ROMANIAN ACADEMY
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- Summary of PhD Thesis -

Malaria diagnosis in human archaeological samples: A biological analysis of (bio)archaeological evidence

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CONTENTS

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1. CURRENT STATE OF SCIENTIFIC LITERATURE

1.1. PALEOPATHOLOGY

The first definition of palaeopathology, given in 1913 by Sir Marc Armand Ruffer, remains relevant to this day: **"the science of diseases whose existence can be demonstrated on the basis of human and animal remains".** (quoted by Dutour, 2013). Palaeopathology developed as a subfield of bioarchaeology and initially consisted of describing anomalies by observing skeletal remains. Over time, with the progress and subsequent availability of scientific methods, palaeopathology studies have methodologically diversified and allowed for a truly multidisciplinary approach involving archaeology, biological anthropology, biochemistry, imaging and genetics.

For a pathological condition to be diagnosed in palaeopathology it must produce changes affecting bone and/or teeth (Waldron, 2009). Sometimes soft tissues may be preserved following natural (in a dry or anaerobic environment) or artificial mummification (Fernández, 2012). However, the majority of human remains studied in bioarchaeology are completely skeletonized due to their age.

Human remains provide direct evidence of the lifestyles and experiences of the people themselves. A great deal of information about the daily existence of people and communities (which would otherwise be inaccessible, not being documented in written records) can be discovered when their skeletons are analysed. Once human remains are discovered in archaeological excavations, their subsequent condition is influenced (positively or negatively) by the manner in which they are handled (excavated, transported and stored, analysed). This raises certain ethical issues/considerations: all human remains should be treated with dignity and respect, but because of their importance for knowledge of the past they should also be available for research. In view of these aspects, it is desirable to have a balance between invasive procedures of analysis and the concern for the optimal preservation of human remains. This is an important issue to be mindful of in all research on human remains and is a topic that continues to be debated (see Larsen and Walker, 2004; Licata and Monza, 2017; Lambert and Walker, 2019; Buikstra et al., 2022).

Ancient DNA analysis has become an essential component of bioarchaeological studies as it enables a variety of topics related to the historical and evolutionary past to be addressed. The first ancient DNA sequences were obtained more than 30 years ago, and since then the research techniques have been constantly developing, both methodologically and theoretically. For instance, *Mycobacterium tuberculosis* (Salo et al., 1994), *Mycobacterium leprae* (Rafi et

al., 1994), *Yersinia pestis* (Drancourt et al., 1998; Raoult et al., 2000), *Plasmodium falciparum* (Taylor et al., 1997) and *Treponema pallidum* (Kolman et al., 1999) infections have been confirmed using ancient DNA. These studies have paved the way for a more rigorous diagnosis in palaeopathology, even in the case of imprecise skeletal markers.

Ancient proteins can be identified by immunological methods. These rely on the detection of a reaction between an antibody and a specific antigen to reveal the presence or absence of the target antigen. Immunological methods used for protein detection in paleopathology were summarized by Tran et al. (2011). In intact tissues, antigens can be visualized by immunohistochemistry, and proteins extracted from various sources are most commonly analysed by immunochromatography or ELISA (Enzyme-Linked Immunosorbent Assay).

Other molecules, such as lipids, are found in smaller quantities in bones, and seldom the subject of bioarchaeological studies. A noteworthy exception is the identification of *Mycobacterium* spp. on the basis of specific cell wall lipids (mycolic acids) (Mark et al., 2010; Tran et al., 2011; Donoghue, 2016; Spigelman and Rubini, 2016; Buzic and Giuffra, 2020).

1.2. MALARIA

Malaria is an infectious disease caused by parasites of the genus *Plasmodium* and transmitted by mosquitoes of the genus *Anopheles* (Meibalan and Marti, 2017). Four species of *Plasmodium* are specific parasites that cause malaria in humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, and *Plasmodium malariae*. Molecular phylogeny studies have shown that *Plasmodium* parasites evolved over millions of years alongside humans and spread out of Africa with their hosts (Conway et al., 2000; Loy et al., 2017). *Plasmodium* populations have expanded rapidly in the last 6000 years, due to a series of changes in human lifestyle with the emergence of agricultural societies and settlements of sufficient size to allow transmission by mosquitoes (Joy et al., 2003; Hume et al., 2003; Siddle and Quintana-Murci, 2014).

The study of malaria in historical populations and the methods used for diagnosis/identification of malaria in paleopathology have been comprehensively reviewed in literature reviews (Setzer, 2014; Bianucci et al., 2015; Nerlich, 2016; 2018; Boualam et al., 2021; Schats, 2023).

Malaria cannot be identified by macroscopic examination of the human skeleton as it does not lead to specific morphological changes in the bones, unlike other infectious diseases.

The primary mechanism by which malaria may affect the skeleton is haemolytic anaemia caused by the massive and simultaneous destruction of red blood cells (Ghosh and Ghosh, 2007; Sinden, 2016). Skeletal markers associated with acquired and inherited anaemia are used as indirect evidence of the disease (Gowland and Western, 2012).

The most common skeletal lesions associated with anaemia, and therefore considered indicators of malaria, are *cribra orbitalia* (the appearance of porous lesions on the upper wall of the orbital cavity) and *cribra cranii* (porotic hyperostosis on the outer surface of the cranial bones, particularly the parietal bones) (Waldron, 2021; Setzer, 2014). Other lesions associated with the presence of malaria are *cribra femora* (porous lesions on the femoral neck) and *cribra humeralis* (porous lesions on the neck of the humerus) (Smith-Guzmán, 2015).

There are two established approaches to malaria diagnosis in most molecular paleopathology studies. The first approach focuses on the recovery of *Plasmodium*-specific DNA and its analysis by conventional PCR or NGS (Next Generation Sequencing) techniques. The second approach is the immunological identification of malaria, most commonly with rapid diagnostic tests.

The first successful ancient DNA-based malaria diagnostic was made by Taylor et al. in 1997. They were able to identify a single *Plasmodium*-specific DNA sequence from a 60-year-old sample. Recovery and analysis of short specific DNA fragments (using different primers, targeting different genes) continue to be used as a diagnostic method for malaria. Recently, *Plasmodium*-specific sequences have been retrieved and described using High-Throughput Sequencing (HTS) techniques.

Fornaciari et al., 2010a; Fornaciari et al., 2010b used rapid diagnostic tests in the analysis of the skeletal remains from four members of the Medici family in order to confirm the malaria diagnostic from the autopsy reports. In a similar manner proteins from ancient Egyptian bone specimens were analysed using rapid tests (Al-Khafif, 2018). Despite the fact that the application of the immunological tests to degraded ancient protein samples has been found to be susceptible to false positive results, the use of these rapid tests remains a screening method for cases where *Plasmodium* infection is suspected.

An alternative marker that can be used to identify malaria is hemozoin. Hemozoin is produced by *Plasmodium* organisms but also by other hematophagous organisms: *Schistosoma mansoni* (parasitic helminths) and *Rhodnius prolixus* (hematophagous insects) following the digestion of haemoglobin. Hemozoin formation is the main pathway of heme detoxification in these organisms (De Villiers et al., 2012). In the intraerythrocytic phase of its life cycle, *Plasmodium* utilizes between 60-80% of the haemoglobin in erythrocytes (Krugliak et al.,

2002; Sun et al., 2016). Amino acids that result from the breakdown of haemoglobin protein chains are utilized by the parasite. Haemoglobin digestion releases the heme as well. The parasite lacks the ability to excrete free heme and does not possess a heme oxygenase to recover iron and detoxify heme (Olivier et al., 2014). Therefore, detoxification is achieved by bio-crystallization in the form of hemozoin.

The fact that hemozoin is a highly stable product that accumulates in the human body means that it could be a suitable marker for detection in archaeological context. However, very few attempts to detect hemozoin in archaeological human remains have been reported. The presence of hemozoin crystals has been described using MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization - Time Of Flight) mass spectrometry, ATR-FTIR (Attenuated Total Reflectance - Fourier Transform Infrared Spectroscopy) and SEM electron microscopy (Cox, 2018), using a method pioneered by Dr. Jamie Inwood at Yale University (https://news.yale.edu/2015/03/17/creating-malaria-test-ancient-human-remains). To date, these results have not been published in peer-reviewed journals.

1.3. MALARIA IN ROMANIA

Malaria was historically present in most parts of Europe, from the Mediterranean Sea to the southern shore of the Baltic Sea and from the south of the UK to European Russia (Huldén, Huldén and Heliövaara, 2005) before its eradication in the second half of the 20th century.

Malaria was also present in Romania and claimed numerous victims before malaria eradication and control campaigns began. The earliest data on the incidence of malaria in Romania were recorded starting in the late 19th century (Ciucă, 1966 cf. Neghina et al., 2011). Before World War II, approximately 300,000 cases of malaria were recorded annually for a population of approximately 19,000,000, with an accelerated rise during the war, due to a shortage of anti-malarial treatments. The reported species are those occurring in the temperate zone: *P. vivax*, *P. falciparum* and *P. malariae* (Ciuca, 1956). George Zotta's studies on the habitat of *Anopheles* mosquitoes showed that about 30% of Romania's territory, including all of Dobrogea, presented favourable conditions for endemic malaria (Neghina et al., 2011). The Malaria Commission was formed by the Ministry of Health in 1947 to organise the campaign against malaria. As a result of the eradication campaign, from 1962 onwards there were no more indigenous cases of malaria, so the disease was declared eradicated in Romania in 1963.

The chronology of the emergence of malaria in present-day Romania is not known, nor is the prevalence of the species and vectors involved, but it is obvious that historical populations were exposed to the disease. Supplementing historical information with palaeopathological data can clarify, at a local level, the understanding of the history of malaria in Europe. With these considerations in view, this thesis was designed to explore the hypothesis that malaria was present in medieval Dobrogea and that it can be diagnosed based on archaeological evidence. The hypothesis is addressed from a bioarchaeological perspective through the direct analysis of human remains.

2. AIM AND OBJECTIVES OF THE PHD THESIS

The general aim of the thesis is the study of malaria in a bioarchaeological context, by analysing human remains from two archaeological sites in south-eastern Romania (Mireasa and Capidava, Constanța county). Specifically, we assumed that malaria affected the populations present in Dobrogea in the medieval and pre-modern periods. In order to test this hypothesis, we analysed human skeletal material from 38 individuals, following several research objectives:

- Radiocarbon dating of the samples and establishing the period of use of the burial sites.
- Observation of skeletal markers indicative of anaemia.
- Immunological identification of specific *Plasmodium* proteins in the samples.
- Identification of DNA markers specific to the genus *Plasmodium*.
- Detection of *Plasmodium*-specific pigment (hemozoin).

3. MATERIALS AND METHODS

3.1. MATERIALS

The specimens are human skeletal remains from two archaeological sites in S-E Romania (Mireasa, Constanța county, and Capidava, Constanța county). A total of 38 sets of skeletal remains were analysed: 34 from the Mireasa site (33 from the medieval cemetery, one from the Bronze Age tumulus) and 4 from the Capidava site. The Mireasa cemetery was identified during the construction of the Siliștea eolian park, in 2012. 162 graves were identified as a result of archaeological documentation, of which 14 dated to the Bronze Age and 148 estimated to be from the medieval period. Skeletal remains from the burial ground associated with the Capidava fortress were discovered during systematic archaeological excavations carried out between 2010 and 2014 (Sector X extramuros site). They were dated according to burial inventory and burial rituals to the period between the 10th and 11th centuries (Pinter et al., 2011). Furthermore, one burial complex from each presumed time period (Bronze Age and medieval for Mireasa; 10th century for Capidava) was radiocarbon dated using the Beta Analytic, Inc. dating service. Table 3.1. contains a summary of the specimens included in the analyses.

Table 3.1. Summary of tissues included in the analyses

ID code	Site	Age	Anatomical elements
M37	Mireasa	17th century	2 teeth
M40	Mireasa	17th century	2 teeth, 1 femur
M43	Mireasa	17th century	2 teeth, 1 femur
M44	Mireasa	17th century	2 teeth, 1 femur
M47	Mireasa	17th century	4 teeth, 1 femur
M49	Mireasa	17th century	2 teeth
M54	Mireasa	17th century	2 teeth, 1 humerus
M56	Mireasa	17th century	2 teeth
M62	Mireasa	17th century	2 teeth
M65	Mireasa	17th century	2 teeth
M67	Mireasa	1635–1670 cal. AD*	4 teeth
M69	Mireasa	17th century	4 teeth, 1 femur
M71	Mireasa	17th century	2 teeth
M72	Mireasa	17th century	2 teeth, 1 femur
M77	Mireasa	17th century	2 teeth, 1 femur
M88	Mireasa	17th century	2 teeth
M92	Mireasa	17th century	2 teeth, 1 humerus
M95	Mireasa	17th century	2 teeth
M96	Mireasa	17th century	2 teeth
M97	Mireasa	17th century	2 teeth
M100	Mireasa	17th century	2 teeth, 1 femur
M107	Mireasa	17th century	2 teeth
M111	Mireasa	17th century	2 teeth
M112	Mireasa	17th century	2 teeth
M116	Mireasa	17th century	2 teeth
M118	Mireasa	17th century	2 teeth
M126	Mireasa	17th century	2 teeth
M131	Mireasa	17th century	2 teeth
M133	Mireasa	17th century	2 teeth
M134	Mireasa	17th century	2 teeth, 1 femur
M142	Mireasa	17th century	2 teeth
M144	Mireasa	17th century	2 teeth
M152	Mireasa	17th century	2 teeth
Cap M1	Capidava	10th century	4 teeth
Cap M4	Capidava	880–990 cal. AD*	4 teeth
Cap M8	Capidava	10th century	4 teeth
Cap M17	Capidava	10th century	4 teeth

*radiocarbon dating (Beta Analytic Inc.).

3.2. ANCIENT DNA ANALYSIS

Using this method, ancient DNA was purified from all 33 individuals from the 17th-century burial at the Mireasa site (see Table 3.1.). To purify ancient DNA, the powder obtained from dental tissue was used. Dental tissue powder was obtained from dental pulp, accessed through the root end, using a drill attached to an electric micromotor at low speed to avoid degradation of biological material by heating. Purification of ancient DNA was conducted using a protocol for the recovery of very short, degraded fragments of ancient DNA (Dabney et al., 2013).

To test for the presence of *Plasmodium falciparum* DNA in ancient DNA extracts, PCR amplification was performed using primers designed to amplify a partial fragment (196 bp) of the AMA1 (Apical Membrane Antigen 1) gene, as successfully used by Lalremruata et al. (2013). In order to monitor for possible contamination, a PCR control sample (without DNA) was introduced for every 3 samples of ancient DNA amplified.

The presence of PCR products was checked by 2% agarose gel electrophoresis. Bands of the expected size were purified from gel using FavorPrep GEL/ PCR Purification Kit (Favorgen). DNA fragments obtained from agarose gels were inserted into pJet1.2 plasmids using the CloneJet PCR Cloning Kit (Thermo Fisher Scientific). Plasmids were used for KCM transformation (Chung and Miller, 1988) of competent E. coli strain DH5α cells. Plasmids containing AMA1 amplification products were isolated using the Fast-n-Easy Plasmid Mini-Prep Kit (Jena Bioscience). Each PCR product obtained was cloned and sequenced in sets of 6 clones.

The resulting plasmids were sent to Macrogen Europe (https://www.macrogen-europe.com/) for sequencing using the standard primer pJET1.2R. Sequence files were analyzed using BioEdit (Hall, 1999) and compared with the AMA1 reference sequence (Gene ID: 810891, GenBank). Sequences were also aligned with the GenBank non-redundant nucleotide database using the blastn algorithm (NCBI BLAST 2.7.0+).

3.3. ANCIENT PROTEIN ANALYSIS

Proteins from dental pulp from 30 individuals from the Mireasa site were purified using this method. The samples consisted of powder obtained from dental tissue. The powder was obtained from the dental pulp, accessed through the root tips, using a drill attached to an electric

micromotor (Strong 209B, Saeshin Inc.) at low speed, using a sterilized different dental bur for each sample.

The protein purification method was designed specifically for this study and was based on a method for protein extraction from dental pulp samples (Tran et al., 2011), which was adjusted to allow recovery of both nucleic acids and proteins from the same dental powder sample. Since for the 30 samples the ancient DNA analysis was already performed, at this stage the entire sample was used for protein purification.

The concentration of the proteins obtained was determined by 2 methods: spectrophotometric (absorbance at 280 nm or A_{280}) and colorimetric (Bradford method).

3.4. Dual DNA-protein extraction

Four sets of human remains from the medieval burial ground at Capidava (samples M1, M4, M8 and M17), one from the Bronze Age burial ground at Mireasa (M6) and three from the pre-modern burial ground at Mireasa (M47, M67 and M69) were included in this study. Samples from different ages were selected in order to test the age limit for the recovery of biomolecules (DNA and proteins) by the proposed method, but also to assess whether the visually determined state of preservation determines the extraction yield. The samples consisted of dental powder obtained from teeth, preferably multi-radicular.

The simultaneous extraction was performed by modifying a protein extraction protocol described by Tran et al. (2011). DNA degradation can be avoided and, in this way, both types of biomolecules can be recovered from the same source, reducing the amount of anatomical elements destroyed during molecular analysis.

To confirm the functionality of the new extraction method, DNA from samples (teeth) belonging to the same set of remains was extracted independently using silica-based columns according to a protocol described by Yang et al. (1998). This protocol was adapted to suit the conditions and facilities available in the ancient DNA laboratory and was the protocol routinely used at that time. We tested whether the new method can be applied to access information encoded in DNA by amplifying hypervariable regions of the mitochondrial genome (HVR), one of the most widely used molecular markers in population genetics. HVR-I sequences of the mitochondrial genome were obtained by amplification of four overlapping segments covering this region and ranging from 126 base pairs (bp) to 170 bp (Gabriel et al, 2001). All of the amplified fragments were cloned (CloneJet PCR Cloning Kit, Thermo Scientific) and a minimum of five clones containing the insert were sequenced (Macrogen) using the standard

primer pJET1.2R. The resulting sequences were aligned using BioEdit software and compared to the mtDNA reference sequence according to the Revised Cambridge Reference Sequence (rCRS), GenBank: NC_012920.1.

After the validation of the method for the extraction of ancient DNA, AMA1 primers were used for the identification of *Plasmodium* DNA in the same samples using the protocol described in chapter 3.2.3 *Plasmodium* identification by ancient DNA analysis.

Similarly, to evaluate the efficiency of the simultaneous method we used two alternative protein purification methods. The first is a method previously described by Schmidt-Schultz and Schultz (2004) for protein extraction from archaeological bone tissue. The second alternative method is a solution for the extraction of proteins from animal cells (ProteoJET™, Fermentas). This has been used according to the manufacturer's instructions for protein extraction from tissue samples.

For a quick determination of the amount of protein obtained we measured A280 for proteins obtained by all three methods described above. In addition, the Bradford method (Bradford, 1976) was used for more accurate estimation of protein concentration. Subsequently, proteins obtained by all three methods were separated according to their molecular weight by SDS-PAGE in a discontinuous system (Biometra Minigel - Analytik Jena).

3.5. RAPID DIAGNOSTIC TESTS

All 38 samples for which total proteins were obtained by any of the methods described previously were tested for the presence of *Plasmodium*-specific proteins using two rapid diagnostic tests: BinaxNOW Malaria (Alere Inc.) and the SeeNow Malaria test (Camp Medica).

3.6. HEMOZOIN

Hemozoin is a metabolic product of hematophagous parasites (including *Plasmodium*) resulting after the digestion of haemoglobin and is a biomarker of these infections. The fact that hemozoin is a very stable product that accumulates in the body suggests that it could be a suitable marker for detection in archaeological remains. In order to detect the presence of hemozoin, intact long bones, preferably the femur bone, were selected. The long bone diaphysis was opened and dark deposits were visually identified. Where available, deposits were scraped off bone tissue using a plastic spatula and stored for further investigation.

3.6.1. Optical microscopy

The appearance of the deposits collected from the bone marrow cavity of long bones was observed using a Zeiss Stemi 2000-C stereomicroscope for a rapid confirmation of the specific appearance (black deposits on cancellous bone tissue).

Small fragments of these deposits were mounted on glass microscope slides and inspected by standard light microscopy and polarized light microscopy using a Zeiss Axio Imager M2 microscope.

3.6.2. Scanning Electron Microscopy (SEM) and Energy-dispersive X-ray spectroscopy (EDS or EDX)

In order to investigate the morphological appearance of the hemozoin deposits as well as the elemental content of the samples, samples taken from bone marrow cavity deposits were directly deposited, without any further processing, on aluminium supports and metalized by sputtering with a 10 nm gold layer.

1. SEM analysis was performed using a Hitachi SU8230 high-resolution electron microscope.
2. SEM-EDX analysis was performed using an FEI Quanta 3D FEG scanning electron microscope equipped with an Apollo-X SDD EDX detector.

3.6.3. Transmission Electron Microscopy (TEM) and Selected Area Electron Diffraction (SAED)

Transmission electron microscopy (TEM) and selected area electron diffraction (SAED) techniques were used to determine the morphology and structure of the presumed hemozoin deposits. A Jeol JEM-1400 microscope was used to investigate samples taken from bone marrow cavity deposits.

Samples were collected by scraping the black deposits from the surrounding bone tissue. Subsequently, the primary material was ground using a pestle and mortar grinder to a fine homogeneous powder.

Two routine techniques were used to prepare TEM samples:

1. The fine-grained powder was transferred directly onto TEM grids coated with formvar.

2. The ground powder was separated by centrifugation. First, an aqueous suspension of powder was sonicated and centrifuged at 3,000 rpm for 1 minute to disperse and remove the larger size fractions of the sample. The supernatant was then centrifuged at 15,000 rpm for 10 minutes. A portion of the sediment obtained after the second centrifugation was loaded onto a TEM grid.

Samples processed by both methods were viewed in both image and diffraction modes. The images and corresponding diffraction spectra were saved as images that were analysed and compared in Adobe Photoshop CC 2019.

For a more accurate determination of hemozoin presence, the SAED spectra obtained were indexed based on available data (Klonis et al, 2010). For this purpose, the TEM camera of the Jeol JEM-1400 microscope was calibrated using a standard polycrystalline gold foil.

4. RESULTS AND DISCUSSION

4.1. PHYSICAL ANTHROPOLOGY

In order to determine the period of utilisation of the cemeteries and the age of the burial complexes, one sample/burial complex from each cemetery was radiocarbon-dated (Beta Analytic, Inc.). Thus, the M4 Capidava complex was dated to 880-990 cal. AD, as previously estimated by funerary inventory analysis (Pinter et al., 2011). On the other hand, the M67 Mireasa complex was dated to 1635-1670 cal. AD, and is more recent than originally assumed from the archaeological analysis.

Osteological analysis recorded the bone changes most commonly associated with anaemia: *cribra orbitalia* and *cribra cranii*. *Cribra orbitalia* lesions were observed in 85% of the skulls analysed, while *cribra cranii* lesions were observed in 70%. Bone changes are not conclusive enough to be certain of the presence of malaria, but an association has been shown between haemolytic anaemia caused by malaria and and *cribra orbitalia* and *cribra cranii* has been shown (Smith-Guzmán, 2015). This, along with the fact that Dobrogea was one of the areas favourable for endemic malaria in Romania, supports the initial hypothesis that these populations were likely affected by malaria.

4.2. ANCIENT DNA

Amplification using AMA1 primers produced clear bands of the expected size for 10 of the 33 samples. Following sequence analysis (insert and primer identification, and alignment with the AMA1 sequence (NCBI Gene ID: 810891)) the plasmids were found to contain inserted sequences ranging from 122 to 202 bp. Also, the presence of used primers was noted, but the amplicons were not the expected 196 bp AMA1 fragment. Instead, by interrogating the GenBank nucleotide database using the blastn algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi) amplicons matched non-specific sequences amplified from contaminants (mainly soil bacteria).

Some of the sequences consisted of mosaics of DNA molecules that cannot be identified with certainty (alignments with databases for less than 40% of the sequence length or alignments with several different, unrelated organisms). The majority of sequences that could be identified (96%) were from soil bacteria. Another source of identified DNA was insects (*Coleoptera*), presumably from the decay processes.

Since PCR negative controls were always clean (no amplification), contamination with exogenous DNA seems unlikely. The occurrence of these sequences in the recovered total ancient DNA mixture appears to be consistent with the bacterial load described from bones and teeth derived from human remains decomposed in soil (Margaryan et al., 2018; Kazarina et al., 2019). Therefore, these results are more likely to be attributed to the survival of biomolecules in the osteological material.

4.3. ANCIENT PROTEINS

The analysis of the results reveals that A280 overestimates the protein concentration compared to the Bradford method. The average observed ratio is 2.61 times. Given the complex protein mixture, it is difficult to determine the exact concentration, as both methods are approximations compared to a BSA standard (a pure protein, different from the mixture obtained from dental pulp). A plausible explanation might be the co-purification of other substances that are extracted along with the proteins and show absorbance at 280nm (peptides, nucleic acids, etc.) but are not detected by the Bradford test.

4.4. DUAL DNA - PROTEIN EXTRACTION

The main reason for developing the described dual extraction method is to recover DNA and proteins from a single archaeological sample at the same time, reducing the destruction of the archaeological material while maximizing the information obtained. Experiments have shown that the simultaneous extraction protocol is suitable for recovering both categories of biomolecules, which then can be used in subsequent analyses. The details of the method and the results have been published in the paper *Dual DNA-protein extraction from human archaeological remains* (Rusu et al., 2019).

Complete HVR sequences were obtained for all DNA samples purified using silica columns (the alternative method). HVR sequences obtained by the dual extraction method were then compared with the former. The pattern of molecular polymorphism observed in the analogous sequences was identical for almost all samples. The only exception is the HVR-IA fragment of the M6 Mireasa individual, the oldest specimen included in this study. Being dated to the Bronze Age, this is most likely explained by poor nucleic acid preservation.

Repeated attempts to identify *Plasmodium* parasite-specific DNA resulted in amplifications for samples M47 and M69. After cloning (minimum 5 clones/ amplicon) and sequencing, the same results as described in chapter 4.2 were obtained: either amplifications from soil contaminants or unidentifiable sequences were obtained.

The yields of all protein extraction methods (mg protein/g tissue) were calculated based on the concentrations determined by the Bradford method. The graph in Figure 4.5. contains a comparison of the amount of protein obtained by all three extraction methods.

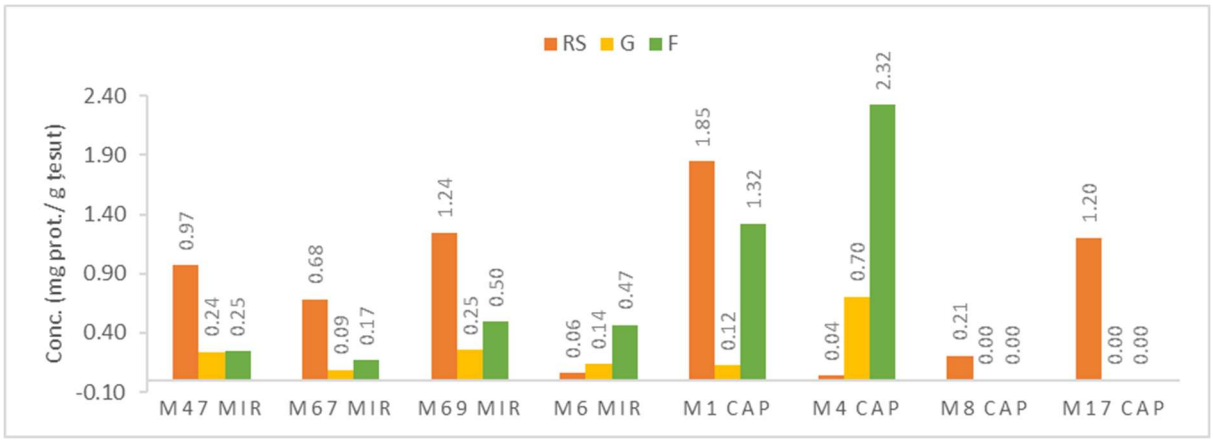

Fig 4.5. Comparison of the yields of the 3 protein extraction methods (RS - simultaneous extraction; G - Schimdt-Schultz; F - ProteoJET).

In general, the highest yield was observed when proteins were isolated by the dual DNA-protein extraction method. For two of the samples, M4 Capidava and M6 Mireasa, the yield was higher when ProteoJET was used. For two other samples, M8 Capidava and M17 Capidava, proteins could only be extracted by the dual DNA-protein extraction method.

All samples contain highly degraded proteins and separation in SDS-PAGE is not possible. Although all samples extracted by all three methods contain the same pattern of degraded proteins appearing as a smear without specific bands, differences between the three alternative methods can be seen in Figure 4.7. Simultaneous extraction forms a smear throughout the size range, while alternative methods tend to predominantly recover either the large (Schmidt-Schultz and Schultz, 2004) or the small (ProteoJET kit) fractions.

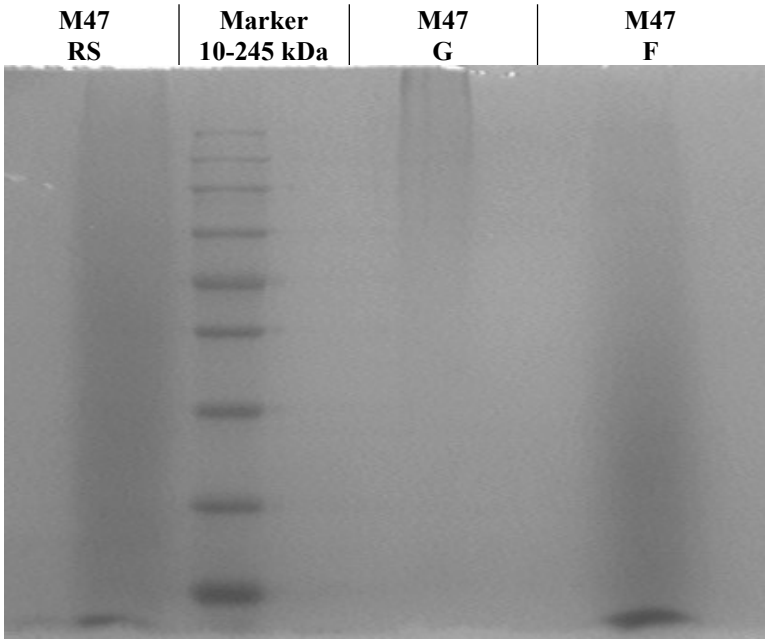

Fig. 4.7. SDS-PAGE: M47 Mireasa (RS – dual extraction,
G – Schimdt-Schultz, F – ProteoJET)

4.5. RAPID DIAGNOSTIC TESTS

The 38 samples were all tested for the presence of Plasmodium-specific proteins. A minimum of three rapid diagnostic tests were repeated for each sample. Of the 38 samples, 20 (53%) gave at least one positive result in the rapid diagnostic tests. The BinaxNOW Malaria test was used for 14 samples (of which 5 were positive), while the SeeNow Malaria test was used for 26 samples (of which 16 were positive). Two of the samples were tested using both tests and showed congruent results: one sample (M88) was positive while the other (M112) was negative. Also, all extraction control samples were tested using rapid tests and showed no positive results.

4.6. HEMOZOIN

Black deposits (Fig. 4.11.), presumed to be hemozoin, have been found in the long bones of individuals sampled for this type of investigation. These black deposits were subsequently collected and analysed to assess whether microscopic techniques are suitable for the identification of hemozoin.

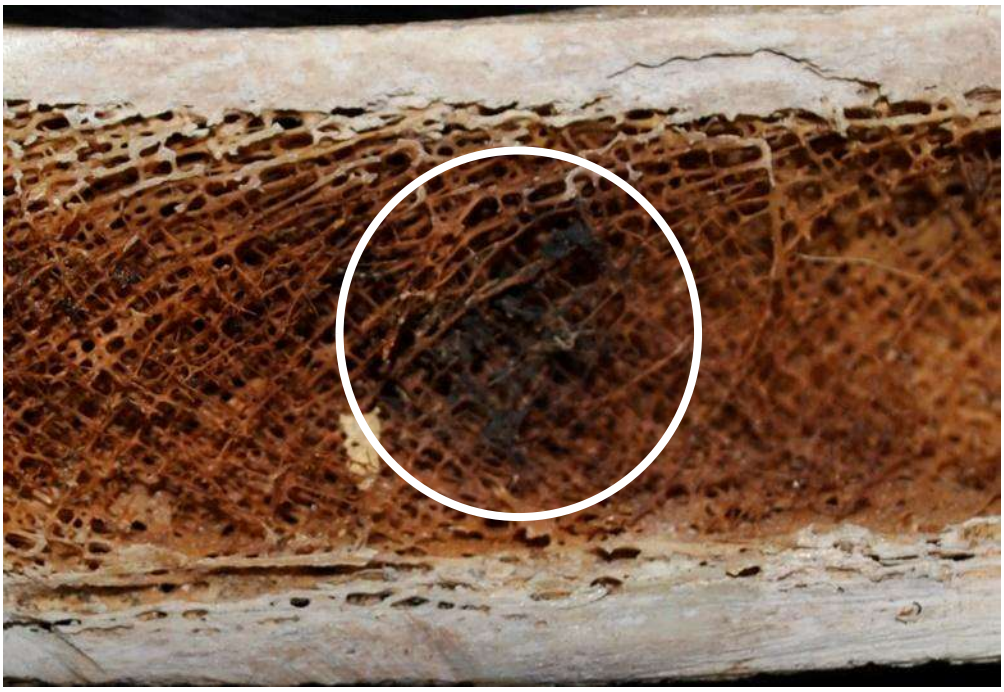

Fig. 4.11. Presumed hemozoin deposits (inside the white circle) are visible on the trabecular bone of a femur (M69).

4.6.1. Optical microscopy

The appearance of the deposits was observed under a stereomicroscope. The deposits consist of conglomerates of irregular shapes closely associated with the surrounding bone tissue.

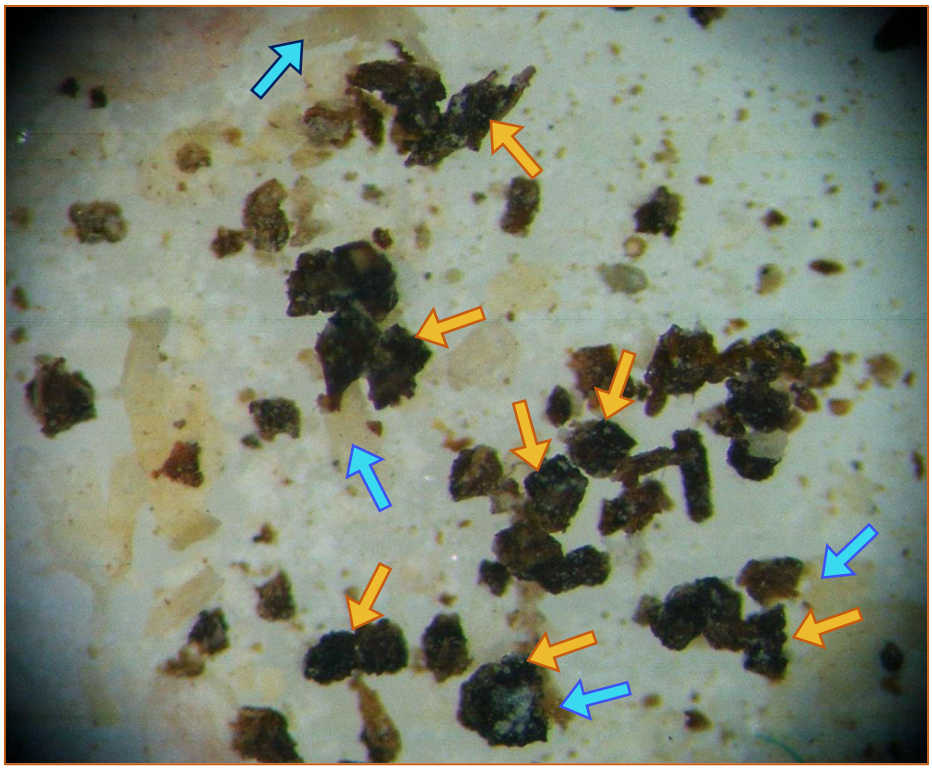

Fig. 4.12. The appearance of hemozoin deposits (black, marked with orange arrows) associated with bone tissue (white/yellow, marked with blue arrows) (50X magnification)

Deposits show a distinctive birefringence under polarized light microscopy (Lautenschlager et al., 2019), as can be seen in Figure 4.13. Polarized light microscopy highlights the presence of the pigment very effectively, which is why it has been used to identify these parasites in fresh blood samples since the 1980s (Cho et al., 2012; Pirnstill and Coté, 2015).

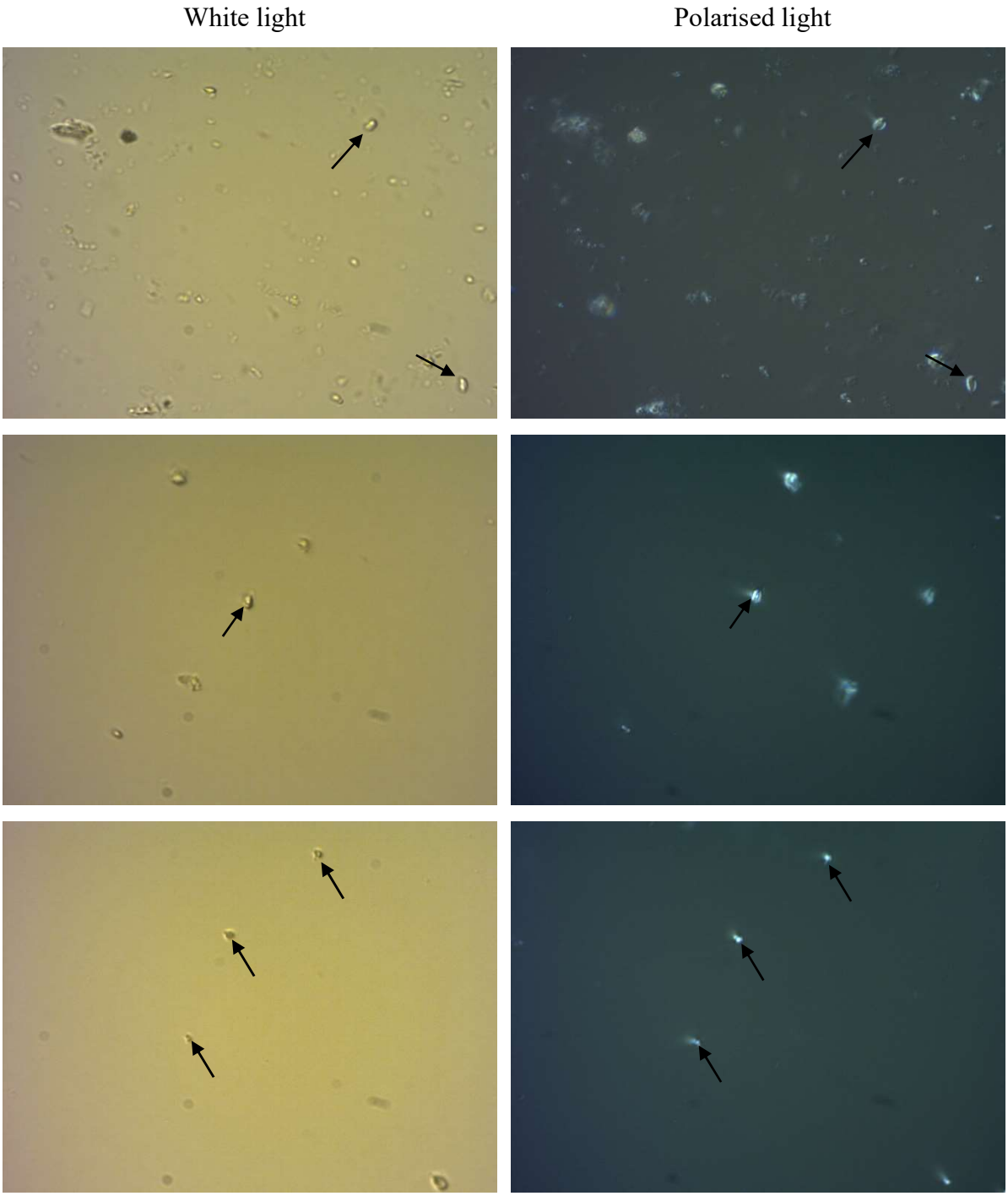

Fig. 4.13. Small fragments of deposits collected from archaeological bones are refractive in polarized light (1000 X magnification).

4.6.2. Scanning Electron Microscopy (SEM) and Energy-dispersive X-ray spectroscopy (EDS or EDX)

SEM analysis of deposits from bone marrow revealed crystals with a length of 100-150 nm (Fig. 4.15.) and a width of approximately 30 nm.

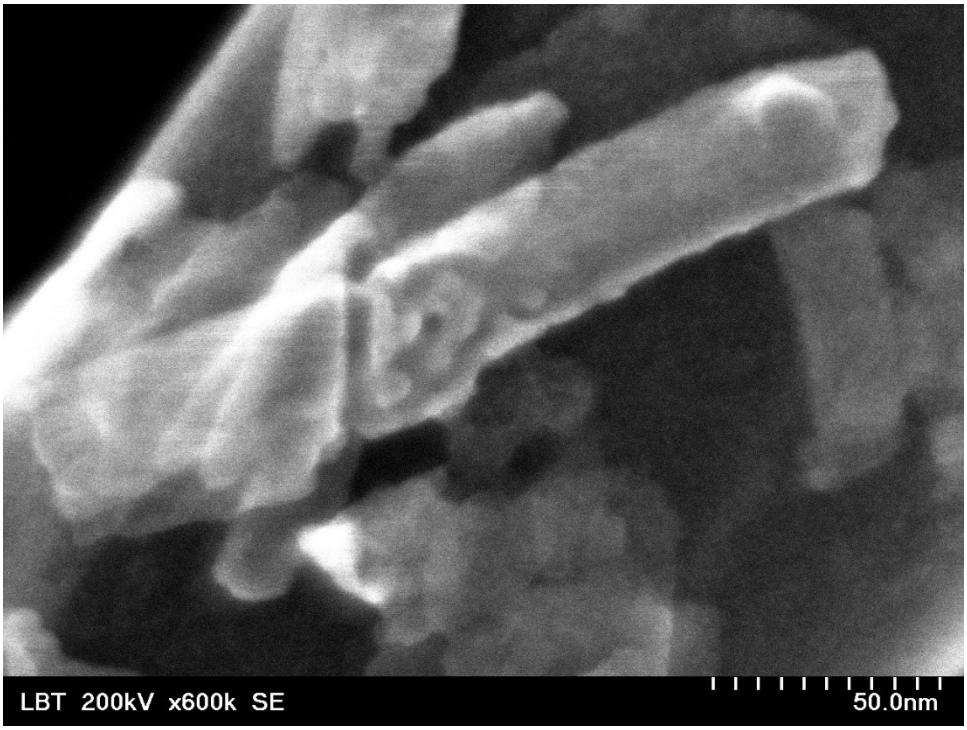

Fig.4.15. Morphology of a crystal identified in hemozoin deposits

The elemental composition and spatial distribution reveal the distribution of iron and its spatial correlation with oxygen (Fig. 4.16.). The elemental composition detected in this way is congruent with the chemical nature of hemozoin (Wendt et al., 2021), a heme polymer containing carbon, oxygen, nitrogen, and iron. Calcium and oxygen, specific to the organic matrix, are detected over the entire analysed area, as is expected given the presence of bone tissue.

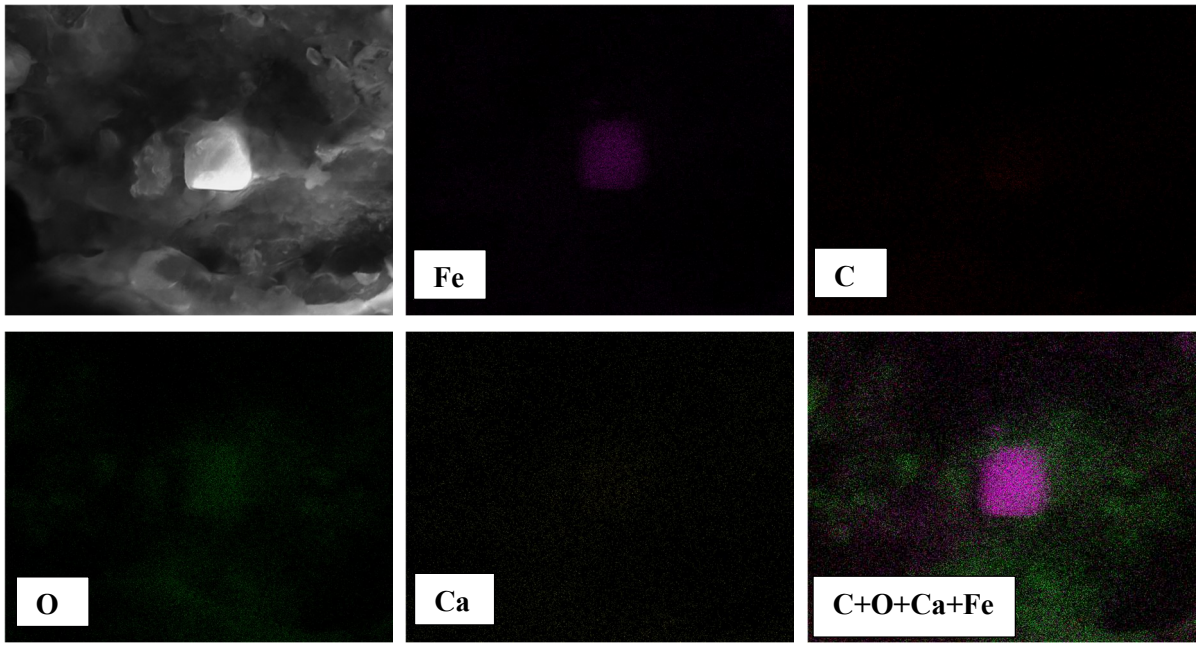

Fig. 4.16. SEM/EDX analysis for element mapping

4.6.3. Transmission Electron Microscopy (TEM) and Selected Area Electron Diffraction (SAED)

The specimens (fine powder, obtained by grinding the deposits) that were deposited directly on the grid showed that the particles in both analysed samples exhibited considerable morphological heterogeneity (Fig. 4.17.A. and Fig. 4.17.D.). In the less transparent areas (marked with red arrows) conglomerates of needle-like particles as well as more transparent particles (marked with blue arrows) of irregular shapes and sizes can be observed.

A more uniform aspect of the particles was observed when specimens (from the same source) were deposited on the grid after sonication and centrifugation (Fig. 4.17.B. and Fig. 4.17.E.). Crystal morphology is similar in both samples investigated. The observed particles have a needle shape of about 150 nm length and 25 nm diameter, as can be distinguished in some less dense areas.

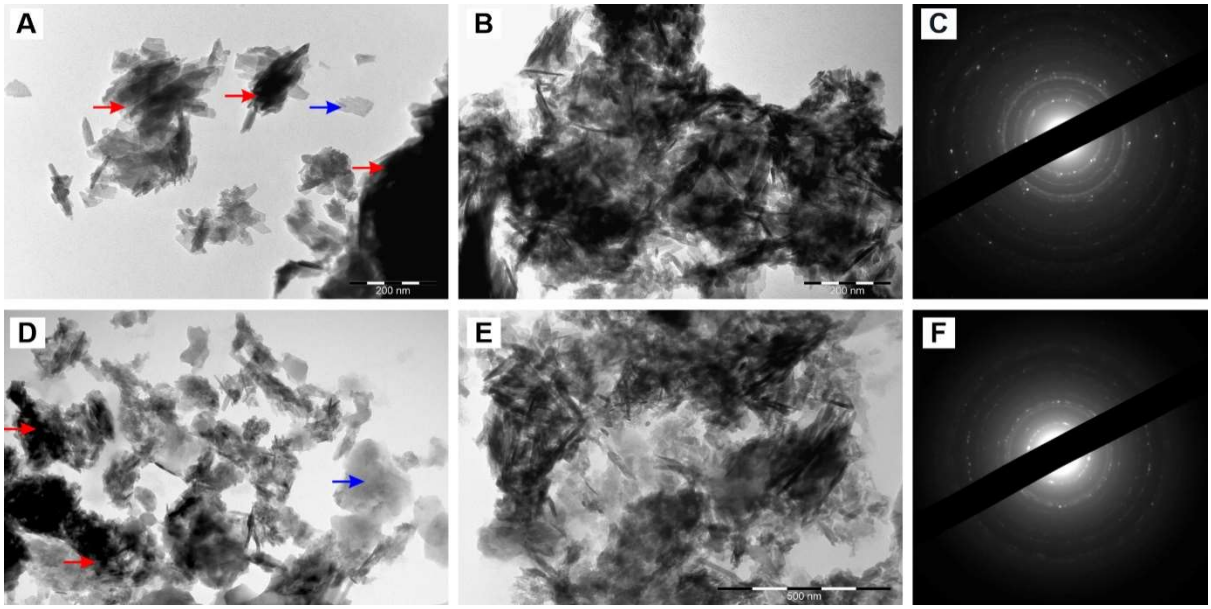

Fig. 4.17. A,D Samples deposited directly on the grid; B,E Ultrasonicated and centrifuged samples; C,F SAED patterns associated with the samples (from Paica et al., 2023 © Elsevier Inc.).

The general appearance is comparable to previous descriptions of hemozoin produced by Plasmodium (Noland et al., 2003; Orbán et al., 2014), but the particle size is smaller. On the other hand, the sizes of these particles are similar to those seen in samples visualized using SEM. Research on the structural and morphological characterization of hemozoin is limited to fresh samples (cell cultures, blood, bone marrow), whereas no data regarding archaeological samples could be found in the literature. It is unclear how the historical age of samples, the processing protocols or the different post-mortem environments affect the morphology of the crystals.

Archaeological samples included in the TEM and SAED analysis yielded electron diffraction patterns. The appearance of these patterns is evidence that the investigated specimens have a crystalline structure. Diffraction patterns of dispersed hemozoin particles, consisting of concentric rings, are specific to randomly oriented crystallites (Fig. 4.17.C. and Fig. 4.17.F.). Because hemozoin is adherent to the trabecular bone tissue and cannot be separated, bone fragments appear as impurities in TEM images. In addition, cryptocrystalline hydroxyapatite in bone tissue as well as other amorphous impurities appear as amorphous background in electron diffraction.

By superimposing the experimental model (Fig. 4.18.A.) with the reference data (Slater et al., 1991 - Fig. 4.18.B.; Oliveira et al., 2005 - Fig. 4.18.C.) it can easily be noticed that the obtained diffraction patterns coincide both in terms of angular position and diffraction line intensities. In addition, the X-ray diffractogram (XRD) resembles both diffraction patterns regardless of the analysis method.

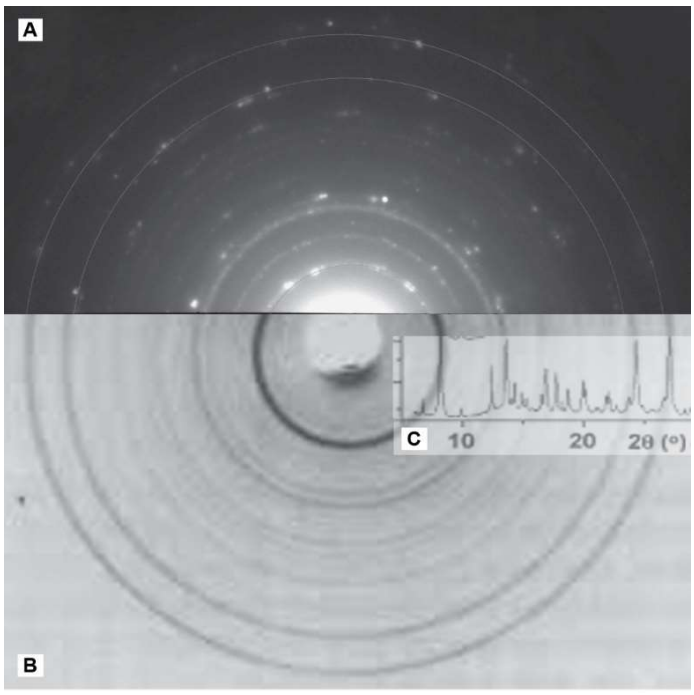

Fig. 4.18. Sector of a diffraction pattern compared with reference data:
A. M44 SAED model
B. Reference X-ray diffraction pattern modified from (Slater et al., 1991)
C. X-ray diffractogram performed on hemozoin, modified from Oliveira et al., 2005.

To provide additional insight into the crystal structure, interplanar distances were calculated for the planes corresponding to the most intense electron diffraction peaks, denoted d_{exp}. The experimental data (d_{exp}) was compared to theoretical distances (d_{hkl}) calculated based on data from Oliveira et al. (2005), as shown in Table 4.7. The differences between the calculated inter-planar distances for our experimental data and the theoretical data are negligible, therefore we can consider that the analyzed samples contain hemozoin crystals.

Table. 4.7 Interplanar distances calculated from theoretical (d_{hkl}) and experimental (d_{exp}) data for planes defined by the main Miller indices (hkl).

[hkl] planes			**Interplanar distance (Å)**	
h	**k**	**l**	**d_{hkl}**	**d_{exp}**
1	0	0	11.98	11.96
0	2	0	7.28	7.31
1	2	0	5.87	5.85
2	1	1	4.19	4.23
0	3	1	4.11	4.11
1	3	1	3.69	3.71
3	1	0	3.72	3.73
3	1	1	3.23	3.23

These results indicate that deposits found in the long bones of archaeological remains could be used as an alternative method for diagnosing malaria. In addition, hemozoin is easily visualized using polarized light microscopy without any additional sample processing, allowing for confirmation of its presence within minutes. Elemental analysis of the deposits also confirmed the presence of Fe, C, and O, which is consistent with the known chemical composition of hemozoin. In addition, the electron diffraction patterns obtained for the investigated specimens can be attributed to hemozoin. Therefore, following various microscopic analyses we have provided compelling evidence that hemozoin deposits survive in the long bones of skeletal remains of malaria-affected individuals and that they are available for sampling and identification.

4.7. OVERVIEW OF RESULTS

A total of 38 sets of skeletal remains from two archaeological sites were analysed: four from the Capidava site and 34 from the Mireasa site (of which one from the Bronze Age tumulus and 33 from the pre-modern burial ground). The results obtained are presented in summary in Table 4.8.

For all 38 samples DNA and protein were purified, either independently or by the dual DNA-protein extraction method. The DNA and proteins obtained were used to detect Plasmodium-specific biomarkers in order to identify the presence and frequency of malaria in the populations studied.

Immunological test results were positive for 20 (53%) of the samples analysed, suggesting the presence of malaria. However, diagnostic tests are designed for fresh blood diagnosis, and the results should be regarded as an indication rather than an accurate diagnosis. The composition of protein samples extracted from archaeological tissues is unknown and cross-reactions with bacterial or fungal residues from the burial environment or other substances in the soil could not be excluded.

In total, DNA sequences amplified with Plasmodium-specific primers were obtained for 10 of the 38 individuals (26%), but their analysis revealed only the presence of contaminating DNA from the environment. The ancient DNA results demonstrate the limitations of the PCR-based approach to palaeopathological research, imposed by the minute amount of parasite DNA preserved in archaeological human remains.

Hemozoin deposits could only be identified for a part of the skeletons analysed, depending on the state of preservation and the presence of intact long bones. The hemozoin-related analyses were destructive and were not considered necessary for all individuals, as they aimed to prove the concept that this biomarker can be identified in an archaeological context. The presence of hemozoin deposits was observed for 10 individuals. Eight of them had positive immunological test results and two had negative results. The occurrence of these partially incongruent results can be explained by differences in the preservation and recovery of biological molecules.

Table 4.8. Overview of the results

No.	Sample	Immunological tests		*Plasmodium* DNA	Optical microscopy	Electron microscopy	
		BinaxNOW	SeeNow			TEM	SEM
1.	M37		+	×			
2.	M40		+	non-specific			✓
3.	M43		-	×			✓
4.	M44	-		non-specific		✓	
5.	M47	+		non-specific	✓		
6.	M49	-		×			
7.	M54	+		non-specific	✓		
8.	M56		-	×			
9.	M62		+	×			
10.	M65		+	non-specific			
11.	M67		-	non-specific			
12.	M69	+		non-specific	✓	✓	
13.	M71	-		×			
14.	M72		+	non-specific	✓		✓
15.	M77		-	×			
16.	M88	+	+	×			✓
17.	M92		-	non-specific			
18.	M95		+	×			
19.	M96		+	×			
20.	M97		+	×			
21.	M100		+	×			✓
22.	M107		-	×			
23.	M111		+	non-specific			
24.	M112	-	-	×			
25.	M116		+	×			
26.	M118		-	×			
27.	M126		+	×			
28.	M131	-		×			
29.	M133		+	×			✓
30.	M134		-	×			
31.	M142		+	×			
32.	M144		+	×			
33.	M152		-	×			
34.	M1 Cap	-		×			
35.	M4 Cap	-		×			
36.	M8 Cap	+		×			
37.	M17 Cap	-		×			
38.	M6	-		×			

5. CONCLUSIONS

In this thesis, the presence of the Plasmodium parasite and/or its residues in human skeletal material of archaeological origin was investigated. The interdisciplinary approach to the topic proved necessary because of the complexity of parasite biology, further complicated by the inherent state of degradation of archaeological evidence.

1) One sample from each site included in the study was radiocarbon dated in order to establish with certainty the age of the samples. While the age of the medieval burial ground at Capidava and the Bronze Age tumulus at Mireasa suggested by the archaeological context were confirmed by 14C dating, the burial ground at Mireasa is more recent than initially presumed, being dated to the 17^{th} century.
2) Anthropological analysis recorded bone lesions indicative of anaemia in most of the skeletons, supporting the hypothesis that the populations studied could have been affected by malaria.
3) We sought to determine if there was a reasonable amount of *Plasmodium* spp. DNA in our samples for it to be identified. Although ancient human DNA could be recovered, attempts to recover parasite DNA were hindered by the presence of contaminating DNA in the soil (bacteria and insects that inevitably occur in remains that have been buried).
4) In addition to ancient DNA analysis, rapid diagnostic tests were used to detect the presence of specific *Plasmodium* spp. proteins, the results of which suggest the presence of the parasite in the investigated populations.
5) Research on both types of molecules (DNA and proteins) has led to the development and testing of an original method for isolating DNA and ancient proteins from the same source of tissue, thus reducing the amount of archaeological tissue destroyed for molecular analysis. Details of this method were published in Rusu I*, Paica I* et al, Dual DNA-protein extraction from human archaeological remains. *Archaeological and Anthropological Sciences*, 11, 3299-3307 (2019).
6) An additional metabolite produced by *Plasmodium*, hemozoin, could be a suitable biomarker when the results of ancient DNA and protein analyses are insufficient or inconclusive. Detection of hemozoin in archaeological remains is theoretically possible, but few attempts to identify this metabolism product are reported, and those are inconclusive. During these investigations, we identified hemozoin deposits and analysed them extensively using optical and electron microscopy methods, based on characteristics

such as birefringence in polarized light, elemental composition (SEM/EDX), or specific electron diffraction pattern (TEM/SAED). The results of these investigations were described in Paica et al, Tentative indicators of malaria in archaeological skeletal samples, a pilot study testing different methods. *International Journal of Paleopathology*, 40, 109-116 (2023).

The results of the research outline the challenges and limitations of malaria diagnosis in paleopathology based on ancient proteins and DNA. Meanwhile, the results of the pilot study demonstrate that the detection and characterization of hemozoin using microscopy methods is a promising strategy that could be used in malaria diagnosis as a complement to genetic and immunological evidence. Further evaluation of the availability and preservation of hemozoin in bone samples from more diverse archaeological contexts (in terms of preservation, age, environmental conditions, etc.) would be helpful for the future development on such a diagnosis strategy in paleopathology.

List of publications

1. Rusu, I., **Paica, I.,** Vulpoi, A., Radu, C., Mircea, C., Dobrinescu, C., Bodolică, V., Kelemen, B. (2019) Dual DNA-protein extraction from human archeological remains. *Archaeological and Anthropological Sciences,* 11, 3299–3307.
2. **Paica, I. C.**, Rusu, I., Popescu, O., Brînzan, A., Pencea, I., Dobrinescu, C., & Kelemen, B. (2023). Tentative indicators of malaria in archaeological skeletal samples, a pilot study testing different methods. *International Journal of Paleopathology*, 40, 109-116.

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