-group comparisons, the Bonferroni correction was applied. The bivariate correlation analysis was performed to determine the association between the various cortical and trabecular microarchitectural parameters in each of the macroscopic stages.

All statistical analyses were performed using SPSS for Windows, version 15. The null hypothesis was rejected at the level of $\alpha = 0.05$.

4. RESULTS

4.1 Macroscopic morphological analyses

The macroscopic morphological analysis of the attachment sites encompassed 19 entheses of the upper and lower limbs, both fibrous and fibrocartilaginous (Table 1). The current study analysed the different appearance of EC scores (A, B, C) for a total of 82 individuals, of which 34 (41.5%) belonged to of the agricultural population and 48 (58.5%) to the horse rider population.

Table 1. Entheses under study.

Limb	Bones	Entheses	F	FC
Upper limbs	humerus	m. subscapularis		+
		mm. supraspinatus and infraspinatus		+
		muscles attached on the epicondilus medialis humeri		+
		muscles attached on the epicondilus lateralis humeri		+
		m. pectoralis major	+	
		m. deltoideus	+	
	radius	m. biceps brachii		+
		m. pronator teres	+	
	ulna	m. triceps brachii		+
Lower limbs	pelvis	Common origin of the m. biceps femoris, semidenndinosus, semimembranosus		+
	femur	m. gluteus medius		+
		m. gluteus minimus		+
		m. ilio-psoas		+
		m. gluteus maximus	+	
		muscles attached on the linea aspera	+	
		m. adductor magnus	+	
	tibia	m. soleus	+	
	patella	m. quadriceps femoris		+
	calcaneus	m. triceps surae		+

F-fibrous entheses; FC-fibrocartilaginous entheses.

In the horse rider population, females were represented in 56.3% of cases, while males comprised 43.7% (Table 2). The senilis age category was the least frequent within the horse-rider sample (12.5%), while the adult and maturus age categories encompassed 39.6% and 47.9%, respectively (Table 3).

Table 2. Sex distribution among investigated sample.

Sex	Sample population			
	Horse riders		Agricultural	
	N	%	N	%
Males	27	43.7	22	64.7
Females	21	56.3	12	35.3
Total	48	100	34	100

Table 3. Distribution of age categories among investigated sample.

Age group	Investigated sample			
	Horse riders		Agricultural	
	N	%	N	%
Adultus	19	39.6	21	61.8
Maturus	23	47.9	10	29.4
Senilis	6	12.5	3	8.8
Total	48	100	34	100

In the agricultural population, females were represented in approximately two thirds of cases (64.7%), while males comprised 35.3% (Table 2). The analyses of age distribution within the agricultural population sample demonstrated the following results: 61.8% of individuals belonged to the adult age category, 29.4% to matures, while only 8.8% belonged to the senilis age category (Table 3).

In order to analyse the difference of the EC scores between the limbs of both sides, the Wilcoxon signed-rank test was performed. The results suggested that there is no statistically significant difference between the sides, except in the case of the pectoralis major muscle (p=0.047), whose attachment was more pronounced on the right side.

4.1.1 Relationship between age and EC scores

The influence of age on the prevalence of the EC scores was studied in the sample of 82 adult individuals of both sexes. The analysis was conducted on the horse riders as well as the agriculturals, separately. The evaluation of the age dependence of the EC scores included the limbs of both sides (pooled), with the exception of the pectoralis major muscle due to this muscle demonstrating a difference between the sides (p=0.047).

The significant differences in the EC scores were verified between three age groups (Adult, Maturus and Senilis) within the whole sample. As expected, in the majority of cases the prevalence of EC scores was higher in individuals of a more advanced age.

In the group of farmers the statistical analyses demonstrated the age dependence of the EC scores among the different age groups. The Kruskal-Wallis One-Way analysis of variance was applied to test the prevalence of EC scores between the three investigated age groups revealing an age dependence in only three attachment sites: mm. supraspinatus and infraspinatus (p=0.019), muscles attached on the epicondilus lateralis humeri (p=0.018) and m. gluteus maximus (p=0.000) (Table 4). The more pronounced EC scores of the aforementioned enthesis were recorded in the older age groups.

Table 4. Prevalence of EC scores between the three investigated age groups for the agricultural population (Kruskal-Wallis One-Way analysis of variance)

Limb	Bones	Entheses	*p*
Upper limbs	humerus	m. subscapularis	0.637
		mm. supraspinatus and infraspinatus	0.019*
		muscles attached on the epicondilus medialis humeri	0.871
		muscles attached on the epicondilus lateralis humeri	0.018*
		m. deltoideus	0.291
	radius	m. biceps brachii	0.227
		m. pronator teres	0.162
	ulna	m. triceps brachii	0.566
Lower limbs	pelvis	Common origin of the m. biceps femoris, semidenndinosus, semimembranosus	0.230
	femur	m. gluteus medius	0.224
		m. gluteus minimus	0.125
		m. ilio-psoas	0.497
		m. gluteus maximus	0.000*
		muscles attached on the linea aspera	0.176
		m. adductor magnus (tuberculum adductorium)	0.373
	tibia	m. soleus	0.305
	patella	M. quadriceps femorais	0.472
	calcaneus	M. triceps surae	0.165

* Statistically significant difference.

In the horse rider population the Kruskal–Wallis One-Way analysis of variance demonstrated a significant difference in the EC scores among the age groups between the following attachment sites: m. subscapularis (p=0.046), mm. supraspinatus and infraspinatus (p=0.036), muscles attached to the epicondilus medialis humeri (p=0.010), m. pronator teres (p=0.012), m. soleus (p=0.021), and m. iliopsoas (p=0.029) (Table 5). Once again, as expected, the more pronounced EC scores were recorded in the older age groups for all attachment sites, except in the case of the iliopsoas muscle. Concerning the iliopsoas muscle, the prevalence of traits was predominant in younger ages.

Table 5. Prevalence of EC scores between the three investigated age groups for horse rider's population (Kruskal-Wallis One-Way analysis of variance)

Limb	Bones	Entheses	*p*
Upper limbs	humerus	m. subscapularis	0.046*
		mm. supraspinatus and infraspinatus	0.036*
		muscles attached on the epicondilus medialis humeri	0.010*
		muscles attached on the epicondilus lateralis humeri	0.217
		m. deltoideus	0.332
	radius	m. biceps brachii	0.138
		m. pronator teres	0.012*
	ulna	m. triceps brachii	0.225
Lower limbs	pelvis	Common origin of the m. biceps femoris, semidenndinosus, semimembranosus	0.097
	femur	m. gluteus medius	0.179
		m. gluteus minimus	0.224
		m. ilio-psoas	0.029*
		m. gluteus maximus	0.547
		muscles attached on the linea aspera	0.092
		m. adductor magnus (tuberculum aductorium)	0.399
	tibia	m. soleus	0.021*
	patella	m. quadriceps femoris	0.656
	calcaneus	m. triceps surae	0.165

* Statistically significant difference.

4.1.2 Sexual dimorphism

The differences in the prevalence of EC scores between the sexes of 82 individuals were studied separately in the agricultural population and the horse rider population. The evaluation of the sex dependence of the EC scores included the limbs of both sides (pooled), except the pectoralis major muscle due to this muscle demonstrating a difference between the sides (p=0.047).

Evaluating the individual morphological appearance of the attachment sites among the sexes in the group of the agricultural population, the difference was statistically significant only for two entheses. The area of the greater tubercle of the humerus (p=0.015) and the insertion of the iliopsoas (p=0.004) muscle suggested a more pronounced EC in males (Table 6).

Table 6. Prevalence of EC scores among sexes for agricultural population (Chi-squared test)

<table>
<tr><th>Limb</th><th>Bones</th><th>Entheses</th><th>p</th></tr>
<tr><td rowspan="8">Upper limbs</td><td rowspan="5">humerus</td><td>m. subscapularis</td><td>0.229</td></tr>
<tr><td>mm. supraspinatus and infraspinatus</td><td>0.015*</td></tr>
<tr><td>muscles attached on the epicondilus medialis humeri</td><td>0.976</td></tr>
<tr><td>muscles attached on the epicondilus lateralis humeri</td><td>0.418</td></tr>
<tr><td>m. deltoideus</td><td>0.243</td></tr>
<tr><td rowspan="2">radius</td><td>m. biceps brachii</td><td>0.161</td></tr>
<tr><td>m. pronator teres</td><td>0.126</td></tr>
<tr><td>ulna</td><td>m. triceps brachii</td><td>0.197</td></tr>
<tr><td rowspan="10">Lower limbs</td><td>pelvis</td><td>Common origin of the m. biceps femoris, semidenndinosus, semimembranosus</td><td>0.432</td></tr>
<tr><td rowspan="6">femur</td><td>m. gluteus medius</td><td>0.686</td></tr>
<tr><td>m. gluteus minimus</td><td>0.386</td></tr>
<tr><td>m. ilio-psoas</td><td>0.004*</td></tr>
<tr><td>m. gluteus maximus</td><td>0.195</td></tr>
<tr><td>muscles attached on the linea aspera</td><td>0.883</td></tr>
<tr><td>m. adductor magnus (tuberculum aductorium)</td><td>0.850</td></tr>
<tr><td>tibia</td><td>m. soleus</td><td>0.245</td></tr>
<tr><td>patella</td><td>m. quadriceps femoris</td><td>0.321</td></tr>
<tr><td>calcaneus</td><td>m. triceps surae</td><td>0.801</td></tr>
</table>

* Statistically significant difference.

Sexual dimorphism was more accentuated in the horse rider sample and reached statistical significance only in the lower extremities (Table 7). All attachment sites which demonstrated significant differences showed greater markers of EC in the male population.

Table 7. Prevalence of EC scores among sexes for horse rider population (Chi-squared test)

Limb	Bones	Entheses	*p*
Upper limbs	humerus	m. subscapularis	0.272
		mm. supraspinatus and infraspinatus	0.368
		muscles attached on the epicondilus medialis humeri	0.480
		muscles attached on the epicondilus lateralis humeri	0.398
		m. deltoideus	0.189
	radius	m. biceps brachii	0.121
		m. pronator teres	0.379
	ulna	m. triceps brachii	0.318
Lower limbs	pelvis	Common origin of the m. biceps femoris, semidenndinosus, semimembranosus	0.025*
	femur	m. gluteus medius	0.005*
		m. gluteus minimus	0.208
		m. ilio-psoas	0.020*
		m. gluteus maximus	0.114
		muscles attached on the linea aspera	0.022*
		m. adductor magnus (tuberculum aductorium)	0.171
	tibia	m. soleus	0.621
	patella	m. quadriceps femoris	0.010*
	calcaneus	m. triceps surae	0.010*

* Statistically significant difference.

4.1.3 Horserider vs. agricultural population

The analyses of EC scores between individuals (pooled sexes) from the horse rider and the agricultural population revealed a statistically significant difference concerning the subscapularis muscle (p=0.009) on the upper limbs, as well as muscles attached at the tuberculum ishiadicum (p=0.012), vastus medialis muscle, adductor brevis, and adductor longus (p=0.017), and adductor magnus on the lower limbs (p=0.000) (Table 8). The morphological changes of the aforementioned entheses were more pronounced in the male Avarian population of horse riders.

Table 8. Prevalence of EC scores between horse rider and agricultural populations (Chi-squared test)

Limb	Bones	Entheses	*p*
Upper limbs	humerus	m. subscapularis	0.009*
		mm. supraspinatus and infraspinatus	0.223
		muscles attached on the epicondilus medialis humeri	0.487
		muscles attached on the epicondilus lateralis humeri	0.183
		m. deltoideus	0.439
	radius	m. biceps brachii	0.716
		m. pronator teres	0.247
	ulna	m. triceps brachii	0.708
Lower limbs	pelvis	Common origin of the m. biceps femoris, semidenndinosus, semimembranosus	0.012*
	femur	m. gluteus medius	0.768
		m. gluteus minimus	0.877
		m. ilio-psoas	0.108
		m. gluteus maximus	0.266
		muscles attached on the linea aspera	0.017*
		m. adductor magnus (tuberculum aductorium)	0.000*
	tibia	m. soleus	0.197
	patella	m. quadriceps femoris	0.394
	calcaneus	m. triceps surae	0.821

* Statistically significant difference.

4.2 Bone microarchitecture at muscle attachment sites

In a further part of the study, the microarchitecture of the muscle attachment sites of the lower extremity was analysed, since it was assumed that these sites should demonstrate significant differences in macroscopic appearance between the horse riding and agricultural population.

4.2.1 Internal architecture in various attachment sites of lower limbs and its correlation with different macroscopic stages

To depict the geometry and overall morphology of the entheses, Figs. 7-10 show cross-sections made at the soleal line (origin of the soleus muscle) (Fig. 7), lesser trochanter (insertion of the iliopsoas muscle) (Fig. 8), tuberculum adductorium (insertion of the adductor magnus muscle) (Fig. 9) and gluteal tuberosity (insertion of the gluteus maximus) (Fig. 10). All these bony prominences, except the soleal line, consist of cortical and trabecular bone tissue that protrudes relative to the surrounding bone surface.

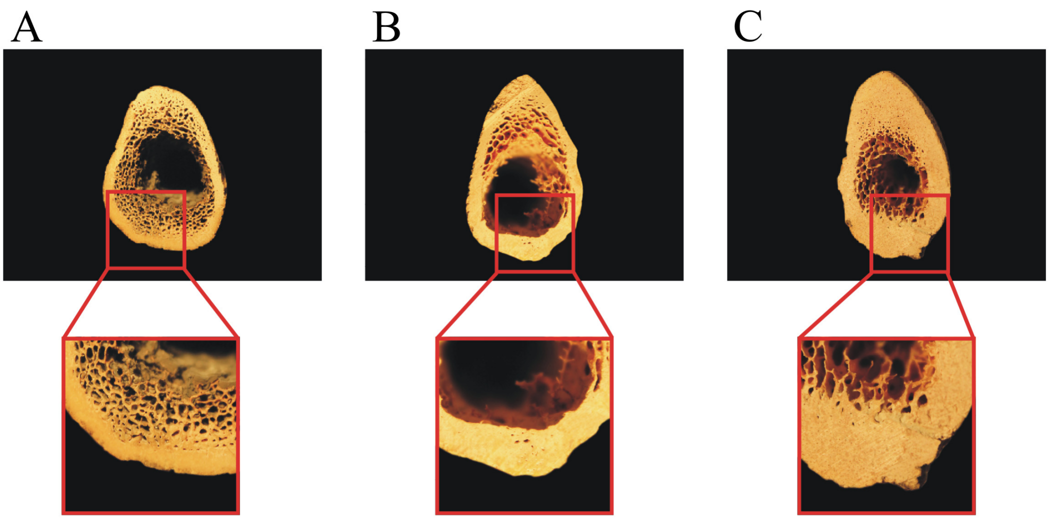


Figure 7. Cross-sections made at soleal line (origin of soleus muscle):

A - stage A (see classification in material and method section).

B - stage B (see classification in material and method section).

C - stage C (see classification in material and method section).

*Cross-sections were cut at the most prominent point of the attachment site.

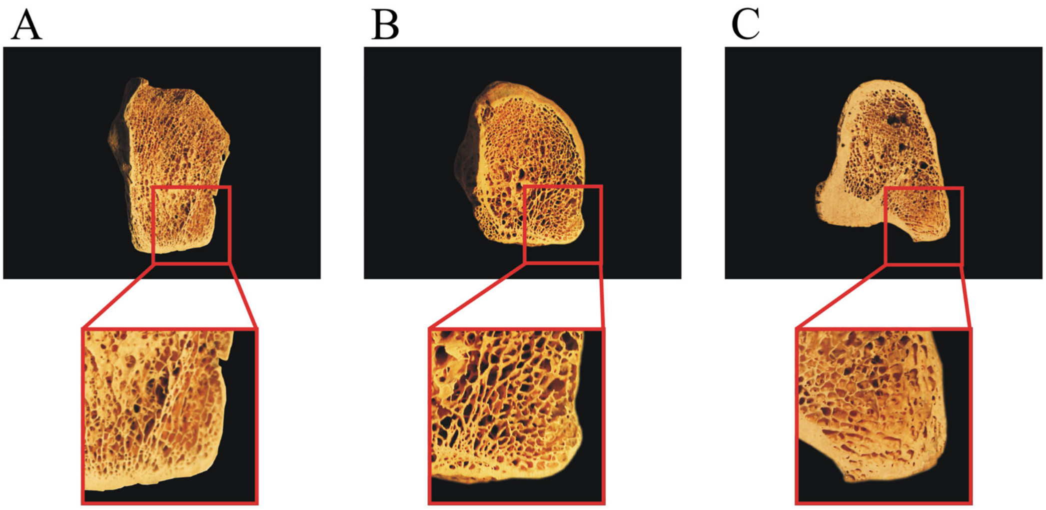


Figure 8. Cross-sections made at lesser trochanter (insertion of iliopsoas muscle).

A - stage A (see classification in material and method section).

B - stage B (see classification in material and method section).

C - stage C (see classification in material and method section).

*Cross-sections were cut at the most prominent point of the attachment site.

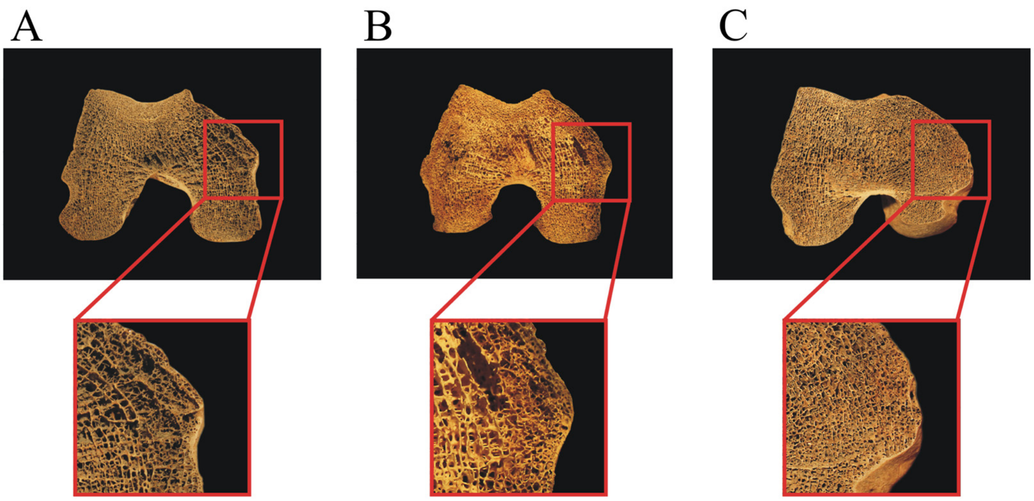


Figure 9. Cross-sections made at tuberculum adductorium (insertion of adductor magnus muscle).

A - stage A (see classification in material and method section).

B - stage B (see classification in material and method section).

C - stage C (see classification in material and method section).

*Cross-sections were cut at the most prominent point of the attachment site.

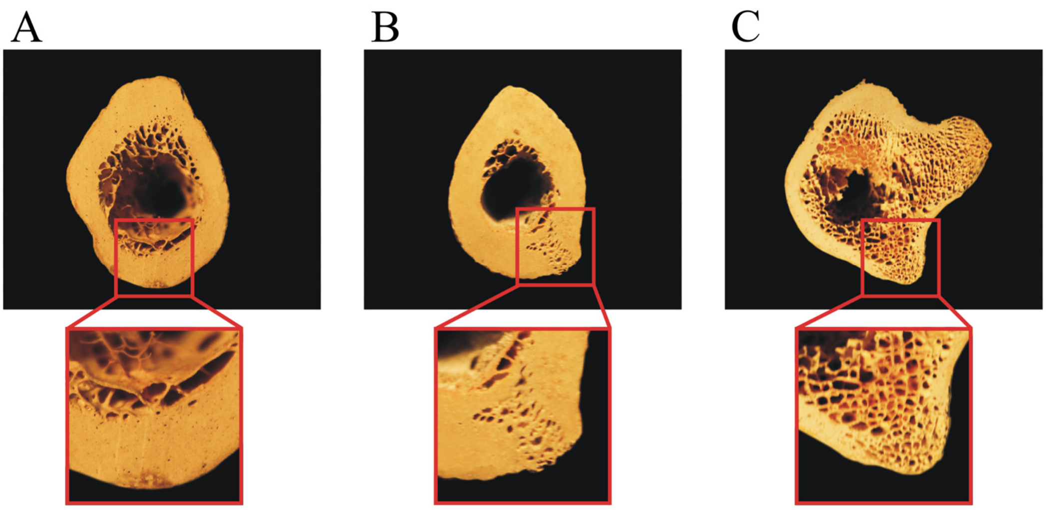


Figure 10. Cross-sections made at gluteal tuberosity (insertion of gluteus maximus).

A - stage A (see classification in material and method section).

B - stage B (see classification in material and method section).

C - stage C (see classification in material and method section).

*Cross-sections were cut at the most prominent point of the attachment site.

4.2.2 Bone microarchitectural design of entheses and the relationship with the macroscopic scores of the entheseal morphology

The conducted micro-CT analysis of the entheses of different macroscopic stages showed an overall lack of dependence of cortical microarchitecture at the attachment sites on the macroscopic stages (Table 9). Cortical pore diameter was the only cortical microstructural parameter showing a negative trend with an increasing macroscopic score of the enthesis, however, statistical significance was not reached.

ANOVA revealed that cortical thickness expressed significant inter-stage differences (Table 9). However, post-hoc tests revealed that stage B presented a significantly higher mean cortical thickness than stages A and C (Table 10). In particular, a significant decline in stage C in comparison to stage B emphasises the lack of a gradual (successive) increase in cortical thickness from macroscopic score A to C.

Further analysis showed the significant effect of the interaction of “site” and “stage” on mean cortical thickness ($p<0.001$) (Table 9). In this context, the gluteal tuberosity (insertion of the gluteus maximus muscle) showed the opposite pattern regarding cortical thickness, where macroscopic stage C expressed a thinner cortex than stage A (Table 9).

Analysis of trabecular microarchitecture (Fig. 11) showed a lack of significant change related to the "successive" stages of EC (Table 11). Generally, entheses classified as macroscopic stages B and C presented with a higher trabecular number and increased trabecular thickness, as well as a decreased trabecular separation than the entheses of stage A (Table 11). However, any dependence of these microstructural parameters on the macroscopic stages was not found, given that stage C always showed slightly worse microarchitecture than stage B. Only the degree of anisotropy seemed to express a tendency towards a gradual reduction together with an increase in the macroscopic stage of the enthesis (p=0.056).

With the simplified macroscopic scoring ("no changes" corresponding to the original stage A *vs.* "any type of change present" corresponding to the original stages B and C combined), cortical pore diameter showed a tendency towards decreased values in the case of entheseal changes (p=0.087), whereas the degree of anisotropy showed a significant decrease in entheseal changes (p=0.015).

Table 9. Cortical microarchitectural parameters in entheses of macroscopic stages A, B and C (Analysis of variance)

	Stage	Gluteal tuberosity	Lesser trochanter	Soleal line	Tuberculum adductorium	All sites						ANOVA[1] (*p*)
		Mean	**Mean**	**Mean**	**Mean**	**Mean**	**SE**	**95% CI (lower, upper)**		**Min**	**Max**	
Cortical bone volume fraction	A	0.934	0.907	0.890	0.882	0.903	0.017	0.862	0.944	0.825	0.903	0.738
	B	0.920	0.879	0.967	0.820	0.873	0.029	0.804	0.942	0.730	0.873	
	C	0.913	0.930	0.946	0.868	0.918	0.014	0.885	0.951	0.862	0.918	
Cortical porosity [%]	A	0.066	0.093	0.110	0.118	0.097	0.017	0.056	0.138	0.036	0.097	0.738
	B	0.080	0.121	0.033	0.180	0.127	0.029	0.058	0.196	0.032	0.127	
	C	0.087	0.070	0.054	0.132	0.082	0.014	0.049	0.115	0.024	0.082	
Pore separation [mm]	A	0.485	0.336	0.521	0.299	0.410	0.052	0.286	0.534	0.205	0.410	0.337
	B	0.287	0.406	0.395	0.207	0.329	0.034	0.248	0.410	0.163	0.329	
	C	0.243	0.504	0.527	0.348	0.398	0.049	0.283	0.512	0.190	0.398	
Pore diameter [mm]	A	0.217	0.136	0.212	0.140	0.176	0.036	0.091	0.261	0.057	0.176	0.372
	B	0.149	0.155	0.069	0.126	0.140	0.024	0.085	0.196	0.058	0.140	
	C	0.065	0.123	0.128	0.161	0.110	0.018	0.068	0.152	0.057	0.110	
Mean cortical thickness [mm]	A	3.034	0.496	1.995	0.585	**1.527**	0.408	0.563	2.492	0.338	1.527	*0.003* [2]*
	B	5.408	0.923	3.207	0.383	**2.347**	0.701	0.690	4.003	0.262	2.347	
	C	0.363	1.426	3.047	1.102	**2.047**	0.847	0.045	4.049	0.271	2.047	
Minimum cortical thickness [mm]	A	2.023	0.097	1.426	0.263	**0.952**	0.320	0.195	1.709	0.080	0.952	0.003*
	B	4.130	0.196	2.467	0.097	**1.555**	0.575	0.196	2.915	0.072	1.555	
	C	0.174	0.556	1.757	0.464	**1.342**	0.773	-0.485	3.169	0.100	1.342	
Maximum cortical thickness [mm]	A	4.228	1.435	2.809	0.964	**2.359**	0.497	1.183	3.535	0.827	2.359	0.02*
	B	6.313	1.944	4.045	1.182	**3.315**	0.755	1.529	5.101	0.543	3.315	
	C	0.869	2.680	4.182	1.734	**2.903**	0.889	0.801	5.006	0.757	2.903	

[1] Analysis of variance (ANOVA): overall difference between the stages A, B and C (irrespective of the site) (for post-hoc tests see Table 10)

[2] In addition, there was also a significant effect of interaction of stage and site on mean cortical thickness ($p<0.001$). Note that gluteus maximus showed a different pattern of relationship between the cortical thickness and EC stages

* Statistically significant difference.

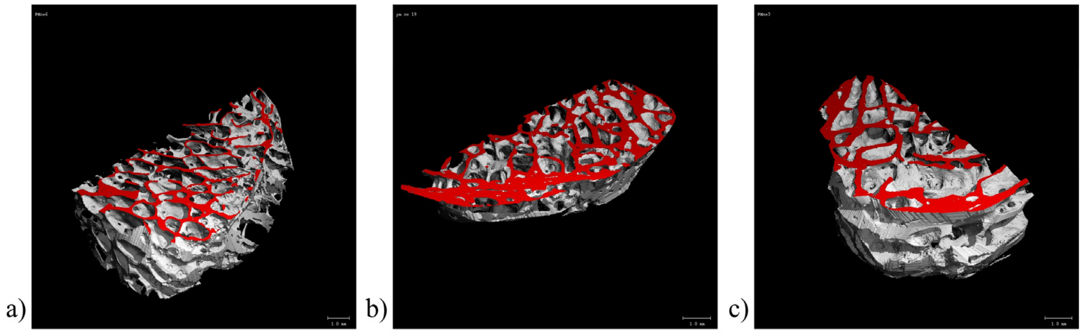


Figure 11. Presentation of 3D reconstruction of trabecular bone in the region of the lesser trochanter.

a - stage A

b - stage B

c - stage C

*The top slice is marked in red.

Table 10. Post-hoc Bonferroni pairwise comparisons of the cortical microarchitectural parameters between the entheseal macroscopic stages A, B and C (irrespective of the site)

Parameter	**Inter-stage comparison**	**Direction of differences**	***p***
Mean cortical thickness [mm]	A *vs.* B	**A < B**	0.007*
	A *vs.* C		1
	B *vs.* C	**B > C**	0.007*
Minimum cortical thickness [mm]	A *vs.* B	**A < B**	0.012*
	A *vs.* C		0.981
	B *vs.* C	**B > C**	0.003*
Maximum cortical thickness [mm]	A *vs.* B	**A < B**	0.034*
	A *vs.* C		1
	B *vs.* C	**B > C**	0.047*

*Statistical significant difference.

Table 11. Trabecular microarchitectural parameters in entheses of macroscopic stages A, B and C (Analysis of variance)

	Stage	Gluteal tuberosity	Lesser trochanter	Soleal line	Tuberculum adductorium	All sites						ANOVA[1] *(p)*
		Mean	**Mean**	**Mean**	**Mean**	**Mean**	**SE**	**95% CI (lower, upper)**		**Min**	**Max**	
Trabecular bone volume fraction	A	0.262	0.184	0.380	0.383	0.302	0.049	0.186	0.418	0.166	0.554	0.911
	B	0.331	0.333	0.318	0.257	0.307	0.027	0.242	0.372	0.210	0.410	
	C	0.419	0.316	0.213	0.365	0.344	0.038	0.252	0.436	0.213	0.472	
Connectivity density [1/mm^3]	A	11.775	10.897	43.893	35.940	25.626	10.998	-0.379	51.631	4.134	82.394	0.295
	B	35.589	30.166	84.290	13.260	41.574	12.858	10.111	73.038	8.470	108.000	
	C	36.144	21.013	5.815	7.241	19.230	5.029	6.923	31.536	5.815	37.349	
Structure model index	A	-1.219	-0.395	-5.735	-2.006	-2.339	1.334	-5.494	0.817	-11	0.138	0.305
	B	-3.122	-0.523	4.153	-0.019	0.586	1.143	-2.211	3.382	-3.122	6.411	
	C	-1.186	-3.019	1.243	-0.765	-1.242	0.618	-2.754	0.270	-3.672	1.243	
Trabecular number [1/mm]	A	1.626	1.494	2.506	1.869	1.874	0.276	1.220	2.527	1.191	3.550	0.717
	B	2.126	1.996	2.886	1.749	2.198	0.285	1.500	2.897	1.650	3.815	
	C	2.805	1.760	1.543	1.748	2.024	0.218	1.490	2.558	1.543	3.123	
Trabecular thickness [mm]	A	0.207	0.138	0.207	0.248	0.200	0.017	0.159	0.241	0.138	0.260	0.594
	B	0.170	0.221	0.237	0.177	0.206	0.014	0.172	0.240	0.150	0.247	
	C	0.208	0.190	1.184	0.240	0.352	0.139	0.010	0.693	0.183	1.184	
Trabecular separation [mm]	A	0.828	0.712	0.611	0.662	0.703	0.063	0.555	0.851	0.430	1.031	0.470*
	B	0.614	0.672	0.478	0.649	0.602	0.054	0.469	0.734	0.337	0.808	
	C	0.473	0.733	0.642	0.638	0.619	0.043	0.512	0.725	0.445	0.737	
Degree of anisotropy	A	2.234	1.738	2.111	1.442	1.881	0.162	1.497	2.265	1.240	2.585	0.056
	B	2.045	1.219	1.481	1.651	1.535	0.110	1.267	1.804	1.146	2.045	
	C	1.219	1.121	1.968	1.715	1.440	0.133	1.115	1.765	1.088	1.968	

[1] Analysis of variance (ANOVA): overall difference between the stages A, B and C (irrespective of the site).

* Statistical significant difference.

4.2.3 Correlations between microarchitectural parameters

Correlation analysis of microarchitectural parameters in entheses of macroscopic stage A showed that cortical thickness was directly related only with cortical microarchitecture, reaching statistical significance in the case of separation between cortical pores (for mean Ct.Th: R=0.725, p=0.042; min Ct.Th: R=0.8, p=0.017; max Ct.Th: R=0.714, p=0.047). There was no relationship between the cortical and trabecular microarchitectural parameters (Table 12).

In stage B entheses, cortical pore diameter correlated with cortical porosity (R=0.812, p=0.014). Cancellous bone volume fraction correlated with the trabecular number (R=0.763, p=0.046). The trabecular number also correlated positively with the trabecular connectivity (R=0.824, p=0.023), and negatively with trabecular separation (R=-0.917, p=0.004). However, again, no cortical – trabecular associations were found (Table 13).

In stage C entheses, cortical thickness showed a tendency towards a positive correlation with cortical BV/TV (mean Ct.Th: R=0.669, p=0.069; min Ct.Th: R=0.659, p=0.075; max Ct.Th: R=0.695, p=0.056). Moreover, cortical thickness showed a significant direct relationship with trabecular thickness (mean Ct.Th: R=0.839, p=0.018; min Ct.Th: R=0.921, p=0.003; max Ct.Th: R=0.758, p=0.048), while trabecular connectivity was correlated negatively with cortical pore diameter (R=-0.775, p=0.041), both indicating that the cortex and trabeculae follow the same adaptation patterns in stage C entheses (Table 14).

Table 12. Bivariate correlations between the cortical and trabecular parameters in entheseal changes stage A (R – coefficient of correlation, *p* – significance level)

		trabecular bone volume fraction	connectivity density	structure model index	trabecular number	trabecular thickness	trabecular separation	degree of anisotropy
Cortical bone volume fraction	R	0.114	0.111	-0.296	0.151	0.213	0.153	-0.028
	p	*0.787*	*0.793*	*0.476*	*0.722*	*0.613*	*0.718*	*0.947*
Cortical porosity	R	-0.114	-0.111	0.296	-0.151	-0.213	-0.153	0.028
	p	*0.787*	*0.793*	*0.476*	*0.722*	*0.613*	*0.718*	*0.947*
Pore separation	R	0.349	0.243	-0.559	0.462	0.290	-0.155	0.287
	p	*0.397*	*0.563*	*0.150*	*0.249*	*0.486*	*0.714*	*0.491*
Pore diameter	R	-0.167	-0.288	0.238	-0.080	-0.329	-0.149	0.395
	p	*0.693*	*0.489*	*0.570*	*0.850*	*0.427*	*0.725*	*0.333*
Mean cortical thickness	R	0.129	0.006	-0.248	0.196	0.218	0.152	0.569
	p	*0.760*	*0.989*	*0.553*	*0.642*	*0.604*	*0.719*	*0.141*
Minimum cortical thickness	R	0.189	0.077	-0.324	0.234	0.332	0.187	0.572
	p	*0.653*	*0.856*	*0.434*	*0.577*	*0.422*	*0.657*	*0.139*
Maximum cortical thickness	R	0.091	0.014	-0.257	0.216	0.097	0.113	0.529
	p	*0.831*	*0.974*	*0.540*	*0.608*	*0.820*	*0.789*	*0.177*

Table 13. Bivariate correlations between the cortical and trabecular parameters in entheseal changes stage B (R – coefficient of correlation, p – significance level)

		trabecular bone volume fraction	connectivity density	structure model index	trabecular number	trabecular thickness	trabecular separation	degree of anisotropy
Cortical bone volume fraction	R	0.224	**0.839**	0.508	0.514	0.557	-0.369	0.024
	p	*0.629*	*0.018**	*0.244*	*0.238*	*0.194*	*0.415*	*0.959*
Cortical porosity	R	-0.224	**-0.839**	-0.508	-0.514	-0.557	0.369	-0.024
	p	*0.629*	*0.018**	*0.244*	*0.238*	*0.194*	*0.415*	*0.959*
Pore separation	R	0.407	0.610	0.277	0.288	**0.897**	0.028	-0.542
	p	*0.364*	*0.146*	*0.547*	*0.532*	*0.006**	*0.953*	*0.208*
Pore diameter	R	0.067	-0.698	-0.750	-0.586	-0.187	0.663	-0.028
	p	*0.887*	*0.081*	*0.052*	*0.167*	*0.688*	*0.105*	*0.953*
Mean cortical thickness	R	0.133	0.418	0.083	0.248	0.069	-0.241	0.553
	p	*0.776*	*0.351*	*0.859*	*0.591*	*0.883*	*0.603*	*0.198*
Minimum cortical thickness	R	0.063	0.434	0.146	0.236	0.064	-0.239	0.596
	p	*0.893*	*0.330*	*0.754*	*0.611*	*0.891*	*0.606*	*0.158*
Maximum cortical thickness	R	0.181	0.374	0.054	0.234	0.116	-0.228	0.485
	p	*0.698*	*0.408*	*0.908*	*0.614*	*0.804*	*0.622*	*0.270*

* Statistical significant difference.

Table 14. Bivariate correlations between the cortical and trabecular parameters in entheseal changes stage C (R – coefficient of correlation, p – significance level)

		trabecular bone volume fraction	connectivity density	structure model index	trabecular number	trabecular thickness	trabecular separation	degree of anisotropy
Cortical bone volume fraction	R	-0.304	0.240	-0.072	0.108	0.424	0.104	-0.251
	p	*0.507*	*0.604*	*0.878*	*0.818*	*0.343*	*0.825*	*0.587*
Cortical porosity	R	0.304	-0.240	0.072	-0.108	-0.424	-0.104	0.251
	p	*0.507*	*0.604*	*0.878*	*0.818*	*0.343*	*0.825*	*0.587*
Pore separation	R	-0.190	-0.491	-0.296	-0.522	0.444	0.529	0.080
	p	*0.682*	*0.263*	*0.519*	*0.230*	*0.318*	*0.222*	*0.865*
Pore diameter	R	0.131	**-0.775**	-0.131	-0.550	0.160	0.346	0.408
	p	*0.780*	*0.041**	*0.780*	*0.201*	*0.731*	*0.448*	*0.363*
Mean cortical thickness	R	-0.445	-0.668	0.256	-0.613	**0.839**	0.395	0.530
	p	*0.318*	*0.101*	*0.579*	*0.143*	*0.018**	*0.380*	*0.221*
Minimum cortical thickness	R	-0.424	-0.610	0.393	-0.501	**0.921**	0.239	0.593
	p	*0.343*	*0.146*	*0.383*	*0.252*	*0.003**	*0.606*	*0.161*
Maximum cortical thickness	R	-0.585	-0.615	0.153	-0.711	**0.758**	0.576	0.400
	p	*0.167*	*0.142*	*0.744*	*0.073*	*0.048**	*0.175*	*0.374*

* Statistical significant difference.

4.2.4 Differences between "proliferative" and "resorptive" entheseal changes at the microarchitectural level

The macroscopic appearance of entheses in stages B and C involves morphological changes showing either predominant signs of "bony formation" (stage B(a) and stage C(a)) or pronounced signs of bony resorption (stage B (b); stage C (b)). To investigate the differences between the "proliferative" and "resorptive" phases of entheseal changes, we further performed micro-CT analyses of the gluteus maximus attachment site with predominant signs of "bone formation" (i.e. "bony proliferation") and those with dominant traces of "bone resorption" at the level of macromorphological entheseal appearance (stage B(a); stage B(b); stage C(a) and stage C(b)).

The results suggested that the microarchitectural parameters of cortical bone in entheses of the gluteus maximus muscle did not demonstrate any general trend between the "proliferative" and "resorptive" entheseal changes (Table 15). In stages B (Ba vs. Bb) there was a trend of a 30% increase in cortical porosity in the □resorptive" phase of stage B, whereas in stage C (Ca vs Cb) there was a 47.27 % reduction in cortical porosity in the bony □resorptive" phase (Chart 1).

Table 15. Cortical microarchitectural parameters in entheses of gluteus maximus muscle among different macroscopic stages (mean value)

	MACROSCOPIC STAGE			
	B(a)	**B(b)**	**C(a)**	**C(b)**
Cortical bone volume fraction [%]	62.130	50.149	42.155	69.498
Cortical porosity [%]	37.869	49.851	57.845	30.502
Pore diameter [mm]	0.543	0.974	0.630	0.483

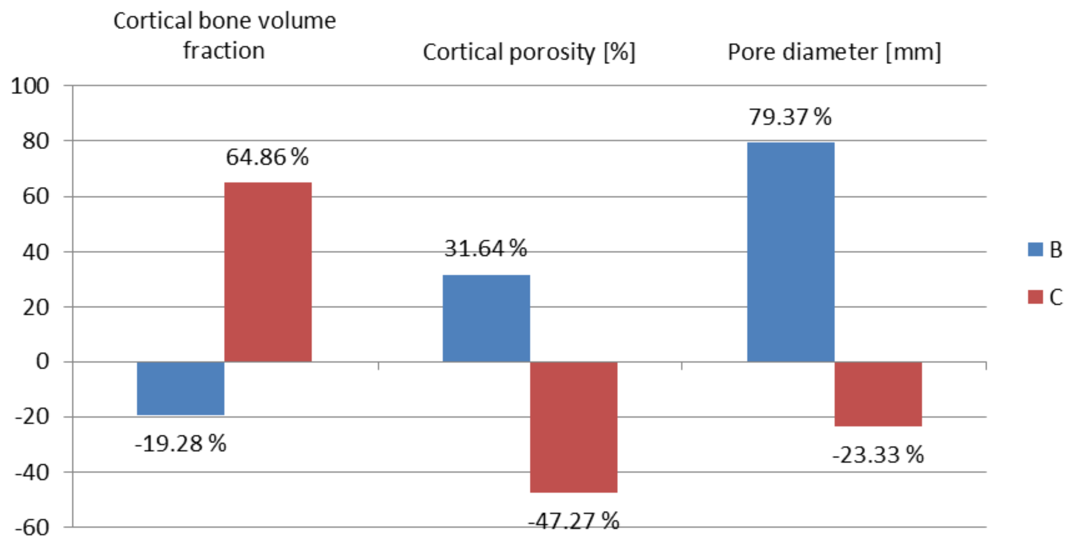
B(a) - stage B(a) (see classification in material and method section).

B(b) - stage B(b) (see classification in material and method section).

C(a) - stage C(a) (see classification in material and method section).

C(b) - stage C(b) (see classification in material and method section).

Chart 1. Microarchitectural parameters of the cortical bone: Percentage of difference between the "resorptive" and "proliferative" entheseal phases.



B - stages B(a) and B(b) (see classification in material and method section).

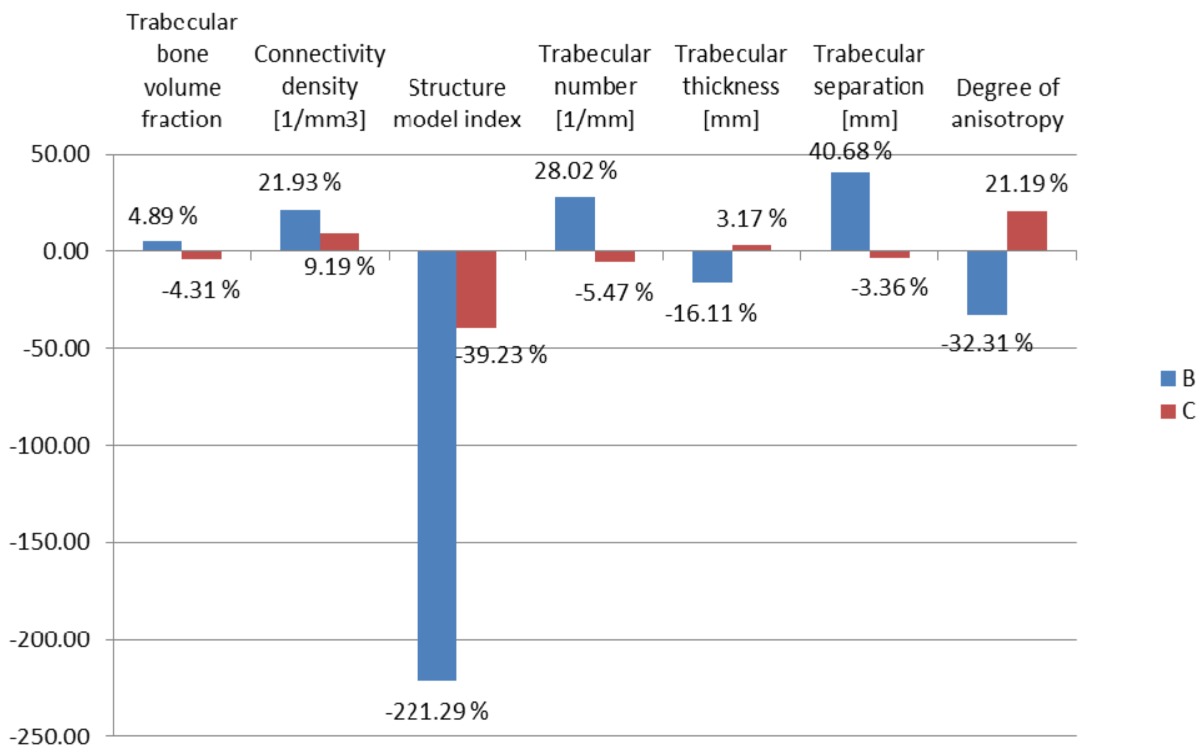
C - stages C(a) and C(b) (see classification in material and method section).

A somewhat similar situation was also observed in the case of the trabecular microarchitectural parameters. Analysing microarchitectural parameters of the trabecular bone in entheses of the gluteus maximus muscle, the only regularity between "resorptive" and "proliferative" entheseal phases was observed in the case of connectivity density. The connectivity density shows higher values in stages of "bony resorption" compared to the stages of "bony formation" (Table 16). Analysis of the percentage of difference in different micro-CT parameters of trabecular bone between "bony formation" and "bony resorption" phase suggested that the most significant difference was found in the structure model index (Chart 2). In stage B, the structure model index was significantly higher in the phase of "bony proliferation", while in stage C, the percentage of difference was lower in the "proliferative" phase.

Table 16. Trabecular microarchitectural parameters in entheses of gluteus maximus muscle among different macroscopic stages (mean value)

	MACROSCOPIC STAGE			
	B(a)	**B(b)**	**C(a)**	**C(b)**
Trabecular bone volume fraction [%]	21.743	22.807	26.890	25.730
Connectivity density [1/mm^3]	14.754	17.990	15.572	17.003
Structure model index	0.883	-1.071	-2.483	-1.509
Trabecular number [1/mm]	1.028	1.316	1.425	1.347
Trabecular thickness [mm]	0.211	0.177	0.189	0.195
Trabecular separation [mm]	0.590	0.830	0.863	0.834
Degree of anisotropy	2.863	1.938	2.006	2.431

Chart 2. Microarchitectural parameters of the trabecular bone: Percentage of difference between the “resorptive” and “proliferative” entheseal phases.



B - stages B(a) and B(b) (see classification in material and method section).

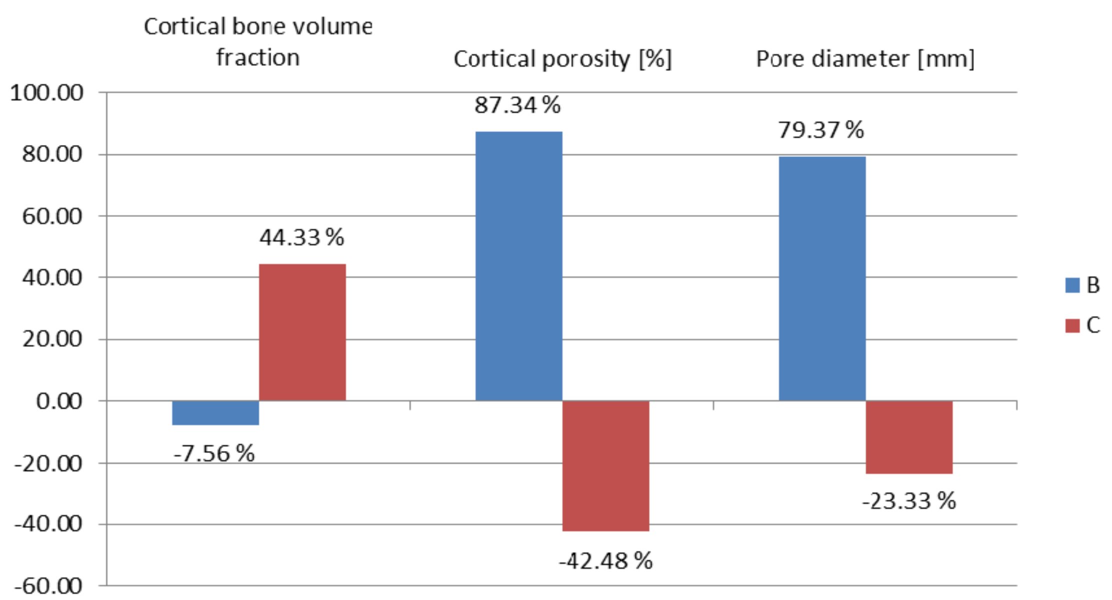
C - stages C(a) and C(b) (see classification in material and method section).

Analyses of bone volume per tissue volume and the total porosity of the whole specimen in entheses of the gluteus maximus muscle in the "resorptive" and "proliferative" phases demonstrated no regularity in the results (Table 17). The percentage of difference between the different micro-parameters of the "bony formation" and "bony resorption" phases in the whole entheses showed an 87.34 % increase in porosity in stage B and a 42.48 % decrease in stage C.

Table 17. Bone volume per tissue volume and total porosity of the whole specimen in entheses of gluteus maximus muscle among different macroscopic stages (mean value)

	MACROSCOPIC STAGE			
	B(a)	**B(b)**	**C(a)**	**C(b)**
Bone volume per tissue volume [%]	37.457	39.331	30.041	40.070
Total porosity [%]	52.370	63.418	62.010	57.731

Chart 3. Percentage of difference in different mct-parameters between "bony formation" and "bony resorption" phases in whole entheses.

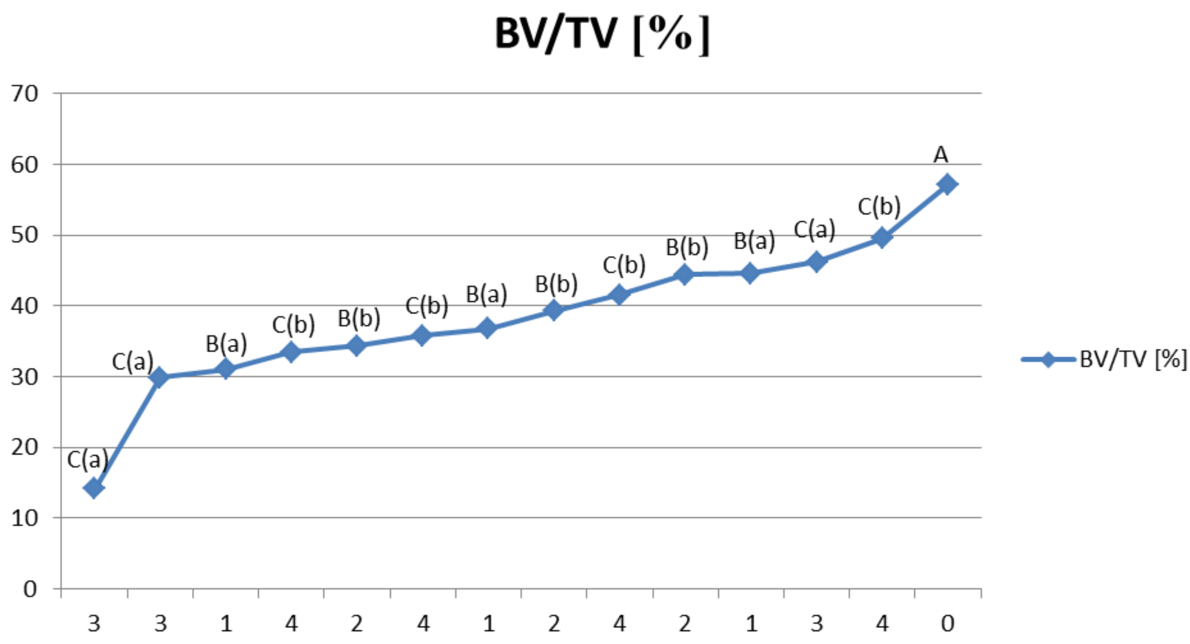


B - stages B(a) and B(b) (see classification in material and method section).

C - stages C(a) and C(b) (see classification in material and method section).

It was expected that bone volume per tissue volume of the whole specimen would grow in proportion to the stages that maintain higher physical activity; however, proof of this was not obtained. In order to analyse this situation, the mean value of bone volume per tissue volume of the whole enthesis was set to grow from the lowest to the highest value. It was expected that stage A should be at the lowest position while stage C(b) should take the highest position. Stage A clearly showed a higher BV/TV than all other stages, however, there was no regular pattern of relationship between all stages and bone volume per tissue volume (Chart 4). Additionally, the same procedure was repeated using the mean value of the degree of anisotropy of the trabecular bone (Chart 5).

Chart 4. Bone Volume per tissue volume of all entheses among different EC scores.



BV/TV - Bone Volume per tissue volume

A - stage A (see classification in material and method section).

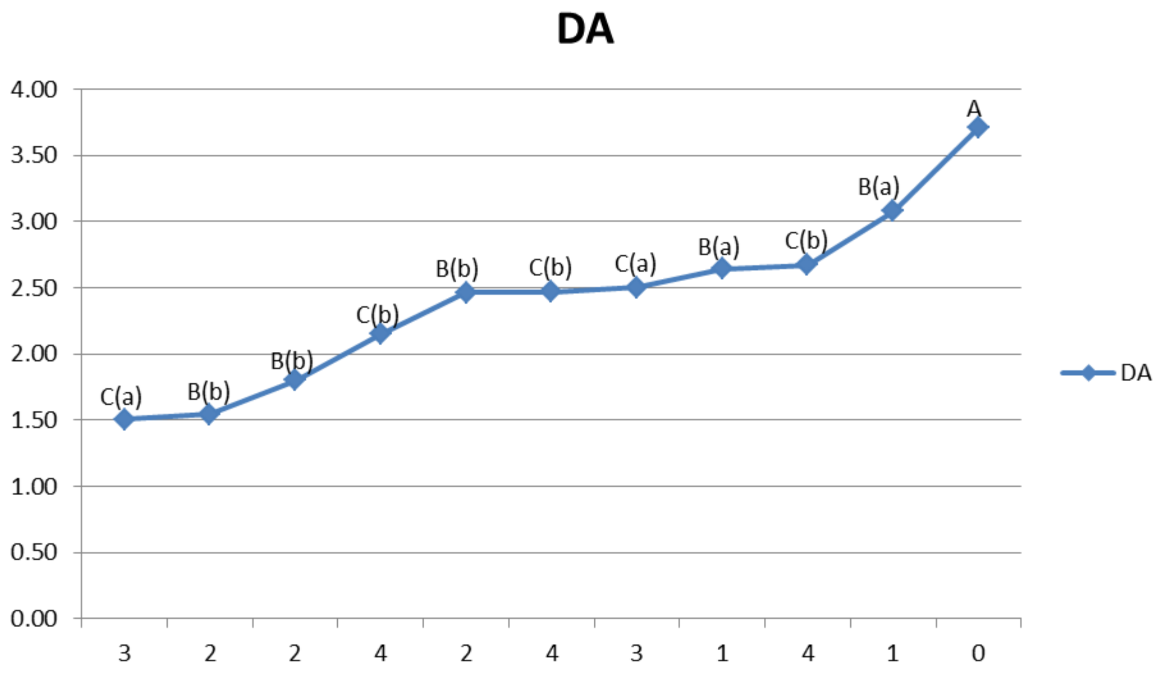
B(a) - stage B(a) (see classification in material and method section).

B(b) - stage B(b) (see classification in material and method section).

C(a) - stage C(a) (see classification in material and method section).

C(b) - stage C(b) (see classification in material and method section).

Chart 5. Degree of anisotropy of trabecular bone among different EC scores.



DA - Degree of anisotropy.

A - stage A (see classification in material and method section).

B(a) - stage B(a) (see classification in material and method section).

B(b) - stage B(b) (see classification in material and method section).

C(a) - stage C(a) (see classification in material and method section).

C(b) - stage C(b) (see classification in material and method section).

5. DISCUSSION

Historical background of horse riders population: the Avars arrived in the Pannonian Basin in the latter part of the seventh decade of the 6th century and, shortly afterwards, emerged as a stronger force than the mutually hostile Germanic tribes (54). They established the Avar Khaganate, which spanned the Pannonian Basin and considerable areas of Central and Eastern Europe from the late 6th to the early 9th century. They were ruled by a khagan, who was assisted by an entourage of professional warriors (74). In history and archaeology Avars are known as the people whose equestrian skills were very well developed. In Avarian society the horse-riders were a very important part not only during warlike activities but also in complex economic and social circumstances. The importance of the horse did not decrease, even after arriving in the Pannonian Basin, although in that moment, the Avars crossed from a nomadic life to a sedentary economy. This important role of horses in the Avar Khaganate is certainly caused by the fact that the main Khaganate power lies in military organisation, where the cavalry has a primary role. Since the need for horses was particularly significant for the Avars in the Pannonian Basin, an intensive rearing of horses was well developed (74). They mostly used fast and durable tarpan whose trappings consisted mostly of the saddle, stirrups, bridles, halters, reins, bits, harnesses and breastplates (74).

For certain, the tactics used by Avars considerably influenced their daily life and physical particularities. Similar to most of the steppe peoples, the bulk of the Avar combatant force was formed of the heavily armoured (horse) lancers and lightly armoured horse archers, together with a number of foot archers. The tactics of most of the steppe peoples was essentially marked by the hit-and-run use of the horse archers and a final rush by heavy lancers, which occurred only after the enemy was reduced enough by the previous offensive. However, unlike this practice, it seems that the mainstream of Avar tactics was different. In almost all battle descriptions preserved in contemporary Greek and Latin sources, the Avars are primarily depicted as heavy lancers storming at the enemy from the very beginning of the battle, and their horse and foot archers are usually shown as a background support to the lancers. This predominant use of heavy lancers certainly contributed to the Avarian ability to engage with the heavy Byzantine and Lombard cavalry. Even before settling in Pannonia, the heavy lancers` attack appears as the main battle tactic, particularly of the Avars, as seen in their battles with the Austrasian Franks in 562 (at Regensburg) and ca. 568, in which they were repelled by King Sigebert I (Greg. Tur. IV.29)[2]. In these battles, the Avars were engaged by the heavy Frankish infantry, on soil relatively unfavourable for cavalry. Their settlement and temporary alliance with the Byzantine Empire in 578 was succeeded by the great series of campaigns of the emperor Mauricius. In the battle of Tomi (598), the Avars smashed the Byzantine army (commanded by the Patricius Comentiolus) with their immediate heavy lancer's charge (Theoph. Sym. VII.13-14)[3]. This Byzantine force, sent by the Emperor to help Patricius Priscus besieged in Tomi, was unable to withstand the swiftness and shocking force of the Avar cavalry, as the source clearly points out. In the next battle with Mauricius` armies, on the banks of The Danube, opposite Viminacium (599), the Avars also started the battle with a heavy cavalry charge, but were beaten by Patricius Priscus (Ibid, VIII.2-3). The source shows that this defeat was due to the ingenuity of Priscus who, using special set of manoeuvres, induced the Avars to cross terrain less favourable to them. Despite the ground being relatively unfavourable for an equestrian frontal shock attack, the Avars decided to put all of their trust in a heavy lancers charge. After having been pursued by Priscus for two weeks, the Avars formed a defensive position (near the Tisza), but were defeated by a Byzantine heavy cavalry attack. Two decades later, there was an Avarian attempt to conquer Northern Italy. After their battlefield victory (ca. 611) over Gisulf II, the Lombardian Duke of Friuli, and while struggling around the Cividale fortress, the Avarian khagan and his entourage are depicted as armoured cavalrymen, charging with lances (Paul Diac. IV.37)[4]. About half a century later, King Grimoald I of the Lombards was forced to call upon the Avars to punish Lupus, the rebel Duke of Friuli, to whom he had temporarily entrusted the government of the northern parts of his kingdom, while he was fighting the Byzantines. The source clearly shows that during the three-day battle of Flovius (ca. 666), the Avars were using cavalry charges against the troops of Lupus,

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[2] (Greg. Tur. IV.29) – Gregorii episcopi Turonensis libri historiarum, ed. B. Krusch - W. Levison, Hannover 1951.

[3] (Theoph. Sym. VII.13-14) – Theophylacti Simocattae historia, ed. C. de Boor, Stuttgart 1972.

[4] (Paul Diac. IV.37) – Pauli historia Langobardorum, ed. L. Bethman - G. Waitz, Hannover 1878.

bringing him ultimately to defeat and death (Ibid, V.19). A rare mention of the Avarian infantry in contemporary written sources can be traced in a description of the great perso-avarian siege of Constantinople (626), where the Avarian cavalry and infantry is mentioned as being transported and deployed by the Khagan (Syncellus VIII)[5], but it is uncertain if it refers to Avarian infantry as such or to Avar-dependent Slav infantry.

Like the contemporary battle descriptions, the only preserved contemporary theoretical sketches of Avarian armament and tactics show the predominant role of the Avarian lancers. The Strategicon of Pseudo-Mauricius asserts the equestrian lance with a belt on it as the main Avarian weapon, recommending that Byzantine cavalrymen should also possess it. It is not clear what the exact function of this belt was, but as contemporary lances were still used in uncouched fashion, there are three main possibilities, all concerning better balance - function to tie the middle zone of the lance to the elbow (most probable), to tie the lance to the wrist, or to secure the lance to the shoulder (whereby lance is still used at waist height, using a belt as a means to transfer part of its burden of weight to the shoulder). As peculiar pieces of Avarian armour, this manual mentions the cord-dangling collets and wide, thick-padded tunics, with linen on the outside and wool on the inside, which he also recommended to the Roman lancers (Strategikon I.2)[6]. Thick-padded tunics, if worn as external armour, are known to be one of the most effective protection from arrows; they were relatively rare in contemporary Europe and in the Middle East, but were much more common in Central and Eastern Asia, from where the Avars certainly adopted it. Iron and felt horse breast-armour, which the author also ascribes to Avarian cavalrymen, is typical for contemporary heavy lancers whose only attacking direction was frontal (unlike the horse archers, who could attack from both sides and even from the back, and whose horses therefore had padded protection from all sides or had no armour protection at all). The widespread use of armour among the Avars, which the author stresses, most probably refers to both lamellar and thick-padded linen/woollen armour, usual for steppe cavalry. The previously mentioned collets, judging by their description, seem very much like the famous Avarian waistbands, protective belts with a ranking insignial function.

[5] (Syncellus VIII) – Theodori Syncelli Homilia, ed. F. Combefisius, Paris 1648.

[6] (Strategikon I.2) – Das Strategikon des Maurikios, ed. G. Dennis - E. Gamillscheg, Wien 1981

However, the manual defines them as necklets, which leaves two main possibilities - that the said collets were part of the neck and breast armour, or they were the waistbelts whose thick dangling cords resembled the Byzantine necklets. The author stresses that most of the Avarian horsemen were simultaneously skilled in lance and bow use, preferring to attack from a distance if possible, but admits that a frontal cavalry charge was their most preferred combat practice, if the terrain was suitable (Ibid, XI.2). He hints that the Avars used a special type of bow, but doesn't give details regarding its precise structure or specific method of use. As an equestrian bow, it certainly had a smaller range than those of the infantry. Thus, a need to engage the enemy infantry archers was the most probable reason for the Avars to develop their own, as the Huns did (who existed independently from the Avarian-dependent Slav infantry). Finally, the said manual does not denote stirrups as military items of an Avarian style, as in the case of the Avarian fashioned lance, padded tunic, collet and tent, which suggests the possibility that stirrups were adopted by the Byzantine army from some other foreign force, prior to the Avars. The evidence of the Avarian use of stirrups entirely relies on material evidence, which is quite abundant; the same is true of the matter of the Avarian saddle. It is undisputable, however, that both of them enabled a greater shock force for the Avarian lancers, and greater stability and shooting durability for the Avarian cavalry archers.

Historical background of agricultural population (Vinča sample): the Byzantine authority was restored in Belgrade and its hinterland (including Vinča) by 1018, and lasted until 1195, when it was taken by the Bulgarians (56). From then, Bulgarian and Hungarian authority over Belgrade changed several times until 1246, when it finally became part of the Kingdom of Hungary. The period of the late 13th and early 14th century was characterised by the increased progress of Belgrade, Vinča and the wider region. During this time, this area was an integral part of the territory historically known as Northern Serbia (Regnum Sesvie), ruled by King Dragutin (56).

The particular quality of some grave goods, especially silver jewellery, indicates that Vinča in the 11th and 12th centuries had to have been economically well developed. Since the settlement and its surroundings lay on fertile land along the river, the inhabitants were mainly engaged in farming, livestock breeding and fishing. In graves from the 13th and 15th centuries, archaeologists have discovered an extremely low number of grave finds. This could point to a general impoverishment of the Vinča inhabitants (56).

Although the series of human osteological excavations conducted before 2011 were anthropologically processed, information about the health status of the medieval Vinča population is, unfortunately, quite poor. According to the findings in the osteological material excavated in the time between 2011 and 2014, almost 25% of the full sample were non adults. The majority of pathological conditions were trauma and degenerative changes, while enamel hypoplasia (31.3%) and caries (34.4%) appear occasionally. When summarising the preliminary data regarding the health status of the medieval Vinča population, it can be concluded that the health status of this population was of a relatively high level.

Historical background of agricultural population (Sirmium sample): during the 12th century, the history of the Balkan Peninsula was filled with the difficult struggle of the Byzantium Empire to suppress the influence of the regions of Hungary and to consolidate the boundaries of its territory. However, the power of the Byzantines in Srem lasted only until the end of the reign of Emperor Manuel I Comnenus. Shortly after the change on the throne, the Hungarian King Bela III resigned as patron of Manuel's son Alexius II, and attacked the Byzantine assets, taking *Sirmium* first (75). In the territory of *Sirmium*, during the 13th century, a new medieval settlement of artisans and traders was formed and *Sirmium* was given a new name, after the monastery dedicated to the town patron St. Demetrius, *Civitas Sancti Demetrii* (55). The basic core of the village in the 13th century represented a monastery, officials and dependent people. In the beginning it was a village of farmers who were joined over time by "guests" (*hospites*), merchants and craftsmen and sometimes immigrants from distant places (76). The most favourable period in the development of *Sirmium* as a commercial centre was created in 1358, when Dubrovnik came under the rule of the Hungarian kings. This political change made it easier for the people of Dubrovnik, who had already been tied to the business of mining sections of Serbia and Bosnia, to expand their operations to the opposite bank of The Sava and to link Srem to the Adriatic coast. Dubrovnik, then in Srem, created the first and the largest colony in the Hungarian

Empire (76). Mitrovica was destroyed shortly after the great battle of Nikopol on 25 September 1396, which completed the heavy defeat of the Christian army. The Turks crossed the Sava River near Mitrovica and occupied the town. Mitrovica, on that occasion, was burned and devastated, while a large proportion of the population was taken into slavery, together with Christians from other devastated areas (76). From the 15th century, there is more data regarding political and military developments around Mitrovica. On several occasions a castle was mentioned. The castle belonged to King Sigismund, but later, although it is not possible to determine exactly when, Mitrovica came into the possession of Despot Đurađ Branković. It is assumed to have been in his hands from 1451 until 1458, when it again came under the direct rule of the king (76). In a large Turkish offensive in the summer of 1521, during which Šabac and Belgrade fell, the Turks crossed Srem and captured its cities. Soldiers from Mitrovica tried to disrupt the construction of the bridge near Šabac, but without success. It was the last desperate attempt of active resistance. After the construction of the bridge, it would only be a matter of time before Mitrovica would fall into Turkish hands. Mitrovica finally fell under Turkish rule in 1526 (76).

In contrast to the very scant information regarding the health of the medieval Vinča population, we have many more details about *Sirmium* citizens. In the area of *Sirmium,* historical sources state that, in the year 1202, many people died due to infectious diseases. It was also noted that there was a famine during 1217. In 1242, during the Tartar invasion, a tremendous famine ensued, the likes of which the chroniclers had never before witnessed. “There were numerous instances of people committing suicide in order to provide meat. Corpses of people murdered by the Tartars, or who had died of hunger were scattered everywhere. Animals which fed on human flesh multiplied. Unburied corpses were rotting, causing an unbearable stench, while poisoning and disease spread quickly. The famine was so intense that human flesh was being sold in markets. This state of affairs lasted for three years” (77). In the years 1263, 1264, 1270 and 1305, a great famine and plague surfaced. In 1311, the Danube rose after heavy floods, polluting drinking water. Malaria, dysentery and enteric fever ensued. In the following years, plague existed in these parts, but never to such an extent as in the rest of Europe. During the years 1365, 1375, 1386, 1410 and 1435, famine and contagious diseases returned (77). In the years 1444 and 1456, the plague raged throughout the

Balkans. It arrived in Vojvodina via Belgrade. After the crusaders' victory over the Turks in 1456, the plague decimated the victorious army. There was a severe drought in 1473 and a few years later, in 1479, the plague resurfaced. In 1485, an unidentified disease struck (*Sudor anglicus*, English sweating sickness), with written records from 1486 mentioning scurvy, whilst in 1495 and 1509–1511, the plague, once again, re-emerged (77).

Horse-riding as a general activity pattern: during prehistoric and historic periods, horse riding was a very common activity. Horses were not only used in battle, but also for agricultural work, transport, hunting, and even for pleasure. This need for horses in everyday life made them very valuable and cherished animals. As well as direct care, such as daily feeding, cleaning and treatment, the horse was given additional attention, which would have included training. In order to be useful the horse had to be healthy and in good shape.

The western steppe peoples of Classical and Late Antiquity (such as the Scythians, the Sarmatians, the Alans, the Huns or the Avars) are well known for the dominant role of horse riding in their wartime and daily life**.** The possession of one or more horses was exclusive to the higher social strata of society. However, someone had to take care of these horses and also to train them. Consequently, the use and, therefore, the training of horses for various activities was, in many ways, a complex process that certainly involved more than one person. The burials of some of the riders' population reflect the importance of their horses. The remains of horses have been recovered from Scythian royal tombs, decorated in ornamental bridles. The importance of the role of the horses is also reflected in the fact that they were even embalmed as part of the 40-day-long celebrations following the death of important rulers (78). In the Avarian population, horse sacrifice stems from the belief that a warrior needed a horse in the afterlife (74). The horse was buried at the same time as its owner. Several necropolises are known in Avarian archaeology where equestrian graves have been discovered. These include Sturovo in Slovakia, Bolj in Hungary and Vojka (74) and Čenej in Serbia. The practice of burials including a horse or even graves of horses without a person is very common in the Indo-European such as for instance Turkish peoples etc. However, if we do not have a horse burial connected with human graves or grave goods connected with riding, can we even assume horse riding practices based on skeletal remains?

Riding requires very different muscle actions (79). Horse riding uses all of the body's muscle groups, but specifically requires very strong and athletic postural muscles. During riding, the body activates the muscles of the abdominal musculature, some back muscles and the muscles of the upper and lower limbs. Horse riding is a combination of static postures (dressage or riding for pleasure) and dynamic postures (jumping, hunting and combat riding) (79). The muscular action depends on the rider's style. Different movements of the horse require different muscular acts (Fig. 12). Nevertheless, in every rider's style the lower limb muscles are certainly involved and some could be considered as specific for riding. Riding involves profound symmetrical stresses between the left and right side musculature, as both legs are responsible for riding movements. The main muscles of the lower extremities used in riding are: the quadriceps muscle, the hamstrings (posterior femoral muscles), the gluteals, the adductors, the hip rotators and the calf muscle (Figs. 13 and 14) (79).

Figure 12. Some horse riding styles.

All parts of the quadriceps femoris extend the knee (Fig. 15). In addition, the rectus femoris helps to flex the thigh in relation to the pelvis and, if the thigh is fixed, it helps to flex the pelvis in relation to the thigh. The rectus can flex the hip and extend the knee simultaneously. There is little or no activity in the quadriceps during standing.

When riding with long stirrups the heels are pushed down by putting more weight through the stirrups. In short stirrups, the quads work extremely hard to hold the body weight and control the position of the seat in and out of the saddle. Basically, training the quads in sustained postures should improve stability, especially when jumping (79).

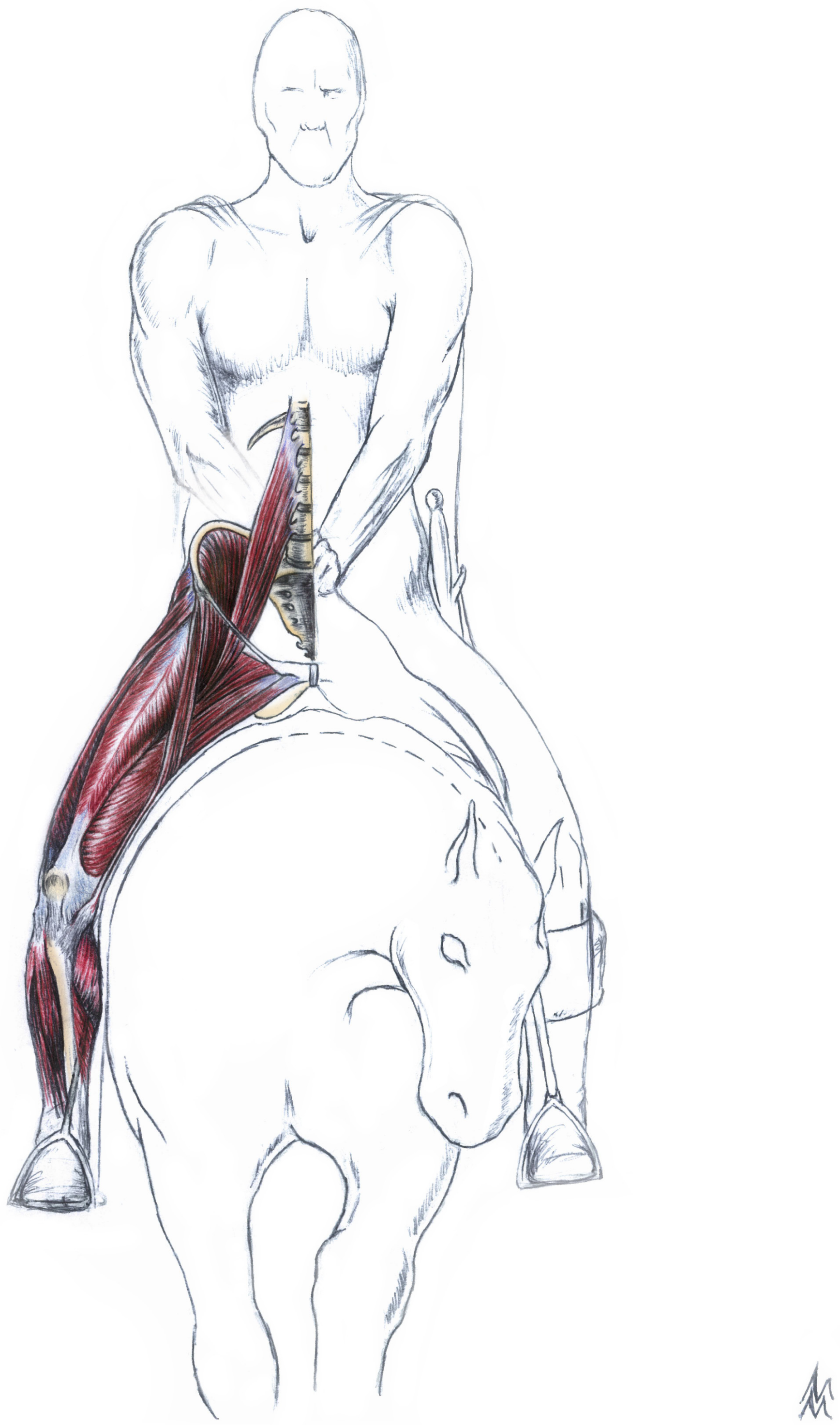

Figure 13. Muscles of lower limb active during horse riding (frontal view).

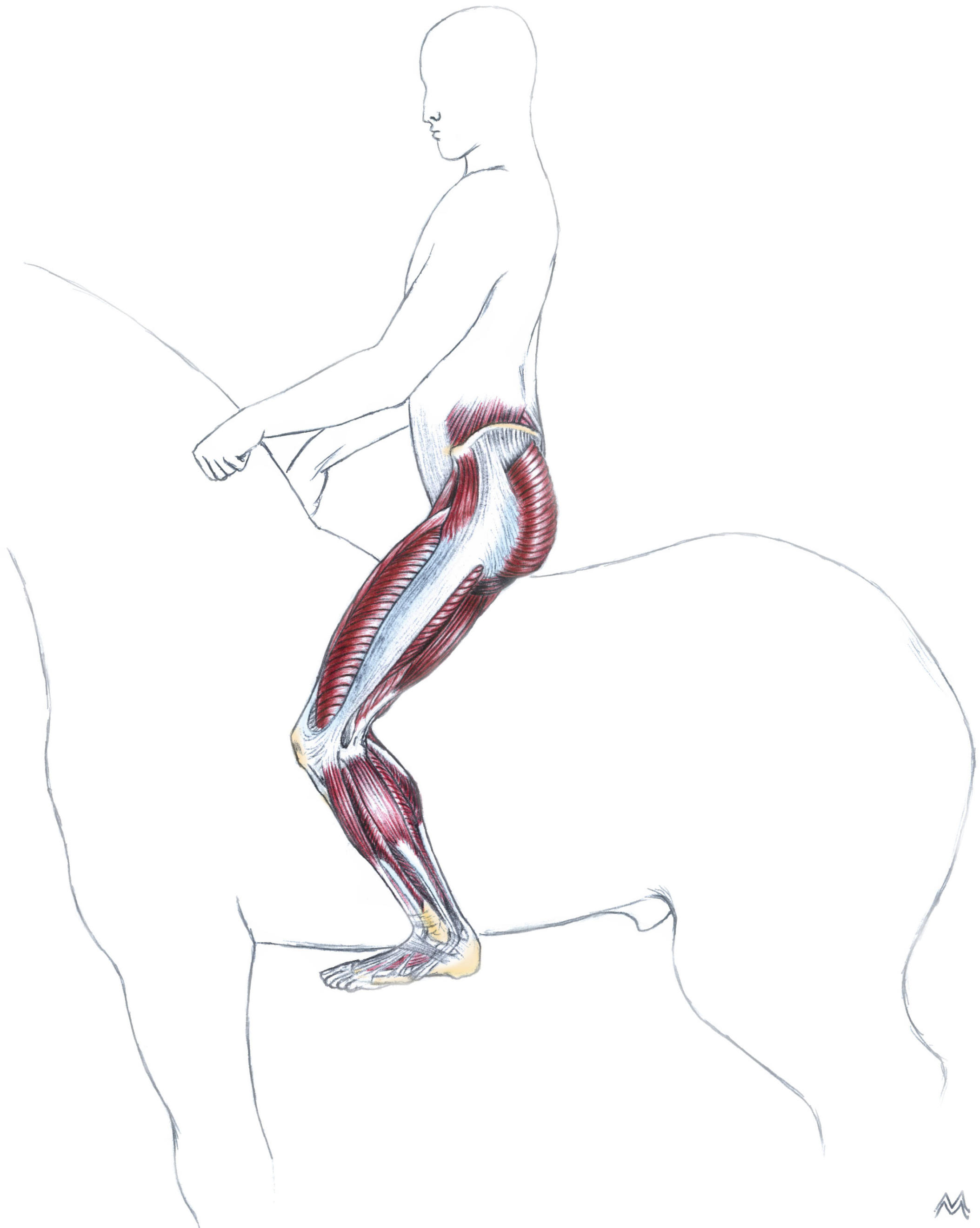

Figure 14. Muscles of lower limb active during horse riding (profile view).

The hamstrings are situated in the posterior compartment and comprise three separate muscles: the biceps femoris, semitendinosus and semimembranosus (Fig. 16). The hamstrings basically bend the knee and extend the hip (79). Acting from above, the posterior femoral muscles flex the knee and, acting from below, they extend the hip joint, pulling the trunk upright from a stooping posture against the influence of gravity, with the biceps being the main agent. When the knee is semiflexed, the biceps femoris can act as a lateral rotator and the semimembranosus and semitendinosus as the medial rotators of the lower leg. When the hip is extended, the biceps is a lateral rotator and the semimembranosus and semitendinosus are the medial rotators of the thigh. However, any action that moves the centre of gravity in front of a transverse axis through the hip joints, (e.g. a forward sway at the ankle joints or a forward bending at the hips), is immediately accompanied by a strong contraction of the hamstrings (80).

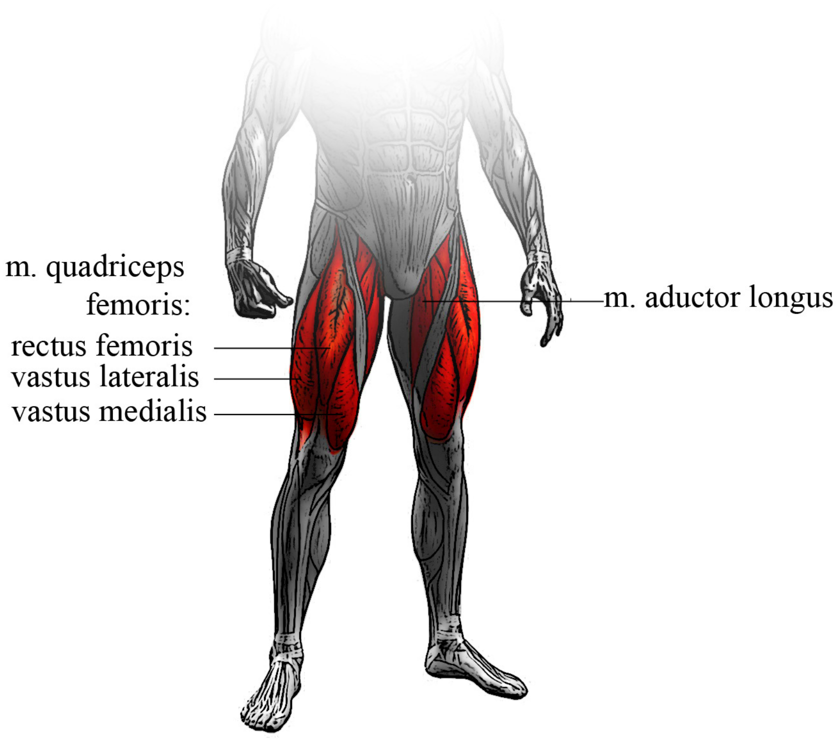

Figure 15. Muscles of upper lower limb (frontal view).

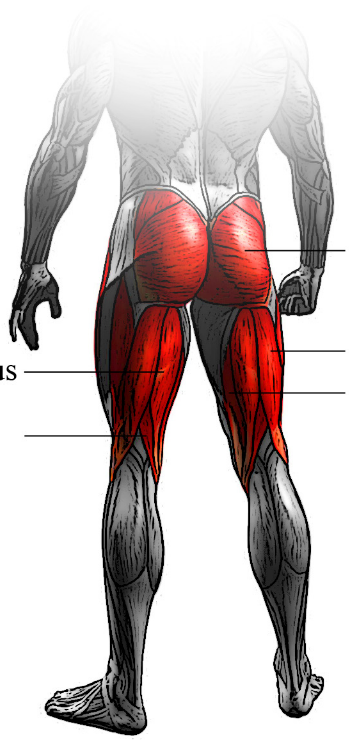

Figure 16. Muscles of upper lower limb (posterior view).

During riding the hamstrings are essential for obtaining a deep stable seat. By stabilising the heel and lower leg against the horse's side, the hamstrings are used to keep the seat deep in the saddle in combination with the abdominal muscles (79).

The gluteal muscles are a group of three muscles which make up the buttocks: the gluteus maximus, gluteus medius and gluteus minimus. The gluteus maximus is the largest of the gluteal muscles (Fig. 16). Acting from the pelvis the gluteus maximus can extend the flexed thigh and bring it into line with the trunk. Acting from the distal attachment, it may prevent the forward momentum of the trunk from producing flexion at the supporting hip during a bipedal gait. The muscle is inactive during standing, swaying forwards at the ankle, or when bending forwards at the hip joints to touch the toes. However, it acts with the hamstrings to raise the trunk after stooping, by rotating the pelvis backwards on the head of the femur. It is intermittently active during the walking cycle and when climbing stairs, but it is continuously active during the strong lateral rotation of the thigh. Its upper fibres are active during the powerful abduction of the thigh. It is also a tensor of the fascia lata and, through the iliotibial tract, it stabilises the femur on the tibia when the knee extensor muscles are relaxed (80).

Both the gluteus medius and minimus, acting from the pelvis, abduct the thigh and their anterior fibres rotate it medially. Acting from the femur, they play an essential part in holding the trunk upright when the foot of the opposite side is raised from the ground during walking and running. In this phase the body weight tends to make the pelvis sag downwards on the unsupported side. This is counteracted by the gluteus medius and minimus of the supporting side which, acting from below, exert such a powerful traction on the hip bone that the pelvis is actually raised a little on the unsupported side (80). Additionally, riders use the gluteal muscles in the jumping position and in the rising trot (79).

Although the adductor magnus, longus and brevis can act as adductors, they more commonly act as synergists in the complex patterns of gait activity and, to some degree, as controllers of posture (Fig. 15). They are active during the flexion and extension of the knee. The adductors are inactive during the adduction of the abducted thigh in an erect posture (when gravity assists), but active in other postures, such as the supine position or during the adduction of the flexed thigh when standing. They are also active during the flexion (longus) and extension (magnus) of the thigh at the hip joint. In a position of symmetrical standing their activity is minimal (80). It should be kept in mind that an extensive or forcible adduction of the femur is not often required (80). Basically, these muscles serve the function of keeping the legs pressed against the horse whilst riding. This group of muscles, in conjunction with the hip rotators, controls the position of the whole leg against the saddle and the horse's side. Riders tend to over use these muscles and grip the saddle in modern riding techniques (79).

The calf muscle is actually made up of two muscles: the gastrocnemius and soleus muscles. These muscles are the primary plantar flexors of the foot. The gastrocnemius is also the flexor of the knee. They are usually large and correspondingly powerful. The gastrocnemius provides force for propulsion in walking, running and jumping. The soleus, acting from below, is more concerned with steadying the leg on the foot while standing. This postural role is also indicated by its high content of slow, fatigue-resistant muscles fibres. In many adult mammals the proportion of this type of fibres in the soleus approaches 100%. However, such a rigid separation of functional roles seems unlikely in man: the soleus participates in locomotion, and the gastrocnemius in posture. The ankle joint is loose packed in the erect posture and, since the weight of the body acts through a vertical line that passes anterior to the joint, a strong brace is required behind the joint to maintain stability. Electromyelography shows that these forces are supplied mainly by the soleus: during symmetrical standing the soleus is continuously active, whereas the gastrocnemius is recruited only intermittently (80). These two muscles are mostly used during walking and running, allowing the heel to be pushed down below the level of the toes (dorsal flexion). As a result, all riders need this muscle to be longer than it is in the general population (79).

Apart from the fact that, based on the different appearance of a muscle’s bony imprints, an assumption can be made regarding a person’s daily activities, it should not be forgotten that life on horseback had other associated risks such as injuries. For instance, nowadays, horseback riding carries a higher injury rate than motorcycle riding (http://www.hughston.com/hha/a.horse.htm 28.10.2015. 15:14). The most common location of horse related injuries is in the upper extremities, followed by lower extremity injuries, while the head and face sustain 20% of injuries (81). If we assume that today's jockeys spend much less time on horseback than the horsemen of ancient times, we can also assume that the risk of injury was much higher among the ancients. However, a high frequency of calcaneal fractures associated with frequent dismounting is noted in the archaeological horseback population (82). Also, when describing the lives of two Scythian horsemen from Alexandropol, Wents and Grummond (2009) reported fractures of the clavicle and the shaft of the humerus, along with damage of the elbow (83). Another bony change that can be related to habitual riding is a variety of asymmetries between the two sides of the body.

Changes in skeletal morphology associated with horseback riding have been noted in several studies (83-85). However, are the bony imprints that are indicative of habitual riding reliable indicators, and which muscle or muscle groups identify horsemen from the other members of the investigated population?

The interpretation of significant differences among the subgroups can cause problems. In this paper, the focus has been on population differences, rather than trying to assign specific occupations to individual skeletons. This has been approached in two ways, trying to identify general patterns of activity, and to reconstruct daily activities. The attempt to reconstruct daily activities is based on two combined viewpoints: an attempt has been made to identify the repetition of motions for specific entheses with a high frequency of lesions, and to interpret these body movements.

Macromorphological analysis of EC (relationship between age and EC): the correlation between the occurrence of entheseal morphological changes and biological age has been reported in numerous studies worldwide which were based on the evaluation of skeletal collections with known ages (1, 12, 48). This correlation has also been analysed in skeletal material where the age has been assessed according to some morphological characteristics of the skeleton, but has not been documented (24, 51, 86). The majority of authors concluded that entheseal changes are more pronounced in older individuals (1, 12, 24, 48, 57). The stand point for this assumption is that the muscles of elderly people have been in use for a long time, and that more pronounced muscle markers arise as a result of continuous and repeated muscular work (39). However, it would be logical to suppose that in older individuals the development path of morphological changes occurring on the place of muscles attachment sites is actually affected not only by mechanical stress but also by the changes in bone quality associated with age. Therefore, analyses of the micro structural proprieties of the underlying bone at the place of muscle attachment sites could clarify general patterns associated with morphological changes of entheses.

This study has also recorded a statistically significant correlation between some attachment sites and age. Analysing horse riders and the agricultural population separately, a statistically significant difference between dissimilar EC scores and age was noted in both investigated samples. On the horse riders' upper limbs a statistically significant difference between age categories was recorded in the attachment sites of the m. subscapularis, mm. supraspinatus and infraspinatus, m. pectoralis major, m. pronator teres and muscles attached on the epicondylus medialis humeri. All of these entheses show a more pronounced morphological appearance in older age groups. On the lower limbs, a statistically significant difference was noted among different age groups only in the case of the m. iliopsoas. The results may suggest that the physical activities of the upper limbs were more or less consistent, i.e. performed from younger to older age, while the activity of the iliopsoas is, perhaps, related to the intensive use of this muscle in younger individuals. The activity of the iliopsoas muscle could be related to horseback riding, since this muscle is active in bending the trunk and pelvis forward against resistance, as well as in the raising of the trunk from a recumbent to a sitting position when undertaking a "sit-up" (80).

The development of the muscles attached to the lesser trochanter (iliopsoas muscle) is reported in, among other activity patterns, horseback riders where the stabilisation of the hip is necessary (82). Enthesopathies of this attachment site were found in individuals from the predynastic Royal Cemetery of Ur, which suggests that horseback riding and cart driving could account for its development (87).

Among the agricultural population, on the upper limbs a difference is observed only in the attachment sites of the m. supraspinatus and infraspinatus, as well as the muscles which are attached to the epicondylus lateralis humeri. The activity patterns related to the action of the m. supraspinatus and infraspinatus will be explained in more detail hereinafter. The activity patterns related to the action of the muscles which are attached to the epicondylus lateralis humeri, in anthropological literature, were linked with the sling (13). The use of the sling in agricultural communities can be related with the herders to tend flocks or with farmers to protect crops from scavengers (13). On the lower limbs, only the entheses of the gluteus maximus muscle demonstrate a statistically significant difference among the age categories. The results show that the entheseal changes are more pronounced in the older age categories. This pattern, which suggests that the entheseal changes are always more pronounced in the older age category, can point to the assumption that the agrarians' level of physical activity increases with age. An alternative could lead to the conclusion that, during the individual's lifetime, the bone quality reduces with the result that a resulting muscle load of the same intensity influences a more dramatic entheseal change in the elderly.

Macromorphological analysis of EC (relationship between sex and EC scores): sexual dimorphism of EC scores in the group of horse-riders was noted on the lower limbs only. All attachment sites which demonstrated significant differences showed greater markers of stress in males.

According to the literature, the situation where EC scores proved higher for males than females, seems to follow a general pattern and was reported in several EC studies (14, 26, 50, 51, 86). For instance, investigating a gender based division of labour in the Natufian and the Neolithic populations, Eshed et al. (2004), found that the males of both populations appear to differ significantly in the muscles of the upper extremity attached to the humerus (the teres major, the pectoralis major, and the deltoideus) (50). The skeletal remains from the Middle Neolithic (2750–2300 BC) burial at Ajvide, Gotland, were analysed in order to explore musculoskeletal patterns of this prehistoric population, and significant positive correlations were observed in male individuals in muscle groups associated with archery and, to some extent, harpooning (51). Bearing in mind that the upper limbs have no difference between the sexes and that, in the case of the lower extremities, men suffered larger loads, the physical activities which were performed by the upper limbs were probably relatively equally represented. In contrary, the results for the lower limbs suggested that males were more exposed to greater physical activity. If riding was exclusive for Avar men, it could be concluded that this physical activity caused these differences. In particular, among other lower limb entheses, statistically significant differences were also noted in the cases of muscles that are specific for riders.

In the agricultural population, the area of the greater tubercle of the humerus and the insertion of the iliopsoas muscle demonstrates a statistically significant difference between the sexes, suggesting more a pronounced EC in males. The mm. supraspinatus and infraspinatus, together with the m. subscapularis and m. teres minor (rotator cuff) assists in the stabilisation of the head of the humerus in the glenoid fossa during shoulder movements (80). Individually, the m. supraspinatus is involved in the abduction of the shoulders and assists the deltoid in the abduction thereafter, while the m. infraspinatus is a lateral rotator of the humerus (80). The population groups from these archaeological sites mainly consisted of individuals whose daily activities were associated, probably, with building operations and activities relating to farming. However, among those activity patterns, specific behaviours which were often linked with rotator cuff muscles are, for example, archery and harpooning (51). The archer's drawing of the bowstring mainly involves the supraspinatus and infraspinatus of the string-arm, while the flexors of the same side would also be active. The deltoideus and triceps brachii are affected in the opposite arm, which would be the arm that extends to hold the bow in this movement (51). If we assume that the everyday activities of women also involved the aforementioned muscles and if we know that drawing a bowstring mainly involves the mm. supraspinatus and infraspinatus of the string arm, it is debatable whether this finding is strong enough to conclude that the cause of this difference is archery. Moreover, given that in our agricultural sample we do not have archaeological findings which imply the frequent or widespread use of archery, the idea that this activity pattern causes a more pronounced EC of the greater tubercle of the humerus is not sustainable.

Harpooning mainly uses the deltoideus and pectoralis major muscles and the rotator cuff (m. teres minor, mm. supraspinatus and infraspinatus, and m. subscapularis) of the working arm (51). Since the investigated archaeological sites are near large rivers (The Sava and The Danube), the use of harpoons could have been an everyday activity. However, archaeological confirmation of the use of harpoons on these sites is lacking.

Our results in the lower limbs suggest that only the iliopsoas muscle shows statistically significant differences among the sexes, with higher EC scores in men. The activity of the iliopsoas muscle has been documented in football players, skiers and horse riders (82). However, the activity of the iliopsoas is related to all motions that include flexion of the hip joint. The variety of agricultural activities comprising bending the body forward (flexion of the hip joint) is significant and it is involved in almost every movement in agricultural work. Additionally, riding could also provide this difference among the investigated sample.

Loading of the individual entheses of the upper limbs and the possible associated activities: the evaluation of the entheseal changes of the upper limbs can tell us much about the everyday physical activities of ancient populations. Most of the muscles of the shoulder, elbow and forearm work as functional groups, either as synergists or antagonists.

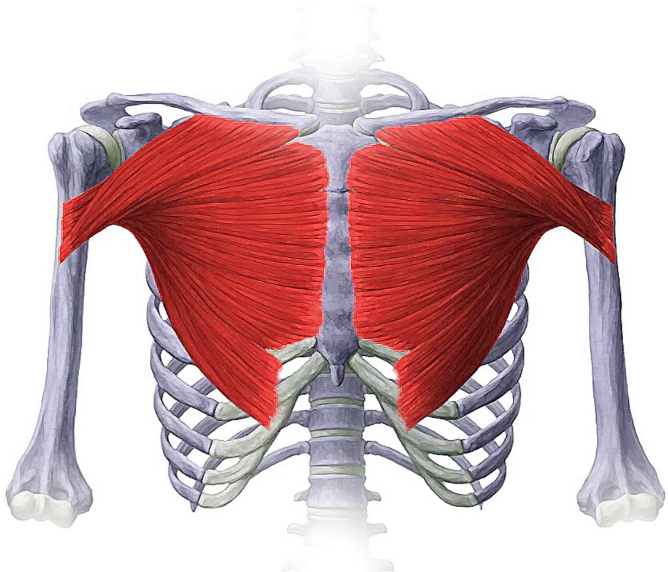

Figure 17. Pectoralis major muscle (frontal view).

The only difference between the sides in the whole sample was the pectoralis major muscle (Fig. 17), which demonstrated predominance on the right side i.e. higher EC scores. Furthermore, this difference was found only in the agricultural population. The pectoralis muscle assists the humeral adduction and medial rotation (80). In anthropological literature, the specific activities which mainly engage the pectoralis major muscle and the muscles of the rotator cuff are associated with harpooning (51), kayaking using a double-bladed paddle (9, 26), lifting a load from the a squatting position (82) and the scraping of animal hides where the opposite arm is holding the heavy skins with the humerus adducted toward the chest and the elbow at an obtuse angle (26, 82). All of these activities could be part of the everyday activity pattern among the analysed population. Side differences found in the EC score of the pectoralis major suggests that, in everyday life, agriculturals have different uses of the left and right arm. However, when considering kayaking, entheseal changes on both sides should be expected and, therefore, an almost equal degree of EC on both sides should also be expected. In harpooning, dexterity should probably be considered. Knusel (2000) noted that dexterity can be complex to trace in skeletal remains (88). However, it can only be assumed that approximately 90% (both today and recorded in historical times) of the total population were right handed (51). Lifting a load from a squatting position was considered to be a common activity in everyday life, particularly because this movement could often be performed in the agricultural community. In literature it is suggested that lifting a load from a squatting position should be associated with the activation of the pectoralis major muscle and brachialis muscle (82) and, therefore, hypertrophy of both attachment sites should be excepted, but was not found in our material.

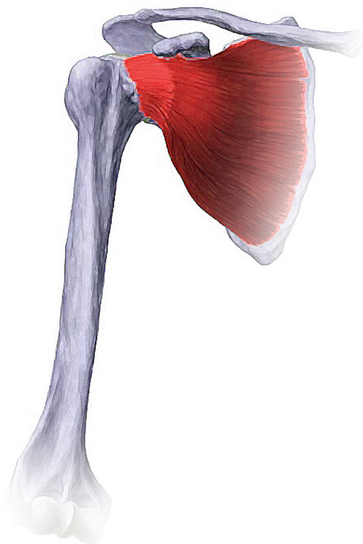

Figure 18. Subscapularis muscle (frontal view).

In addition, the scraping of animal hides should include activity of the pectoralis major and teres major muscles (82). Given that this activity was very common in the prehistoric population with the predominance of a hunter-gatherer economy, in the majority of medieval populations it was probably not so customary. Therefore, the possibility of significant EC due to the scraping of animal hides in medieval populations was not tenable.

A statistically significant difference of the entheseal morphological appearance between riders and the agricultural population was found in the upper limbs only in the subscapularis muscle (Fig. 18), indicating a greater exposure to stress for the riders' population group. Entheseal changes of the attachment sites of the subscapularis, the flexors and the extensors of the elbow and wrist occur today in several sports and occupations involving repetitive and/or forceful tasks (89). It appears difficult to identify specific motions according to the entheseal changes of one attachment site, especially because the majority of motions of the upper limbs mobilise several muscles.

Loading of the individual entheses of the lower limbs and the possible associated activities: most of the attachment sites that showed significant differences in the EC score between the two investigated populations were associated with the lower limbs. This indicates a greater exposure to load stress among the population of riders compared to the agricultural population.

Due to the lack of studies related to entheses of the lower limbs and the fact that the lower limbs are involved in more generic movements, the reconstruction of activities associated with the lower limbs is much more complex. In the comparison of individuals (pooled sexes) of the horse riders' and agricultural populations, statistically significant differences were noted in muscles attached to the tuberculum ishiadicum (hamstrings muscles), vastus medialis muscle, and three adductor muscles. All these entheses show a more pronounced EC in the horse riders' population. Several observations can be made with regard to the anticipated musculature and concomitant bony changes associated with horseback riding.

Since, in the investigated sample, only the muscles which are specific for horse riders were singled out, it can be concluded that the Avarian population used riding as an everyday physical activity more than the agricultural population. However, can we isolate the most important rider muscle group which is used only during riding? Although riding is actually a movement supported by several muscles groups, the adductors are the most specific for riding. The adductors are the most specific for riding because the action of keeping the legs together is not common in everyday activities, while during riding this action is almost continuous and every rider has felt pain in these muscles after riding (79).

Bone microarchitecture at muscle attachment sites (development of entheses - lessons from embryology): the proper understanding of entheseal morphology and the potential relationship to muscle contraction requires the recognition of the developmental pathways. In this context, recent developmental studies of mice showed that the formation of bone ridges is initiated irrespective of muscles and is governed by molecular signals deriving from Scx+ tendon cells which cause BMP4 expressions in bone and subsequent entheseal bone growth (90, 91). It seems that bone ridge initiation is predetermined, indicating the necessity of the attachment site to provide sufficient initial anchoring capabilities for the muscle. However, when a certain ridge size is achieved, the muscle is connected and the further regulation of bone ridge growth depends primarily on muscular activity (90). Many bone ridges are formed through the endochondral ossification pathway; however, considering the large variability in the morphology of bone ridges, it is not known whether they all develop in the same way (90, 91). There is some evidence from animal studies that fibrous entheses are formed postnatally and that parathyroid hormone related protein (PTHrP) is an essential regulator of their development (92); PTHrP is used as a "load-induced modelling tool that directs osteoclasts to excavate the root system by which these sites attach to the cortical surface" (92, 93). We are just beginning to understand the development of entheses, and many questions regarding these processes are still unanswered (91). However, the current understanding of entheseal development suggests that their initial growth follows a particular genetic program.

Bone microarchitecture at muscle attachment sites (understanding load transfer to bone): a very early mention of the mechanical significance of bone form was done by Galileo (1638). He understood that bones in large animals were not simple scaled up version of bones in small animals, but they are also a different shape (94).

The crucial mechanical function of bones is to provide rigid levers for muscles to pull against, as well as to remain as light as possible to allow efficient locomotion. Consequently, bones must adapt their shape and architecture to make efficient use of material. Bone adaptation during skeletal growth and development continuously adjusts skeletal mass and architecture to different mechanical environments. There are three fundamental rules that manage bone adaptation. First, mechanical adaptation is driven by dynamic, rather than static loading. Second, only a short duration of mechanical loading is necessary to initiate an adaptive response, and third, bone cells accommodate to a customary mechanical loading environment, making them less responsive to routine loading signals (95). The famed Wolff's law states that bone in a healthy person will adapt to the loads under which it is placed. If loading on a particular bone increases, the bone will remodel itself over time to become stronger to resist that type of loading. Also, if the loading on a bone decreases, the bone will become less dense and weaker due to the lack of the stimulus required for continued remodelling (96). Therefore, the internal architecture undergoes adaptive changes, both in the trabeculae and cortical portion of the bone. However, alternative loading directions also can cause internal architecture adaptive changes, particularly where the trabeculae realign accordingly. Providing only the horizontal loads are reduced, the vertical trabeculae stay intact, but some of the horizontal trabeculae become disconnected and finally resorbed (97). This suggests that the trabeculae are mostly aligned with the principal stress direction which maintains bone strength (97). For instance, investigating age related changes of the proximal femoral cancellous bone, Djuric et al. (2009) found that an increased anisotropy in this region suggests that the trabeculae are mostly aligned with the principal stress direction (98).

Bone microarchitecture at muscle attachment sites (mechanical adaptation of the bone at an enthesis): it is generally believed that the entheses are structurally designed to meet particular mechanical demands (99). It was suggested that the microscopic structure of the whole enthesis is "optimized to minimise stress and strain concentrations associated with load transfer from the relatively compliant tendon to the relatively rigid bone" (100). This is visible in compositional differences of entheseal histological layers, ensuring gradual changes in stiffness and accepting/transferring mechanical stress from muscle to bone (5). It is believed that differential cortical and trabecular microstructural patterns may not only be a consequence of mechanical stress but also that they may be directly involved in how the stress is dissipated. In this context, it is encouraging that recent studies have started using histomorphometric approaches to assess bone turnover history when investigating the relationship between mechanical strain and enthesis development (101).

It is generally believed that bone ridges provide a stable anchoring point for tendons. Since most of the load generated by muscle contraction and transduced by the tendon encounters the bone ridge before the rest of the bone volume, the bone ridge is likely to absorb/dissipate some of the stress concentrated at the hard-soft tissue interface, diminishing the risk of avulsion fractures (5, 102, 103). Further simulation studies should also clarify whether and how exactly the formation of a bony prominence for tendon attachment is mechanically favourable over the direct attachment to the flat bone surface.

Although muscle activity was frequently linked to overall bone cross-sectional geometry (104-106), further experimental or simulation studies are necessary to check whether, or how, mechanical strains derived from the muscle contraction dissipate along the diaphysis, or if they mostly concentrate at the insertion sites (107). To this end, the comparison of cortical thickness between muscle attachments sites and the surrounding bone might offer some clues. Using pQCT scanning of humeri, Niinimaki et al. reported that cortical thickness of the humeral shaft was greater at muscle attachment sites than at non-attachment points (108). However, our findings showed that the cortex is not necessarily thicker at the entheseal sites than at non-attachment sites within the same cross-section, especially in stage C in the case of the gluteal tuberosity and lesser trochanter (see Figures 7-10). Although this might be partly dependent on the type of entheses (fibrous vs. fibrocartilaginous) (109), further studies are necessary to clarify the origin of variability in cortical thickness within a cross-section.
Though tubercles are more accepted as occurring on real bones than are pits, pits are not rare. As a response to the stress generated by a tendon attached to them, the existing bony tubercles will adapt, with time, in accordance with the changes in the direction of pull, Currey (1968). This theory was based on the fact that tubercules adapt their morphology to realign their long axis with the new direction of pull. In these circumstances bone needs to deposit in the place where the stress is high (110).

In order to analyse the effect of the shape of the bone on the stress, Hirschberg (2000) generated a Flac[7] model. Hirschberg studied the effects of the presence of a tubercle on longitudinal compressive stress existing in the bone using Algebraic Stress. His findings suggest that the stress increases at the bases of tuberculum, but decreases the stress within it. The compressive stress reduces with height within the tubercle and, in the area near the top of the tubercle, was almost negligible. In the region of the corners beneath the tubercle's base, the stress was slightly increased. Therefore, regardless of the existing pattern in the bone, tubercules serve to reduce the stress. The pit has the opposite effect. It increases the stress near its lowest part, but reduces the stress at its

[7] Fast Largangian Analysis of Continua (Flac) is an explicit finite difference program that performs a Largangian analysis to model the behavior of a physical system. In this particular case Flac model represents a bar loaded uniformly in compression along the left and right edges, with tubercle or pit in the middle of the upper edge.

edges. If the edges of a pit are observed as convexities (tubercles), then a stress reducing effect in this region is expected.

This analysis leads to an assumption that if there has been no recent change in loading and mechanical equilibrium is achieved, the surface of the bone should be at the remodelling threshold. Given that there should be no depositing or resorbing, the bone surface stays flat. Also, if a small pit were formed, then the stress in the bottom surrounding of the pit would be increased. This new increased stress situation now causes bone deposition, so the pit would be eliminated. Otherwise, if a small amount of bone were deposited on the surface, it would serve as a tubercle with the aim of reducing the stress. This reduced stress would now cause the process of resorption, thus eliminating the tubercle. This simple mechanism ensures that a long bone's surface remains flat (110).

Bone microarchitecture at muscle attachment sites (as incurred pit or tuberculum): where tendons attach to bone the state of stress has two main sources: tension (pull of the tendon) and longitudinal compression (from joint reaction forces) (110). During the movement that is produced by the action of the muscle, if the change in the angulation of the tendon to the bone surface is small, then the state of tension may protrude some distance into the bone, leading to the formation of the pit (94). This is because the load producing tension at the small region of attachment of a large tendon is very much greater than the load producing compression resulting from body weight. Conversely, if there is a large change in the angulation of the tendon to the bone surface, then the situation may change. That is, though there will certainly be tension in the tendon some distance from the bone, this will be reduced close to the bone because the tendon fibres are under compression laterally, being constrained at their attachment. When the larger the change in angulation during muscle function the consequence will be development of tuberculum at the place of attachment site (94).

Bone microarchitecture at muscle attachment sites (what can bone microarchitecture tell us): analysis of bone microstructure may offer additional clues related to the structural effects of muscle loading, given that persistent or repeated mechanical stress may drive bone microarchitectural adaptation - as suggested by the "bone functional adaptation law" (104, 111-115). In this context, bone microarchitecture may better reflect bone loading history (17, 112, 114), and such a link was evaluated at various skeletal sites experiencing different loading conditions (16, 18, 116). However, the microarchitecture of the bony parts of entheses has been widely neglected, and the relationship between the macroscopic and microscopic appearance at the sites of muscle attachments has not been established.

Relationship between bone macro and microstructure at the enthesis: the microarchitectural evaluation of the entheses with various macromorphological expressions (stages A, B and C) revealed a lack of any direct and consistent relationship between the macro and microstructural features, indicating that macroscopic EC stages do not represent distinct successive phases in bone adaptation to mechanical loading.

Our results demonstrated that macroscopic stage C entheses seemed to show a tendency towards an improved cortical and trabecular microarchitecture (higher cortical thickness, decreased cortical porosity and pore diameter, increased trabecular bone volume fraction and trabecular thickness) in comparison to stage A entheses; however, the consideration of all three entheseal stages (A, B, C) showed that microarchitecture did not demonstrate consistent trends with increasing macroscopic entheseal scores. Particularly, there was a lack of further improvement in the cortical and trabecular microarchitecture of entheses in stage C compared to stage B. Specifically, cortical thickness differed significantly between the EC macroscopic stages, but there was no gradual increase in cortical thickness with increasing entheseal scores, given that stage C showed significantly lower thickness than the stage B. Although macroscopic stages B and C presented an improved trabecular microarchitecture in comparison with stage A, our microarchitecture findings failed to support the successive nature of macroscopic stages. Only two parameters showed a tendency (non-significant) to a continuous decrease with increasing entheseal scores: cortical pore diameter and trabecular degree of anisotropy.

With simplified macroscopic scoring ("no changes" *vs.* "any type of change present"), only the degree of anisotropy was significantly decreased, suggesting that trabeculae are more randomly oriented in the cases of higher entheseal changes.

Correlations between microarchitectural parameters, depending on EC score: mutual relationships between the microarchitectural properties differed depending on the macroscopic stage of EC. In stage A entheses, cortical thickness was associated with separation between the pores but not with pore diameter, which might indicate that it is more associated with the number of pores. Stage B showed that cancellous bone volume fraction depended mostly on trabecular number, while cortical porosity was mainly determined by the diameter of cortical pores. However, no cortical–trabecular associations were ascertainable in stages A and B. Yet, the stage C entheses displayed most cortical–trabecular associations: cortical thickness showed a significant direct relationship with trabecular thickness, and smaller cortical pores were associated with better connected trabeculae, both relationships indicating that the cortex and trabeculae follow the same adaptation patterns (positive bone balance) in stage C entheses.

Therefore, our microstructural findings did not provide support to the theory that macroscopic scores A to C really represent different phases (temporal stages) in the activity induced entheseal modelling. Moreover, mutual correlations between the microarchitectural parameters were not consistent and differed depending on the macroscopic stage. Hence, it seems that the relationship between a bone's microstructural design and the macroscopic scores used in anthropological analysis is not straightforward, which challenges the established anthropological practice of musculoskeletal markers and warrants further investigation of bone adaptation patterns to external loading.

Patterns of microstructural parameters in "proliferative" and "resorptive" phases at the enthesis: in further analysis patterns of microstructural parameters were evaluated in the "proliferative" and "resorptive" phases at the enthesis**.** According to Villotte (2006), the fibrous entheses among the EC expression scores' three stages were distinguished (stage A; stage B; and stage C). While stage A represents a completely regular surface, according to the morphological appearance of bone in stage B, two "subphases" are distinguished: B(a) is characterised by the presence of significant irregularity in most of the insertion zone and the absence of enthesophytes, while B(b) is characterised by the presence of lacuna of cortical bone with a length of less than 20 mm. Likewise, in stage C, two "subphases" could be recognised: C(a) with domination of a significant irregularity in most of the insertion zone and the presence of one large or several smaller reliefs which are distinguished from the insertion zone; phase C(b) is characterised by the presence of lacuna of cortical bone with a length of greater than or equal to 20 mm (10). It could be summarised that the macroscopic appearance of entheses in stages B and C involves morphological changes showing either predominant signs of "bony formation" (stage B(a) and stage C(a)), or pronounced signs of bony resorption (stage B (b); stage C (b)) (Fig. 19). It is unclear whether these two "phases" result from different loading patterns or they are successive steps in the adaptation to mechanical loading at the enthesis. To investigate the internal architectural differences between the "proliferative" and "resorptive" phases of entheseal changes, a micro-CT analyses was performed of the gluteus maximus attachment sites with predominant signs of "bone formation" (i.e. "bony proliferation") and those with dominant traces of "bone resorption" at the level of macromorphological entheseal appearance (stage B(a); stage B(b); stage C(a) and stage C(b)).

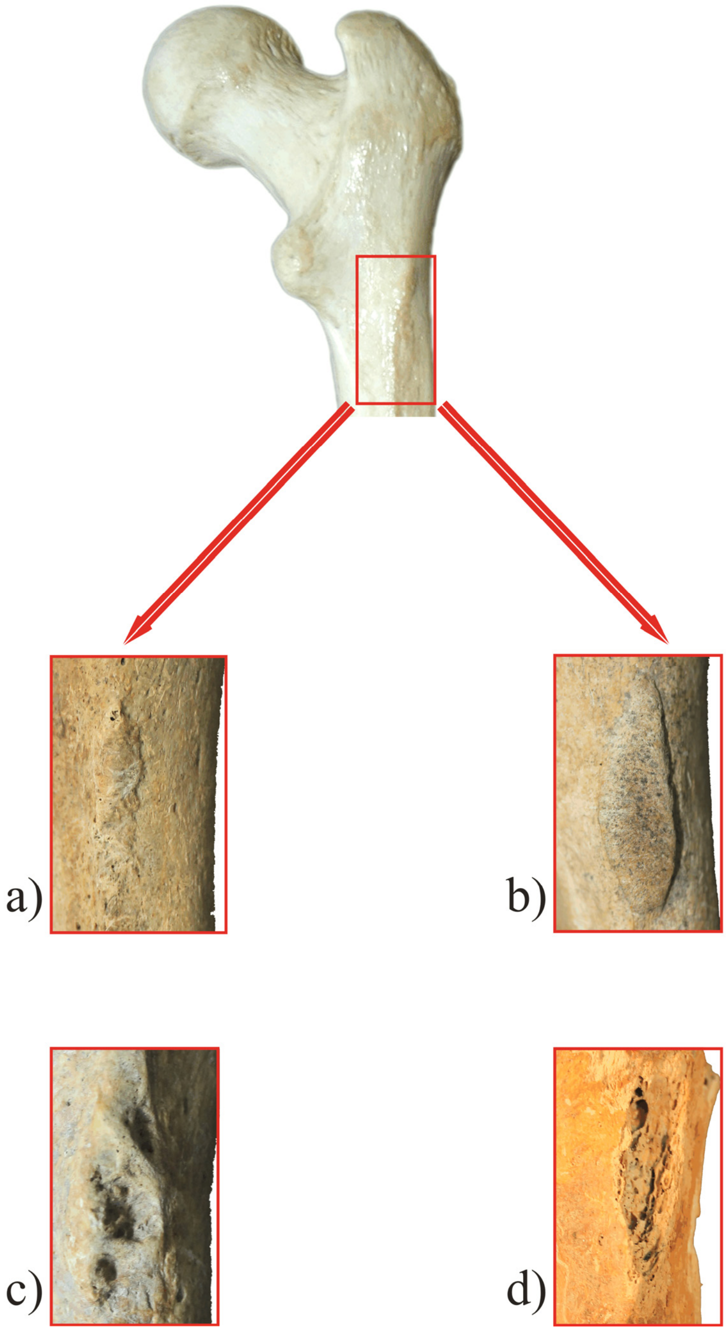

Figure 19. Macromorphological appearance of enthesis in "proliferative" and "resorptive" phases:

a) Stage B(a) (see classification in material and method section)
b) stage C(a) (see classification in material and method section)
c) stage B(b) (see classification in material and method section)
d) stage C(b) (see classification in material and method section)

Comparison between the "proliferative" and "resorptive" phases among different stages of EC suggested that there is no consistent difference among them at the level of trabecular and cortical microarchitecture (Table 15 and 16). For instance, the "resorptive" phase of stage B showed a 30% increase in cortical porosity when compared to the corresponding "proliferative" phase, whereas the "resorptive" phase of stage C had a 47.27 % reduction in cortical porosity (Chart 1). Actually, in both phases of macroscopic stages B and C, the trabecularisation (increased porosity) of cortex exists and grows in the following direction: B(a)→B(b)→C(a). However, such a trend of growing cortical porosity did not continue to stage C(b), where lower cortical porosity was found than in C(a). It was also noticed that behind those macroscopic changes whose appearance corresponds to C(b), actually two different microarchitectural patterns were evident. The first microarchitectural pattern is characterised by the defect of the cortical surface with increased trabecularisation of the underlying bone while in the second microarchitectural pattern a defect of the cortex is not accompanied by trabecularisation of the underlying bone (Fig. 20).

Our results could suggest that from the initial flat surfaces at the place of a muscle attachment site, two directions of EC development are possible. One is a clearly expressed prominence and trabecularisation of the cortical bone inside the prominence. This trabecularisation of cortical bone can lead sporadically to visible porosity on the cortical external surface. In the second option, a surface cortical bone defect develops with the regular cortical bone morphology under the lacuna (Fig. 21).

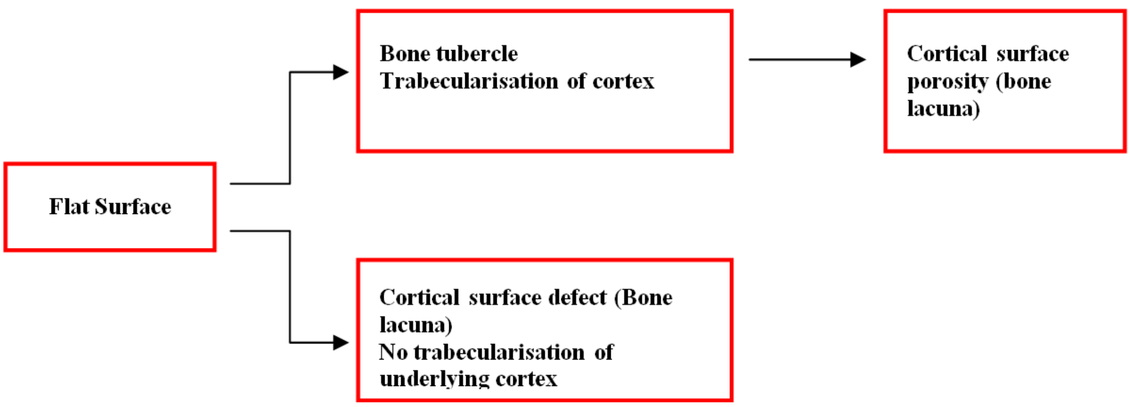

Figure 20. Two possible directions in the development of EC.

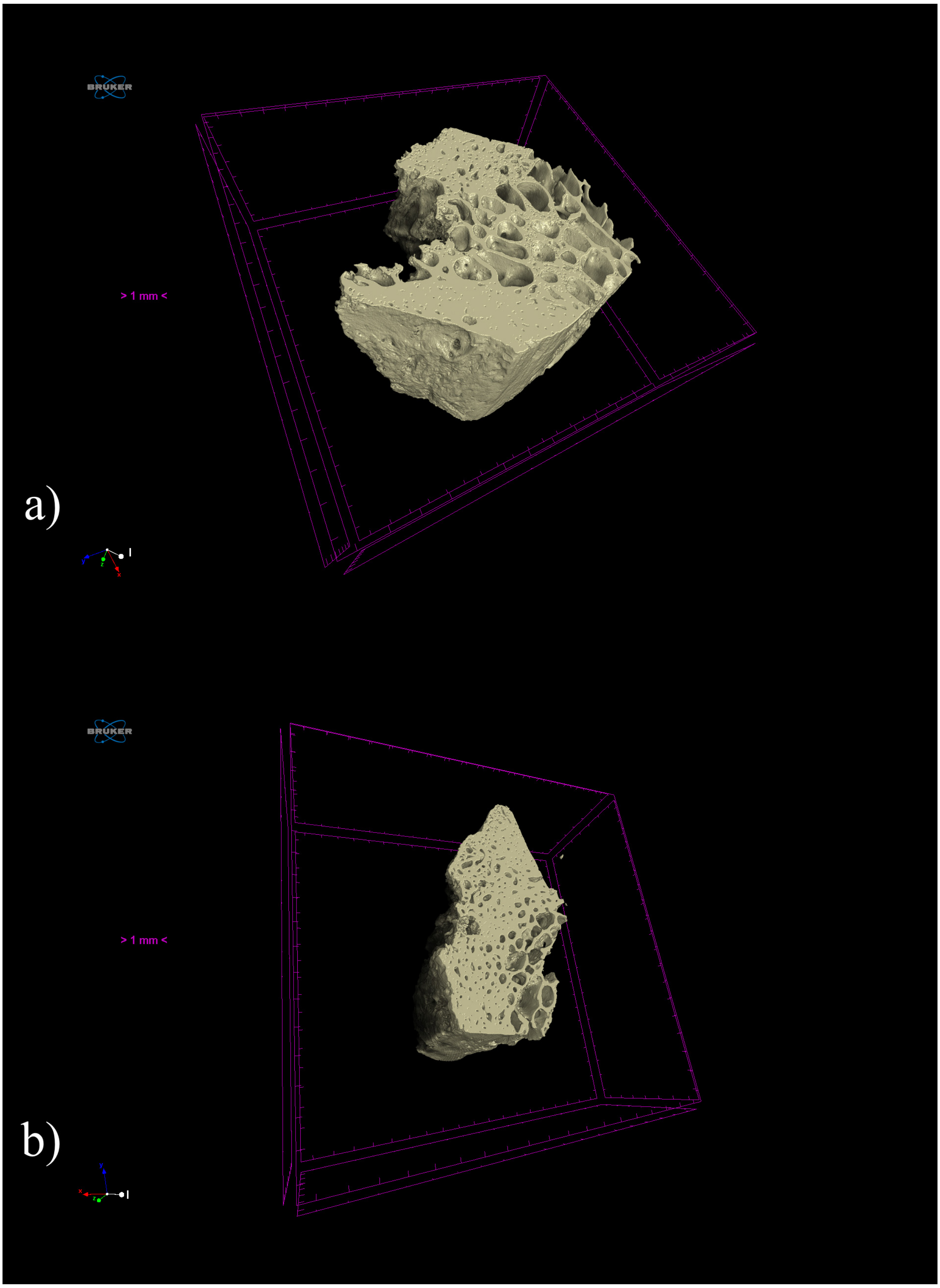

Figure 21. Volume rendering of acquired micro-CT tomographs: **a)** trabecularisation of the cortical bone under the lacuna **b)** regular cortical bone morphology under the lacuna.

Generally, in line with the bone adaptation principle (111, 113), increased loading would be expected to reduce cortical porosity in the case of axial loading. For instance, it was shown experimentally on an animal model that a lack of mechanical loading due to botulinum induced paralysis caused cortical thinning along with increased cortical porosity (117, 118).

However, our microarchitectural findings in all entheseal stages at the gluteus maximum insertion site showed an increased porosity of the cortical bone. This finding may suggest that transmission of the load arising from muscle activity on the enthesis requires a different structural adaptation than compressive axial loads. In this context, a more trabecularised cortex may be a more favourable entheseal microarchitectural design for load transfer, reflecting local adaptation to increased strains, as suggested by Schlecht (2012) (101).

Further studies should clarify how, and why, exactly two developmental directions occur from the initially flat bone surface. It could be speculated that these differences in development of EC are a consequence of variations in magnitude of load, different loading pattern or changes in tendon/bone angle during motion.

Further questions arise (evidence of relationship of muscle activity and bone structure): the microstructural effects of a lack of muscle loading were shown on an animal model where, a few weeks following the local administration of botulinum toxin to the supraspinatus muscle, the muscle volume was decreased, fibrocartilage development delayed and the accumulation of mineralised bone was impeded (100). However, although the subchondral bone might undergo a functional adaptation to exercise due to the changed magnitude and type of load, Frizziero et al. found no significant differences in the enthesis area and adjacent subchondral bone volume between trained and untrained rats. The authors suggested that "moderate exercise" did not affect the subchondral bone volume at the enthesis, however, the tendon thickness was increased in trained rats (119). This raises one of the most crucial questions in the field of bone structural adaptation: what is the osteogenic potential of high intermittent stresses vs. habitual moderate stress, i.e., which type and magnitude of stress drives bone structural adaptation (20, 104, 107, 120)? Following this question, another arises as to whether the structural adaptation to muscle loads is different across lifespan? Does the entheseal morphology mostly reflect the activities in youth, or do the activities in advanced age also determine the appearance of attachment sites, or are both the case (104, 107)? In that sense, it seems too simplistic to just observe the general occupation of an individual when analysing enthesis morphology, and correct interpretations may require a more precise investigation of loading activities. Answering these questions is essential for any interpretative approach to entheseal morphology.

Our findings suggest that the structural patterns of entheses (as well as their relationship with muscle activities) are far more complex than usually thought. Specifically, in order to profoundly understand the relevance of the macroscopic scoring of entheses (in the form of musculoskeletal markers), we have to understand several more issues which are important for stress transfer, such as: different histological types of entheses (99, 107), the angle of muscle attachment to the bone surface and the pennation angle (100, 121), the skeletal region where the enthesis is located, the movement of the enthesis location during growth, caused by constant resorption and new deposition (92, 122), the difference in body size, and the embryological development of the entheses. Although differences in body size may also be important for entheseal morphology, we observed that an individual can present different macroscopic stages at different insertion sites even on the same bone.

6. CONCLUSION

The present study analysed the macromorphological and microstructural characteristics of the bone at the muscle attachment sites, hypothesising that mechanical loading influences the microarchitecture and macromorphology of the bone at the entheses.

1. With horse riders the results suggest that the physical activities of the upper limbs were more or less consistent during their whole life, while the activity of the lower limbs was related to the intensive use of muscles in younger individuals.

2. Among the agricultural population, the results show that the entheseal changes are more pronounced in the older age categories, suggesting that the agrarians' level of physical activity increased with age. An alternative could lead to the conclusion that during the individual's life time the bone quality reduces, with the result that an ensuing muscle load of the same intensity influences a more dramatic entheseal change in the elderly.

3. The sexual dimorphism of EC scores was evaluated in both the horse-rider and agricultural groups and showed that all attachment sites which demonstrated significant differences revealed greater markers of stress in males in both investigated groups.

4. Between the rider and agricultural populations, among the lower limbs, only the muscles which are specific for horse riders were singled out, showing a more pronounced EC in the horse riders' population. This suggests that the evaluation of attachment sites of adductor muscles can provide the best criteria for the identification of riders among the general population.

5. The possible successive nature of the widely used three-stage scoring system of entheseal macroscopic changes was investigated, by comparing EC scores with the microarchitectural features at the musculoskeletal attachment sites on the lower extremities. Overall, the results showed a lack of a consistent correlation between the established stages of the macroscopic scoring system and the microarchitecture at the entheses, suggesting that the macroscopic entheseal

stages might not represent distinct successive phases in bone adaptation to mechanical loading. Therefore, the evaluation of EC should be simplified using only two macromorphological stages: complete regular attachment site and presence of irregularity at the place of the attachment site.

6. The mutual relationships between the microarchitectural properties differed depending on the macroscopic stage of EC. Specifically, while in stages A and B no significant relationship between the characteristics of cortical and trabecular microarchitecture was found, stage C showed significant positive correlations between the cortical and trabecular microarchitectural parameters, suggesting that both bony compartments still followed the same adaptation pattern.

7. It was found that from the initial flat surfaces at the point of the muscle attachment site, two directions of EC can be developed. One is the clearly expressed prominence and trabecularisation of the cortical bone inside the prominence. This trabecularisation of the cortical bone can lead to visible porosity on the external cortical surface. In the second developmental direction, a surface defect exists with the regular cortical bone under the lacuna. It could be speculated that these differences in development of EC are a consequence of variations in the magnitude of the load, a different loading pattern or changes in the tendon/bone angle during the motions. However, further studies are needed to demonstrate how the stress transfer process influences the bone microstructure of the entheses.

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PROFESSIONAL BIOGRAPHY

Đukić Ksenija was born in Belgrade, in 1980. After finishing elementary and high school in Belgrade, she was enrolled in, University of Belgrade, in 2000. Since October 2002 she was involved in the research team of the Laboratory for Anthropology, Institute of Anatomy, led by Professor Marija Djuric. This entailed activity in the anthropological analyses of skeletal remains, the examination of the dental condition of individuals from a forensic and archaeological context, investigation of bone factors affecting the pattern of preservation of immature skeletal remains, paleopathological analyses, macro and micro photographing of skeletal samples, observing and processing results and critical analyses of literature during the preparation of scientific papers.

She graduated from the Faculty of Philosophy, Department of Archaeology in 2008 with GPA 8.36 Afterward, in 2009, she was enrolled in Doctoral Studies at School of Medicine, University of Belgrade – course: Skeletal Biology. Since 2009 until present she participated in the project "Functional, functionalised and advanced nano materials" (No 45005) funded by Ministry of Education, Science and Technological Development of Republic of Serbia as Research Assistant (scientific category A3).

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