INSTITUT ZA ANTROPOLOGIJU
INSTITUTE FOR ANTHROPOLOGICAL RESEARCH

Journal of Bioanthropology

ISSN 2787-8201 (Online)
UDK 572
vol. 2, 2022
https://doi.org/10.54062/jb

INSTITUT ZA ANTROPOLOGIJU
INSTITUTE FOR ANTHROPOLOGICAL RESEARCH

Journal of Bioanthropology

Publisher: Institute for Anthropological Research

Address: Ljudevita Gaja 32, 10000 Zagreb, Croatia

Mail: editors@inantro.hr

Web page: https://inantro.hr/en/journal-of-bioanthropology-2/

First publication: december 2021

Frequency of publication: semi-annually

ISSN 2787-8201

UDK 572

DOI https://doi.org/10.54062/jb

Cover image taken with the permission of the organizing committee, Paleoradiology meets Archeology 3

About the journal:

Journal of Bioanthropology is a multi and interdisciplinary scientific journal that focuses on scientific research in the field of biological anthropology, bioarchaeology, biomechanics, biomedicine, ergonomics, forensics, genetics, human evolution, molecular anthropology, public health and related subjects. Official language of the Journal is English.

All articles published by the Journal of Bioanthropology are made freely and permanently accessible online immediately upon publication, without subscription charges or registration barriers.

Journal of Bioanthropology operates a double-blind peer-review system, where the reviewers do not know the names or affiliations of the authors and the reviewer reports provided to the authors are anonymous.

Submitted manuscripts will generally be reviewed by two to three experts who will be asked to evaluate whether the manuscript is scientifically sound and coherent, whether it duplicates already published work, and whether or not the manuscript is sufficiently clear for publication. Reviewers will also be asked to indicate how interesting and significant the research is. The Editors will reach a decision based on these reports and, where necessary, they will consult with members of the Editorial Board.

INSTITUT ZA ANTROPOLOGIJU
INSTITUTE FOR ANTHROPOLOGICAL RESEARCH
Journal of Bioanthropology

INSTITUT ZA ANTROPOLOGIJU
INSTITUTE FOR ANTHROPOLOGICAL RESEARCH

Journal of Bioanthropology

IAR Publishing

Journal of Bioanthropology

Volume 2, 2022. p. 4-5

ISSN 2787-8201; UDK 572

Paleoradiology, future perspectives

Mislav Čavka

Guest Editor

Third Issue December 16th, 2022

Editorial

In the introduction editorial of the special issue of Canadian Association of Radiologists Journal dedicated to Paleoradiology in 2004 Chhem defined Paleoradiology as the study of bioarcheological materials using modern imaging methods, such as x-ray radiography, computed tomography (CT), magnetic resonance imaging (MRI), and micro-CT. I would like to add that it is the study of all archeological materials and not just bio-archeological. Paleoradiology got its name in 1987, when Notman published his famous study of sailors frozen during Franklin's Arctic expedition.

Paleoradiology as a science is almost as old as radiology; established when a child mummy in Frankfurt was scanned only three months after the discovery of x-rays. Dragutin Gorjanović Kramberger has put Croatia on the paleo-radiological map of the world by scanning the fossilized remains of Neanderthal man from Krapina at Sisters' of Mercy Hospital in Zagreb and published the results in Vienna at an Anthropology conference in 1901. Following the pioneers from the 80's, our group began paleoradiological research in 2008 with the analysis of the Egyptian Collection from the Archaeological Museum in Zagreb which led to important published findings; we were the first to use CT guided endoscopy, the first to scan the entire ancient Egyptian mummy and Late Bronze Cremation urns using MRI and we were first to use MRI in differential diagnosis.

Despite the use of paleoradiology through three centuries it is still under-utilized in everyday settings. One of the reasons is that the paths of anthropologists, archaeologists, historians, and radiologists do not often cross. We have tried to rectify this, by organizing workshop in Zagreb annually during the first week of Advent. The first such introductory workshop was held in Zagreb during the 2018 Paleopathology Association (PPA) event. The workshops, under the organization of the Croatian Society for Medical Anthropology and the University Hospital Centre in Zagreb are held as „hands-on" training/learning sessions in a hospital en-vironment. Frank Rühli, Nataša Šarkić, Fabio Cavalli, Fabrice Dédouit, Patrick Eppenberger, Igor Erjavec, Ivan Jerković and Katherine Van Schaik have participated as lecturers through these past 5 years. In addition to professional workshops, the Croatian Society for Medical Anthropology, along with the Institute of Archaeology (Hrvoje Kalafatić

as *spiritus movens*) are responsible for organizing a bi-annual scientific event called “Paleoradiology meets Archaeology”, which is now looking forward to its 4th such gathering in 2023.

Now is the moment for Paleoradiology to take a step forward and to bring the research focus to a new level. Case reports and radiology as just one feature of anthropology can be educational and visually attractive, however, goals for this decade should include: „Paleo PACS (picture archiving and communicating system), population studies, standardization of technical parameters and artificial intelligence (AI) for paleoradiology.

I hope that this special edition of Journal of Bioanthropology will foster a collaborative spirit between other paleoradiologists and bio-anthropologists worldwide, similarly to that which has grown between Mario Novak and myself over the last 13 years.

Assist. prof. Mislav Čavka, M.D.,

Ph.D, specialist of radiology

IAR Publishing

Journal of Bioanthropology

Volume 2, 2022. p. 6-13

ISSN 2787-8201; UDK 572
https://doi.org/10.54062/jb.2.1.5

A micro-computed tomography examination of primary double teeth from the Muslim necropolis of Macael Viejo (13th-16th centuries; Almeria, Spain)

Nataša Šarkić [1*], Santiago Moreno [2], Igor Erjavec [3], Jesus Herrerin [4], Laura González-Garrido [5]

[1] Aita Bioarchaeology, Caporal Fradera 17, P-02, 08380 Malgrat del Mar (Barcelona), Spain

[2] Independent investigator, Concepción Bahamonde 9, 28028 Madrid

[t] Laboratory for Mineralized Tissue, Department for Anatomy, School of Medicine University of Zagreb, Croatia

[4]Department of Life Sciences, Faculty of Sciences, Campus Universitario, Ctra. Madrid-Barcelona km, 33, 600, 28805 Alcalá de Henares

[5]Área de Antropología Física, Departamento de Biodiversidad y Gestión Ambiental, Universidad de León, Campus de Vegazana, 24071 León, Spain; Institute of Biomedicine (IBIOMED), Universidad de León, Campus de Vegazana, 24071 León, Spain; Bahamonde 9University of Coimbra, Research Centre for Anthropology and Health (CIAS), Department of Life Sciences, Calçada Martim de Freitas, 3000-456 Coimbra, Portugal

* Corresponding author: Email: nsarkic@gmail.com

Received July 12th, 2022
Accepted for publication November 7th, 2022
Online First November 9th, 2022

Keywords: dental anomalies, gemination, dental fusion, Micro-CT, dental anthropology.

Abstract

A child aged 5±1 years with abnormally developed mandibular incisors was discovered during the analysis of a skeletal assemblage from the late medieval/early modern necropolis of Macael Viejo (c. 13th–16th, Almería, Spain). The mandibular right deciduous incisors (81 and 82) were found to have developed a rare abnormality – a fused crown and single root. The identification, descriptions, and differential diagnoses of the anomaly were performed using morphological analyses and radiographic means. This article aims to present a case of fusion of two adjacent primary teeth displayed by a non-adult and analyse the applications and the potential of microcomputed tomography (Micro-CT) in the analysis of tooth crown and root canal morphology in dental anomalies.

Introduction

The double-crowned tooth is among the suite of congenital anomalies of the single-rooted deciduous dentition. Double teeth are a developmental anomaly that describes adjacent teeth joined by the dentin or pulp and occur in two different ways: by division and by fusion (Brabant, 1967; Aguiló et al., 1999; Schuurs & van Loveren, 2000). The division (twinning or gemination) is an attempt to divide a germ (Aguiló et al., 1999; Scheid, 2007). The gemination results in two wholly separated crowns; or a large, incompletely separated crown having a single root canal. The anomalous tooth has a larger mesial-distal diameter than normal and is counted as one (Schuurs & van Loveren, 2000). Fusion is the union of two normally separated tooth germs. There may be a complete union to form one abnormally large tooth, a union of crowns, or a union of roots only. The tooth count reveals a missing tooth with the anomalous tooth is counted as one unless the fusion occurred with a supernumerary tooth (More & Tailor, 2012).

As a phenomenon, fused or geminated teeth are more common in the primary dentition and have a worldwide clinical frequency range of 0.5 - 4.1% (Scheid, 2007), ranging from 0.4% to 0.9% in the mandible (Schuurs & van Loveren, 2000). Most reports conclude that there is no sex preference for this anomaly (Santos et al., 2003). Fusion in the primary teeth more frequently occurs

unilaterally in the mandibular central and lateral incisors, followed by lateral incisor and canines, whereas gemination mainly affects the primary maxillary lateral incisor (Aguiló et al., 1999; Santos et al., 2003; Benazzi et al., 2010). They may also contribute to aesthetic concerns, problems with space, occlusal disturbances, and delayed eruption of permanent successors (Gomes et al., 2014). It has been reported that approximately 50% of fused or geminated primary teeth result in abnormal permanent teeth like hypodontia; hence careful monitoring of the condition is recommended (Yuen et el., 1987). Although understanding how genetic, epigenetic, and environmental influences interplay during odontogenesis has been greatly advanced (Townsend et al., 2012), the aetiology of these abnormalities remains unclear.

This variability in prevalence suggests a potential bioarchaeological utility as a discrete trait. However, tooth fusion is rare in archaeological populations and only a small number of cases are documented in Europe (Benazzi et al., 2010; Tritsaroli et al., 2018; Sperduti et al., 2021) and Iberian samples in particular (Silva & Silva, 2007).

This report aims to present a case of fusion of two adjacent primary teeth displayed by a non-adult dating to the last Islamic period of the Iberian Peninsula from the Late Medieval period to the Early Modern necropolis in central Almería (Spain) and make this data available for comparative analysis and illustrate the applications and the potential of microcomputed tomography (Micro-CT) in the analysis of tooth anatomy and root canal morphology in dental anomalies.

Materials and methods

The site of Macael Viejo dates from the Late Medieval to the Early Modern period (13th–16th century). Geographically located in the interior mountains at Southeast of Almeria (Spain), it lies 2.5 km from the current town of Macael (Fig.1). The foundation of Macael Viejo took place in the middle of the 13th century after the Christian conquest of the neighbouring Kingdom of Murcia (Valladares & García, 2015). The population moved to the hardly accessible mountainous zone of Almeria. The site remained inhabited until the end of the War of the Alpujarra in 1571, an event that determined its abandonment and the deportation of its population (Valladares & García, 2015).

The research carried out so far on this cemetery (2018-2021) allowed the recovery of 47 individuals. The

Figure 1. Location of Macael in the province of Almería (south-eastern Spain) (wikiwand.com) and photo of the necropolis of Macael Viejo.

Figure 2. Remains of a non-adult individual 16 in the site of Macael Viejo, dated to the Late Medieval period to Early Modern periods (13th -16th century).

organization of the maqbara is characterized by the alignments of graves of equivalent morphology and orientation, which determines the uniformity of the landscape and funerary rite, consistent with the Muslim religion.

During the analysis of an assemblage from Macael Viejo (Almeria, Spain), the remains of a non-adult (referred to as Individual 16) with the presence of double deciduous teeth (81 and 82) were registered. Individual 16 was in a nearly supine position, facing south/east orientation (Fig. 2). The skull and arms were not in their anatomical position. Preservation can be defined as a medium, with a Preservation Index of 40.9% (Safont et al., 2000). The skull was highly fragmented; therefore, its orientation could not be established. The legs were straight and parallel but slightly bent. The recovered joints were in close anatomical connection. No grave goods were associated with the burial. All the elements of the burial correspond to the Muslim rite.

In the present work, it has been decided not to determine the sex since the secondary sexual characteristics have not yet been developed (Ferembach, Schwidetzky & Stlovkal, 1980). Standard anthropological methods for non-adult individuals were used for age estimation. These methods were based on dentition (AlQahtani, 2012), length of long bones (Maresh, 1970), and fusion of bones (Schaefer, 2008).

Regarding dental pathology, all teeth were examined under standardized lighting conditions by careful visual inspection. Dental wear and caries were recorded according to Hillson (2001). Possible linear enamel hypoplasia (LEH) and dental anomalies were recorded according to Goodman and Rose, (1990) and Ansari et al., (2019), respectively. The identification and characterization of deciduous double-crowned single-rooted teeth is based on the four descriptive categories reported by Aguiló et al. (1999). Fédération Dentaire Internationale (FDI) notation system was used.

Micro-CT is a non-invasive detection tool that employs radiation and digital X-ray detectors to capture images of a sample's internal structure (on a micro-level) without damaging the sample. Micro-CT scan was performed in the Laboratory for Mineralized Tissue, Department for Anatomy at the University of Zagreb (Croatia). The teeth sample were scrutinized through 1076 Micro-CT (Bruker, Belgium) with the following parameters: voltage of 40 kV and electric current of 250 µA, corresponding to a resolution of 9 µm. The beam hardening was reduced by using a 0.025 mm thick titanium filter. The rotational step was set to 0.3° with a frame averaging set at 2. The acquired data were reconstructed using the NRecon software (Bruker, Belgium) through a dedicated GPU. Reconstructed data was visualized using CTAn and CTVox software (Bruker, Belgium).

Results

The estimated age of death based on dentition (AlQahtani, 2012) is given as the value of 5±1 years. Based on the length of preserved long bones (Maresh, 1970), the age was estimated to be 3.5 years, and the age based on the fusion of bones (Schaefer, 2008) corresponds to an interval between 4–5 years. Since dentition is the indicator that is least sensitive to changes (Prieto, 2008), it has been used as a reference method to estimate the age of non-adult individuals. Therefore, according to the method of AlQahtani (2012), it could be said that Individual 16 is a non-adult aged 5±1 years.

As we mentioned before, the skull was heavily damaged and very fragmented. Maxillary bone was not preserved, and the mandible was divided into 3 parts, with 3 teeth in sockets (Fig. 3). Apart from that, 13 loose teeth were also recovered, but none showed the sign of fusion.

Figure 3. Heavily fragmented mandible with preserved teeth *in situ*.

The individual shows unilateral double-teeth among the primary mandibular central and right lateral incisors (81, 82) (Fig. 4). The double teeth reveal a bifid crown with a well-defined buccal and lingual groove that extends from the incisal edge to the apex of the root that appears separated at least 2 mm (Fig. 4). The lateral incisor was rotated to the mesial aspect forming a 90º angle on the distal aspect of the central incisor. Since no other anomaly was observed in the left mandibular quadrant, this represents a unilateral event. The central incisor was an interproximal dental wear facet on the mesial region equivalent to grade 1 on Hillson (2001), corresponding to dental ware limited to the enamel (Fig. 4).

Figure 4. Primary maxillary teeth of Macael Viejo showing a fused central and lateral incisor (81, 82) on (a) buccal view and, (b) lingual view show an interproximal dental wear facet on the mesial region of central incisor (arrow). Observed the apex between the fused roots that appears separated at the last 2 mm (arrow).

Conventional X-ray examination revealed that the double tooth has two separate pulp chambers and root canals (Fig. 5), but obtaining more information about the union is impossible. Additionally, the Micro-CT images showed two separate canals leaving the pulp chamber, then joined to form one canal to the exiting site but still with a double form canal (Fig. 6).

Micro-CT reveals the central incisor was an interproximal dental wear facet on the mesial region equivalent to grade 2 on Hillson (2001) which corresponds to the awareness that it is limited to the dentin on the occlusal and middle part of the crown (Fig. 5).

Additionally, the enamel thickness at the labial fused portion in the deciduous central/lateral incisor tended to decrease from the incisal edge towards the cervical region. Enamel thickness at the lingual fused portion tended to increase gradually from the incisal edge

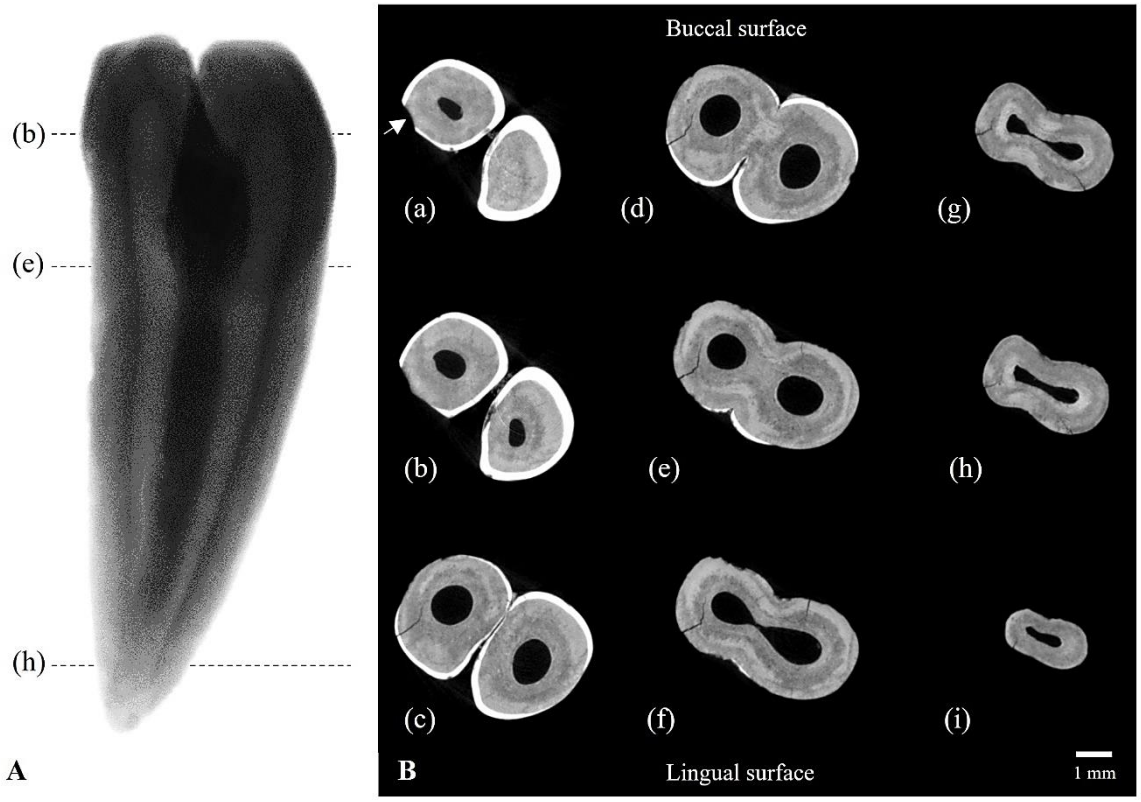

Figure 5. X-ray of primary maxillary teeth of Macael Viejo showing the partial fusion of crowns and roots.

towards the cervical region (Fig. 5). Micro-CT also reveals the presence of incomplete radial microcracks. Originates in the enamel of the interproximal mesial wear facet and continues to the dentine microcrack without reaching the root canal (Fig. 5). These radial microcracks initiate from the base of flexing brittle enamel with in-dentine microcracks originating from the root wall without reaching the root canal (Fig. 5).

Macroscopic and microscopic techniques showed that the teeth are joined by crowns and along the root and fall within clearly distinct but joined roots, with two separate root canals at the last distal part of the root.

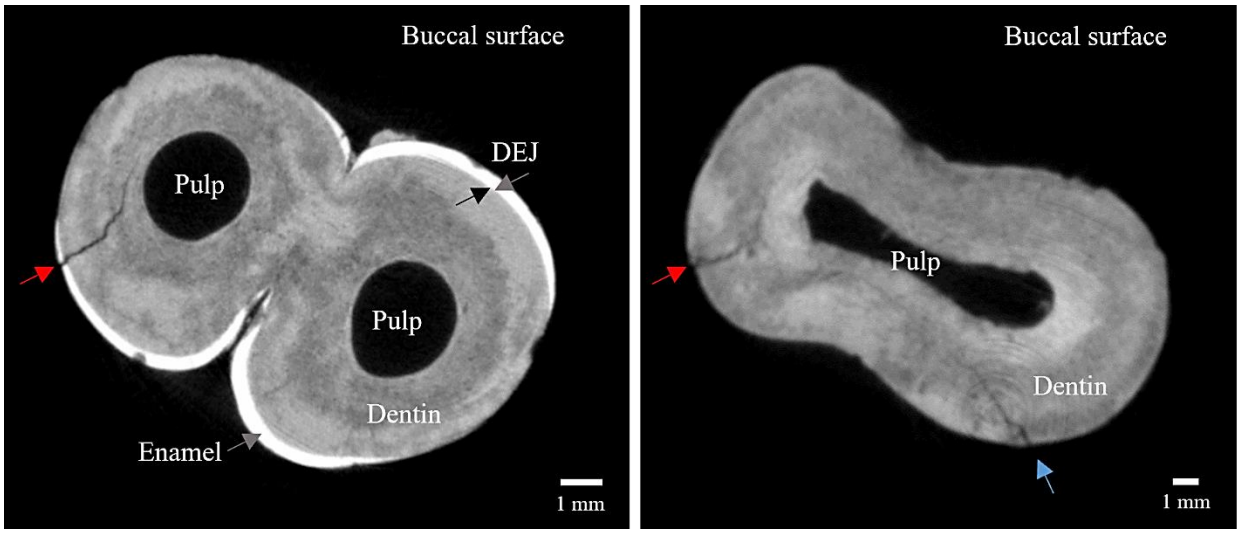

Figure 6. Representative cross-section of micro-CT data, (a-i) changes of the Micro-CT sequential images. (a) The arrow shows the interproximal dental wear facet on the mesial region of central incisor.

Individual 16 corresponds to type-IV described by Aguiló et al. (1999). The Type IV anomaly is two fused crowns with two distinct but fused roots. The Micro-CT images (Fig. 6) show a difference from the conventional X-ray in interpreting the fusion of the root canals, which helps estimate the precise diagnosis.

Fused teeth usually show an increased risk for dental caries due to of conditions favouring dental plaque accumulation along the grooves between the crowns, but in this case, they were not recorded (Fig. 4).

No other bony or dental anomaly, such as supernumerary teeth, were detected in the skeletal remains of this child. The only condition observed in this individual was Linear Enamel Hypoplasia (LEH), visible in the permanent canine (2 well-defined lines), still without a formed root. LEH is a disruption in the enamel formation process that indicates the existence of a period of physiological stress experienced by the individual during the formation of the dental crown (Goodman and Rose, 1990). This process can last from the prenatal period up to 12 months in deciduous teeth, from birth to 7 years for permanent teeth (Hillson, 2008), and up to 16.5 years for third molars (AlQahtani et al., 2010), which makes it a good indicator of non-specific stress in childhood. These lines of hypoplasia tell us of at least two episodes of metabolic stress that this child survived, while the position of those lines indicates that those episodes occurred at the ages of 3.4 and 4.3 years (Reid & Dean, 2000).

Discussion

Because gemination and fusion can create teeth that appear morphologically and physiologically similar, it can be difficult to correctly diagnose the mechanism involved, necessitating reliance on tooth count (Mahendra et al., 2014). The use of Micro-CT was the key to reveal the fusion, the most probable cause of these double teeth cases. Most clinical literature agrees that there has to be a union of the dentine, and the process can result in a shared pulp chamber and root canal, or both elements can remain separate (Benazzi et al., 2010; Koszowski et al., 2014). In the clinical literature geminated teeth are usually not found in the mandible and are the minority crown form in the maxilla (Duncan and Helpin, 1987). Population differences have also been noted; the fusion of the mandibular incisors is evidently more prevalent in Spanish samples (Aguiló et al., 1999).

The primary teeth suffered more numerous and extensive subsurface microcracks. Microcracks occur when enamel surfaces contact very small hard particles. While the particles slide on the surface, a combined process of micro-cutting, cutting of enamel crystals at the level of rods and inter rods microstructures, and microcracking of both the surface and the subsurface takes place (Low et al., 2008). The microcracks readily formed in the deciduous enamel even at low loads, which indicates the poor fracture resistance of deciduous teeth by virtue of their lower hardness and fracture toughness, compared to the permanent tooth (Low et al., 2008).

LEH is often associated with early mortality (decreased life expectancy) and frequently is found in individuals who die at a young age (Šlaus, 2002). LEH points to physiological stress that this child survived at different stages of his/her life. Although not pointing to growth delay, the slight discrepancy between dental eruption and length of a preserved long bone shows that the child was at the lower limit of its age group, which can be connected to physiological stress. However, no correlation could be made between physiological stress and mentioned dental anomaly, as the lines of hypoplasia were noted on a permanent tooth, that starts its formation at 10 months, and the mentioned anomaly was noted on deciduous incisive, forming in utero.

Conclusion

Tooth fusion is a rare condition, and archaeological cases have only occasionally been reported. The present example adds to the documentation of a mandibular primary dental fusion of teeth abnormality in past populations. The presence of dental anomalies and correct diagnosis is always a challenge for a dental anthropologist. Therefore, morphological and radiological features should be carefully evaluated, and a description must be made. Micro-CT can provide new and accurate image data that have not been possible to obtain fully with conventional diagnostic methods and are essential for the definitive diagnosis of significant

dental anomalies. Regular Micro-CT methodology application would further help understand and distinguish this phenomenon from others with similar characteristics.

References

Aguiló, L., Gandia, J. L., Cibrian, R., & Catala, M. (1999). Primary double teeth. A retrospective clinical study of their morphological characteristics and associated anomalies. International journal of paediatric dentistry, 9(3), 175–183. https://doi.org/10.1046/j.1365-263x.1999.00131.x

AlQahtani, S. J. (2012). The London Atlas: developing an atlas of tooth development and testing its quality and performance measures (Doctoral dissertation, Queen Mary University of London).

Ansari, G., Golpayegani, M. V., & Welbury, R. (2019). Atlas of pediatric oral and dental developmental anomalies. John Wiley & Sons.

Armelagos, G. J., Goodman, A. H., Harper, K. N., & Blakey, M. L. (2009). Enamel hypoplasia and early mortality: Bioarcheological support for the Barker hypothesis. Evolutionary Anthropology: Issues, News, and Reviews: Issues, News, and Reviews, 18(6), 261-271.

Arrieta, J.J., Bartolomé, B., (1999). Anomalías dentarias. In: M. Varela (Ed.). Problemas bucodentales en pediatría. (pp. 43-58). Madrid: Ergón

Bei, M. (2009). Molecular genetics of tooth development. Current Opinion in Genetics & Development, 19(5), 504–510.

Benazzi, S., Buti, L., Franzo, L., Kullmer, O., Winzen, O., & Gruppioni, G. (2010). Report of three fused primary human teeth in an archaeological material. International Journal of Osteoarchaeology, 20(4), 481-485.

Benazzi, S., Buti, L., Franzo, L., Kullmer, O., Winzen, O., & Gruppioni, G. (2010). Report of three fused primary human teeth in an archaeological material. International Journal of Osteoarchaeology, 20(4), 481–485.

Brabant, H. (1967). Comparison of the characteristics and anomalies of the deciduous and the permanent dentition. Journal of Dental Research, 46(5), 896-902. Doi: 10.1177/00220345670460054701. PMID: 5234030.

Brook, A. H., & Winter, G. B. (1970). Double teeth. A retrospective study of'geminated'and'fused'teeth in children. British Dental Journal, 129(3), 123-130.

Chen, R., Wang, C. (1990) Gemination of a maxillary premolar. Oral Surg Oral Med Oral Pathol 69:656.

Duncan, W. K., & Helpin, M. L. (1987). Bilateral fusion and gemination: a literature analysis and case report. Oral Surgery, Oral Medicine, Oral Pathology, 64(1), 82-87

Ferembach, D., Schwidetzky. I., Stloukal, M. (1980). Recommedations for age and sex diagnoses of skeletons. Journal of Human Evolution, 9, 517-549.

Gomes, R. R., Fonseca, J. A. C., Paula, L. M., Acevedo, A. C., & Mestrinho, H. D. (2014). Dental anomalies in primary dentition and their corresponding permanent teeth. Clinical oral investigations, 18(4), 1361-1367.

Goodman, A. H., & Rose, J. C. (1990). Assessment of systemic physiological perturbations from dental enamel hypoplasias and associated histological structures. Yearbook of Physical Anthropology 33:59-110.

Hagman, F. T. (1985). Fused primary teeth: a documented familial report of case. ASDC Journal of Dentistry for Children, 52(6), 459-460.

Hillson, S. (2001). Recording dental caries in archaeological human remains. International Journal of Osteoarchaeology, 11(4), 249-289.

Koszowski, R., Waśkowska, J., Kucharski, G., & Śmieszek-Wilczewska, J. (2014). Double teeth: evaluation of 10-years of clinical material. Open Medicine, 9(2), 254-263.

Low, I. M., Duraman, N., & Mahmood, U. (2008). Mapping the structure, composition and mechanical properties of human teeth. Materials Science and Engineering: C, 28(2), 243-247.

Mahendra, L., Govindarajan, S., Jayanandan, M., Shamsudeen, S. M., Kumar, N., & Madasamy, R. (2014). Complete bilateral gemination of maxillary incisors with separate root canals. Case reports in dentistry, 2014. (425343), 1–4. https://doi.org/10.1155/2014/425343

Maresh, M. M. (1970). Measurements from roentgenograms, heart size, long bone lengths, bone, muscles and fat widths, skeletal maturation. Human growth and development, 155, 200.

More, C. B., & Tailor, M. N. (2012). Tooth fusion, a rare dental anomaly: analysis of six cases. International. J Oral Maxillofac Pathol, 4, 50-53.

Nik-Hussein, N. N., & Salcedo, A. H. (1987). Double teeth with hypodontia in identical twins. ASDC Journal of Dentistry for Children, 54(3), 179-181.

Phillips, E. L., Irish, J. D., & Antoine, D. (2021). Ancient anomalies: Twinned and supernumerary incisors in a medieval Nubian. International Journal of Osteoarchaeology, 31(3), 456-461.

Prieto, J. (2008). La maduración del tercer molar y el diagnóstico de la edad: Evolución y estado actual de la cuestión. Cuadernos de Medicina Forense, 14(51), 11-24.

Puy, L., Pizarro, C., & Navarro, F. (1991). Double teeth. The Journal of clinical pediatric dentistry, 15(2), 120-124.

Reid, D. J., & Dean, M. C. (2000). Brief communication: the timing of linear hypoplasias on human anterior teeth. American Journal of Physical Anthropology, 113(1), 135-139.

Safont, S., Malgosa, A., & Subirà, M. E. (2000). Sex assessment on the basis of long bone circumference. American Journal of Physical Anthropology, 113(3), 317-328.

Santos, L. M., Forte, F. D. S., & Rocha, M. J. C. (2003). Pulp therapy in a maxillary fused primary central incisor–report of a case. International Journal of Paediatric Dentistry, 13(4), 274-278.

Santos, B. M. D., Ribeiro, R. R., Stuani, A. S., Silva, F. W. G. D. P., & Queiroz, A. M. D. (2006). Kabuki make-up (Niikawa-Kuroki) syndrome: dental and craniofacial findings in a Brazilian child. Brazilian Dental Journal, 17, 249-254.

Schaefer, M. C. (2008). A summary of epiphyseal union timings in Bosnian males. International Journal of Osteoarchaeology, 18(5), 536-545.

Scheid, R.C. (2007). Woelfel's dental anatomy: its relevance to dentistry. 10th ed. Philadelphia PA: Lippincott Williams & Wilkins

Schuurs, A. H. B. (2000). Anomalies. Double teeth: review of the literature. Journal of Dentistry for Children, 67, 313-325.

Silva, A. M., & Silva, A. L. (2007). Unilateral fusion of two primary mandibular teeth: Report of a Portuguese archeological case. Dental Anthropology Journal, 20(1), 16-18.

Sperduti, A., Fattore, L., Botto, M., Cavazzuti, C., Cicala, L., Garau, E., ... & Candilio, F. (2021). Dental twinning in the primary dentition: new archaeological cases from Italy. Bulletin of the International Association for Paleodontology, 15(1), 6-20.

Šlaus, M. (2000). Biocultural analysis of sex differences in mortality profiles and stress levels in the late medieval population from Nova Rača, Croatia. American Journal of Physical Anthropology, 111(2), 193-209.

Smith, M. O., & Wojcinski, M. C. (2011). Anomalous double-crowned primary teeth from Pre-Columbian Tennessee: A meta-analysis of hunter–gatherer and agriculturalist samples. International Journal of Paleopathology, 1(3-4), 173-183.

Sperduti, A., Fattore, L., Botto, M., Cavazzuti, C., Cicala, L., Garau, E., Interlando, S., Fentress, E., Candilio, F. (2021). Dental twinning in the primary dentition: new archaeological cases from Italy. Bulletin of the International Association for Paleodontology, 15(1). Retrieved from https://hrcak.srce.hr/ojs/index.php/paleodontology/article/view/16625 Accessed 25 May 2022.

Townsend, G., Bockmann, M., Hughes, T., & Brook, A. (2012). Genetic, environmental and epigenetic influences on variation in human tooth number, size and shape. Odontology, 100(1), 1–9. https://doi.org/10.1007/s10266-011-0052-z

Tritsaroli, P. (2018). A case of dental fusion in primary dentition from late bronze age Greece. Balkan Journal of Dental Medicine, 22(2), 102-105. https://doi.org/10.2478/bjdm-2018-0018

Valladares, M. A., & García, J. A. G. (2015). La explotación de los recursos cárnicos en la frontera del Reino Nazarí de Granada.: Un estudio de caso en el yacimiento de La Moraleda (Antequera, Málaga). Revista del Centro de Estudios Históricos de Granada y su Reino, (27), 21-39.

Yuen, S. W., Chan, J. C., & Wei, S. H. (1987). Double primary teeth and their relationship with the permanent successors: a radiographic study of 376 cases. Pediatric Dentistry, 9(1), 42-48.

IAR Publishing

Journal of Bioanthropology

Volume 2, 2022. p. 14-21

ISSN 2787-8201; UDK 572
https://doi.org/10.54062/jb.2.1.4

Bipartite parietal bone: a case from Croatian population

Željana Bašić[1], Ivan Jerković[1*], Tina Bareša[1], Nika Jerković[2], Saša Mladenović[2], Ivan Skejić[3], Krešimir Dolić[3], Ivana Kružić[1]

[1]University of Split, University Department of Forensic Sciences, Ruđera Boškovića 33, 21000 Split

[2]University of Split, Faculty of Science, Ruđera Boškovića 33, 21000 Split

[t] University Hospital Center Split, Clinical Department for Diagnostic and Intervention Radiology, Spinčićeva 1, 21000 Split

* Corresponding author: Email: ivanjerkovic13@gmail.com

Received July 21st, 2022
Accepted for publication October 17th, 2022
Online First October 27th, 2022

Keywords: cranial variation, epigenetic traits, bipartite parietal bone, MSCT, Croatia.

Abstract

This study reports the first case of the bipartite parietal bone in the Croatian modern and archaeological population. We have examined 458 skulls from Multi-Slice Computed Tomography (MSCT) images and found only one case of this trait (0.22%; 95%CI 0.01%-1.21%). The bone exhibiting an additional suture was the left parietal; the type of the trait was complete, horizontal, and asymmetrical. Some additional sutural bones were observed, including asterion and lambda ossicle and two lambdoid ossicles. Among the other epigenetic variants, parietal, zygofacial, oval, and mastoid foramina, frontal grooves, supratrochlear notch, and condylar canal were visible. The principal components analysis (PCA) of features extracted from the frontal, lateral, and posterior view of 110 female crania using a convolutional neural network (CNN) for image analysis demonstrated that the skull deviated from the average female skull in the population. The visual and metric examination of the skull showed ultrabrachycephaly, mild lateral deformational plagiocephaly, and depression on the posterior part of the skull.

The rarity of this case in the Croatian population indicated that this variant could be interesting for both clinicians and forensic anthropologists, as it can be used as means of comparative identification. Further studies will show its importance in assessing population affinities and relations inside the graveyards.

Introduction

Bipartite parietal bone (divided parietal bone, double parietal bone, or sutura parietalis) is one of the variants in mammalian skulls, which is more frequent in other mammals than in humans (Shapiro, 1972). According to Hrdlička, the first mention of the bipartite bone occurred in 1753 by Tarin (Hrdlička, 1903), but its etiology has not been clarified to date. Some authors consider that it occurs due to several (usually two) ossification centers (Bhatt, Hunsaker, & Kalina, 2014; Goss, 1954; Hauser & De Stefano, 1989; Sanchez, Stewart, Walvick, & Swischuk, 2010), while others believe that it develops from one (Gray, 1901; Shapiro, 1972). This variant can be extremely important when analyzing human skeletal remains in a forensic and archaeological context, especially in children, where it can be mistaken for trauma (especially when unilateral) when analyzing head X-rays. This is more pronounced in children because in adults sclerosis or sutural seriation can be good indicators of the absence of trauma (Shapiro, 1972). On the other hand, considering its frequency, it could be one of the individual traits used for analyzing unidentified human remains when comparative identification is possible.

There are several types of expression of the trait, which include: complete horizontal (symmetrical and asymmetrical), complete vertical (symmetrical and asymmetrical), and incomplete form (Shapiro, 1972). In some cases, it can be tripartite or even quadripartite, as published by Fusari, Maggi and Rannke and discussed by Shapiro (Shapiro, 1972). Additionally, Hauser and De Stefano suggest that divided parietal bone has to be recorded by the completeness of division (complete; partial – if longer than 1 cm; trace – if less than 1cm long), by direction (horizontal, vertical, or oblique), and by the number of parts (two, three, four) (Hauser & De Stefano, 1989).

This variance can be accompanied by skull asymmetry, hydrocephalus, plagiocephaly, additional sutures (os suturarum, metopic suture, mastoid suture, sutura mendosa) as well as the obliteration of some other sutures and the teeth retention (Abdel-Salam et al., 2014; Becker, Cheverud, Govier, & Kane, 2005; Berry, 1909; Bessell-Browne & Thonell, 2004; Fenton, Sirotnak, & Handler, 2000; Hauser & De Stefano, 1989; Hrdlička, 1903; Shapiro, 1972).

The frequency of bipartite parietal bone in studied populations is low; for example, Hauser and De Stefano estimate that its frequency is less than 1% (Hauser & De Stefano, 1989). In his clinical experience, Shapiro estimated that this trait was extremely rare (3 of around 25,000 examined RTG images), which was additionally supported by Hrdlička, who found 8 cases of parietale bipartita in the sample of 34,000 humans (Shapiro, 1972). Almeida Prado et al. have done an extensive literature review and found three cases in 711 skulls; and 80 cases reported in previously published literature from 1753 to 2016 (Hrdlička, 1903; Prado et al., 2016).

To our knowledge, our paper is first to present the finding of the bipartite parietal bone in the sample of the Croatian population.

Materials and methods

Settings

The Multi-Slice Computed Tomography (MSCT) images of crania were obtained at the Department of Diagnostic and Interventional Radiology of the University Hospital Center Split (Croatia) using device Definition Edge (Siemens AG Medical Solutions, Erlangen, Germany) with a slice thickness of 0.75 mm. The soft tissue convolution kernel and original slice thickness were used for image reconstruction.

Crania were examined using OsiriX MD 12.5 (Pixmeo SARL, Geneva, Swiss 2021) in 2D views and 3D volume rendering techniques (VRT). Visualizations were provided using 3D Cinematic Rendering mode.

Anatomical analysis

The epigenetic variants of the crania were analyzed according to Hauser and De Stefano (Hauser & De Stefano, 1989). The skull was measured, and cranial vault asymmetry index (CVAI) (Callejas Pastor et al., 2020), skull shape (Langley, Jantz, Ousley, Jantz, & Milner, 2016; Looman & Flannery, 2012; Topinard, 1885), and transcranial diameter difference (TDD) were calculated (Looman & Flannery, 2012).

Image analysis and Principal components analysis

MSCT database consisted of 274 skull scans from University Hospital Split (137 females and 137 males), age of females ranging from 18 to 93, and males from 18 to 88. Only patients without head trauma and other anomalies that could affect the skull size and morphology are included into the collection. We used 110 female crania with visible region of interest from our MSCT database to compare our patient to the other female patients without the described condition. As we did not have population osteometric or landmark data for comparison, we captured in 3D VRT three images of each cranium in frontal, lateral, and posterior views and conducted image analysis.

Classic computer graphics-based methods rely on per-pixel-based multi-scale feature computation, resulting in complex and timely modeling. To address the complex and timely modeling, we used a convolutional neural network (CNN) for image analysis. We employed the transfer learning approach, which also can be used for image classification when dealing with restricted sample sizes (Shaha and Pawar, 2018), to represent

images as arrays of numerical values, each describing a specific extracted feature.

Images were imported into Orange Data Mining, version 3.32.0, a visual programming tool for quick prototyping (Demšar et al., 2013). Among different CNN architectures commonly used for image analysis, we selected VGG16, a CNN model for image feature extraction. VGG16 model achieves a 92.7% top-5 test accuracy on the ImageNet dataset. Furthermore, the network has 16 layers and multiple 3X3 kernel-sized filters resulting in increased depth of the neural network facilitating the understanding and recognition of more complex features and patterns (Simonyan & Zisserman, 2015). As the feature extractor provided 4096 features for each cranial image, we used principal component analysis (PCA) to reduce the dimension of the data and reveal where the cranium with bipartite parietal will cluster within other female crania sample.

Ethical declarations: Approved by the ethical committees of the University Hospital Centre Zagreb (Class: 8.1-21/216-3; Number: 02/21 AG.), University Hospital Centre Split (Class: 500-03/17-01/56; Number: 2181-147-01/06/M.S.-17-2), and University Department of Forensic Sciences (Class: 024-04/17-03/00026; Number: 2181-227-05-12-17-0003).

Results and Discussion

The patient in whom we found a unilateral bipartite parietal bone was a female, age 55. She was admitted to the emergency room due to strong headaches and vertigo. Brain MSCT was administered, but no acute focal changes were found.

The skull length was 159.9 mm, and breadth 149.5 mm, producing a cranial index of 93.5, which defines the skull as ultrabrachycephalic (extremely wide and short). There was central posterior deformity and widening of the posterior skull characteristic of posterior deformational plagiocephaly. CVAI was 3.25, which did not indicate plagiocephaly. However, the skull exhibited fronto-lateral differences between the left and right side, with visible bulging on the right side of the occipital bone (Figure 1). TDD was 5.5 mm, implying the lateral deformational plagiocephaly of mild severity (flattening restricted to the back of the skull).

The left parietal bone was bipartite and unfused; the type was complete, horizontal, and asymmetrical. It was located at the first third closest to the squamous suture/border of the parietal bone starting at the lambdoid suture, 2.98 cm from asterion and 3.20 cm from pterion (Figure 2).

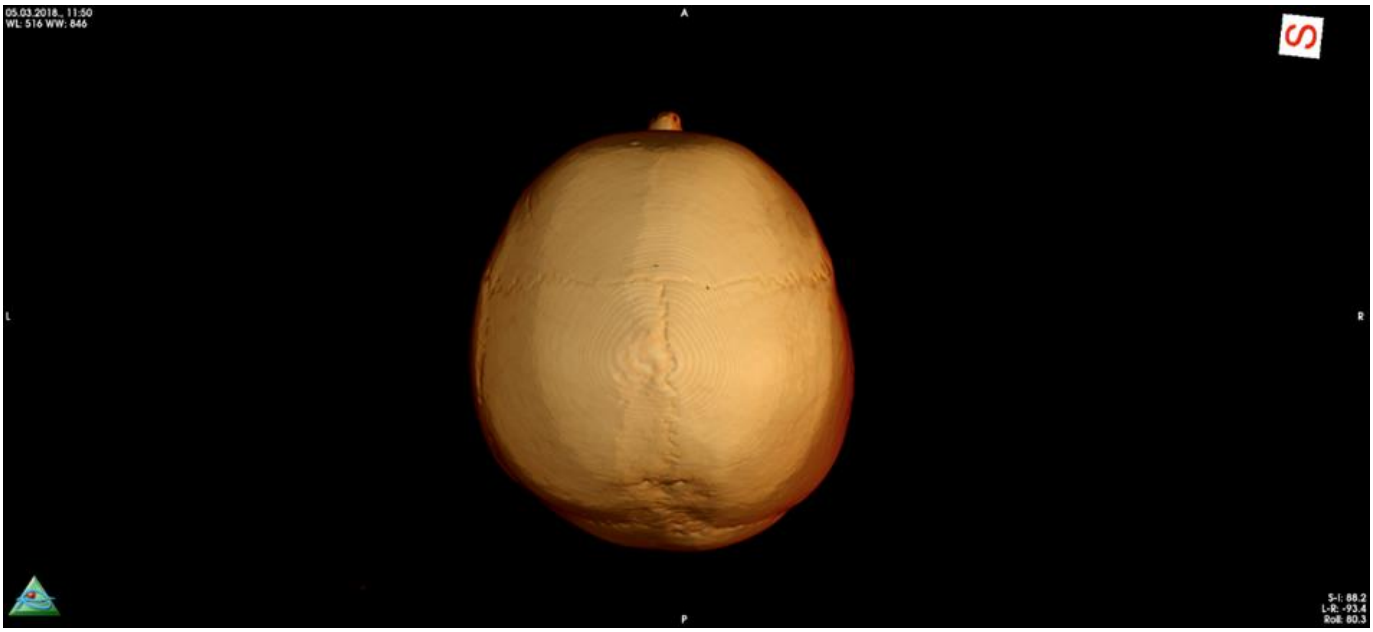

Figure 1. Superior view of the skull indicating occipital bulging on the right side and unsymmetrically positioned sagittal suture.

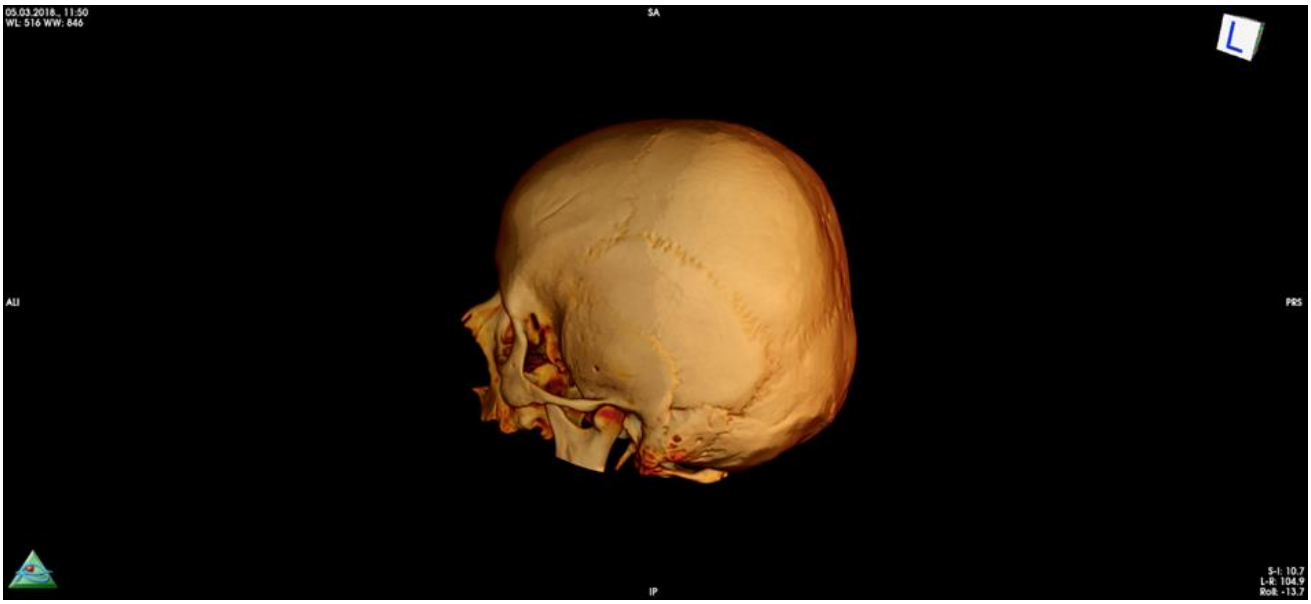

Figure 2. Lateral view of the skull showing the position of the additional suture and pronounced occipital flattening.

The sagittal suture was shifted slightly right from the midsagittal plane (Figure 1). The depression was visible on the posterior part of the skull, in the area from the posterior half of the sagittal suture to the external occipital protuberance, concentrating on the area around the midline (Figure 3). Mild, unsymmetrical flattening of the entire occiput was visible (Figure 2). The other sutures showed no asymmetrical closures or pathological obliterations.

An asterion ossicle was visible on the left side. There was a lambda ossicle and two lambdoid ossicles, one on the right side of the junction with the additional sagittal suture and the other on the right side of the lambda (Figure 4). Among other variants of human skull, we identified bilaterally: parietal foramen, frontal groove, supratrochlear notch, mastoid foramen, and foramen ovale. Four zygofacial foramina, one on the left and three on the right bone, were also visible. The condylar canal was visible on the left side.

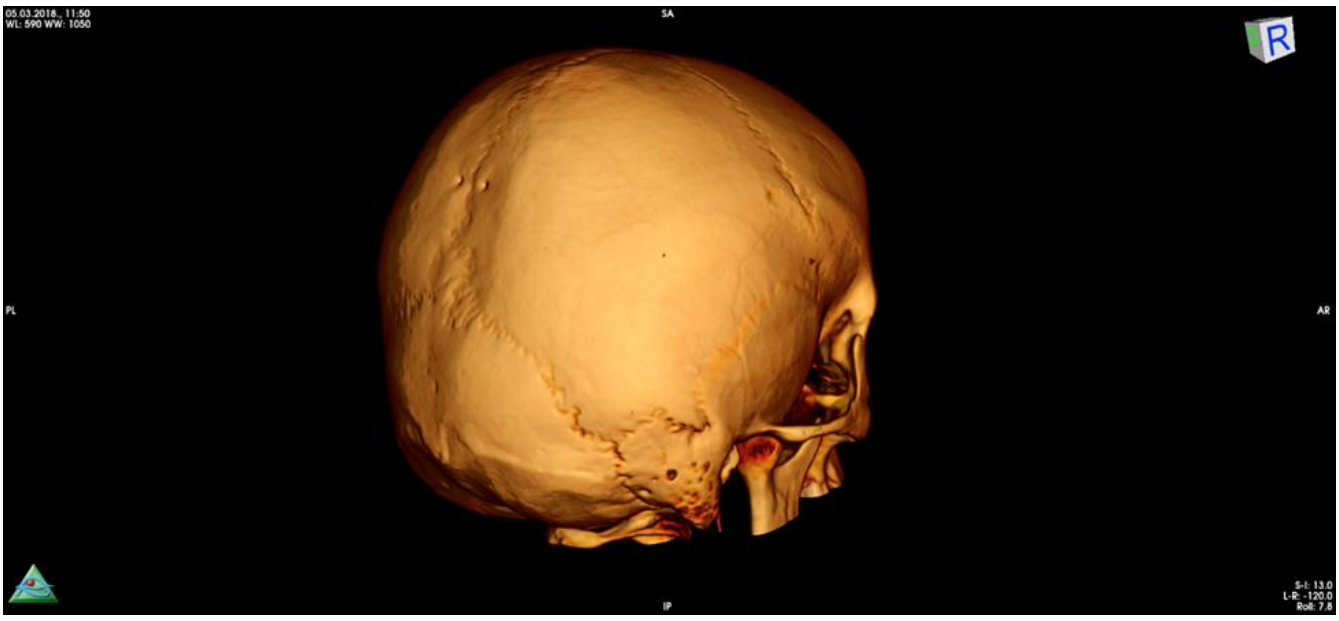

Figure 3**.** Lateroposterior view showing the depression on the posterior part of the skull.

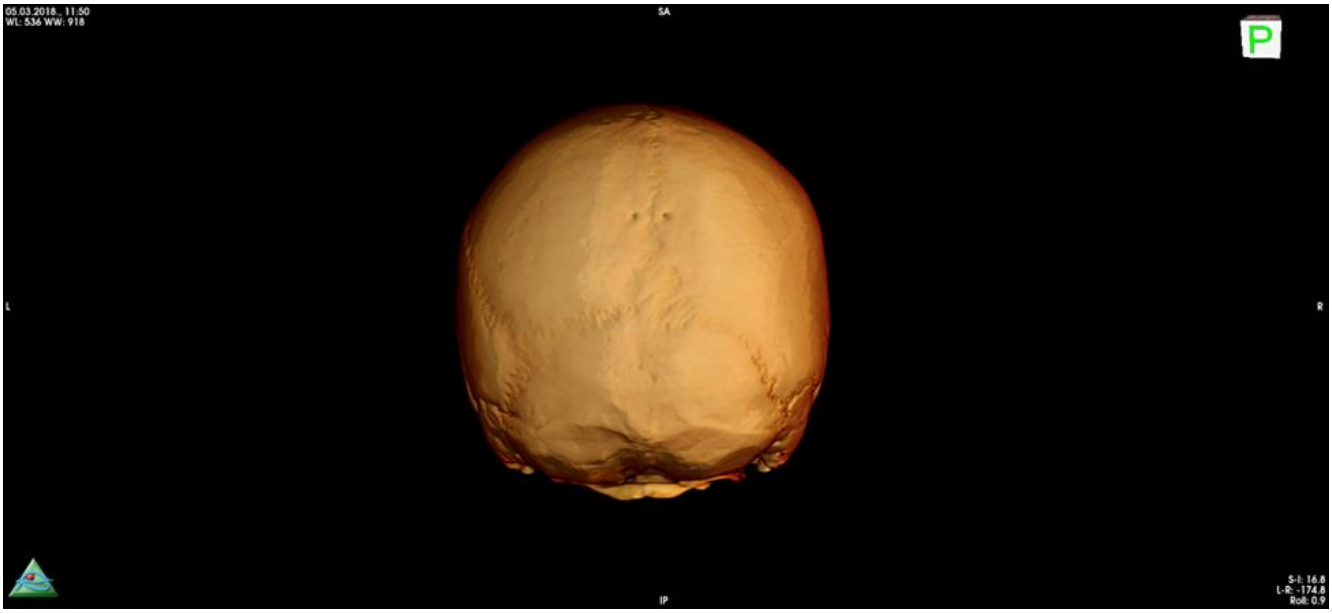

Figure 4. Posterior view of the skull showing the additional ossicles.

Among unrelated findings, we identified, a button osteoma was visible on the left part of the frontal bone and deviated nasal septum.

The depicted case was the only case of bipartite parietale in the virtual collection of 458 skull MSCTs, so the frequency of this trait in the examined population was 0.22% (95%CI 0.01%-1.21%).

To analyze this specific skull within the referent population, features obtained by image analysis (of frontal, lateral, and posterior views) were transformed using the first two principal components that explained the highest amount of variance. The first two components explained 25% of the variance in frontal, 21% in lateral, and 30% in posterior view. While in lateral view examined skull did not remarkably deviate from the rest of the sample, in frontal and posterior views, the skull was positioned at the edges of the quadrants (Figure 5).

Discussion and Conclusion

Our literature search found no similar case in the Croatian population, making this the first reported case of bipartite parietal bone in the Croatian population from both modern and archaeological settings. This is one of the less-represented studies that report findings at the population level, which is essential for studying such low-frequency anatomical traits. The low frequency of this trait in the examined population suggests that the trait could have identification weight, and it could be important for comparative identification purposes in forensic context when antemortem images are available. This finding could also be of value in clinical forensic medicine and expert opinion report when it could possibly be mistaken for a skull fracture in a case of suspected head trauma.

On the population level, this person's cranium was one of the 458 MSCT cranial images examined through this project, suggesting the trait prevalence of 0.22%. This frequency should be taken with caution considering the sample size and previous studies that showed much lower frequency ranging from 0.012% (Shapiro, 1972) to 0.024% (Hrdlička, 1903). The most recent study reported a frequency of 1.94% in the population of Cyprus, while the total sample of four examined countries (including Cyprus) was 0.42%. However, if this sample was divided by population, that would indicate that Brazil, Portugal, and Greece had no cases of this trait (Prado et al., 2016). Interestingly, the highest frequency of this trait was observed in samples from modern Croatian and Cypriot populations, which are geographically not distant. Population frequency in other European or Mediterranean populations is still

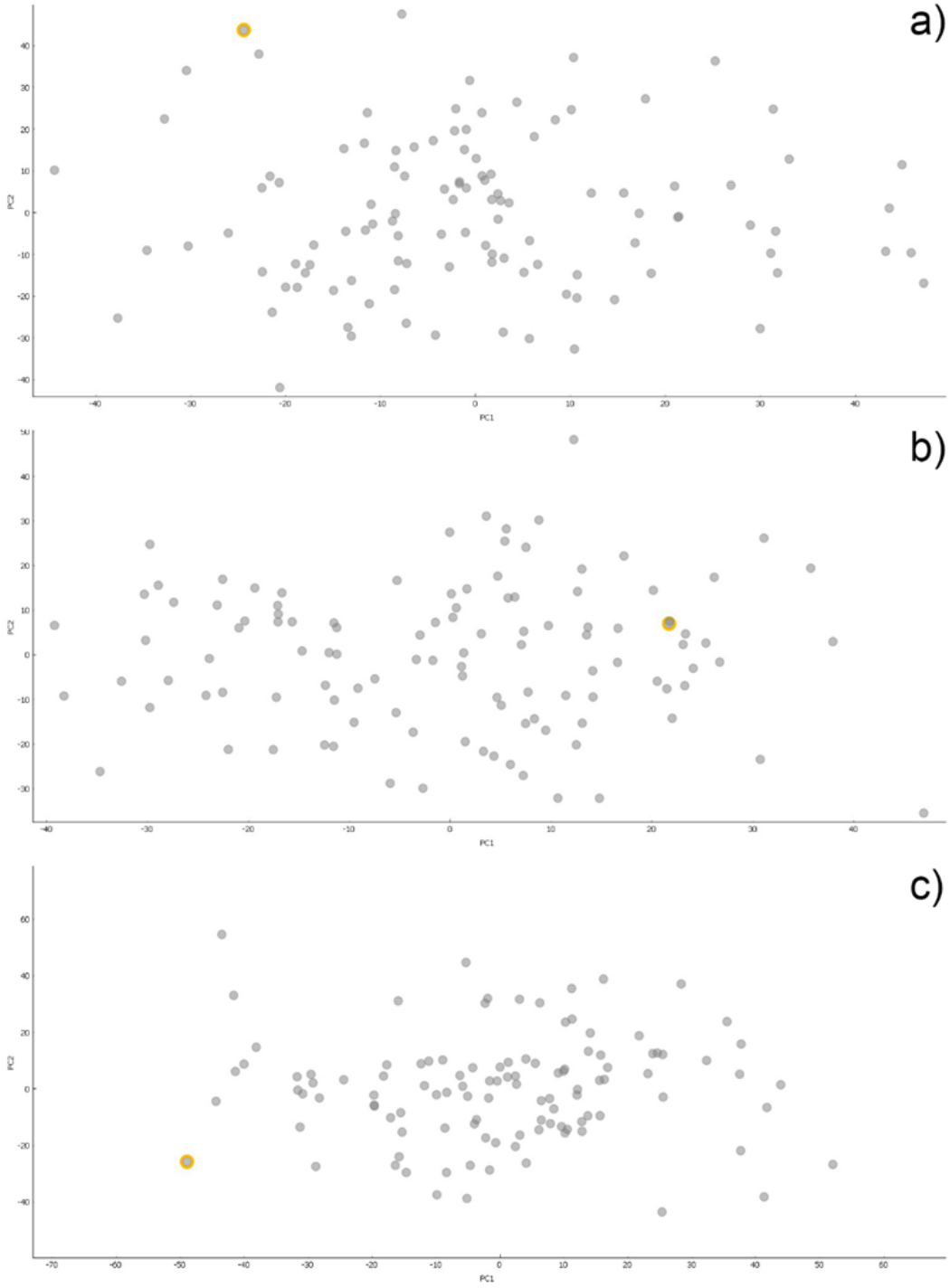

Figure 5. PCA analysis of frontal (a), lateral (b), and posterior (c) view of crania indicating position of the skull with divided parietal bone within referent population of female crania (n=110).

unknown as this trait is mainly found in case reports in clinical or medico-legal contexts, usually concerning infants and discussing clinical implications (Abdel-Salam et al., 2014; Angonese, Sonnaert, Rassart, Gauquier, & Cavatorta, 2010; Becker et al., 2005; Flössel, Hahn, Schmitter, & Erfurt, 2013; Weir, Suttner, Flynn, & McAuley, 2006; Wiedijk et al., 2016).

To analyze this specific cranium on a population level, we have performed PCA of the crania's frontal, lateral

and occipital views using image analysis based on convolutional neural networks. The skull in question was compared to 110 other female crania without this condition. The results revealed that the patient deviated from the rest of the female samples in frontal and posterior views, performing as an outlier. As this analysis is based on the black box approach, it could not identify traits or ratios that could lead to such results. However, specificities of the cranium could be explained by the visual inspection results and indices calculated from cranial measurements.

The skull shape was ultrabrachycephalic, which is not a frequent finding in populations ranging from 0.7% to 11% (Garson, 1887; Hossain, Lestrel, & Ohtsuki, 2004). The skull exhibited depression on the posterior part, and unsymmetrical flattening of the entire occiput indicated that this could be a case of plagiocephaly (Bessell-Browne & Thonell, 2004). Although plagiocephaly was not detected using CVAI that was below the threshold of 3.5, TDD indicated that this person had lateral deformational plagiocephaly in the mild form. This could be related to the divided parietal bone, the unsymmetrical position of the sagittal suture, and depression located at the posterior half of the sagittal suture.

Except for the divided parietal bone, this skull also exhibited several distinct cranial anatomical variations: asterion, lambda, and lambdoid ossicles, parietal foramen, frontal groove, supratrochlear notch, mastoid foramen, foramen ovale, zygofacial foramina, and condylar canal. It is interesting to notice that most of these traits were bilateral, thus probably not attributed to the divided parietal bone. In contrast, an asterion and a lambdoid ossicle were positioned on the left half of the skull and were probably related to this finding.

This person exhibited no clinical manifestations that could be related to these cranial variations. Nevertheless, it allowed us to study the rare condition and related manifestations in cranial variations. This is important for assessing its potential identification value in forensic anthropology and when conducting bioanthropological population studies and studying possible familiar relationships in historical identifications or intracementrary relations (Zupanič Slavec, 2012). The usefulness of this trait in the archeological context for accessing family relationships or population affinity is still unknown. Although Hauser and De Stefano presume that there is a genetic background for the expression of this trait (Hauser & De Stefano, 1989), the heritability of this trait has not been confirmed. Since the present study reported the trait prevalence on limited sample size, further studies planned within the project Forensic Identification of Human Remains Using MSCT Image Analysis (CTforID) should reveal more details on this and other cranial variation both of modern and ancient population that inhabited the area of Croatia.

Funding

This study was funded by the Croatian Science Foundation, Installation Research Project no. HRZZ-UIP2020-02-7331 - Forensic identification of human remains using MSCT image analysis (CTforID).

References

Abdel-Salam, G. M. H., Abdel-Hadi, S., Thomas, M. M., Eid, O. M., Ali, M. M., & Afifi, H. H. (2014). Gómez-López-hernández syndrome versus rhombencephalosynapsis spectrum: A rare co-occurrence with bipartite parietal bone. *American Journal of Medical Genetics Part A*, *164*(2), 480–483.

Angonese, A., Sonnaert, M., Rassart, A., Gauquier, N., & Cavatorta, E. (2010). Skull fracture or bipartite parietal bone. *Archives de Pediatrie: Organe Officiel de La Societe Francaise de Pediatrie*, *17*(4), 391–393.

Becker, D. B., Cheverud, J. M., Govier, D. P., & Kane, A. A. (2005). Os parietale divisum. *Clinical Anatomy: The Official Journal of the American Association of Clinical Anatomists and the British Association of Clinical Anatomists*, *18*(6), 452–456.

Berry, R. J. A. (1909). A case of os parietale bipartitum in an Australian aboriginal skull. *Journal of Anatomy and Physiology*, *44*(Pt 1), 73.

Bessell-Browne, R. J., & Thonell, S. (2004). Bipartite parietal bone: a rare cause of plagiocephaly. *Australasian Radiology*, *48*(2), 248–250.

Bhatt, A. A., Hunsaker, J., & Kalina, P. (2014). Pearls and pitfalls of pediatric head trauma imaging. *Applied Radiology*, *43*, 10.

Callejas Pastor, C. A., Jung, I.-Y., Seo, S., Kwon, S. Bin, Ku, Y., & Choi, J. (2020). Two-dimensional image-based screening tool for infants with positional cranial deformities: A machine learning approach. *Diagnostics*, *10*(7), 495.

Fenton, L. Z., Sirotnak, A. P., & Handler, M. H. (2000). Parietal pseudofracture and spontaneous intracranial hemorrhage suggesting nonaccidental trauma: report of 2 cases. *Pediatric Neurosurgery*, *33*(6), 318–322.

Flössel, U., Hahn, G., Schmitter, S., & Erfurt, C. (2013). Fallstrick: Os parietale bipartitum. *Rechtsmedizin*, *23*(6), 482–484.

Garson, J. G. (1887). The Cephalic Index. *The Journal of the Anthropological Institute of Great Britain and Ireland*, *16*, 11–17.

Goss, C. M. (1954). *Gray's Anatomy*. Philadelphia: Lea & Febiger.

Gray, H. (1901). *Gray's Anatomy* (1995th ed.). New York: Barnes and Noble.

Hauser, G., & De Stefano, G. F. (1989). *Epigenetic variants of the human skull*. Stuttgart: Schweizerbart'sche Verlagsbuchhandlung.

Hossain, M. G., Lestrel, P. E., & Ohtsuki, F. (2004). Secular changes in head dimensions of Japanese females over eight decades. *Anthropological Science*, 407120011.

Hrdlička, A. (1903). Divisions of the parietal bone in man and other mammals. *Bulletin of the American Museum of Natural History*, *9*, 231–386.

Langley, N. R., Jantz, L. M., Ousley, S. D., Jantz, R. L., & Milner, G. (2016). *Data collection procedures for forensic skeletal material 2.0*. Knoxville, Tennessee: The University of Tennessee.

Looman, W. S., & Flannery, A. B. K. (2012). Evidence-based care of the child with deformational plagiocephaly, Part I: assessment and diagnosis. *Journal of Pediatric Health Care*, *26*(4), 242–250.

Prado, P. S. A., García-Donas, J. G., Langstaff, H., Cunha, E., Kyriakou, P., & Kranioti, E. F. (2016). Os parietale partitum: Exploring the prevalence of this trait in four contemporary populations. *Homo*, *67*(4), 261–272.

Sanchez, T., Stewart, D., Walvick, M., & Swischuk, L. (2010). Skull fracture vs. accessory sutures: how can we tell the difference? *Emergency Radiology*, *17*(5), 413–418.

Shapiro, R. (1972). Anomalous parietal sutures and the bipartite parietal bone. *The American Journal of Roentgenology Radium Therapy and Nuclear Medicine*, *115*(3), 569–577.

Simonyan, K., & Zisserman, A. (2015). Very deep convolutional networks for large-scale image recognition. *3rd International Conference on Learning Representations, ICLR 2015 - Conference Track Proceedings*, 1–14.

Topinard, P. (1885). Éléments d'anthropologie générale. *Bulletins de La Société d'anthropologie de Paris*, *1*(8), 14–17.

Weir, P., Suttner, N. J., Flynn, P., & McAuley, D. (2006). Normal skull suture variant mimicking intentional injury. *British Medical Journal*, *332*(7548), 1020–1021.

Wiedijk, J. E. F., Soerdjbalie-Maikoe, V., Maat, G. J. R., Maes, A., Van Rijn, R. R., & De Boer, H. H. (2016). An accessory skull suture mimicking a skull fracture. *Forensic Science International*, *260*, e11–e13.

Zupanič Slavec, Z. (2012). *New method of identifying family related skulls: Forensic medicine, anthropology, epigenetics*. Springer Science & Business Media.

IAR Publishing

Journal of Bioanthropology

Volume 2, 2022. p. 22-28

ISSN 2787-8201; UDK 572
https://doi.org/10.54062/jb.2.1.8

Ankylosis of knee joint in human skeletal remains from Istria, case report from St. Teodor (15th-18th centuries)

Elvira Krešić[1], Magdalena Linić[2], Igor Erjavec[3], Željana Bašić[4], Siniša Pamić[5], Tihomir Percan[5] †, Ivana Kuhtić[1], Nataša Šarkić[6]

[1]University Hospital Center "Zagreb", Department of Diagnostic and Interventional Radiology

[2]University of Bradford

[3]University of Zagreb, School of Medicine, Center for Reproductive and Regenerative Medicine, Laboratory for Mineralized Tissue

[4]University of Split, University Department of Forensic Sciences

[5]Croatian Conservation Institute

[6]Aita Bioarchaeology

* Corresponding author: magdalena.linic@gmail.com

Received September 26th, 2022
Accepted for publication December 8th, 2022
Online First December 13th, 2022

Keywords: knee, ankylosis, CT, paleoradiology, Croatia.

Abstract

Objective: To present a case of knee joint ankylosis in human skeletal remains found in Istria, Croatia.

Materials and methods: A fused knee joint was found at archeological site St Teodor. We analyzed the knee changes using macroscopic observation, using digital radiography and computed tomography.

Results: Macroscopic analysis and both digital radiography and computed tomography imaging show knee ankylosis with cortical thickening.

Conclusion: Knee ankylosis with cortical thickening suggests that the person still used the leg despite the disability.

Introduction

X-ray scanning of a child mummy in AD 1896 in Frankfurt Museum, only three months after the invention of the X-ray, is considered to be a birthdate of paleoradiology (Rühli et al., 2004; Chhem and Brothwell, 2007; Zesch et al., 2016). However, the name was coined by Notman almost a century later; in AD 1987 when frozen sailors from the Franklin expedition were studied (Notman et al., 1987). Dragutin Gorjanović Kramberger, a famous Croatian paleontologist was the first to scan fossilized remains of Neanderthal man from Krapina in 1901 in the Sisters of Mercy Hospital in Zagreb (Gorjanović-Kramberger, 1899). Since then X-ray has remained a golden standard in paleoradiology, particularly for bony structures as it is easily available to archeologists and bioanthropologists (Chhem and Brothwell, 2007). On the other hand, computed tomography (CT) provides superior contrast and spatial resolution and has the capacity to remove superimposed embalming materials from images of internal structures, which is very important in the study of mummies (Braunstein et al., 1988). Ankylosis is a condition when a complete or partial fixation of a joint, by an osseous or fibrous union of the bones, is caused by disease or injury (Roberts & Manchester, 2010).

We present a case report of an ankylosed knee joint, found in St. Teodor graveyard, Istria, Croatia (15th-18th

Figure 1. Excavations carried out in AD 2019 on grave 5

century) scanned on X-ray and CT, and provide an overview of 3D reconstructions on CT.

Materials and Methods

Digital radiography is a fundamental imaging method in the skeletal analysis of human remains. It is a widely accessible imaging method for archeologists and bioanthropologists. In clinical medicine and scientific study of mummies, CT is essential for obtaining information about the soft tissues and internal body cavities, or in this case, for the quantitative evaluation of the cortical bone thickness. The benefits of CT include the capacity to remove superimposed embalming material from images of internal structures and also the superior contrast and spatial resolution (in comparison with X-ray). A possible limiting factor for the use of CT in paleoradiology can be its availability to archeologists and bioanthropologists.

The church of St. Teodor, located on the border of the municipalities of Rakalj and Krnica (Istria, Croatia) was first mentioned in AD 690. In the sources known so far, it was not mentioned again until the 17th century. The church was abandoned in the 19th century, after which it quickly decayed, and the sacred inventory was moved to the parish church of St. Blaža in Vodnjan. (Milotić, 2010). Since AD 2018, archaeological research has been carried out by the Croatian Conservation Institute.

During the excavations carried out in AD 2019 in grave 5, (Figure 1) which is located in the western part of the church, among many dislocated bones, a part of the upper and lower right leg (femur and tibia), completely fused, was discovered (Figure 2). Based on coins and jewelry found in that grave, it is most likely that grave 5 dates to the 15th-18th century.

The fused right knee has been scanned in University Hospital Centre Zagreb on Axiom Aristos MX (Siemens, Erlangen, Germany) X-ray unit and Multidetector computerized tomography (MDCT). CT scans were obtained using a Siemens Sensation 16 unit (Siemens, Erlangen, Germany) with the following scan

Figure 2. Part of the upper and lower right leg (femur and tibia), completely fused

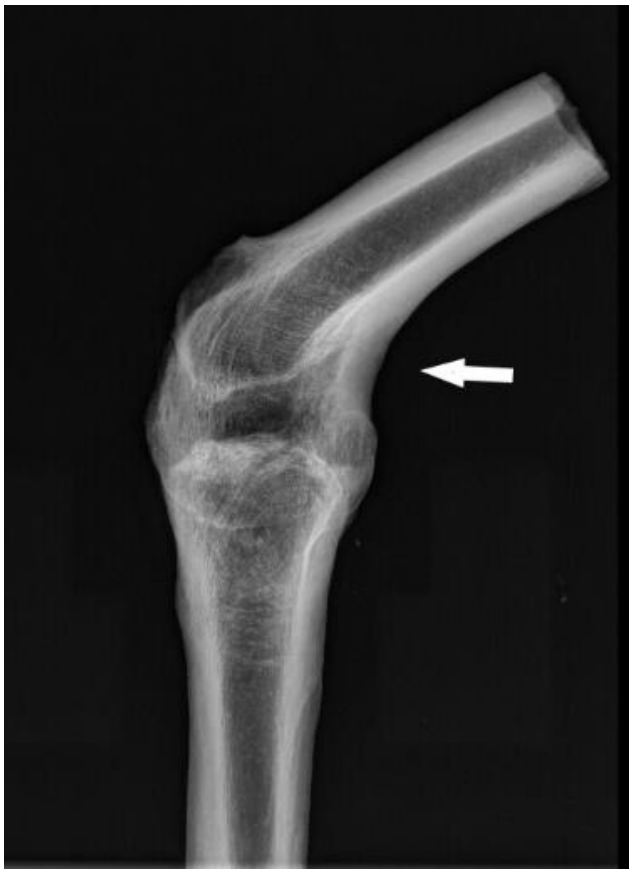

Figure 3. Part of the upper and lower right leg (femur and tibia), completely fused x-ray

parameters; 16 × 0.75 mm collimation, 130 KVP, and 300 mAs with a field of view (202 mm). Post-processing (3D volume rendering technique - VRT) and multiplanar reconstructions (MPR) were done using imaging processing software (Horos MD v. 8.1.5. Pixmeo Software, Geneve, Switzerland).

Results

Part of a right femur and tibia were preserved with a partially ankylosed knee joint in the lateral compartment. The knee was bent in a flexed position at 45° degrees. Unfortunately, due to the large number of individuals buried in this grave (Minimal Number of Individuals was 7), as well as it was the case of the commingled remains, it was not possible to determine whether any other bones from this assemblage belonged to the individual who had this pathological change, and consequently, neither the sex nor age at death could be estimated. Based on closed epiphyses and cortical thickness, we concluded that the knee

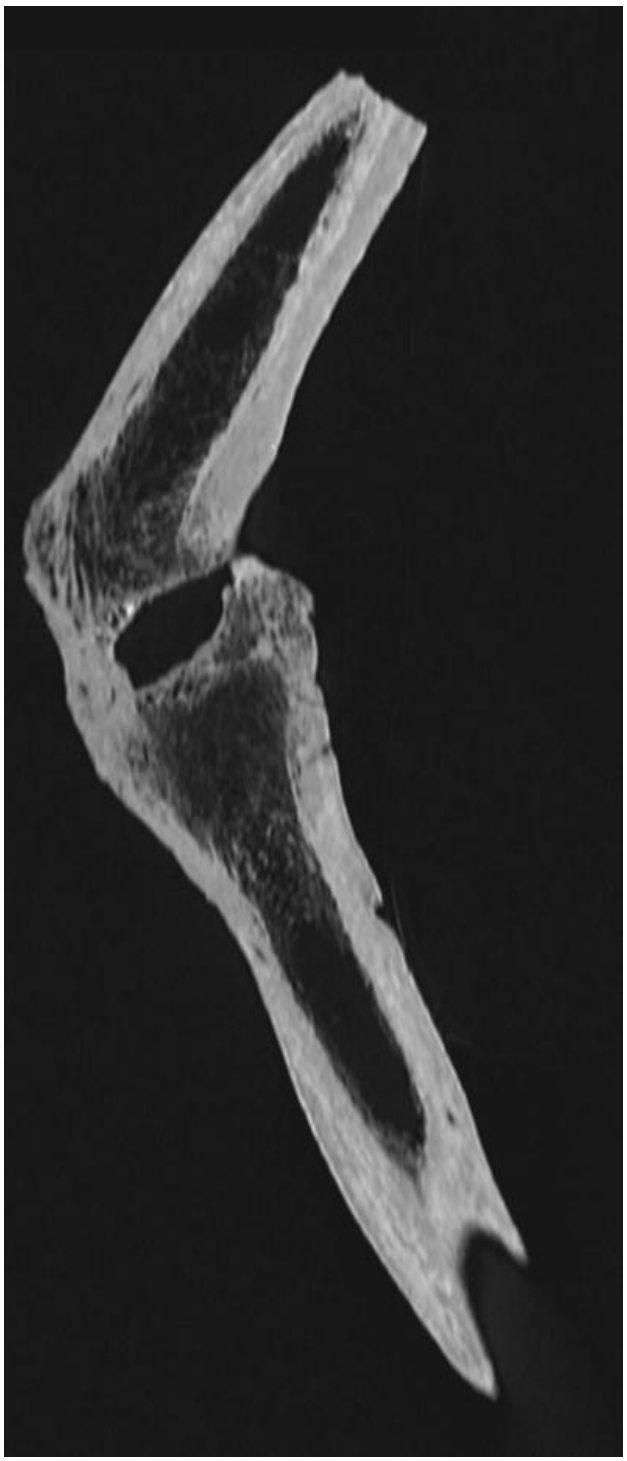

Figure 4. Part of the upper and lower right leg (femur and tibia), completely fused CT imaging

belonged to an adult individual. Macroscopic analysis and both x-ray (Figure 3) and CT imaging (Figures 4 and 5) showed tibiofemoral joint fusion with cortical thickening up to 13 mm and normal trabecular bone architecture.

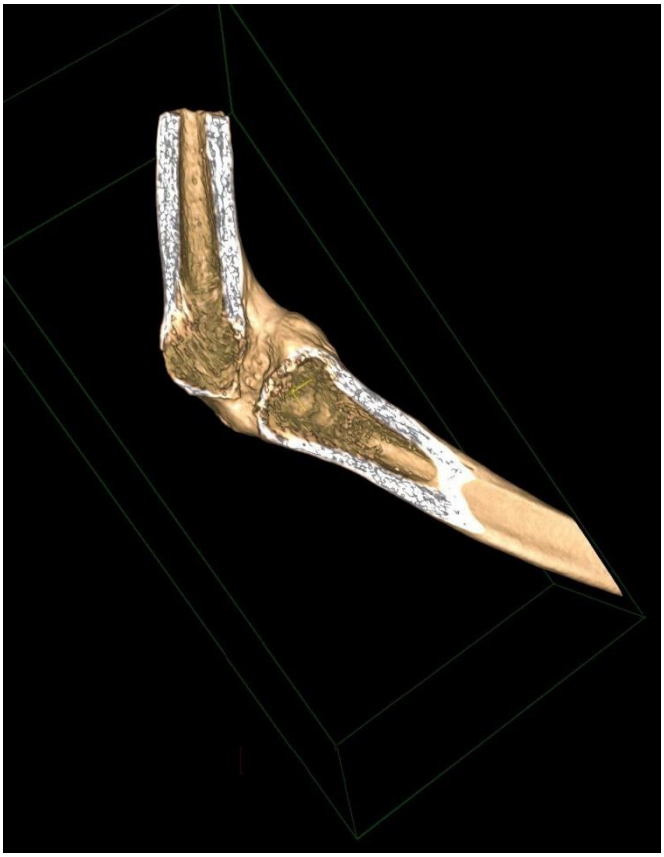

Figure 5. Part of the upper and lower right leg (femur and tibia), completely fused CT imaging

Discussion

Despite the condition of the knee joint, the individual has used the leg throughout life. The absence of osteoporosis and thinning of the cortical bone support this statement. (Brickley et al., 2020).

Nowadays in Western societies ankylosis of the knee is rather a rare condition as diseases are treated as early as possible, but in the past when treatments were not so widely available this was not the case. Still, ankylosis of limb joints is a rarely described alteration in paleopathological literature (Ortner, 2003; Roberts & Manchester, 2010). Lately, ankylosis of a knee joint from Medieval London has been reported (Redfern & Austin, 2020). Their re-analysis of a female skeleton with knee joint fusion suggests that the ankylosis was most likely formed due to a congenital condition, but non-adult trauma and osteoarthritic changes were discussed as well. In our case, no other visible signs of fracture are detectable, although they could be hidden in medial parts of the knee which are missing. Several underlying conditions could cause the ankylosis of a knee joint and should be taken into consideration for a differential diagnosis.

One of the outcomes of untreated rheumatoid arthritis (RA) could be ankylosis of the limb. The most prominent feature of rheumatoid arthritis is polyarthritis which is manifested with pain and swelling of the feet, wrists, hands, and knees. Individuals with longstanding, inadequately treated RA develop joint damage and deformities. Characteristic deformities for RA are ulnar deviation, swan neck, and boutonniere deformities of the hands, and flexion contractures of the knees and elbows. Those classic late-stage deformities are less common in the modern age due to improved treatment options (Sparks, 2019).

Although there is no agreement on the antiquity of the RA, the first modern description is found in the dissertation of Augustin Jacob Landré-Beauvais from AD 1800 (Entezami et al., 2011). For the cases of RA before AD 1800, we present the evidence of its existence in chronological order (Table 1 and Table 2) (Aceves-Avila

Table 1. The most convincing descriptions of chronic polyarthritis before 1800.

Source	Place	Time
Caraka Samhita	India	500 BC–AD 100
Scribonius Largus	Rome	Circa 100 BC
Michael Psellus	Rome	Circa AD 1000
Alonso López de Hinojosos	México	AD 1578
Thomas Sydenham	England	AD 1676
William Heberden the Elder	England	AD 1710–1801
Jon Pétursson	Iceland	AD 1782

Table 2. Pictorial evidence of the existence of chronic symmetric polyarthritis before the 19th century. The artistic representations are from Europe.

Painting	Artist	Year
The Temptation of St. Anthony	Unknown	1500–1670
The Donators	Jan Gossaert	Possibly 1530
Portrait of Siebrandus Sixtius	Unknown	1538–1631
Various paintings	Peter Paul Rubens	1577–1640
The Painter's Family	Jacobo Jordaen	1593–1678

et al., 2001). In Table 2 there is artistic evidence and we always have to be careful when analyzing art as it is always questioned to what extent the created painting is a realistic review or just a representation of the style (Čavka et al., 2010; Rühli et al., 2016; Bianucci et al., 2020).

In our case what makes RA unlikely as a cause of ankylosis is a lack of bone erosions. RA causes chronic inflammation which leads to peri-inflammatory bone lesions (erosions). In other words, the damage to the cortical bone with the destruction of the barrier between the bone marrow compartment and the extra-skeletal tissue (Lorenzo et al., 2015).

Another possible cause of knee joint ankylosis is tuberculosis (TB). The oldest cases of human tuberculosis date from 7000 BC in the site of Atlit Yam (Eastern Mediterranean, Israel). Remains of an immature individual and an adult female presented paleopathological evidence of TB, and were confirmed by aDNA analyses and lipid biomarkers (Hershkovitz et al., 2008). Among the earliest cases of TB in skeletal evidence in Europe, there are cases of Pott's disease in two individuals from the Early Neolithic (5400-4800 BC) from three sites in Germany (Halberstadt, Derenburg and Karsdorf) (Nicklisch et al., 2012).

As for skeletal manifestations of TB, the most affected site is the spine. Vertebral bodies are affected by lytic lesions resulting in ankylosis, body collapse and kyphosis (Pott's disease). Articular lesions are second in frequency. The large and medium-sized joints are involved preferentially, the hip and the knee particularly. It is rare for TB to affect more than one joint. TB presents with synovitis (which includes synovial hypertrophy and effusion), marginal erosions and juxta-articular osteopenia. Untreated TB can progress to joint ankylosis (Chapman et al., 1979). As mentioned before, in our case there are no visible erosions, so TB is probably not a cause of knee ankylosis.

Untreated syphilitic arthritis can also lead to joint ankylosis. Syphilis, according to most scientists, spread to Europe immediately after the discovery of America (Naranjo, 1994). Many artists became victims of this highly contagious disease, some of them are Franz Schubert, Robert Schumann, Bedrich Smetana, Ernst Theodor A. Hoffmann, Arthur Schopenhauer, Charles Baudelaire, Gustave Flaubert, Edouard Manet, Friedrich W. Nietzsche, Paul Gauguin, etc. (Rietschel et al., 2004). Neuropathic arthropathy of the Charcot joint was first described by Charcot as an arthritic sequela of neurosyphilis (Badazadeh et al., 2010). Neurosyphilis or tertiary syphilis often affects large weight-bearing joints such as the knee. Changes in the affected joint include destruction of articular surfaces, joint debris (loose bodies), dislocation and deformity (Pinzur, 2000).

Today we know that Charcot neuropathic osteoarthropathy is seen in individuals with various peripheral neuropathies, such as diabetes, trauma, and metabolic abnormalities (Harris & Violand, 2022). In the modern age, diabetes mellitus has taken over as the major cause of the Charcot joint (Sella & Barrette, 1999; Parvizi et al., 2003). However, diabetes is more likely to affect the foot and ankle (Lee et al., 2003). Midfoot collapse, described as a "rocker-bottom" foot is the hallmark deformity associated with this condition (Rogers et al., 2011).

As for the antiquity of diabetes mellitus, Aretaeus of Cappadocia AD 100 gave the first accurate description

of the disease, and introduced the term ‘diabetes’ (from the Greek diabainein which means passing through; a large discharge of urine) (King & Rubin, 2003). Dupras et al. (2010) described the earliest case of diabetes mellitus in skeletal remains from Dayr al-Barsha, Egypt dated to ca. 2050-1911 BC.

Recently, the so-called Medici syndrome has raised much attention in the history of medicine. The Medici, a famous family from Florence, ruled the city for most of the period from the 1430s to the 1730s. Given their wealth, political power, often poor health and the fact that they were patrons of arts, the Medici rulers and their relatives have attracted the attention of biomedical researchers as well as historians. Males in the Medici family suffered from a triple pathology of stenotic spinal ankylosis, recurrent peripheral joint disease and erythematous skin disease. Authors mention knee ankylosis as a final complication (sequel) of peripheral joint disease. (Lippi et al., 2014). Although this would be highly improbable, as only part of a right femur and tibia with a partly ankylosed knee joint was available for analysis, the Medici syndrome, as well as Charcot neuropathy, should be included in the differential diagnosis.

Conclusion

Our case shows an adult individual with knee joint ankylosis. Based on bone morphology and cortical thickness we can conclude with great certainty that this individual has used the leg in everyday life despite the disability caused by a fusion of the knee joint.

References

Aceves-Avila, F.J., Medina, F. & Fraga, A. (2001) The Antiquity of Rheumatoid Arthritis: A Reappraisal. J Rheumatol 28(4), 751–7.

Babazadeh, S., Stoney, J.D., Lim, K., & Choong, P.F.M. (2010) Arthroplasty of a Charcot knee. Orthop Rev (Pavia) 2(2), e17. https://doi.org/10.4081/or.2010.e17.

Bianucci, R., Kirkpatrick, C.L., Perciaccante, A., Galassim F.M., Lippim D., Appenzeller, O., & Nerlichm A,G. (2020) A case of congenital Horner syndrome from the 16th century. Lancet Neurol 19(8), 646-647. https://doi.org/10.1016/S1474-4422(20)30214-3

Braunstein, E.M., White, S.J., Russell, W., & Harris, J.E. (1988) Paleoradiologic evaluation of the Egyptian royal mummies. Skeletal Radiol 17, 348–352. https://doi.org/10.1007/BF00367181

Brickley, M., Ives, R., & Mays, S. (2020) The bioarchaeology of metabolic bone disease. Second edition. Amsterdam: Academic Press.

Chhem, R.K., & Brothwell, D.R. (2007) Paleoradiology: Imaging Mummies and Fossils. Springer Science & Business Media.

Čavka, M., Kelava, T., Čavka, V., Bušić, Ž., Olujić, B., & Brkljačić, B. (2010) Homocystinuria, a Possible Solution of the Akhenaten’s Mystery. Coll. Antropol. 34 Suppl 1, 255-258.

Chapman, M., Murray, R.O., & Stoker, D.J. (1979) Tuberculosis of the Bones and Joints. Seminars in Roentgenology 14 (4), 266-282. https://doi.org/10.1016/0037-198X(79)90024-5

Dupras, T.L., Williams, L.J., Willems, H., & Peeters, C. (2010) Pathological skeletal remains from ancient Egypt: the earliest case of diabetes mellitus? Pract Diab Int 27(8), 358-363a. https://doi.org/10.1002/pdi.1523

Entezami, P., Fox, D., Clapham, P., & Chung, K. (2011) Historical perspective on the etiology of rheumatoid arthritis. Hand Clin 27(1), 1-10. https://doi.org/10.1016/j.hcl.2010.09.006

Gorjanović-Kramberger, D. (1899). Der paläolitische Mensch und seine Zeitgenossen aus dem Diluvium von Krapina in Kroatien. Mitteilungen der Anthropologischen Gesellschaft in Wien 29, 65-68.

Harris, A., & Violand, M. (2022) Charcot Neuropathic Osteoarthropathy. StatPearls [Internet]. https://www.ncbi.nlm.nih.gov/books/NBK470164 Accessed 29 July 2022.

Hershkovitz, I., Donoghue, H.D., Minnikin, D.E., Besra, G.S., Lee, O.Y.C., Gernaey, A.M., Galili, E., Eshed, V., Greenblatt, C.L., Lemma, E., Bar-Gal, G.K., & Spigelman, M. (2008)

Detection and Molecular Characterization of 9000-Year-Old Mycobacterium tuberculosis from a Neolithic Settlement in the Eastern Mediterranean. PLOS ONE 3(10), e3426. https://doi.org/10.1371/journal.pone.0003426

King, K.M., & Rubin, G. (2003) A history of diabetes: from antiquity to discovering insulin. Br J Nurs 12(18), 1091–5. https://doi.org/10.12968/bjon.2003.12.18.11775

Lee, L., Blume, P.A., & Sumpio, B. (2003) Charcot joint disease in diabetes mellitus. Ann Vasc Surg. 17(5), 571–80. https://doi.org/10.1007/s10016-003-0039-5

Lippi, D., Matucci-Cerinic, M., Alburyc, W.R., & Weisz, G.M. (2014) Inherited knee disorders in the Medici family. The Knee 21(1), 2-5. https://doi.org/10.1016/j.knee.2013.11.006

Lorenzo, J., Horowitz, M., Yongwon, C., Takayanagi, H., & Schett, G. (2015) Osteoimmunology Interactions of the Immune and Skeletal Systems. 2nd Edition, Elsevier: Academic Press.

Milotić, I. (2010.) Crkva u Istri, povijesna i kulturna baština. Pazin – Poreč: Porečkopulska biskupija.

Naranjo, P. (1994) On the American Indian origin of syphilis: fallacies and errors. Allergy Proc. 15(2), 89–99. https://doi.org/10.2500/108854194778703044

Nicklisch, N., Maixner, F., Ganslmeier, R., Friederich, S., Dresely, V., Meller, V., Zink, A., & Alt, K.W. (2012) Rib lesions in skeletons from early neolithic sites in Central Germany: On the trail of tuberculosis at the onset of agriculture. Am J Phys Anthropol 149, 391-404. https://doi.org/10.1002/ajpa.22137

Notman, D.N., Anderson, L., Beattie, O., & Amy, R. (1987). Arctic paleoradiology: portable radiographic examination of two frozen sailors from the Franklin expedition (1845-1848). AJR Am J Roentgenol. 149(2), 347-50. https://doi.org/10.2214/ajr.149.2.347

Ortner, D.J. (2003) Identification of pathological conditions in human skeletal remains. Second edition. San Diego, CA: Academic Press.

Parvizi, J., Marrs, J., & Morrey, B.F. (2003) Total knee arthroplasty for neuropathic (Charcot) joints. Clin Orthop Relat Res. 416, 145–50. https://doi.org/10.1097/01.blo.0000081937.75404.ed.

Pinzur, M.S. (2000) Charcot's foot. Foot Ankle Clin 5(4), 897-912. Redfern, R.C., & Austin, A. (2020) Ankylosis of a knee joint from Medieval London: Trauma, congenital anomaly or osteoarthritis? Int J Paleopathol 28, 69-87. https://doi.org/10.1016/j.ijpp.2019.10.002

Rietschel, E.T., Rietschel, M., & Beutler, B. (2004) How the mighty have fallen: fatal infectious diseases of divine composers. Infect Dis Clin North Am 18(2), 311–339. https://doi.org/10.1016/j.idc.2004.02.002

Roberts, C.A., & Manchester, K. (2010) The archaeology of disease. Third edition. Stroud: The History Press.

Rogers, L.C., Frykberg, R.G., Armstrong, D.G., Boulton, A.J., Edmonds, M., Van, G.H., Hartemann, A., Game, F., Jeffcoate, W., Jirkovska, A., Jude, E., Morbach, S., Morrison, W.B., Pinzur, M., Pitocco, D., Sanders, L., Wukich, D. K., & Uccioli, L. (2011) The

Charcot Foot in Diabetes. Diabetes Care 34(9), 2123-2129. https://doi.org/10.2337/dc11-0844

Rühli, F.J., Chhem, R.K., & Böni, T. (2004). Diagnostic paleoradiology of mummified tissues: interpretation and pitfalls. Can Assoc Radiol J. 55, 218-227.

Rühli, F.J., Galassi, F.M., & Haeusler, M. (2916) Palaeopathology: Current challenges and medical impact. Clin Anat. 29(7), 816-22. https://doi.org/10.1002/ca.22709

Sella, E.J., & Barrette, C. (1999) Staging of Charcot neuroarthropathy along the medial column of the foot in the diabetic patient. J Foot Ankle Surg. 38(1) 34–40. https://doi.org/10.1016/S1067-2516(99)80086-6

Sparks, J.A. (2019) Rheumatoid Arthritis. Annals of Internal Medicine 170(1), ITC1-ITC16. https://doi.org/10.7326/AITC201901010

Zesch, S., Panzer, S., Rosendahl, W., Nance, J.W. Jr, Schönberg, S.O., & Henzler, T. (2016) From first to latest imaging technology: Revisiting the first mummy investigated with X-ray in 1896 by using dual-source computed tomography. Eur J Radiol Open. 3, 172-181. https://doi.org/10.1016/j.ejro.2016.07.002

IAR Publishing

Journal of Bioanthropology

Volume 2, 2022. p. 29-36

ISSN 2787-8201; UDK 572
https://doi.org/10.54062/jb.2.1.7

The life and death of Faust Vrančić – What could his bones tell us? A prelude to paleoradiological analysis

Zlatka Rodin

Memorial Centre Faust Vrančić, Ulica I 1a, 22 233 Prvić Luka, Croatia

* Corresponding author: zlatkarodin@yahoo.com

Received August 19th, 2022
Accepted for publication November 29th, 2022
Online First December 8th, 2022

Keywords: Faust Vrančić, paleoradiology, bioarhaeology, forensic facial reconstruction.

Abstract

The text was prepared as a response to a collaboration proposal of dr. Mislav Čavka from the University Hospital Centre Zagreb to the Memorial Centre Faust Vrančić, the institution that I work at. The collaboration would concern the potential analysis of the remains of Faust Vrančić that are buried at the church Sv. Marija od Milosti (Church of St. Mary of Grace) in the village of Prvić Luka on the island of Prvić near Šibenik and that would be conducted with the approval of the parish vicar, Božo Škember. The purpose of the text is to clarify the circumstances surrounding the death of Faust Vrančić, details related to the execution of his will, interesting facts related to his burial, opening and robbing of his crypt as well as the facts about his physical appearance. The analysis of the remains of Faust Vrančić could help us gain new insights into his life and death, which would shed a new light to his biography. Therefore, we need these data in order to use them as a background for further investigation. The relevant analysis could reveal facts such as: his height, the diseases that he suffered from, the cause of his death, if he suffered any bone fractures, his nutrition habits and his dwelling places (this part of his life is well documented, but it would be interesting to see if the biographic data match with the results of the paleoradiological analysis). Eventually, the bone analysis would tell us how he looked like and we would be able to compare the results of the forensic facial reconstruction with the only known "official" portrait of Faust Vrančić.

Introduction

I have been thinking for quite some time if the curiosity is my only motivation to pursue this project and if the only purpose of this project would be to satisfy the curiosity, be it my own, as a person who is professionally occupied with Faust Vrančić, be it the scientific curiosity of dr. Čavka and his colleagues. My doubts got event bigger following my first discussion with the local vicar about the topic, when he raised the question that was left hanging in the air and that concerned of the purpose of the project. I must admit that reconsideration was a useful tool to filter the actual motivation for this project. However, I found the answer to the posed question in Faust Vrančić himself, in what he actually was: a family man, a priest, a politician and a diplomat, a patriot, a linguist, a lawyer, a philosopher and, above all, an inventor and scientist driven by curiosity that has never been an end in itself. The efforts he put into preparing a dictionary intended for the European people that jointly fought against the Ottoman Turks resulted in one of the first multilingual dictionaries that was also the first dictionary of the Croatian and Hungarian ever. Therefore, Faust Vrančić is rightfully considered the father of the Croatian and Hungarian lexicography. He was the man who, in his forties, already had patents for specific mills and sawing machines. At the end of his life he published a book

comprising the results of his life-long work on inventions and provided certain solutions that appeared for the first time in the technical literature, e.g. the first idea of the suspension bridge, the metal bridge, the cable car, the carriage shock absorber, the precursor of water turbines and the vertical axle mill. These were all very useful inventions aiming at making the everyday life of people easier. However, what really made Faust Vrančić famous was his Homo Volans – the Flying Man, a representation of the first functional parachute ever – an invention that was a product of his restless and curious spirit more than anything else. Faust named his collection of inventions Machine novae – the New Machines. At his time, these inventions were literally new, some of them were even too advanced for that time, so that they became reality and widely accepted much later. Thinking in the context of this text and the achievements in the segment of paleoradiology and similar sciences, the questions raise: If Faust could see all the "new machines" available to scientists nowadays and how much it could be found out about him only based on the analysis of his bones, would he be impressed and delighted? Would he still be curious? Would he approve of the method? Knowing him, as much as it is possible to know a person considering the time distance of four centuries, I am sure that he would. A curious boy from Dalmatia from a noble family who first got to know the world in Bratislava where he continued his education with support of his uncle who worked there as a royal governor, then as a student in the cosmopolitan and university city of Padua where the Anatomical Theatre was not built out of curiosity or fun, Faust Vrančić experienced the peak of his carrier as a secretary at the court of Rudolph II Habsburg in Prague where the European scientific elite of that time gathered. I do not think that anything else needs to be added here to describe his greatness and open-mindedness. Knowing this, it is clear that his perspectives were neither narrowed nor burdened by prejudice or unnecessary rules. And then again, he was a very spiritual man, devoted to the Church, which is why we hope that the Church would offer us their help and grant us the permission to exhume his remains and take them to Zagreb for analysis.

How would we benefit from the new insights today? Since Faust Vrančić is one of the most influential Croats of all times that we can be more than proud of and proudly present and popularise his work, adding new interesting facts to his biography would actually help us do it using all available resources in all scientific segments. Honour the scientist with science! On top of this, this would be the first case of a detailed analysis of remains of a known historical figure in Croatia. So far, the objects of this scientific analysis in Croatia were only museum mummies, religious relics and bones of mostly anonymous deceased buried at specific cemeteries and a couple of saints. A collaboration like this could result in a scientific colloquium, a documentary, a new museum exhibit, increased number of museum visitors… the options are numerous. What would the bones tell us – I hope that we will soon find out.

Last wishes comprised in the codicil; death and burial

The location: Venice, sestiere Castello, the home of Jeronim Jubete, the reverend of the Church of San Provolo. The time: 15 January 1617. Faust Vrančić, at his deathbed, aware that his life is coming to an end, wrote a supplement (a codicil) to his will that he left two years earlier in Rome. He requested in his will to be buried in his homeland, on the island of Prvić and a wooden box to be placed in the coffin next to his dead body that would comprise his literal works. Additionally, he decided on his epitaph. Faust Vrančić died five days later on 20 January 1617.

Ivan Tomko Mrnavić, a protégé of Faust Vrančić, a historian and novelist, escorted Faust's remains from Venice to Šibenik. He also held a funeral speech (known today as the *Faust Vrančić Euology*) at the Šibenik Cathedral of St. James and it was published in Venice in that same year. According to the records prepared by the Šibenik bishop Fosco, Mrnavić held the speech on 22 February 1617. Therefore, it is to presume that the burial in Prvić Luka was held close to that date. It seems that the people of Šibenik could not easily let that the greatest son of their city of that time be buried near his birthplace without having a deserved and dignified commemoration. Faust wanted to be taken to Prvić and buried there straight away. He requested to be laid to

rest in a grave in front of the church door. When mentioning this request in his speech, Mrnavić romanticised it saying: "*…his last wish was to be laid to rest on the cemetery on the island of Prvić in the bare soil among the dead bodies of the common people, where he thought he would find the peace that he longed for in his life*." Still, he was buried in the central part of the church, as it was usual place of burial of dignitaries of that time.

The reason why Faust Vrančić chose the island of Prvić for his last resting place was his close connection with the island that was established during his stays there in his family mansion situated in the village of Prvić Šepurine. It is a Renaissance villa now declared a cultural property. The mansion is still owned by the Draganić-Vrančić family, i.e. the descendants of the Faust's brother Kažimir.

The tombstone comprises the epitaph mentioned in the codicil prepared by Faust Vrančić himself:

FAUSTUS VERANTIUS EPISC. CHANADIENSIS NOVORUM PREDICAMENTORUM ET NOVARUM MACHINARUM AC FRAGMENTORUM HISTORIAE ILLYRICAE AC SARMATIAE COLLECTOR

(Faust Vrančić, the Bishop of Csanád, collector of new facts and devices as well as fragments concerning the history of Illyrians and Sarmatians).

Below is the list of books that were buried together with the remains of Faust Vrančić (the left column includes the list of titles as Vrančić informally described them in the codicil, and the right column lists the known titles of these books i.e. their official titles) named in Table 1.

It is interesting that Alberto Fortis in his work titled *Travels into Dalmatia* was astonished by the Faust's wishes related to the burial with his books, and he was even more astonished that his descendants respected his so unusual wishes.

Circumstances preceding the death of Faust Vrančić

To better understand the circumstances preceding the death of Faust Vrančić, it must be mentioned that after leaving the Prague court of Rudolph II Habsburg for the second time, Faust spent the last years of his life in Rome where he got sick. In 1615, at the doctor's recommendation Faust decided to leave the Eternal City and to return to his homeland. It was because the air in Rome did not suit his health. Taking into consideration his delicate health and the fact that he was to take off on a long journey, Faust left his will on 12 June 1615. In his will, he took care of his family members, his daughter Alba Rosa and his mother Katarina, as well as the male descendants of his brother Kažimir that were to continue the Vrančić lineage. According to the records of Mrnavić who escorted

Table 1: books that were buried together with the remains of Faust Vrančić

un libro delli Machine nove	*Machinae novae*
un libro delli Nove predicamenti	Most probably *Logica Nova* printed together with *Ethica Cristiana*; so he could refer here to both works
un libretto delle Vergini da me transliterato	*Život nikoliko izabranih divic* (Life of Few Chosen Virgins)
un libro Dittonario cinque lingue	*The dictionary of five most prominent European languages*
un'altro de l'historia di Dalmazia et questo voglio sii stampato con la cura et diligenta de miei essecutori	A manuscript that has never been published; it is possible that he refers to *Illyrica Historia* that was bought together with some other documents from the Draganić-Vrančić Mansion in Prvić Šepurine in 1948 and are kept today in the Collection of Manuscripts of the National University Library.

Faust, Faust was not able to travel by boat from Ancona to Šibenik because of his health condition, so he opted to travel by carriage through Venice, where he had some unfinished business. While in Venice, despite his many friends who would gladly welcome him at their homes, Faust stayed at a hospice held by priests and eagerly worked on publishing his two books: Machinae novae and a joint edition of *Logica nova* and *Ethica cristiana*. Since *Machine novae* does not include the year of publishing it is presumed that it was published in 1616, as Mrnavić mentioned in his *Eulogy*. After the books were published, Vrančić was aware of his health condition and the fact that his return home was not probable. Therefore, he wrote his codicil and died according to Mrnavić: "*twenty days following his sixty sixth birthday*", which also reveals us the precise date of his birth.

The grave of Faust Vrančić – Chronology of openings

The priest don Krsto Stošić, the founder of the first Šibenik museum, reported for the Šibenik newspapers titled *Narodna straža* that the parish church was closed because of the threat of the ceiling falling in, on 14 April 1926. Later, on 18 September 1926, Stošić wrote again about the church in Prvić Luka (Fig. 1), saying that the church was finally repaired and "*the opportunity was used to examine the tomb of the famous scientist Faust Vrančić*". According to Stošić, after the tombstone was removed, the gathered people were somewhat disappointed because there was no trace of any bishop's attributes and the grave only contained "*rotten clothes and a strong skull*". However, the article reveals that allegedly it had not been the first time that the grave of Faust Vrančić was opened. In that very article, Stošić claims that the tomb was opened once out of pure curiosity some 60 years earlier, at the time of abbot Šarac who established that the tomb was broken into on the side from another grave. The word has it that abbot Šarac only found a broken tin box and several damp papers, none of which he managed to preserve.

In 1941, Krsto Stošić published his work *Sela šibenskog kotara* (*The Villages of the Šibenik County*). In the chapter dedicated to Prvić, he mentions again the breaking into the tomb of Faust Vrančić. This time, he said that no box was left in the grave, since the grave was broken into during the night and the burglars took the box with them thinking that they would find treasure in it. However, the box contained "*only published and unpublished works by Faust Vrančić that scattered later in all directions around the village*".

Vladimir Bazala in his work titled *Pregled hrvatske znanstvene baštine* (*Overview of the Croatian Scientific Heritage*) from 1978 says that "*the grave of Faust Vrančić was opened around 1900, and some things found in it were allegedly taken to Budapest, but no one bothered to investigate further what these things actually were*". However, Bazala did not specify the source of this information.

Figure 1: The resting place of Faust Vrančić, Church of St. Mary of Grace in Prvić Luka

In the article titled *Enigma Vrančićeva rukopisa Storia della Dalmazia* (*The Enigma of the Vrančić's Manuscript Storia della Dalmazia*) published in the *Vijenac* magazine, Milovan Buchberger wrote that the scattered manuscripts were collected by the people of Prvić Luka and handed over to Frane Kazimir Draganić-Vrančić, the successor of the Faust's mansion in Šepurine. Still, Buchberger did not specify any other source of this information, besides the two sources specified by don Krsto Stošić that actually did not mention this, as we found out.

In December of the war year 1993, the old tombstone situated in the floor of the church was replaced by a new one as a part of the event titled *Faust Vrančić, naš suvremenik* (*Faust Vrančić, Our Contemporary*) organised by the Juraj Šižgorić library from Šibenik. Then, the old tombstone was exhibited in a niche in a church wall, and the original text of the epitaph was carved in the new one.

So, in chronological order, first there was the robbery of the grave around 1860 or earlier (but we cannot precisely know when), according to the two records by don Krsto Stošić that do not fully match. Then, at approximately the same time, at the time of abbot Šarac, the grave was opened for the first time out of curiosity. The third opening of the grave, and the second opening out of pure curiosity, was documented in a newspaper article from 1926. Krsto Stošić wrote about it saying that there were no books or manuscripts in the grave. The last opening of the grave was the one in 1993 for the purpose of replacing the tombstone that was also blessed on that occasion. We hope that we will soon have an opportunity to remove the tombstone again. This time, the purpose would be different, and I believe that Faust who was so curious himself and fascinated by the science would approve of it.

The books and the manuscripts about the history of Dalmatia have been lost without doubt. It remains unknown if the disappointed “treasure hunters” really scattered them around the village or if the abbot Šarac might have collected them and if they found the way to Faust’s descendants and ended up in the Vrančić mansion and were sold later.

The portrait of Faust Vrančić

So far, this text covered the illness, the death and the grave of Faust Vrančić, which are the topics that the potential bone analysis could shed a new light on. However, this bone analysis also offers another very interesting option – the forensic facial reconstruction of Faust Vrančić. There are several portraits depicting Faust Vrančić, but there are records that he actually posed for only one of them (Fig. 2). This portrait is kept in the family mansion Draganić-Vrančić in Prvić Šepurine. It is a work of an unknown artist painted in oil painting technique in Rome in 1605 (89 x 110 cm). It represents Faust Vrančić at the age of around fifty, sitting, slightly turned to the right. His hair is dark and he has a long black beard, wearing a cross pendant around his neck. There are also another two portraits similar to this original one that can be found in certain publications and on the Internet. They depict Faust Vrančić in the same position and clothes, but his face is somewhat different. The members of Draganić-Vrančić

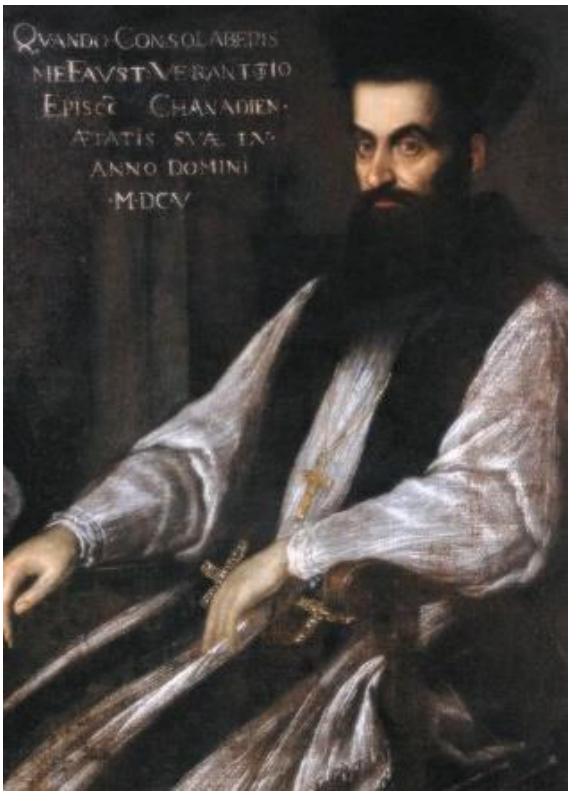

Figure 2: Posed portrait of Faust Vrančić, Rome, 1605

family told us that the portrait was lent earlier quite often for the exhibition purposes, which might explain the origin of these reproductions.

Another known portrait of Faust Vrančić, depicting Faust without the beard (Fig. 3), situated in the hall of the bishop's palace in Timișoara in Romania, served the sculptor Kosta Angeli Radovani as a model for a bust that is now exhibited in the Alley of Sculptures of major figures of Croatian science and technology. Faust Vrančić could never pose for that portrait since it was painted a century after his death on the occasion of expanding the gallery of bishops' portraits. Namely, Faust was appointed the Titular Bishop of Csanád in the period 1598 through 1608. This means that he was not physically there because of the occupation of the territory by Ottoman Turks. Today, the Roman Catholic Diocese of Timișoara is one of the successors of the former historical Diocese of Csanád. We got into contact with the archivist of the Diocese, Dr Claudiu Calin, who told us that he believed that the portrait had been painted in the 19th century by an unknown painter.

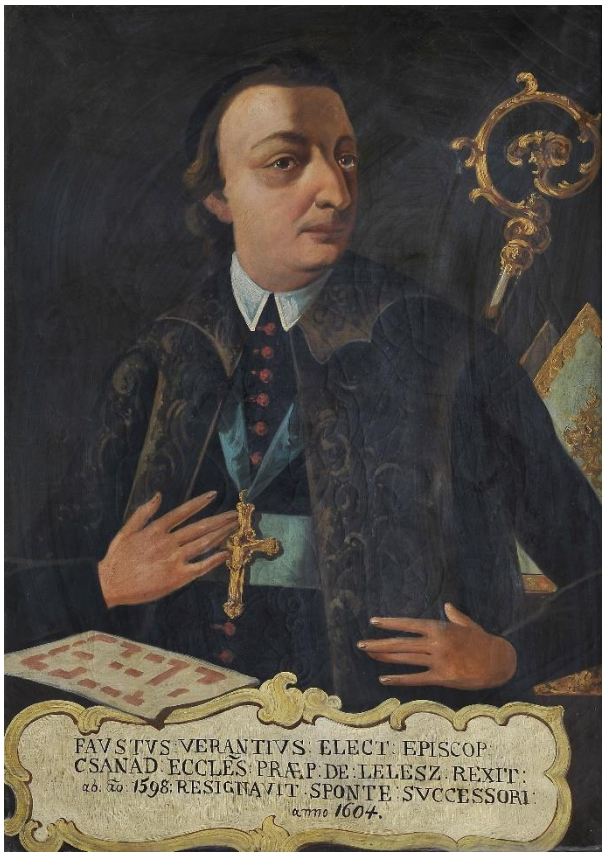

Figure 3: Portrait of Faust Vrančić , 19th century

It is also interesting to add that the ceiling fresco in the Croatian National Theatre in Šibenik also includes a portrait of Faust Vrančić. However, the Faust is painted here in the company of his uncle Antun Vrančić and other influential people of Šibenik: Martin Rota Kolunić, Andrija Medulić Schiavone and Nikola Tommaseo. Apparently, this portrait was painted following the example of the one from Prvić Šepurine. The author of this portrait is the 19th century painter Antonio Zuccaro.

As for the Faust's physical appearance, besides the portraits, there is also a written record – a certificate of the rector of the Faculty of Law of the University of Padua dated 14 April 1569 that confirms the status of Faust Vraničić as a student of law. The rector described Faust as a Dalmatian with a scar on his forehead (Faust Verantius Dalmata habens cicatricem in fronte). We will probably never find out where and how Faust got the scar, but it would be very interesting to see if there are traces of any trauma on his skull.

Future analyses

This text covers mostly the last period of the life of Faust Vrančić, his death and the circumstances after his death in terms of his remains. It also includes all relevant information that could serve as a starting point for a future analyses of the remains of Faust Vrančić that we hope we will able to carry out in the near future.

We would like to scan the skeletal remains on X-ray and on CT. Digital planar radiographs of the body (in several parts) will be done in two projections. CT slices will be obtained using 64x0.6 collimation with “dual energy” scanning parameters; 80 kV and 140 kV respectively (Somatom AS+, Siemens Healthcare, Erlangen, Germany) with 0.5 reconstruction increment (RI).

3D Multiplanar (MPR), Volume Rendering Technique (VRT), Maximum Intensity Projection (MIP) and Cinematic Rendering will be done on Singo.Via workstation (Siemens Healthcare, Erlangen, Germany)

and with OsiriX MD Imaging software, v 7.0.4. (Pixmeo, Geneva, Switzerland).

Assessment of sex, age at death, pathological conditions and trauma will be done based on standards of bioarchaeological practice. Sex assessment will be based on standard methodology and scoring systems described in Buikstra & Ubelaker (1994). Age at death will be assessed based on cranial suture closure and dental attrition scoring systems described in Meindl &Lovejoy (1985) and Brothwell (1981). If possible samples will be dated by means of radiocarbon dating method.

References

Bazala, V. (1978). Pregled hrvatske znanstvene baštine. Zagreb: Nakladni zavod MH.

Borić, M. (2019). Faust Vrančić - Portret izumitelja. Zagreb: Tehnički muzej Nikola Tesla.

Brothwell, D. (1981): Digging Up Bones. Cornell UniversityPress, Ithaca.

Buchberger, M. (2017). Enigma Vrančićeva rukopisa Storia della Dalmazia. Vijenac No. 609.

Buikstra, J.E. & Ubelaker, D.H. (1994): Standards for Data Collection from Human Skeletal Remains. Arkansas Archaeological Survey, Fayetteville.

Čavka, M., Janković, I., Rajić Šikanjić, P., Tičinović, N., Radoš, S., Ivanac, G., Brkljačić, B. (2010). Insights into a Mummy: A Paleoradiological Analysis. Coll. Antropol. 34 (2010) 3: 797–802

Čoralić, L. (1999). Oporuke dalmatinskih patricija u Mlecima (XV.-XVIII. st.). Zbornik Odsjeka za povijesne znanosti Zavoda za povijesne i društvene znanosti HAZU (1330-7134) 17 (1999); 85-109

Fortis, A. (1984). Put po Dalmaciji. Zagreb: Globus.

Fosco, A. G. (1890). Vita di Giovani Tonco-Marnavić. Šibenik.

Janković, I., Balen, J., Ahern, J. C. M., Premužić, Z., Čavka, M., Potrebica, H., & Novak, M. (2017). Prehistoric massacre revealed. Perimortem cranial trauma from Potočani, Croatia. Anthropologischer Anzeiger; Bericht uber die biologisch-anthropologische Literatur, 74(2), 131–141.

Kurelac, I. (2004). Illyrica historia Fausta Vrančića. Zbornik Odsjeka za povijesne znanosti Zavoda za povijesne i društvene znanosti Hrvatske akademije znanosti i umjetnosti, Vol. 22 No., 2004.

Kurelac, I. (2013). Oporuka Fausta Vrančića iz ostavštine plemićke obitelji Draganić-Vrančić u Državnom arhivu u Rijeci. Croatica Christiana periodica, Vol. 37 No. 71, 2013.

Martinović, I. (2016). Marko Antun de Dominis vs. Faust Vrančić: od Logike (1608) do Nove logike (1616). Prilozi za istraživanje hrvatske filozofske baštine 42/2(84) (2016) 293–330

Meindl, R.S. & Lovejoy, C.O. (1985): Ectocranial suture closure: a revised method for the determination of skeletal age at death based on the lateral-anterior suture. Am. J. Phys. Anthropol.68: 57–66.

Miagostovich, V. (1898). Il ritratto di Fausto Veranzio: d' alcune cose intorno alle sue „Machinae novae“ e del suo testamento; Il nuovo Cronista di Sebenico. Trieste.

Mrnavić, I. T. (1993). Govor na pogrebu Fausta Vrančića. Šibenik: Gradska knjižnica „Juraj Šižorić“.

Polić Lj., Petaros A., Cuculić D., Bosnar, A. (2012). Forenzička facijalna rekonstrukcija – između umjetnosti i znanost Medicina fluminensis 2012, Vol. 48, No. 1 , p. 30-40

Prijatelj, K. (2001). Faust Vrančić i arhitektura. Zbornik o Faustu Vrančiću. Šibenik: Gradska knjižnica „Juraj Šižgorić“.

Putanec, V. (2001). Epitaf Fausta Vrančića (1551. – 1617.) i pitanje bibliografije njegovih djela. Zbornik o Faustu Vrančiću. Šibenik: Gradska knjižnica „Jurja Šižgorić“.

Stošić, K. (1926). Zatvorena župska crkva. Šibenik: Narodna straža 17/1926.

Stošić, K. (1926). Grobnica Fausta Vrančića. Šibenik: Narodna straža 37/1926.

Stošić, K. (1941). Sela šibenskog kotara. Šibenik

Vrančić, F. (1992). Dictionarium quinque nobilissimarum Europae linguarum: Latinae, Italicae, Germanicae, Dalmatiae & Ungaricae. Zagreb: Novi Liber.

Vrančić, F. (1993). Machinae novae. Zagreb: Novi Liber ; Šibenik : Gradska knjižnica “Juraj Šižgorić”.

Vrančić, F. (1995). Život nikoliko izabranih divic. Šibenik: Gradska knjižnica „Juraj Šižgorić“.

Vrančić, F. (2018). Nova logika. Šibenik: Gradska knjižnica „Juraj Šižgorić“, Zagreb: Instutut za filozofiju.

Zelić, D. (2018). Izumitelj i poduzetnik – tri nepoznata dokumenta o Faustu Vrančiću iz 1588.–1590. godine // Faust Vrančić i njegovo doba. Zbornik radova s Međunarodnoga znanstvenoga skupa održanoga u povodu 400. obljetnice objavljivanja Novih strojeva Fausta Vrančića, Vodice – Šibenik 22. – 23. rujna 2015. / Borić, Marijana ; Blažević, Zrinka ; Marotti, Bojan (ur.). Prvić Luka: Memorijalni centar »Faust Vrančić«, 2018. str. 41-64

IAR Publishing

Journal of Bioanthropology

Volume 2, 2022. p. 37-44

ISSN 2787-8201; UDK 572
https://doi.org/10.54062/jb.2.1.2

Sex determination of medieval skeletal remains: evaluation of anthropological, odontological and genetic methods

Belma Jusić[1]*, Mirela Džehverović[1], Amela Pilav[1], Samra Terzić[2], Selma Zukić[3], Edin Bujak[4], Jasmina Cakar[1]

[1]Laboratory for Forensic Genetics, University of Sarajevo-Institute for Genetic Engineering and Biotechnology, Bosnia and Herzegovina

[2]Department of Archaeology, Faculty of Humanities and Social Sciences, University of Zagreb, Croatia

[3]Department of Dental Morphology, Dental Anthropology and Forensics, University of Sarajevo-Faculty of Dentistry with Clinics, Bosnia and Herzegovina

[4]Department of Archeology, Faculty of Philosophy, University of Sarajevo, Bosnia and Herzegovina

* Corresponding author: belma.jusic@ingeb.unsa.ba

Received December 28th, 2021
Accepted for publication February 28th, 2022
Online First March 1st, 2022

Keywords: medieval Bosnia, sex assessment, anthropology, odontology, ancient DNA.

Abstract

During 2019, 28 grave constructions were excavated at cemetery Metaljica (Hadzici municipality, Bosnia and Herzegovina). Archaeological excavation has revealed 17 well preserved skeletons that were subjected to anthropological, odontological and genetic analyses. To determine sex by genetic analysis, amelogenin and DYS391 loci were taken into consideration. Concordance between results of anthropological, odontological and genetic analyses applied in this research occurred in one sample out of 17. In four samples, sex was determined only by genetic analysis, since sexual dimorphism indicators were not preserved. Concordance between odontological and genetic determinations was observed in two samples, while affirmative results of anthropological and genetic analysis were obtained in only one sample. Discrepancy in the majority of samples can be atributted to the state of preservation of skeletal remains, interpopulation differences and allele drop-out. Moreover, anthropological and odontological analyses were not applicable to juvenile/subadult skeletons, since sexually dimorphic characteristics relevant for metric and morphological analysis are not developed well at that developmental period. This study emphasizes the importance of combining anthropological, odontological and genetic methods in order to determine sex of archaeological skeletal remains accurately.

Introduction

Alongside age determination, sex determination is an important element within the forensic and bioarchaeological analysis of human archaeological skeletal remains (Tekeli *et al.*, 2020; Navitainuck *et al.*, 2021). According to The Cambrige Dictionary of Human Biology and Evolution by Mai *et al*. (2005), sex represents "biological category based upon reproductive attributes and roles in sexually reproducing species". Traditional anthropological and odontological approach of sex determination is based on sexual dimorphism, which refers to differences in metric and morphological properties of skeletal remains and teeth between males and females (Banerjee *et al*., 2016; Horbaly *et al*., 2019). In general, differences between males and females reflect in size and shape of bones, where male bones are larger and

more robust in comparison with smaller and gracile female bones. The most commonly used bones with traits reliable for sex estimation are postcranial and pelvic bones (Spradley, 2016). A study conducted by Navitainuck *et al*. (2021) showed that morphological traits of the cranium and pelvis are at an advantage compared with metric traits of pelvis and scapula, due to their higher utility in the purpose of sex estimation. The other research conducted by Inskip *et al*. (2018), focused on *os coxae* and skull traits, revealed their high sex estimate accuracy, with an accent on *os coxae* or pelvic traits. In comparison with non-metric, odontometric approach is more accurate and based on the differences of tooth size and proportions, where the linear measurements like buccolingual (BL) and mesiodistal (MD) dimensions of teeth crowns are the most commonly used for sex determination (Joseph *et al*., 2013). If the skeletons are damaged or incomplete, which is common in archaeological skeletal remains, sex assessment by morphological analysis is limited or impossible. In that case, metric methods are considered more appropriate for assessing sex. However, sex assessment equations should be used only when the sample is known to come from the same population from which the functions were derived since the expression of sexual dimorphism is population specific (Chovalopoulou et al., 2018). Therefore, formulae derived from one population group will not be applicable for another group due to differences in sexual size dimorphism (Spradley, 2016). Thus, analysis of genetic markers is a powerful solution for sex assessment when anthropological or odontological analysis is not efficient, especially in cases of non-adult or juvenile skeletal remains, due to the fact that sexual dimorphism development depends on the age of individual (Vaňharová & Drozdová, 2008). Two sex specific genetic markers commonly used for sex determination are amelogenin and SRY (sex determining region Y). Amelogenin exists on both the X and Y chromosome and provides information for the synthesis of amelogenin, which is an essential protein for normal tooth development, while SRY gene occurs on the Y chromosome and codes for transcription factors crucial for development of male specific characteristics (Primorac *et al*., 2014; Stewart *et al*., 2017). An aggravating factor for successful extraction and analysis of aDNA (ancient DNA) from archaeological or recent bones and dental material can be degradation of DNA conditioned by low amount of starting DNA and the presence of polymerase chain reaction (PCR) inhibitors in the soil and environmental factors like soil type, soil pH, temperature, humidity and the presence of microorganisms (Jakubowska *et al*., 2012; Higgins & Austin, 2013). Laboratory procedures, equipment and experience can contribute to the successfulness of DNA analysis (Siriboonpiputtana *et al*., 2018). Furthermore, soil pH and type, bone type and size as well as age and sex of individuals can affect survival of bone (Manifold, 2012). Also, biological, chemical and physical agents can lead to morphological changes of bone material (White, 2005). The aim of this research was to determine the sex of 17 skeletons excavated from medieval cementery Metaljica (Hadzici municipality, Bosnia and Herzegovina) applying anthropological, odontological and genetic analyses

Materials and methods

Samples

In 2019, archaeological excavations within multidisciplinary project “Genetic characteristics of inhabitants of Medieval Bosnia” (No. of project 11/05-14-27684/19) revealed large medieval cemetery at the Metaljica locality near Tarcin (Hadzici municipality, Bosnia and Herzegovina). In 28 excavated graves, 21 human skeletal remains were found, among which 17 skeletons were well preserved for anthropological, odontological and genetic analysis.

Anthropological analysis

Before primary anthropological analysis was carried out, human skeletal remains had been carefully washed in order to preserve skeletal remains (Fig. 1, Fig. 2, Fig. 3). After the process of washing, osteological material was left on paper towels to dry. Drying lasted about seven days. During the entire process of examination (washing, drying, primary anthropological analysis and storage) regulations on temperature and moisture were strictly followed. Temperature ranged from 18 to 21°C, while the humidity was about 50% and not more than 70% (Gob & Drouguet, 2007). Dried osteological material was laid out on the examination table in

Figure 1: Nuchal crest of individual from grave 5

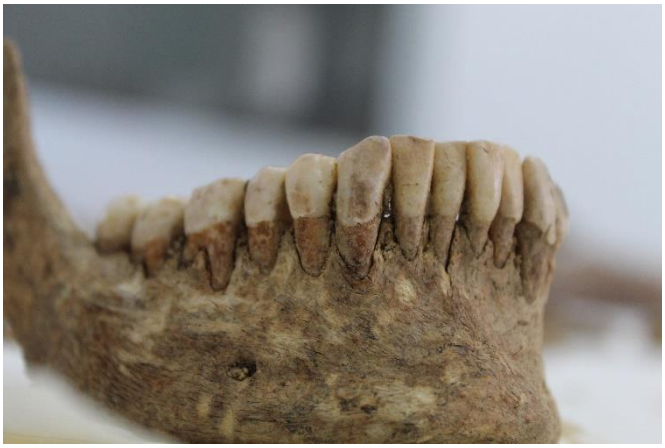

Figure 2: Mandible of individual from grave 18

Figure 3: Fragmented sciatic notch of right innominate

anatomical position in order to build biological profile for each individual. It is necessary to point out that full biological profile was built for none of individuals, due to high fragmentation of human skeletal remains. During anthropological analysis, anthroposcopy method was applied for sex determination. On the other hand, metric methods for sex estimation could not be applied since such formulae have not been even derived for medieval Bosnian population. These formulae are going to be produced when there will be a large enough (and otherwise representative) sample of medieval Bosnian population available for study. Since sexual dimorphism is mostly manifested on pelvis and cranium, these two regions of skeleton were particularly the subject of analysis. Analysis was conducted following already established standards for sex estimation (Buikstra & Ubelaker, 1994). Degree of preservation for the most of the samples was in the range of 10 to 20%, with the exceptions of the grave 10 (1%) and the grave 17 (9%). The highest degree of preservation of skeletal remains was determined in the grave 26 (40%) and the grave 2 (55%). In the case of the samples from graves 4, 8 and 16, fragmented skeletal material or insufficient amount of skeletal material for anthropological analysis was collected.

Odontological analysis

All samples were previously washed, cleaned from remnants of dirt and photographed. For some samples parts of the jaws were present with several teeth (Fig. 4), while in other samples only teeth were recovered (Fig. 5). Condition of material was also different, from well preserved to poorly preserved. Out of 17 individuals recovered, in 4 samples no teeth and/or no jaws were recovered, so there were classified as "Unidentified" by odontological analysis. Out of remaining 13 individuals, two were children with mixed dentition. Even though deciduous teeth were measured as well, for this analysis only measurements of

Figure 4: Well preserved sample with two parts of maxilla and teeth

Figure 5: Sample where only teeth were recovered

permanent teeth were included. Number of teeth and type of teeth was different in different samples. Total number of permanent teeth analyzed was 50. Teeth measurements were done with veneer caliper, performed by single investigator. Crown measurements included mesiodistal (MD) and buccolingual (BL) diameter as recommended by other researchers (Vodanovic, 2007). Additionally, mesiodistal crown width at cervical level was measured as well. Cervicoocclusal diameter was also noted, but excluded in all cases with severe abrasion to avoid false results. Length of the root and length of the whole tooth were also measured. In samples where parts of jaws were present, additional measurements were taken, such as height of mandible at level of foramen mentale, intercanine distance, etc. During odontological analysis, the results of anthropological analysis were unknown to the researcher. Sex estimation was done by comparison to the average tooth dimensions of contemporary population (Konjhodzic Rascic, 1978).

Genetic analysis

Teeth samples were used for DNA analysis. Prior to analysis samples were soaked in 5% w/v Na-hypochlorite for 10 minutes, then rinsed three times with distilled water and soaked in absolute ethanol (Sigma Aldrich, USA) for five minutes. Washed teeth samples were transferred to a clean paper towel and dried for five days. After drying, all samples were irradiated by UV light for three minutes and grounded to a powder using sterilized IKA Tube mill (IKA®-Werke GmbH&Co.KG, Germany). Approximately one gram of powder of each sample was placed in sterile 50 mL polypropylene tube. DNA extraction was performed in a laboratory hood dedicated to ancient DNA work, according to an optimized phenol-chloroform-isoamyl alcohol DNA extraction protocol, preceding decalcification with 0.5 M EDTA (Sigma Aldrich, USA) solution during seven days. In order to detect possible contamination during extraction process, negative extraction control was included. Laboratory hood, work surfaces and laboratory equipment were cleaned with Na-hypochlorite and 70% ethanol, and irradiated by UV light. DNA extracts were purified with DNA-free water using Amicon Ultra 0.5 mL centrifugal filter units (Merck, Millipore, Carrigtwohill, Co.Cork, IRL), transferred into 1.5 mL Eppendorf tubes and stored at -80°C. DNA amplification was performed using Investigator® 24plex QS Kit (Qiagen, Hilden, Germany) which includes 23 autosomal STR loci (amelogenin, TH01, D3S1358, vWA, D21S11, TPOX, DYS391, D1S1656, D12S391, SE33, D10S1248, D22S1045, D19S433, D8S1179, D2S1338, D2S441, D18S51, FGA, D16S539, CSF1PO, D13S317, D5S818, D7S820) in GeneAmp™ PCR System 9700 (Applied Biosystems, USA). To check for possible contamination, for all PCR analyses was used PCR negative control. Products of amplification were detected and separated by capillary electrophoresis using 3500 Genetic Analyzer (Applied Biosystems, USA). Data were collected using 3500 Series Data Collection Software and analysed by GeneMarker® HID Software (Soft Genetics, USA). To determine sex, amelogenin and DYS391 loci were taken into consideration.

Results and Discussion

Seventeen out of 28 skeletal remains excavated from medieval cemetery Metaljica near Tarcin (Hadzici municipality, Bosnia and Herzegovina) were included in this research and investigated by three different methods of determining sex (Table 1). None of individuals had all anthropological indicators of sexual dimorphism preserved. For two individuals from graves 6 and 27 sex was not estimated by using anthropological analysis, since the individuals were subadults. There are no standards for sex diagnosing in juvenile/subadult materials considered acceptable by most osteologists (Buikstra & Ubelaker, 1994). For two

Table 1. Results of sex assessment by anthropological, odontological and genetic analysis conducted on skeletal remains from medieval necropolis Metaljica

Sample	Anthropological sex determination	Level of confidence (1-4)[a]	Odonatological sex determination	Level of confidence (1-4)[b]	Genetic sex determination		Level of confidence (1-4)[c]
					Amelogenin	DYS391	
Grave 1	Male	2	Male	2	Unidentified		1
Grave 2	Female	2	Female	2	X Y	Y	2
Grave 3	Unidentified	1	Male	3	X Y	Y	4
Grave 4	-*	-	Unidentified	1	X Y	Y	3
Grave 5	Male	2	Female	2	X Y	Y	4
Grave 6	Unidentified	1	Male	2	X Y	-	3
Grave 7	Male	2	Female	3	X	-	3
Grave 8	-*	-	Unidentified	1	X Y	Y	3
Grave 11	Female	2	Female	2	X Y	Y	3
Grave 15	Male	2	Male	3	Unidentified		1
Grave 16	-*	-	Unidentified	1	X Y	Y	4
Grave 17	Female	2	Female	3	X	Y	3
Grave 18	Male	2	Male	4	X Y	Y	4
Grave 19	Female	3	Male	4	X Y	Y	4
Grave 23	Unidentified	1	Unidentified	1	X	-	3
Grave 26	Unidentified	1	Male	3	Unidentified		1
Grave 27	Unidentified	1	Male	2	X	-	3

*Fragmented skeletal material or insufficient amount of skeletal material with expressed sexual dimorphism for anthropological analysis

[a]Scale of confidence on the anthropological sex determination: 1- it was not possible to determine sex, 2- sex determination is based on insufficient number of indicators of sexual dimorphism, 3- sex determination is based on more than half indicators of sexual dimorphism, 4- there were present all anthropological indicators of sexual dimorphism

[b]Scale of confidence on the odontological sex determination: 1- it was not possible to determine the sex, 2- sex determination is based on 1-3 teeth, 3- sex determination is based on 4-8 teeth present, 4- there were more than 8 teeth present and/or additional anthropometric analysis of jaws was possible

[c]Scale of confidence on the genetic sex determination: 1- it was not possible to generate DNA profile, 2- partial DNA profile with less than half STR loci amplified, 3- partial DNA profile with more than half STR loci amplified, 4- full DNA profile

individuals (graves 3 and 26) sex could not be determined at all, since indicators for sexual dimorphism have not been preserved. Six individuals had only one indicator of sexual dimorphism while individual from grave 19 had the highest number of preserved indicators of sexual dimorphism: nuchal crest, supra-orbital margin, glabella, mental eminence and sciatic notch. The pelvis is not preserved, but only incomplete iliac bones with partially preserved sciatic notches. It is necessary to emphasize that skeletal remains were highly fragmented and that none of skeletal sets had complete pelvic bones, therefore, features such as ventral arc, the subpubic concavity, the ischiopubic ramus ridge and preauricular sulcus (in female skeletons) were absent. Regarding the sex estimation based on odontological analysis, in our research, the odontometrics was used but the estimation itself was done by comparison to teeth dimensions of contemporary population, therefore certain inaccuracy was expected. Most of the recent researches on odontometric sex estimation worldwide are based on teeth of contemporary populations, therefore certain inaccuracy was expected (Yapes, 2019; Viciano, 2020; Kanchan 2021). For samples from graves 4, 8, 16 and 23 odontological analysis could not be performed because no dental material was available. For samples from graves 18 and 19 sex was estimated with high level of confidence by using odontological

analysis while for samples 3, 7, 5 and 17 determined sex was confirmed with DNA analysis. In general, archaeological samples are too small to represent the base for population odontometric standards. Other researches recommend usage of other dental characteristics (Kazazi, 2018), non-metric dental traits, and/or dental arch dimensions, jaw dimensions, dental indices of specific teeth (Żądzińska, 1999; Gupta, 2016; Kanchan, 2021), which might be impossible to perform in fragmented and poorly preserved archaeological samples. Severe abrasion, usually seen in teeth of archaeological origin, limits the possibility to use all teeth dimensions. Other, non-metric dental traits can be used to establish differences between populations, therefore if noted can help in answering the question of geographic origin of the samples (Vankatesh, 2019; Zukic, 2020).

For samples from graves 1, 15 and 26, genetic analysis could not be done since no markers at amelogenin or DYS391 locus were amplified probably due to the small quantities and degraded DNA molecules present in archeological samples (Dzehverovic et al., 2020). For one sample (grave 6) X and Y alleles were amplified, without amplification at DYS391 locus, while for sample from grave 17 X allele was amplified with observed amplification at DYS391 locus. For those two samples, male sex was established, despite "partial" amplification. Failure to amplify DNA at both loci as well as absence of "complete" amplification (XY at amelogenin and amplified DYS391) in both cases can be explained by the fact that genetic analysis of skeletal remains faces with significant issues such as low amount and quality of aDNA (ancient DNA), presence of contamination and PCR inhibitors as well as small amount of starting material (Quincey *et al.*, 2013). Discrepancy in sex determination between three methods applied in this research can be attributed to highly fragmented skeletal remains, interpopulation differences and allele drop-out (Eliášová and Kubálek, 2009; Bauer, 2013). Also, the fact that juvenile/subadult skeletons do not have developed secondary characteristics relevant for metric and morphological analysis explains non-concordance between results of these analyses and genetic analysis. It is important to have in mind that each of used methods have limitations which are mainly related to sample preservation which is very small in case of archeological samples. It is also necessary to have in mind that incorrect sex identifications can be made because of variation among populations. Some populations are composed of larger and more robust individuals, both sexes, while some populations are much smaller and gracile. Because of such interpopulation differences, sex assessment could be mistaken. Furthermore, all of the morphological techniques used in sexing skeletal remains depend on the preservation of sexually dimorphic elements. All of them share a nontrivial error rate, even for adult remains. However, if aDNA can be recovered from osseous remains, the sex of any individual (regardless of individual age) can be determined with high precision (White & Folkens, 2005). On the other hand, results of DNA analysis should be interpreted very carefully when it comes to aDNA since small amounts of DNA can give inconclusive results due to the presence of different amplification artifacts (allele drop out, null allele, increased stutter peak, etc.) (Harder *et al.*, 2012; Butler, 2015). Therefore, for sex determination for ancient samples it should be imperative to conduct as many available analyses as possible.

Conclusion

Through an evaluation of anthropological, odontological and genetic data, our research demonstrates that combining these three methods contributes to the accurate sex determination of archaeological skeletal remains. Also, in situations involving poorly preserved or highly fragmented skeletal remains as well as juvenile or subadult skeletal remains without expressed sexually dimorphic characteristics relevant for anthropological and odontological analyses, genetic analysis is efficient solution for sex determination of archaeological skeletal samples.

Acknowledgements

This study was part of project "Genetic characteristics of inhabitants of Medieval Bosnia" (No. of project 11/05-14-27684-1/19) supported by Ministry of Education, Science and Youth of Canton Sarajevo.

References

Banerjee, A., Kamath, V. V., Satelur, K., Rajkumar, K., & Sundaram, L. (2016). Sexual dimorphism in tooth morphometrics: An evaluation of the parameters. *Journal of forensic dental sciences*, *8*(1), 22–27. doi:10.4103/0975-1475.176946.

Bauer, C.M., Niederstätter, H., McGlynn, G., Stadler, H., & Parson, W. (2013). Comparison of morphological and molecular genetic sex-typing on mediaeval human skeletal remains. *Forensic science international:Genetics*, *7*(6), 581–586. doi: 10.1016/j.fsigen.2013.05.005.

Buikstra, E.J., & Ubelaker, H.D. (1994). Standards for Data Collection from Human Skeletal Remains Proceedings of a Seminar at the Field Museum of Natural History, *Arkansas Archaeological Survey Research Series* No. 44, Fayetteville, Arkansas: Arkansas Archeological Survey.

Butler, J.M. (2015). Forensic DNA Typing: Interpretation. Massachusetts: Academic Press.

Chovalopoulou, M.E., Bertsatos, A., Zoi, I., Manolis, S.K. & Valakos, E. (2017). Reliability testing of metric methods for sex determination in ancient skeletal remains in Greece. *Mediterranean Archaeology and Archaeometry*, *18*(1), 37-47. doi: 10.5281/zenodo.1069518.

Dzehverovic, M., Cakar, J., Bujak, E., Pilav, A., Ramic, J., Kalajdzic, A., & Pojskic, N. (2021). DNA analysis of skeletal remains of an important historical figure from the period of mediaeval Bosnia. *International Journal of Osteoarchaeology*, *31*(5), 857-865. doi:10.1002/oa.3002

Eliášová, I., & Kubálek, P. (2009). Comparison of genetic and anthropological analyses in sex determination of skeletal remains from Knìževes (Czech Republic). *Anthropologie (Brno)*, 47(1-2), 69-72.

Eliášová, I., Mazura, I., & Smejtek, L. (2010). DNA analysis of ancient skeletal remains. *Folia biologica*, *56*(2), 47-50.

Gob, A., & Drouguet N. (2003). *La muséologie: Histoire, développements, enjeux actuels*. Paris: Armand Colin. Croatian edition: Gob, A., & Drouguet N. (2007). *Muzeologija: Povijest, razvitak, izazovi današnjice* (trans: Horn, J.). Zagreb: Izdanja Antibarbarus.

Gupta, B., & Gupta, M. (2016). Sex identification in forensic odontology-a review of various methodology. *International Journal of Forensic Identification*, *1*(1), 9-13. doi: 10.4103/2542-5013.185693.

Harder, M., Renneberg, R., Meyer, P., Krause-Kyora, B., & von Wurmb-Schwark, N. (2012). STR-typing of ancient skeletal remains: which multiplex-PCR kit is the best? *Croatian Medical Journal*, *53*(5), 416-422. doi: 10.3325/cmj.2012.53.416.

Higgins, D., & Austin, J.J. (2013). Teeth as a source of DNA for forensic identification of human remains: A Review. *Science & Justice*, *53*(4), 433–441. doi: 10.1016/j.scijus.2013.06.001.

Horbaly, H.E., Kenyhercz, M.W., Hubbe, M., & Steadman, D.W. (2019). The Influence of Body Size on the Expression of Sexually Dimorphic Morphological Traits. *Journal of Forensic Sciences*, *64*(1), 52-57. doi: 10.1111/1556-4029.13850.

Inskip, S., Scheib, C. L., Wohns, A. W., Ge, X., Kivisild, T., & Robb, J. (2019). Evaluating macroscopic sex estimation methods using genetically sexed archaeological material: The medieval skeletal collection from St John's Divinity School, Cambridge. *American journal of physical anthropology*, *168*(2), 340–351. doi: 10.1002/ajpa.23753.

Jakubowska, J., Maciejewska, A., & Pawłowski, R. (2012). Comparison of three methods of DNA extraction from human bones with different degrees of degradation. *International journal of legal medicine*, *126*(1), 173–178. doi:10.1007/s00414-011-0590-5.

Joseph, A.P., Harish, R.K., Mohammed, P.K., & Vinod Kumar, R.B. (2013). How reliable is sex differentiation from teeth measurements. *Oral and Maxillofacial Pathology Journal*, *4*(1), 289-292.

Kanchan, T., Chugh, V., Chugh, A., Setia, P., Shedge, R., & Krishan, K. (2021). Estimation of Sex from Dental Arch Dimensions: An Odontometric Analysis. *Journal of Craniofacial Surgery*, *32*(8), 2713-2715. doi: 10.1097/SCS.0000000000007787.

Kazzazi, S.M., & Kranioti, E.F. (2018). Sex estimation using cervical dental measurements in an archaeological population from Iran. *Aarchaeological and Anthropological Sciences*, *10*, 439–448. doi: 10.1007/s12520-016-0363-7.

Konjhodzic Rascic, H. (1978). Prosjecne anatomske mjere zuba jugoslovenske populacije i postojanje seksualnih razlika u velicini zuba (dissertation). Sarajevo: University of Sarajevo.

Mai, L.L., Owl, M.Y., & Kersting, M.P. (2005). *The Cambridge Dictionary of Human Biology and Evolution*. Cambridge: Cambridge University Press.

Manifold, B.M. (2012). Intrinsic and extrinsic factors involved in the preservation of non-adult skeletal remains in archaeology and forensic science. *Bulletin of the International Association for Paleodontology*, *6*(2), 51-69.

Navitainuck, D.U., Vach, W., Alt, K.W., & Schibler, J. (2021). Best practice for osteological sexing in forensics and bioarchaeology: The utility of combining metric and morphological traits from different anatomical regions. *International Journal of Osteoarchaeology*. doi:10.1002/oa.3014.

Primorac, D., Schanfield, M.S., & Marjanovic, D. (2014). Basic Genetics and Human Genetic Variation. In D. Primorac & M.S. Schanfield (Eds.), *Forensic DNA Application- an interdisciplinary perspective* (pp. 3-53). Boca Raton, Florida, USA: CRC Press.

Quincey, D., Carle, G., Alunni, V., & Quatrehomme, G. (2013). Difficulties of sex determination from forensic bone degraded DNA: A comparison of three methods. *Science and Justice*, *53*(3), 253-260. doi: 10.1016/j.scijus.2013.04.003.

Siriboonpiputtana, T., Rinthachai, T., Shotivaranon, J., Peonim, V., & Rerkamnuaychoke, B. (2018). Forensic genetic analysis of bone remains samples. *Forensic science international*, *284*, 167–175. doi: 10.1016/j.forsciint.2017.12.045.

Spradley, K.M. (2016). Metric Methods for the Biological Profile in Forensic Anthropology: Sex, Ancestry and, Stature. *Academic Forensic Pathology*, *6*(3), 391-399. doi: 10.23907/2016.040.

Stewart, N.A., Gerlach, R.F., Gowland, R.L., Gron, K.J., & Montgomery, J. (2017). Sex determination using peptides from tooth enamel. *Proceedings of the National Academy of Sciences*, *114* (52), 13649-13654. doi: 10.1073/pnas.1714926115.

Tekeli, E., Gültekin, T., Doksanalti, E.M., Öztaner, H.S., & Cüneyt, E. (2020). Accurate sex determination using ancient DNA analysis for human skeletal remains from different historical archeological sites in Turkey. *Mediterranean Archaeology and Archaeometry*, *20*(1), 93-106. doi:10.5281/zenodo.3605672.

Vaňharová, M., & Drozdová, E. (2008). Sex determination of skeletal remains of 4000-year-old children and juveniles from Hoštice 1 za Hanou (Czech Republic) by ancient DNA analysis. *Anthropological Review*, *71*(1), 63-70. doi:10.2478/v10044-008-0011-7.

Venkatesh, D., Sanchitha, V., Smitha, T., Sharma, G., Gaonkar, S., & Hema, K.N. (2019). Frequency and variability of five non metric dental crown traits in the permanent maxillary dentitions of a racially mixed population from Bengaluru, Karnataka. *Journal of Oral and Maxillofacial Pathology*, *23*(3), 458-465. doi: 10.4103/jomfp.JOMFP_144_18.

Viciano, J., López-Lázaro, S., & Alemán, I. (2020). Sex estimation based on deciduous and permanent dentition in a contemporary spanish population. *American Journal of Physical Anthropology*, *152* (1), 31-43. doi: 10.1002/ajpa.22324.

Vodanovic, M., Demo, Z., Njemirovski, V., Keros, J., & Brkic, H. (2007). Odontometrics: a useful method for sex determination in an archaeological skeletal population? *Journal of Archaeological Science*, *34*(6), 905-913. doi: 10.1016/j.jas.2006.09.004.

White, D.T., & Folkens, A.P. (2005). The Human Bone Manual. Amsterdam: Elsevier, Academic Press.

Yepes, V.A., Luna, L.H., & Gómez, J. (2019). Sex estimation using coronal measurements of permanent canines in a contemporary mestizo population from Manizales, Colombia. *Revista Facultad de Odontologìa Universidad de Antioquia*, *30*(2), 202-210. doi: 1017533/udea.rfo.V30n2a7.

Żądzińska, E., Frenzel D, & Malinowski, A. (1999). Odontological Analysis of Contemporary Germans from Hamburg. *Zeitschrift Für Morphologie Und Anthropologie*, *82*(2-3), 225–40.

Zukic, S., & Bujak, E. (2020). Enamel extensions on deciduous teeth-an example on late medieval archaeological sample in Bosnia and Herzegovina. *Bulletin of the International Association for Paleodontology*, *14* (2), 130-135.

IAR Publishing

Journal of Bioanthropology

Volume 2, 2022. p. 45-55

ISSN 2787-8201; UDK 572:902

https://doi.org/10.54062/jb.2.1.3

Unilateral Congenital Aural Atresia from an Ychsma Group Burial at the Site of Pachacamac, Peruvian Central Coast

Lawrence S. Owens[1]*, Anna M. Davies-Barrett[2] & Letisha M. C. Service[3]

[1] University of Winchester, UK

[2] School of Archaeology and Ancient History, University of Leicester, UK

[3] Independent Researcher, Market Harborough, Leicestershire, UK

* Corresponding author: lawrence_owens@yahoo.co.uk

Received October 23rd, 2021

Accepted for publication June 02nd, 2022

Online First July 4th, 2022

Keywords: Developmental anomaly, Aural pathology, Palaeopathology, Andean archaeology, Bioarchaeology.

Abstract

Congenital malformations of the human skeleton are a major area of palaeopathological and bioarchaeological interest, although our understanding of such conditions in the ancient world is hampered by their often extreme rarity, and inconsistent reporting in the field. A cranium from a group burial at the Peruvian site of Pachacamac was found to display almost complete absence of the right external auditory meatus, styloid process, vaginal process and tympanic plate of the right temporal bone. Following a differential diagnosis, it was determined that the skeletal pathology likely represents an instance of congenital aural atresia, a developmental anomaly resulting in the partial or complete aplasia of the external acoustic meatus. This condition is often associated with other congenital abnormalities and syndromes. However, as the postcranial remains of this individual could not be distinguished from other remains in the group burial, no further associated skeletal anomalies could be detected. This is the first instance of congenital aural atresia recovered from a defined funerary context in Peru, complementing other bioarchaeological reports of this rare congenital anomaly. The pathology of the condition is described, and compared with clinical and bioarchaeological data. The potential social implications concerning social attitudes of the Pachacamac population towards this individual are also considered, in reference to both historical and archaeological contexts.

Introduction

Congenital aural atresia has been frequently reported in the clinical record but has rarely been recorded in the archaeological literature. Several examples have been recovered from prehispanic Peruvian contexts (Hrdlička, 1933), with other reported cases in the United Kingdom (Knüsel & Bowman, 1996; Wells, 1962), Czech Republic (Vyhnanek & Kuzelka, 1998), United States (Hodges et al., 1990; Hrdlička, 1933), and more recently in Bulgaria, Canada, and Venezuela (Keenleyside, 2011; Swanston et al., 2011; van Duijvenbode et al., 2015). This article will explore the condition, the social context of archaeological cases, and review a new discovery of congenital aural atresia in an Ychsma group burial at Pachacamac, Peru. It also provides information concerning how the Pachacamac population seem to have viewed this individual, whose condition does not seem to have precluded them from social acceptance insofar as burial context is concerned. This finding from the Ychsma is then contrasted with ethnohistorical information reflecting dissimilar behaviour in the subsequent Inka polity.

Figure 1: Map of Peru with the Location of Pachacamac (Eeckhout, 2021)

Congenital aural atresia

Congenital aural atresia is a birth defect causing malformation or absence of the external auditory canal, often associated with severe microtia (underdevelopment of the external ear) (Schuknecht, 1989; van Duijvenbode et al., 2015). It usually manifests unilaterally (Verma et al., 2016). Congenital aural atresia can be associated with facio-auriculo-vertebral spectrum (Goldenhar Syndrome), Pierre Robin Syndrome, Treacher Collins Syndrome, and diverse chromosomal abnormalities (Lo et al., 2014).

Pathological characteristics

Congenital aural atresia should not be confused with the atresia (narrowing) caused by external auditory exostoses, which is particularly associated with coastal populations from the Palaeolithic until the present day (Villotte and Knusel 2016; Trinkaus and Villotte, 2017). Characteristics of congenital aural atresia include partial or total aplasia of the external auditory canal, a hypoplastic middle ear cavity, tympanic bone deformity (bony atresia plate), pinna (external ear) malformation, ossicular erosion and malformation, erosion of the tegmen tympani, anterior location of the descending facial nerve canal, bony atresia, as well as abnormalities of the jaw and alterations to levels of mastoid pneumatisation (Tassano et al., 2015; Todd, 1994; Trojanowska et al., 2012; Verma et al., 2016). In practical terms, congenital aural atresia causes partial or complete deafness on the affected side; modern day interventions may include surgery, electronic implants, and/or auricular reconstruction (Lo et al., 2014).

Modern prevalence and distribution

Congenital aural atresia prevalence is estimated to be between 1:10,000 and 1:20,000 individuals (Karmody and Annino 1995; Lo et al., 2014). It is twice as common in males as females (Kelley & Scholes, 2007), affects the right ear more than the left (De la Cruz & Teufert, 2003), and is most common in Hispanic, Native American, and Western Asian populations (Ali et al., 2017). Prevalence of microtia/anotia (congenital absence of the ear) follows a similar pattern (Harris et al., 1996).

Archaeological Examples

Congenital aural atresia has been identified among archaeological human remains since the 1930s (Hrdlička, 1933). Of the cases summarised in Table 1, 55.6% are Amerindian, 38.8% are European and 5.5% are Western Asian; 81.2% affected the right side, and 75% of affected individuals were female. It is unlikely that these figures reflect ancient realities, as reporting has been inconsistent. Pachacamac has previously yielded several examples of congenital aural atresia (Hrdlička, 1933, p. 356), although these were surface finds without any archaeological context. This makes it impossible to map the frequency of the trait over time or to discuss the possible cultural attitudes towards this very visible disability. The current example is the first case of congenital aural atresia to be discovered in a culturally-specific context not only at Pachacamac, but the Andean area as a whole.

Table 1 Archaeological Cases of Congenital Aural Atresia.

Site	Country	Affiliation	Sex	Age	Side	Ref
Apollonia Pontica	Bulgaria	Classical	F	Adult	R	(Keenleyside, 2011)
Mladá Boleslav	Czech Republic	Middle Ages (13th C)	F	Adult	?	(Vyhnanek and Kuzelka, 1998)
Mladá Boleslav	Czech Republic	Middle Ages (13th C)	F	Adult	?	(Vyhnanek and Kuzelka, 1998)
Chapel Ossuary, Rain	Germany	Modern (1400-1800)	?	Subadult	L	(Panzer et al., 2008)
St Jacob's Chapel	Slovakia	Modern (AD 1500-1800)	?F	MA	R	(Masnicová and Benuš, 2001)
Caister-on-Sea	UK	Anglo-Saxon	F	YA	R	(Wells, 1962)
St Brides Church	UK	Modern (19th C)	M	MA	L	(Knüsel and Bowman, 1996)
Lachish	Israel	Iron Age	?	Adult	R	(Lukaszek et al., 2010)
St Vitals Church	Canada	Modern (19th C)	M	YA	R	(Swanston et al., 2011)
Chicama	Peru	Prehispanic	M	MA	R	(Hrdlička, 1933, p. 360)
Chicama	Peru	Prehispanic	F	YA/MA	R	(Hrdlička, 1933, p. 361)
Pachacamac	Peru	Prehispanic	?F	Subadult	R	(Hrdlička, 1933, p. 361)
Pachacamac	Peru	Prehispanic	F	YA/MA	R	(Hrdlička, 1933, p. 361)
Pachacamac	Peru	Prehispanic	F	YA	R	(Hrdlička, 1933, p. 361)
Pachacamac	Peru	Prehispanic	?M	MA	R	Current Paper
Arkansas	USA	-	F	YA	R	(Hrdlička, 1933, p. 361)
Carlsbad, NM	USA	-	F	YA	R	(Hrdlička, 1933, p. 361)
Jackson County	USA	Late Woodland	F	YA/MA	L	(Hodges et al., 1990)
Los Tamarindos	Venezuela	Prehispanic	M	Adult	R	(van Duijvenbode et al., 2015)

Materials and methods

Pachacamac (Figure 2) is a large multi-period, multi-polity monumental religious and burial site located 30km south of Lima (Figure 1). Founded in the 3rd century AD, Pachacamac rose to prominence as a habitation and ritual site under the Lima group, burgeoning to become the capital of the subsequent Ychsma polity (c. 1000-1476) during the Late Intermediate Period (LIP). It was subsequently conquered by the Inka (Late Period), becoming a prominent pilgrimage and religious site until its eventual conquest by European invaders in 1533 (Eeckhout, 2013; Eeckhout & Owens, 2008; Owens & Eeckhout, 2015).

The site is known for the Sacred Precinct, and its series of highly distinctive dynastic buildings ('pyramids with ramps') that are particularly associated with the Ychsma polity. These designs continued into the Late Period occupation, along with more typically Inka constructions (including a Temple of the Sun, and an Acllahuasi [Temple of the Chosen Women]) associated with the site's important function as a religious focus for the Andean region. Considerably less is known about the residential areas of the site, most previous research having focused upon the monumental architecture of the administrative centre, and the Sacred Precinct.

The site also had a funerary function, with an estimated 80,000 interments spanning all periods of the site's occupation. Ychsma interments are noted for their variety, but range from single pit burials to group/communal tomb interments containing up to 130 individuals. Tomb architecture includes wood and reed supports, holding up a plant fibre roof. Bodies were usually buried wrapped in successive layers of textile and plant fibre, especially reeds. Group burials often follow a 'core and periphery' pattern, focused on older male individuals who are usually associated with the widest range of grave goods (Owens & Eeckhout, 2015).

Individual 2018 B15 (16/193) consists of a single cranium, recovered from a collective tomb containing the remains of over 30 individuals. The tomb was situated within the Sacred Precinct, adjacent to a small temple that – unusually – was decorated with anthropomorphic and geometric designs. The tomb context was looted in antiquity and the human remains commingled, although the remains of prestige textiles,

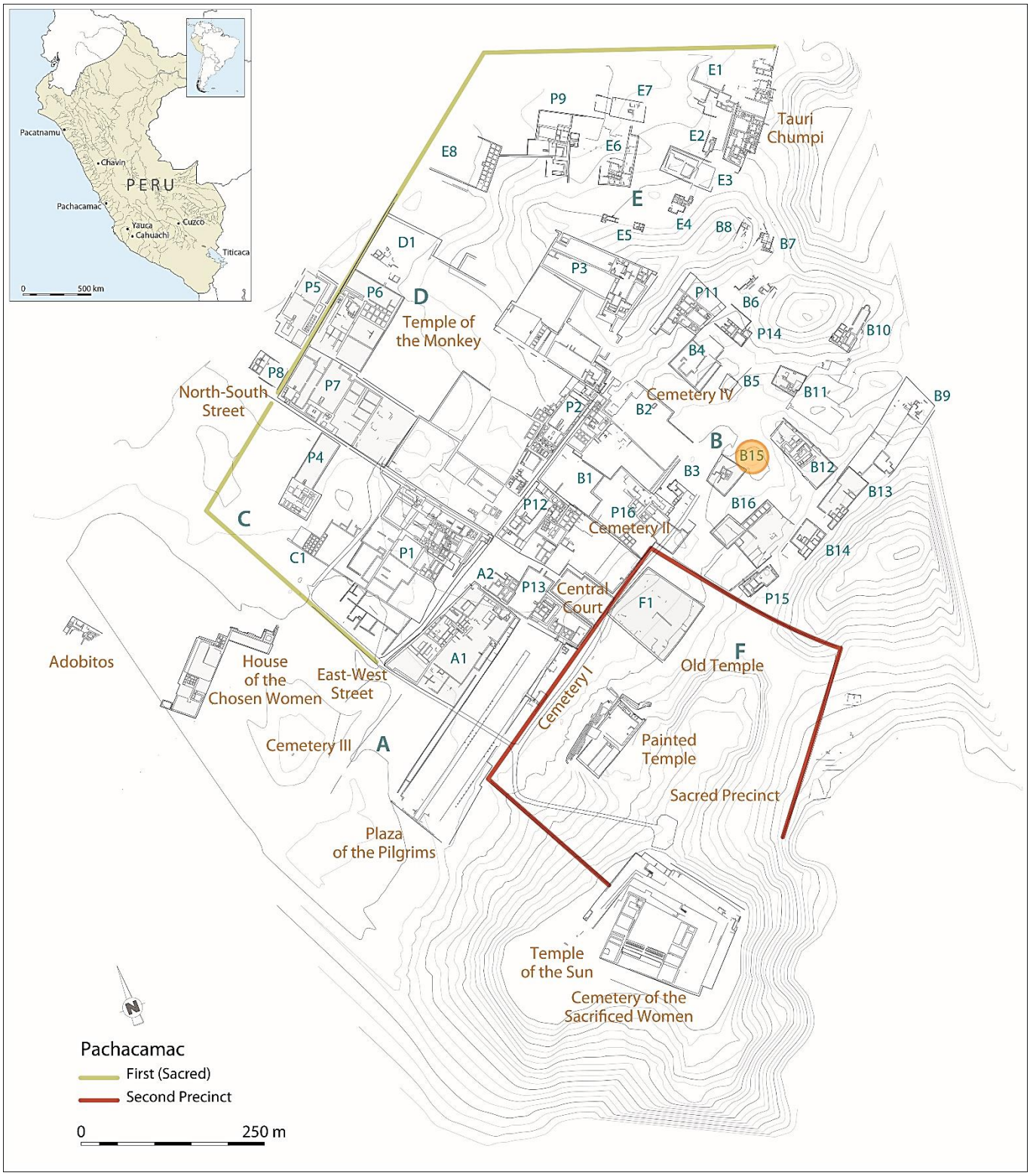

Figure 2: Site map of Pachacamac with the location of B15 (16/193) (Eeckhout, 2021)

Figure 3: Lateral view of the right temporal bone of B15 (16/193). (Owens, 2019)

feather garments, ceramics, and other grave goods were recovered. Ceramic analysis indicates a date in the Early Ychsma, around AD 1100-1200 (Eeckhout, Pers. Comm.). The cranium was not associated with a mandible, nor any postcranial remains. It was in good overall condition with no signs of breakage or extensive weathering.

The individual was analysed according to conventional methods for osteological sex estimation, consisting of observation of the glabella, supraorbital ridges, supraorbital margin, suprameatal crest, mastoid process, and nuchal crest (Acsádi & Nemeskéri, 1970; Bass, 2005; Buikstra & Ubelaker, 1994; Ferembach et al., 1980; Krogman & Iscan, 1986). Age estimation was necessarily restricted to dental wear and sutural closure (Buikstra & Ubelaker, 1994; Lovejoy, 1985), and thus provided only a broad estimation of age. A macroscopic analysis of the cranium was undertaken to examine oral and joint diseases, as well as any other pathological changes or developmental anomalies present.

Results and discussion

The individual was judged to be a probable male, based upon supraorbital anatomy, nuchal development and mastoid process morphology. Ageing was more tentative, given the absence of pelvic remains; tooth wear and sutur

al obliteration patterns suggested the individual was a middle/older adult (35-50+ years). In the right temporal region, the external auditory meatus was almost entirely absent, along with the styloid process of the temporal bone, the vaginal process and the tympanic plate (Figure 3, 4, 5). No abnormal irregular proliferative or destructive bone remodelling processes were observed in this region of the cranium, with the affected area consisting of smooth, uninterrupted cortical bone. The temporal region of the left side of the cranium appeared normal. Right side occipital condylar degeneration/lipping, septal deviation, bilateral cribra orbitalia, and slight asymmetry of the nasal aperture and orbits were also observed, although these are unlikely to be associated with the congenital aural

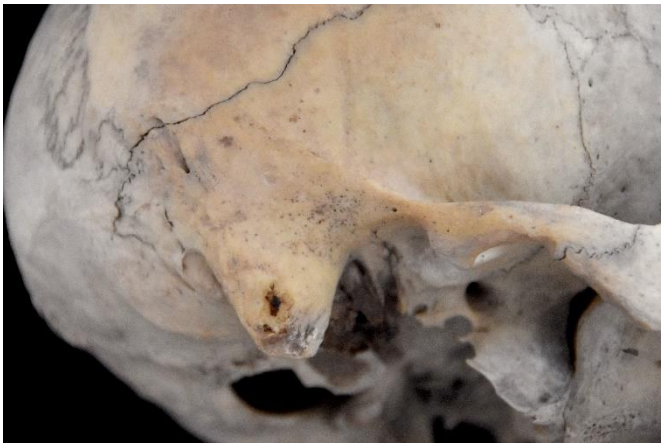

Figure 4: Right temporal region of B15 (16/193). (Owens, 2019)

atresia. Antemortem maxillary tooth loss was extensive, the molars being absent and the alveolar bone remodelled/remodelling. A partially healed dental abscess (left UM2) and a supernumerary tooth (adjacent to the right UI1) were also noted.

Differential diagnosis

A careful consideration of the exact appearance and distribution within the skeleton of skeletal abnormalities or changes is required to accurately determine which pathological processes may have been responsible, and to rule out others (Klaus, 2017). A differential diagnosis was undertaken using the suggested modified nomenclature of the Istanbul Protocol, as proposed by Appleby and colleagues (2015). The right temporal bone of Individual 2018 B15 (16/193) exhibited almost complete absence of the external auditory meatus, tympanic region, vaginal process, and styloid process Differential diagnosis included pseudopathology, stenosis (narrowing) of the external auditory meatus (due to external auditory exostosis), acquired aural atresia (due to trauma, infection or neoplasm), and congenital aural atresia.

The excellent condition of the cranium indicated that the lesions were not consistent with pseudopathology. Partial stenosis of the external acoustic meatus can be caused by benign osseous growth within the canal, known as external auditory exostoses. However, the formation of exostoses within the auditory canal is not likely to result in complete occlusion of the external acoustic meatus and normally occurs bilaterally, and

Figure 5: Inferior view of the cranium of B15 (16/193). (Owens, 2019)

was, therefore, not consistent with the observed absence of the tympanic region, vaginal process, and styloid process of the temporal bone (House & Wilkinson, 2008).

The loss of anatomical features on the temporal bone can potentially be brought about by destruction and new bone formation as a result of trauma, infectious disease, or neoplastic disease, although such cases are rare (Bajin et al., 2015). A traumatic lesion causing severe deformity and structural absences without affecting surrounding areas was considered to be inconsistent with the observed pathology, due to the localised nature of the affected area. Similarly, infectious disease was unlikely to have been restricted solely to the auricular area, while the lack of neoplastic growth (in the event of a benign lesion) or perforative

pathology (suggestive of aggressive metastatic neoplastic processes) were entirely absent. The smooth, intact cortical surface and the lack of any associated abnormal irregular bone reaction, either proliferative or destructive, in this region of the cranium was thus not consistent with these pathological processes.

The absence of anatomical features is most likely to have occurred during the development of the temporal bone itself. The complex development and ossification of the ear and surrounding anatomical features of the temporal bone begin within the first few weeks of embryonic development (Cunningham et al., 2017, pp. 78–85). Thus, the condition observed was typical of a congenital disorder developing in utero.

Due to the complex nature of the anatomical structures of the ear, several developmental anomalies can affect this region. These variously affect the external ear (the pinna), the external acoustic meatus, and the middle and inner ears (Bartel-Friedrich & Wulke, 2007). The observed lack of development of the anatomical structures of the right ear is diagnostic of congenital aural atresia, this being the only condition that results in the complete absence of the external auditory canal, as well as tympanic bone deformity (Tassano et al., 2015; Todd, 1994; Trojanowska et al., 2012; Verma et al., 2016). Congenital aural atresia occurs most frequently in males, with a ratio of 2:1, and is three to five times more likely to occur unilaterally, with the right side most frequently affected (Liaw et al., 2017). This is consistent with our observations of individual 2018 B15 (16/193).

Although it can develop in isolation, congenital aural atresia is also often associated with other developmental anomalies and congenital syndromes (Lo et al., 2014). Malformation of the middle ear and auditory ossicles are often affected in instances of congenital aural atresia (Trojanowska et al., 2012). For example, CT scanning of an archaeological individual from pre-Columbian Venezuela with congenital aural atresia of the right ear demonstrated the fusion of the incus and malleus (van Duijvenbode et al., 2015). However, involvement of the middle ear was unobservable in individual 2018 B15 (16/193) due to the lack of access to CT scanning equipment. Disturbance of the normal development and eruption of teeth has been clinically associated with congenital aural atresia (Boone et al., 2011); nothing abnormal was noted in the dentition of this individual, with the exception of the supernumerary tooth mentioned above.

Facio-auriculo-vertebral syndrome (or Goldenhar syndrome) can also affect the normal development of the ears, resulting in congenital aural atresia. This syndrome also affects the development of the maxilla, zygomatic arch, mandible and teeth, ranging from mild facial asymmetry to severe cranio-facial deformation. This can include skeletal changes such as cleft palate, agenesis of teeth, supernumary teeth, cleft spine and other spinal defects, microcephaly, club foot, radial hemimelia, thumb abnormalities, and various abnormalities of the internal organs (Bogusiak et al., 2017, Table. 1).

The single supernumerary tooth and the aural atresia are consistent with facio-auriculo-vertebral syndrome (D'Alessandro et al., 2006; Hoffman et al., 2019; Tasse et al., 2005). However, while this condition could not be ruled out as a cause of the aural atresia, none of the aforementioned clefting or other cranial abnormalities were found in the current individual (Martelli-Júnior et al., 2010). Further, none of the mandibles, radii, foot/ankle bones or first metacarpals recovered from the funerary context displayed any indications of facio-auriculo-vertebral syndrome.

Discussion

Patients with congenital aural atresia present with deafness on the affected side, cognitive and linguistic issues, and problems with directional hearing (Keenleyside, 2011). The physical deformity of the external ear may result in negative social outcomes in childhood (Friedman, 2009; Lipan & Eshraghi, 2011; Stanley, 2014), requiring social, financial, and emotional support networks (Ear Community, 2020). Archaeologically, ancient social attitudes towards affected individuals may be ascertained by considering pathology, demography, burial location, burial style and lifeway contextualisation (Keenleyside, 2011; Nystrom & Tilley, 2018). As it is not a life-threatening condition,

the 'Bioarchaeology of Care' concept (Tilley, 2012; Tilley & Oxenham, 2011) – used to determine the strength of social and caring networks in antiquity – would not seem to apply.

Significant deviations from standard burial practice have been argued to represent special – and often negative – status for the deceased in the eyes of the burying population (Tsaliki, 2008), yet unusual burials can also signal anthropodeic appeals, marks of honour, or gestures of affection as easily as a gesture of denigration or negativity (Gabelmann and Owens, 2020). Establishing the norm for a population and whether specific individuals fall within or outside it may thus be informative as to social mores regarding the perception of 'otherness' in that population (although the possibility of certain individuals being disposed of beyond the reach of archaeologists should also be considered). There are some signs that ancient peoples recognised – and made funeral format choices based upon – aural deformity and/or deafness.

An affected individual from prehispanic Venezuela (van Duijvenbode et al., 2015, p. 18) was interred with a pair of antlers located beside the head, a unique finding in the Venezuela/Caribbean region. Conversely, the Greek colonial case cited by Keenleyside (2011) was contextually unremarkable other than the head being turned so the affected ear was facing downwards. The prone body position of a deaf child at Romano-British Poundbury was shared by others at the site, while the grave was if anything more prestigious than many others (Roberts & Cox, 2003, p. 115-6). A series of deaf individuals buried together at the Amerindian site of Roffelsen was suggested to represent ancestral relationships (Spence et al., 2014), or as recognition of their condition.

There are no contextualised findings of ancient congenital aural atresia in the Andean area. However, contact-period sources provide Andeanists with unusual access to the social mores of the 16th and 17th centuries, although the cultural specificities (i.e. Inka) of these sources should be considered. The limited records dealing with this issue suggest a measure of negativity towards the profoundly deaf. De Molina notes that all whose "…ears were broken, and all deformed persons" were kept away from ceremonial occasions, as the Inka believed that "they were in that state as a punishment for some fault [and] their ill luck might drive away some piece of good fortune" (De Molina, 2011 [1576], p. 21). At the very least, anyone whose ear "…orifice was broken through by any accident, the man to whom it happened was looked upon as unfortunate…" (De Molina, 2011 [1576], p. 46). Yet the profoundly deaf were not exempt from paying tribute, and seem to have been excluded from the Inka system of supporting those with severe disabilities who were unable to work (Vega, 2016, p. 354, 369, 383). We can therefore conclude that ear deformity 'mattered' to the Inka – which accords with the Inka desire for physical perfection in various of their rituals (Cobo, 1990 [1653]) – while not considering it to be sufficiently serious to merit the charity received by the more physically infirm. Ascertaining whether this policy of social exclusion applied to earlier groups is less certain, as the Ychsma – who preceded the Inka at Pachacamac – are beyond the reach of historical record, and in any case the Inka cannot be relied upon to accurately relate the social conventions of their predecessors. The current individual may be informative in this respect.

B15 (16/193) is likely to have been deaf in one ear, which would have been visibly deformed; yet this individual was interred in a prestigious Ychsma tomb in a key part of one of the Andean world's most important sites. Considering that the Inka discriminated against physical deformity both in the living (see above) and the dead (Cobo, 1990 [1653]), this key location in the Sacred Precinct is an unlikely burial spot for someone who would have been excluded from it in life. We would therefore suggest that the Ychsma did not follow these same precepts, and that the fact that they were buried in a tomb of notable (if not elite) importance suggests social acceptance and – by extension – that physical deformities did not determine social (or at least funeral) treatment in the Ychsma of this period.

Conclusion

This paper presents the first reported discovery of congenital aural atresia in Peru for over eighty years. The differential diagnoses precludes traumatic and infectious causes, and has determined a likely

congenital cause for this auricular deformity. Facio-auriculo-vertebral syndrome could not be ruled out, but seems to be unlikely owing to the absence of major cranial clefting, and while the cranium was admixed with other individuals, none of the postcrania recovered showed any signs of other pathology associated with the condition.

This is also the first Andean case of congenital aural atresia ever to be found in a contextualised funerary setting, and this has proven to be significant for examining the social attitudes of the Ychsma towards those with disabilities or deformities. Published sources from elsewhere in the world suggest that those suffering from the condition are often highlighted culturally in archaeological contexts, to either positively or negatively denote their differing status (see above). In the current case, however, the individual did not receive any such marker, and while the precise details of the interment are lacking owing to looting, the nature and location of the tomb and associated grave goods suggests that they were held in considerable regard by the burying population.

We suggest that the Ychsma therefore differ notably from the Inka of the Late Period. Non-normative burials of 'perfect' children and the social exclusion of those with physical deformities suggests a rather different social landscape among the Inka - given that the Ychsma only used non-normative burial to denote those who died in sacrificial ritual (Eeckhout & Owens, 2008), and seem to have privileged relatively elderly individuals – bearing various markers of trauma and physical degeneration – with the best-equipped burials. Children - by comparison - were marginalised and even buried en masse as grave goods with more wealthy (adult) burials (Owens and Eeckhout, 2015).

This is the first time that this approach has been utilised in analysis of the Ychsma polity; the seeming social acceptance of this individual by contemporary populations casts a positive light on how physically disadvantaged people were viewed in the Andean Late Intermediate Period. Future research should focus on refining and testing this hypothesis - using larger datasets and undisturbed contexts to examine both pathology - and social attitudes towards it - in the ancient Andean world.

Acknowledgements

The authors would like to acknowledge Dr Peter Eeckhout (Director of the Ychsma Project) for his ongoing assistance and contributions of images. We would also like to thank the other project team members for their assistance and support. We would like to thank the two anonymous reviewers for their remarkable attention to detail; all remaining errors are our own.

References

Acsádi, G., & Nemeskéri, J. (1970). History of human life span and mortality. Budapest: Akadémiai Kiadó.

Ali, K., Mohan, K., & Liu, Y. C. (2017). Otologic and Audiology Concerns of Microtia Repair. Seminars in Plastic Surgery, 31(3), 127–133. https://doi.org/10.1055/s-0037-1603957

Bartel-Friedrich, S., & Wulke, C. (2007). Classification and diagnosis of ear malformations. GMS Current Topics in Otorhinolaryngology, Head and Neck Surgery, 6, Doc05. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/22073081

Bass, W. M. (2005). Human Osteology: A Laboratory and Field Manual (5th ed.). Springfield: Missouri Archaeological Society. https://doi.org/9780943414966

Bogusiak, K., Puch, A., & Arkuszewski, P. (2017). Goldenhar syndrome: current perspectives. World Journal of Pediatrics, 13(5), 405–415. https://doi.org/10.1007/s12519-017-0048-z

Boone, B. N., Schuman, T. A., & Eavey, R. D. (2011). Congenital Aural Atresia With Ectopic Tooth. Otology & Neurotology, 32(9), e35. https://doi.org/10.1097/MAO.0b013e318210b8cb

Buikstra, J. E., & Ubelaker, D. H. (1994). Standards for data collection from human skeletal remains. In Fayetteville: Arkansas Archeological Survey. Fayetteville: Arkansas Archaeological Survey.

Cobo, B. (1990). Inca Religion and Customs (First; Roland Hamilton, Ed.). Austin: The University of Texas Press.

Cunningham, C., Scheuer, L., & Black, S. (2017). Developmental Juvenile Osteology (2nd ed.). Amsterdam: Academic Press/Elsevier.

D'Alessandro, L., Kovesi, T., Massoud, S., Lougheed, J., Hunter, A., & Reisman, J. (2006). Horseshoe lung and facio-auriculo-vertebral sequence: A previously unreported association. Pediatric Pulmonology, 41(6), 592–596. https://doi.org/10.1002/ppul.20404

De la Cruz, A., & Teufert, K. B. (2003). Congenital aural atresia surgery: Long-term results. Otolaryngology - Head and Neck

Surgery, 129(1), 121–127. https://doi.org/10.1016/S0194-5998(03)00531-X

De Molina, C. (2011). Account of the fables and rites of the Incas (First; B. S. Bauer, V. Smith-Oka, & G. E. Cantarutti, Eds.). Austin: University of Texas Press. https://doi.org/10.5860/CHOICE.49-1046

Ear Community. (2020). Microtia and Atresia. Retrieved April 7, 2020, from https://earcommunity.org/

Eeckhout, P. (2013). Change and permanency on the coast of ancient Peru: the religious site of Pachacamac. World Archaeology, 45(1), 137–160. https://doi.org/10.1080/00438243.2012.759516

Eeckhout, P., & Owens, L. S. (2008). Human Sacrifice at Pachacamac. Latin American Antiquity, 19(4), 375–398. https://doi.org/10.1017/S104566350000434X

Ferembach, D., Schwindezky, I., & Stoukal, M. (1980). Recommendation for Age and Sex Diagnoses of Skeletons. Journal of Human Evolution, (9), 517–549. Retrieved from https://kupdf.net/download/ferembach-et-al-1980recommendations-for-age-and-sex-diagnoses-of-skeletons_599a96e2dc0d60757c53a1ff_pdf

Friedman, E. (2009). U.K. Model's Surprising Secret. Retrieved April 7, 2020, from Abc News website: https://abcnews.go.com/Health/story?id=4002415&page=1

Gabelmann, O. U., & Owens, L. S. (2019). Good, Bad, or Indifferent? In The Odd, the Unusual, and the Strange (pp. 133–151). University of Florida Press. https://doi.org/10.2307/j.ctvx06wvr.14

Harris, J., Kallen, B., & Robert, E. (1996). The epidemiology of anotia and microtia. Journal of Medical Genetics, 33(10), 809–813. https://doi.org/10.1136/jmg.33.10.809

Hodges, D. C., Harker, L. A., & Schermer, S. J. (1990). Atresia of the external acoustic meatus in prehistoric populations. American Journal of Physical Anthropology, 83(1), 77–81. https://doi.org/10.1002/ajpa.1330830109

Hoffman, S., Sadler, L., Totman, T., & Bagne, L. (2019). A Possible case of Facio-Auriculo-Vertebral sequence (FAVs) in an adult female from medieval Iceland (13th–16th Century). International Journal of Paleopathology, 24(September 2018), 41–47. https://doi.org/10.1016/j.ijpp.2018.08.009

House, J. W., & Wilkinson, E. P. (2008). External auditory exostoses: Evaluation and treatment. Otolaryngology–Head and Neck Surgery, 138(5), 672–678. https://doi.org/10.1016/j.otohns.2008.01.023

Hrdlička, A. (1933). Seven prehistoric American skulls with complete absence of external auditory meatus. American Journal of Physical Anthropology, 17(3), 355–377. https://doi.org/10.1002/ajpa.1330170318

Keenleyside, A. (2011). Congenital aural atresia in an adult female from Apollonia Pontica, Bulgaria. International Journal of Paleopathology, 1(1), 63–67. https://doi.org/10.1016/j.ijpp.2011.01.001

Kelley, P. E., & Scholes, M. A. (2007). Microtia and Congenital Aural Atresia. Otolaryngologic Clinics of North America, 40(1), 61–80. https://doi.org/10.1016/j.otc.2006.10.003

Knüsel, C. J., & Bowman, J. E. (1996). A possible case of neurofibromatosis in an archaeological skeleton. International Journal of Osteoarchaeology, 6(2), 202–210. https://doi.org/10.1002/(SICI)1099-1212(199603)6:2<202::AID-OA263>3.0.CO;2-N

Krogman, W. M., & Iscan, M. Y. (1986). Human Skeleton in Forensic Medicine. Charles C. Thomas Publisher.

Liaw, J., Patel, V. A., & Carr, M. M. (2017). Congenital anomalies of the external ear. Operative Techniques in Otolaryngology-Head and Neck Surgery, 28(2), 72–76. https://doi.org/10.1016/j.otot.2017.03.012

Lipan, M., & Eshraghi, A. (2011). Otologic and Audiology Aspects of Microtia Repair. Seminars in Plastic Surgery, 25(04), 273–278. https://doi.org/10.1055/s-0031-1288919

Lo, J. F. W., Tsang, W. S. S., Yu, J. Y. K., Ho, O. Y. M., Ku, P. K. M., & Tong, M. C. F. (2014). Contemporary Hearing Rehabilitation Options in Patients with Aural Atresia. BioMed Research International, 2014, 1–8. https://doi.org/10.1155/2014/761579

Lovejoy, C. O. (1985). Dental wear in the Libben population: Its functional pattern and role in the determination of adult skeletal age at death. American Journal of Physical Anthropology, 68(1), 47–56. https://doi.org/10.1002/ajpa.1330680105

Martelli-Júnior, H., Miranda, R. T. de, Fernandes, C. M., Bonan, P. R. F., Paranaíba, L. M. R., Graner, E., & Coletta, R. D. (2010). Goldenhar syndrome: clinical features with orofacial emphasis. Journal of Applied Oral Science, 18(6), 646–649. https://doi.org/10.1590/S1678-77572010000600019

Nystrom, K. C., & Tilley, L. (2018). Mummy studies and the bioarchaeology of care. International Journal of Paleopathology, 25(June), 64–71. https://doi.org/10.1016/j.ijpp.2018.06.004

Owens, Lawrence S, & Eeckhout, P. (2015). To the God of Death, Disease, and Healing. Social Bioarchaeology of Cemetery I at Pachacamac. In L. S Owens & P. Eeckhout (Eds.), Funerary Practices and Models in the Ancient Andes : the Return of the Living Dead (1st ed., pp. 158–185). New York: Cambridge University Press. https://doi.org/https://doi.org/10.1017/CBO9781107444928

Roberts, C., & Cox, M. (2003). Health and Disease in Britain: From Prehistory to the Present Day (1st ed.). Sutton Publishing.

Schuknecht, H. F. (1989). Congenital Aural Atresia. The Laryngoscope, 99(9), 908–917. https://doi.org/10.1288/00005537-198909000-00004

Spence, M. W., Williams, L. J., & Wheeler, S. M. (2014). Death and Disability in a Younge Phase Community. American Antiquity, 79(1), 108–127. https://doi.org/10.7183/0002-7316.79.1.108

Stanley, P. (2014). Face the Music: A Life Exposed. HarperOne.

Swanston, T., Carter, Y., Hopkins, C., Walker, E. G., & Cooper, D. M. L. (2011). Developmental Fusion of the Malleus and Incus in a Late 19 th -Century Case of Aural Atresia. International Journal of Osteoarchaeology, 23(5), n/a-n/a. https://doi.org/10.1002/oa.1286

Tassano, E., Jagannathan, V., Drögemüller, C., Leoni, M., Hytönen, M. K., Severino, M., … Gimelli, G. (2015). Congenital aural atresia associated with agenesis of internal carotid artery in a girl with a FOXI3 deletion. American Journal of Medical Genetics Part A, 167(3), 537–544. https://doi.org/10.1002/ajmg.a.36895

Tasse, C., Böhringer, S., Fischer, S., Lüdecke, H.-J., Albrecht, B., Horn, D., … Wieczorek, D. (2005). Oculo-auriculo-vertebral spectrum (OAVS): clinical evaluation and severity scoring of 53 patients and proposal for a new classification. European Journal of Medical Genetics, 48(4), 397–411. https://doi.org/10.1016/j.ejmg.2005.04.015

Tilley, L. (2012). Tilley, L. 2012 The bioarchaeology of care. The Archaeological Record, 12.

Tilley, L., & Oxenham, M. F. (2011). Survival against the odds: Modeling the social implications of care provision to seriously disabled individuals. International Journal of Paleopathology, 1(1), 35–42. https://doi.org/10.1016/j.ijpp.2011.02.003

Todd, N. W. (1994). Mastoid pneumatization in patients with unilateral aural atresia. European Archives of Oto-Rhino-Laryngology, 251(4), 196–198. https://doi.org/10.1007/BF00628422

Trinkaus, E., & Villotte, S. (2017). External auditory exostoses and hearing loss in the Shanidar 1 Neandertal. PLOS ONE, 12(10), e0186684. https://doi.org/10.1371/journal.pone.0186684

Trojanowska, A., Drop, A., Trojanowski, P., Rosińska-Bogusiewicz, K., Klatka, J., & Bobek-Billewicz, B. (2012). External and middle ear diseases: radiological diagnosis based on clinical signs and symptoms. Insights into Imaging, 3(1), 33–48. https://doi.org/10.1007/s13244-011-0126-z

Tsaliki, A. (2008). Unusual Burials and Necrophobia: An Insight into the Burial Archaeology of Fear. In E. M. Murphy (Ed.), Deviant Burial in the Archaeological Record (pp. 1–16). Oxford: Oxbow.

van Duijvenbode, A., Herschensohn, O. J., & Morgan, M. E. (2015). A severe case of congenital aural atresia in pre-Columbian Venezuela. International Journal of Paleopathology, 9, 15–19. https://doi.org/10.1016/j.ijpp.2014.11.002

Vega, G. de la. (2016). Comentarios Reales de los Incas 1609 (1st ed.). Lima: Universidad Inca Garcilaso de la Vega.

Verma, R., Jana, M., Bhalla, A. S., Kumar, A., & Kumar, R. (2016). Diagnosis of osteopetrosis in bilateral congenital aural atresia: Turning point in treatment strategy. World Journal of Clinical Pediatrics, 5(2), 228. https://doi.org/10.5409/wjcp.v5.i2.228

Vyhnanek, L., & Kuzelka, V. (1998). Atresia of the External Auditory Meatus: Two New Cases from Medieval Bone Materials. 1998 12th European PPA Meeting Abstracts August 26-29 Prague and Pilsen, Czech Republic, 23. Detroit: Paleopathology Association. Retrieved from https://paleopathology-association.wildapricot.org/resources/Documents/European PPA Programs/1998 12th European PPA Meeting Abstracts August 26-29 Prague and Pilsen, Czech Republic.pdf

Wells, C. (1962). Three Cases of Aural Pathology of Anglo-Saxon Date. The Journal of Laryngology & Otology, 76(11), 931–933. https://doi.org/10.1017/S002221510006014X

IAR Publishing

Journal of Bioanthropology

Volume 2, 2022. p. 56-61

ISSN 2787-8201; UDK 572
https://doi.org/10.54062/jb.2.1.1

Challenges in obtaining high-quality data from a custom-made panel for the next generation sequencing (NGS) using Ion Torrent GeneStudio™ S5 platform

Lana Salihefendić[1,2], Adna Ašić[2,*], Ivana Čeko[1], Dino Pećar[1], Larisa Bešić[2], Naida Mulahuseinović[1], Amra Džuho[2], Laura-Severina Köhn[2], Rijad Konjhodžić[1]

[1]Alea Genetic Center, Bosnia and Herzegovina

[2]Department of Genetics and Bioengineering, Faculty of Engineering and Natural Sciences, International Burch University, Bosnia and Herzegovina

* Corresponding author: Email: adna.asic@ibu.edu.ba

Received November 4th, 2021
Accepted for publication February 24th, 2022
Online First February 28th, 2022

Keywords: COVID-19, next-generation sequencing, Ion Torrent GeneStudio™ S5, immunological response.

Abstract

The goal of this part of the study was to optimize the sequencing procedure for 16 human genes and their regulatory regions that might be associated with differential immunological response to COVID-19. The study was performed on 60 COVID-19 patients from the General Hospital of Tešanj, Bosnia and Herzegovina, categorized into three groups of mild, moderate, and severe clinical manifestation, based on the diagnosis by the residential physician. Target coding sequences and their regulatory regions were amplified for the following genes: *HLA-A, HLA-B, HLA-C, ACE2, IL-6, IL-4, TMPRSS2, IFITM3, IL-12, RIG-I/DDX58, IRF-7, IRF-9, IL-1B, IL-1A, CD55,* and *TNF-α*. DNA was isolated from the whole blood samples stored at -20°C for six months using QIAamp® DNA Mini Kit according to manufacturer's instructions. Since NGS analysis of target genomic regions was performed on the Ion Torrent GeneStudio™ S5 platforms, libraries were prepared using Ion AmpliSeq™ Library Kit Plus according to manufacturer's instructions in a protocol optimized for low-quality DNA. Due to dissatisfactory sequencing results, further protocol optimization steps were employed through separating two primer pools, increasing the number of PCR cycles, and decreasing the annealing temperature for the primer pool which showed poorer amplification results. In the end, 36 samples produced optimal results, while the remaining 24 samples will be re-sequenced following repeated sample collection and DNA isolation, accompanied by additional protocol modifications.

Introduction

Considering the global impact of COVID-19 in terms of its consequences on healthcare, economy and social norms of behavior, multidisciplinary studies of issues related to this pandemic have been the focus of the scientific research for the past two years.

Coronaviruses belong to the subfamily *Coronavirinae,* a member of *Coronaviridae* family (Luo et al, 2020). The genome of SARS-CoV-2 is an RNA-based genome that is 29,881 bp in size, encoding 9,860 amino acids (Chen et al, 2020). Symptoms and clinical manifestation of COVID-19 show high variability. These manifestations include pneumonia with variable severity, acute respiratory distress syndrome (ARDS), as well as a significant fraction of asymptomatic carriers. Most of the patient's experience fever, cough, dyspnea, myalgia, and fatigue. A poorer outcome and prognosis are associated with older age, pre-existing chronic conditions (diabetes, cardiovascular and respiratory diseases) and male sex (Alahmad et al, 2020).

Next-generation sequencing (NGS) or multiparallel sequencing enables obtaining sequence information for

the entire genomes. There is a number of different NGS platforms which can perform parallel sequencing of millions of small DNA fragments. Each of these fragments is sequenced multiple times, thus providing high sequencing depth with the goal of delivering accurate data and providing information on unexpected and/or novel gene variants. There are various potential uses of NGS in clinical practice, including capturing a broader spectrum of mutations than Sanger sequencing, high sensitivity in detection of mosaic mutations, and detection of rare mutations or mutations of low frequency (for example, in tumor tissues and liquid biopsy) (Behjati & Tarpey, 2013).

We hereby report technical issues encountered while sequencing a panel of 16 genes of interest and their regulatory regions for 60 COVID-19 patients from the General Hospital of Tešanj, Bosnia and Herzegovina. This research was performed using semi-conductor sequencer GeneStudio™ S5 (Ion Torrent, Thermo Fisher Scientific, Waltham, MA).

Materials and methods

Whole blood samples were obtained from 60 COVID-19-positive patients examined at the General Hospital of Tešanj, Bosnia and Herzegovina. All collected samples were classified into three groups

TABLE 1. Classification of the study participants into three clinical manifestation groups (mild, moderate, and severe) based on observed symptoms (table taken and adapted from Baj et al, 2020).

Clinical manifestations	Imaging findings	Other	Classification (Baj et al, 2020)	Classification (current study)
Absence of typical and atypical clinical manifestations	No changes in CT and X-ray scans	Positive tests for SARS-CoV-2	Asymptomatic	Mild
Typical: fever, dry cough, sore throat, runny nose, sneezing, fatigue, myalgia, tiredness, muscle ache, headache, loss of smell Atypical: nausea, vomiting, diarrhea, abdominal pain	Usually, no changes in CT and X-ray scans	Absence of any manifestations of pneumonia	Mild	
Presence of symptoms of pneumonia, fever (usually persistent and higher than 37.8°C) with a dry cough	Usually, the presence of ground-glass opacities and lung consolidation	Crackles or wheezing during pulmonary auscultation	Moderate	Moderate
Dyspnea, hypoxia, and >50% lung involvement, diarrhea, vomiting, nausea	Ground glass opacities, lung consolidation, pleural effusions, lymphadenopathy	Continually worsening clinical outcome of patients	Severe	Severe
Severe difficulty of breathing and shortness of breath, chest pain, movement impairments, loss of speech	Ground glass opacities (usually bilateral), lung consolidation, pulmonary nodules	Continually worsening clinical outcome of patients Most prevalent complications: acute respiratory distress syndrome or respiratory failure, myocardial injury, arrhythmia or heart failure, acute kidney injury, acute liver injury, encephalopathy, disseminated intravascular coagulation, rhabdomyolysis, septic shock, multiple organ dysfunctions	Critical	

according to the clinical manifestation of COVID-19, namely mild, moderate, and severe clinical manifestation (including five deceased patients). Patient classification into the study groups was performed by residential physicians based on the previously published guidelines (Baj et al, 2020; Table 1). Prior to sample collection, ethical clearance to conduct this research was obtained from the Ethics Committee of the Faculty of Engineering and Natural Sciences, International Burch University (Sarajevo, Bosnia and Herzegovina), as well as from the Joint Ethics Committee of the General Hospital of Tešanj (Tešanj, Bosnia and Herzegovina). All participants signed an informed consent form of voluntary participation in the research.

Samples were collected in November 2020, stored at -20°C and delivered to the laboratory for analysis six months later. Following delivery, samples were immediately de-frosted, and DNA was extracted using QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Isolated DNA was quantified using Qubit® 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA), according to manufacturer's instructions, with quantification results ranging from 5 ng/µl to 40 ng/µl. Samples with concentration lower than 10 ng/µl were re-extracted and higher concentrations obtained.

NGS panel was custom-made for 16 genes, including *HLA-A, HLA-B, HLA-C, ACE2, IL-6, IL-4, TMPRSS2, IFITM3, IL-12, RIG-I/DDX58, IRF-7, IRF-9, IL-1B, IL-1A, CD55* and *TNF-α.* Genes of interest were chosen based on their candidate status for modulating patients' response to SARS-CoV-2 infection, either due to their protein products being the members of the host immune system, or encoding host-cell receptors for viral entry into the cell. In addition, previous research on candidate genes for variable response to SARS-CoV or SARS-CoV-2 infection was consulted (Asselta et al, 2020; Chapman & Hill, 2012; de Lang et al, 2006; Lingeswaran et al, 2020; Lipworth et al, 2020; Trowsdale & Knight, 2013). *In silico* coverage was 98% at 100x sequencing depth. Total amplicon number was 185, with one primer pool with 92 and the second pool with 93 amplicons (Table 2).

TABLE 2. The number of primer pairs used for sequencing 16 target genes and their regulatory regions..

Gene	Number of primer pairs (amplicons)
HLA-A	11
HLA-B	10
HLA-C	11
ACE2	22
IL-6	7
IL-4	5
TMPRSS2	19
IFITM3	3
IL-12	7
RIG-I/DDX58	23
IRF-7	15
IRF-9	10
IL-1B	10
IL-1A	8
CD55	17
TNF-α	7
Total	185

According to DNA concentrations, libraries were prepared using Ion AmpliSeq™ Library Kit Plus (Thermo Fisher Scientific). Ion Torrent GeneStudio™ S5 (Thermo Fisher Scientific) was used as a sequencing platform, and it requires a minimum 1 ng of DNA. Sixteen libraries were prepared from the mild clinical manifestation group and protocol was immediately adjusted due to the fact that the blood samples used for DNA isolation were not used immediately after collection. The protocol was adjusted for low-quality DNA, meaning that three cycles were added in the amplification PCR and initial DNA input in the reaction was 20 ng. Following amplification, partial digestion of amplicons was achieved using exonucleases and two primer pools were pooled into one. Following the incubation step, barcodes were ligated on the samples and prepared libraries were purified using Agencourt AMPure XP reagent (Beckman Coulter, Brea, CA) and 70% freshly prepared ethanol. Purified libraries were quantified using Ion Library TaqMan™ Quantitation Kit (Thermo Fisher Scientific) on real-time PCR. Library quantification values were all higher than 100 pM and were diluted to 80 pM, according to manufacturer's instructions.

Results and Discussion

Sequencing results for the first group of 16 samples were satisfactory and no further optimization of the protocol presented above was planned at that point. However, when the second set of 16 samples was sequenced, the conflicting results were obtained. While some of the samples showed high coverage of 100x 100%, others showed 1x 50% coverage, even though all libraries had the same starting concentration. Namely, library quantification analysis gave the results ranging from 100 pM to 250 pM. All libraries were therefore diluted to the final concentration of 100 pM to achieve uniform library concentration in the final pool. Since the primer panel was custom-made, subsequent optimization steps, aimed at improving the coverage, included another library preparation step with protocol modification in which two primer pools were prepared separately for each sample in order to test and optimize the primers. After applying the same protocol, library quantification showed large difference between the library concentrations in two primer pools, which partially explained conflicting coverage on NGS (Table 3). Further optimization of the protocol was made by adding two more PCR cycles to the primer pool 2 and, finally, by lowering the annealing temperature by 2°C for the same pool. Obtained results were better, but still did not reach the desired quality of the output data.

Library preparation of the remaining 28 samples was later initiated to test the quality of samples and whether introduced protocol modifications were satisfactory. Library quantifications were still conflicting, so that out of a total of 60 samples, 36 gave optimal coverage results, while the analyses for the remaining 24 should be repeated.

As presented above, several experimental issues have been encountered while optimizing the NGS protocol for the target gene sequencing in a set of COVID-19 patients. Firstly, whole blood samples were collected from patients and kept frozen at -20°C for six months. While fresh samples are producing better results, it is not unusual to produce high-quality DNA isolated from samples that have been stored for several years (Tagliaferro et al, 2021). Also, QIAamp® DNA Mini Kit that was used in the present study is best valued for its time-effectiveness, lower possibility of sample contamination and ease of use (Chacon-Cortes & Griffiths, 2014). However, previous research has shown that for older samples, other DNA extraction methods, such as modified salting-out method, should be used (Mardan-Nik et al, 2019). Other studies have shown

TABLE 3. Library concentrations for two primer pools in the second set of 16 samples.

Sample no.	DNA concentration (ng/µl)	Library concentration, pool 1 (pM)	Library concentration, pool 2 (pM)
17	14	741	488
18	34	1069	4
19	16	191	1
20	22	100	0
21	15.7	68	9
22	6	1091	138
23	13.9	119	46
24	12	566	0
25	11.5	150	22
26	18	312	45
27	21.9	1102	69
28	9.2	870	15
29	43.6	1035	95
30	17.8	603	100
31	37.4	720	55
32	15.7	102	2

that silica-based DNA isolation using commercial kits, such as QIAamp® DNA Blood Midi Kit (Qiagen) (Mardan-Nik et al, 2019) and DNeasy® Blood & Tissue Kit (Qiagen) (Tagliaferro et al, 2021) are both capable of producing high-quality DNA extracts suitable for subsequent PCR amplification.

Furthermore, the custom-made primer panel that was produced for the purpose of this research has proved itself to be the major experimental obstacle in producing satisfactory data following NGS analysis of the samples. Inconsistent data produced for two primer pools, as well as conflicting results for different samples despite comparable starting DNA concentrations point out to this conclusion. Furthermore, the primers were designed *in silico* for the research-use-only (RUO) application and with the goal of primer panel validation. In order to improve the primer pool performance, protocol modifications were included according to the pre-established practice of increasing the number of PCR cycles to achieve target region amplification. Although the multiparallel sequencing platform was used and 16 genes and their regulatory regions are being analyzed at once, sequencing fragments of around 200 bp in length were still used in this research to achieve better sequence coverage and, therefore, higher result accuracy. Since the sequencing issues were encountered following these modifications, it is suggested that the primer pairs for different genes might differ in their annealing efficiency, as well as that excessive primer-dimer formation was encountered due to sequence complementarity.

Previous studies dealing with NGS data accuracy, sensitivity, specificity, and precision have highlighted the importance of using proper bioinformatics pipeline for the variant calling with the purpose of producing optimum results (Shin et al, 2017), no matter which NGS platform is used as a method of choice with an aim of replacing the Sanger sequencing method (Sandmann et al, 2017). In addition, the importance of proper library preparation is often emphasized as a crucial step in any successful NGS analysis (Forth & Hoeper, 2019), including adding the proper amount of DNA and conversion of that DNA sample into a functional library that can be successfully sequenced. Previously published detailed technical notes emphasize the importance of several critical steps in generation of high-quality NGS data, such as obtaining adequate amount and quality of DNA sample to be sequenced, decontamination of pre-amplification area, primer design, as well as gel test to check for the amplification of expected PCR products, amplicon purification and potential primer dimer formation (Wohlhieter et al, 2021).

Conclusion

Since it is generally accepted that the time elapsed from sample collection to sample analysis does not significantly influence the quality of isolated DNA, and based on the DNA quantification results, we can conclude that the sample quality was not a significant limitation in sequencing challenges encountered during this research. It is reasonable to assume that the custom-made primer panel failed to perform as expected in the NGS protocol. Following the sequencing protocol optimization and consulting literature sources explaining optimization of other custom-made sequencing panels, it was clear that accurate results cannot be obtained for 24 samples using hereby established protocol.

When it comes to future steps aimed at solving the issue of unsatisfactory library quantification results, the first option is to replace those samples with freshly collected whole blood samples and repeat the analyses. Furthermore, if this approach does not contribute towards solving the issue, primer pool separation will be employed in order to obtain satisfactory library quantification data for the amplicons contained in primer pool 1. Finally, missing amplicons will be generated using TruSight One Sequencing Panel (Illumina, San Diego, CA) for the clinical exome sequencing of more than 4,800 genes associated with human disease, including the genes of interest in the present research.

Acknowledgements

This research is a part of the project titled “Personalized approach to COVID-19 infection through analysis of molecular genetic predisposition of the patients for a differential immune response”, that is co-financed by

the Ministry of Science, Education, and Youth of the Sarajevo Canton (decision no. 11/05-34-12880-8/20).

The authors have no conflicts of interest to disclose.

References

Alahmad, B., Al-Shammari, A. A., Bennakhi, A., Al-Mulla, F. & Ali, H. Fasting Blood Glucose and COVID-19 Severity: Nonlinearity Matters. (2020). *Diabetes Care, 43*(12), 3113-3116.

Asselta, R., Paraboschi, E. M., Mantovani, A., & Duga, S. (2020). *ACE2* and *TMPRSS2* variants and expression as candidates to sex and country differences in COVID-19 severity in Italy. *Aging*, *12*(11), 10087–10098. doi:https://doi.org/10.18632/aging.103415.

Baj, J., Karakuła-Juchnowicz, H., Teresiński, G., Buszewicz, G., Ciesielka, M., Sitarz, E., Forma, A., Karakuła, K., Flieger, W., Portincasa, P., & Maciejewski, R. (2020). COVID-19: Specific and Non-Specific Clinical Manifestations and Symptoms: The Current State of Knowledge. *Journal of Clinical Medicine*, *9*(6), 1753. doi:https://doi.org/10.3390/jcm9061753.

Behjati, S., & Tarpey, P. S. (2013). What is Next Generation Sequencing? *Archives of Disease in Childhood: Education and Practice Edition, 98*(6), 236-238. doi:10.1136/archdischild-2013-304340.

Chacon-Cortes, D. F., & Griffiths, L. (2014). Methods for extracting genomic DNA from whole blood samples: current perspectives. *Journal of Biorepository Science for Applied Medicine*, *2014*(2), 1-9. doi:https://doi.org/10.2147/BSAM.S46573.

Chan J. F., Yuan S., Kok K. H., To K. K., Chu H., Yang J., Xing, F., Liu, J., Yip, C. C.Y., Poon, R. W. S., Tsoi, H. W., Lo, S. K. F., Chan, K. H., Poon, V. K. M., Chan, W. M., Ip, J. D., Cai, J. P., Cheng, V. C. C., Chen, H., Hui, C. K. M. & Yuen, K. Y. (2020). A familial cluster of pneumonia associated with the 2019 novel coronavirus indicating person-to-person transmission: a study of a family cluster. *The Lancet*, *395,* 514–523. doi:https://doi.org/10.1016/S0140-6736(20)30154-9.

Chapman, S. J., & Hill, A. V. (2012). Human genetic susceptibility to infectious disease. *Nature Reviews: Genetics*, *13*(3), 175–188. doi:https://doi.org/10.1038/nrg3114.

de Lang, A., Osterhaus, A. D., & Haagmans, B. L. (2006). Interferon-gamma and interleukin-4 downregulate expression of the SARS coronavirus receptor ACE2 in Vero E6 cells. *Virology*, *353*(2), 474–481. doi:https://doi.org/10.1016/j.virol.2006.06.011.

Forth, L. F., & Höper, D. (2019). Highly efficient library preparation for Ion Torrent sequencing using Y-adapters. *BioTechniques*, *67*(5), 229–237. doi:https://doi.org/10.2144/btn-2019-0035.

Lingeswaran, M., Goyal, T., Ghosh, R., Suri, S., Mitra, P., Misra, S., & Sharma, P. (2020). Inflammation, Immunity and Immunogenetics in COVID-19: A Narrative Review. *Indian Journal of Clinical Biochemistry: IJCB*, *35*(3), 260–273. doi:https://doi.org/10.1007/s12291-020-00897-3.

Lipworth, B., Chan, R., & Kuo, C. R. (2020). Predicting Severe Outcomes in COVID-19. *The Journal of Allergy and Clinical Immunology in Practice*, *8*(8), 2582–2584. doi:https://doi.org/10.1016/j.jaip.2020.06.039.

Luo, H., Tang, Q. L., Shang, Y. X., Liang, S. B., Yang, M., Robinson, N. & Liu, J. P. (2020). Can Chinese Medicine Be Used for Prevention of Corona Virus Disease 2019 (COVID-19)? A Review of Historical Classics, Research Evidence and Current Prevention Programs. *Chinese Journal of Integrative Medicine, 26*(4), 243–250. doi:10.1007/s11655-020-3192-6.

Mardan-Nik, M., Saffar Soflaei, S., Biabangard-Zak, A., Asghari, M., Saljoughian, S., Tajbakhsh, A., Meshkat, Z., Ferns, G.A., Pasdar, A., & Ghayour-Mobarhan, M. (2019). A method for improving the efficiency of DNA extraction from clotted blood samples. *Journal of Clinical Laboratory Analysis*, *33*(6), e22892. doi:https://doi.org/10.1002/jcla.22892.

Sandmann, S., de Graaf, A. O., van der Reijden, B. A., Jansen, J. H., & Dugas, M. (2017). GLM-based optimization of NGS data analysis: A case study of Roche 454, Ion Torrent PGM and Illumina NextSeq sequencing data. *PloS one*, *12*(2), e0171983. doi:https://doi.org/10.1371/journal.pone.0171983.

Shin, S., Kim, Y., Chul Oh, S., Yu, N., Lee, S. T., Rak Choi, J., & Lee, K. A. (2017). Validation and optimization of the Ion Torrent S5 XL sequencer and Oncomine workflow for BRCA1 and BRCA2 genetic testing. *Oncotarget*, *8*(21), 34858–34866. doi:https://doi.org/10.18632/oncotarget.16799.

Tagliaferro, S. S., Zejnelagic, A., Farrugia, R., & Wettinger, S. B. (2021). Comparison of DNA extraction methods for samples from old blood collections. *BioTechniques*, *70*(5), 243-250. doi:https://doi.org/10.2144/btn-2020-0113.

Trowsdale, J., & Knight, J. C. (2013). Major histocompatibility complex genomics and human disease. *Annual Review of Genomics and Human Genetics*, *14*, 301–323. doi:https://doi.org/10.1146/annurev-genom-091212-153455.

Wohlhieter, C. A., Uddin, F., Quintanal-Villalonga, À., Poirier, J. T., Sen, T., & Rudin, C. M. (2021). An optimized NGS sample preparation protocol for in vitro CRISPR screens. *STAR Protocols*, *2*(2), 100390.