Molecular diagnosis of paleopathological human remains by using mass spectrometry

Ph.D. Thesis

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1. Contents

2. Abbreviations

AD = Anno Domini

aDNA = ancient DNA

ANX = Annexin

BC = Before Christ

BCL2 = B-cell lymphoma 2-related protein

BP = Before Present

CHCA = α-cyano-4-hydroxycinnamic acid

CID = Collision –Induced Dissociation

DHB = 2,5 dihydroxybenzoic acid

DLC-1 = Deleted in Liver Cancer 1

DTT = Dithiotreitol

EDTA = Ethylenediaminetetraacetic acid

HSP = Heat-Shock Protein

IAA = Iodoacetamide

LID = Laser Induced Decay

MALDI MS = Matrix Assisted Laser Desorption/Ionization Mass Spectrometry

MCP = Microchannel Plate detector

MTC = *Mycobacterium tuberculosis* Complex

PMF = Peptide Mass Fingerprint

PSD = Post Source Decay

RhoGAP = Rho GTPase-activating protein

R. T. = Room temperature

SA = Sinapinic acid

SDS PAGE = Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

TFA = Trifluoroacetic acid

TOF = Time-of-Flight

VIM = Vimentin

Abbreviation of amino acids:

A = Alanine

C = Cysteine

D = Aspartate

E = Glutamate

F = Phenylalanine

G = Glycine

H = Histidine

I = Isoleucine

K = Lysine

L = Leucine

M = Methionine

N = Asparagine

P = Proline

Q = Glutamine

R = Arginine

S = Serine

T = Threonine

V = Valine

W = Tryptophan

Y = Tyrosine

3. Introduction

3.1. Disease biomarkers in saliva

Based on the previous work of our research group, tumor biomarker identification in saliva (Jarai et al., 2012), we started to analyse paleopathological bone samples to find potential biomarkers for two different ancient diseases. A cancerous disease, malignant bone tumor, osteosarcoma and an infectious disease, tuberculosis caused by *Mycobacterium tuberculosis* were analysed.

The analysis of human clinical body fluid samples, searching for disease biomarkers using proteomics is an important research field nowadays. Body fluids flow through the body and come in contact with several tissues of different organs picking up proteins. These proteins serve as possible biomarkers of disease and may provide new clues for drug development.

Oral cancer affects about 360,000 people worldwide (Hu et al., 2007). More than half of oral cancers are advanced by the time they are detected (Gonsalves et al., 2007; Weinberg and Estefan, 2002), due to the poor symptomatology for a long period. There is still no appropriate non-invasive, high-throughput diagnostic technique to develop the early signs of malignant transformation at least at those who are at a high risk. Early diagnosis plays a key role in disease progression, treatment response and ultimately, quality of life and patient survival (Marocchio et al., 2010).

Identifying protein-type biomarkers in body fluids collected at the clinic and analysed using mass spectrometry could solve the problem of the limited early detection of oral cancer (Szanto et al., 2012) as well as head and neck squamous carcinoma (Jarai et al., 2012).

Saliva is protein rich, and may constitute a valuable source of diagnostic and therapeutic markers in oral cancer patients (Drake et al., 2005; Edgar, 1992; Yao et al., 2003). Protein biomarkers derived from oral cancer cells have been identified after SDS-PAGE and tryptic digestion using MALDI TOF/TOF MS instrument by our research group. Comparing to healthy controls we found annexin 1 and 2 and

proteins such as peroxiredoxin1 and 2 and thioredoxin peroxidase in oral tumor patients (Szanto et al., 2012). Increased level of annexin 1, zinc finger protein 28, regulator G-protein 3, indoleamine 2,3-dioxygenase, OFD1 protein and CEP290 protein were found in the saliva samples of malignant cancer patients (Jarai et al., 2012). These proteins may serve as potential tumor markers in the future.

3.2. Tumor Biomarkers

Tumor biomarkers are proteins or genes which are naturally produced in cells but their overexpression or in case of genes increased activity indicate tumorous mutation generated by cancerous cells. These substances are found among others in blood, urine, saliva, tumor tissue and bone. At present there are more than 20 tumor markers in clinical use. The markers are mostly characteristic to the type of the cancer. Nevertheless the tumor marker alone is not acceptable to prove the presence of mutation, further tests and other measurements e.g. biopsy is needed, to set the diagnosis. The reasons are the following, not all of the patients produce elevated level of markers or the increased level of the crucial proteins not always indicates tumorous disease. In addition tumor markers have not been identified for every type of cancer.

3.2.1. Annexin protein family

The name of Annexins derived from the Greek *annex* meaning “bring/hold together” what points to their main functions binding to and possibly holding together membranes. ANXs are soluble, hydrophilic intracellular proteins that bind to negatively charged phospholipids in a Ca^{2+} dependent manner (Ca^{2+} / phospholipid binding proteins). The binding is reversible; ANXs can be liberated from the phospholipid matrix using chelating agents. The different members of this family have different Ca^{2+} sensitivity and phospholipid head group specificity (e.g.: phosphatidic acid, phosphatidylserine, phosphatidylinositol). Ca^{2+} dependent signal transduction pathways are involved in the regulation, growth and apoptosis of the cells (Gerke et al., 2002). Dysregulation of ANXs has been reported in numerous cancers, affecting proliferation, invasiveness, cancer-related signalling pathways assuming its crucial role in tumoral development and progression (Deng et al. 2012; Hata et. al., 2014; Kwon et al, 2013; Lin et al., 2012; Luo et al., 2013; Mogami et al., 2013; Sun et al., 2013; Zeng et al., 2013).

The family member, ANXA10 protein (Figure 1) is highly responsible for the cellular proliferation of oral squamous cell carcinoma (Shimizu et al., 2012). The elevated level of this protein corresponds to the stage of the tumor growth. However, decreased expression was observed in case of gastric (Kim et al., 2009; Kim et al., 2010) hepatocellular (Liu et al., 2002; Peng et al., 2005) and bladder carcinoma (Munksgaard et al., 2011), suggesting a tumor suppressing and apoptosis inducing property of ANXA10 protein.

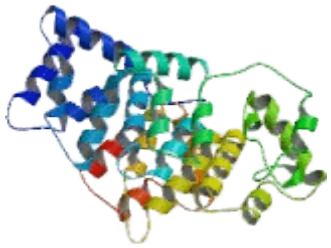


Figure 1: 3D structure of ANXA10 protein (Length: 324 amino acids; 37.3 kDa) (http://www.uscnk.com/directory/Annexin-A10(ANX-A10)-4780.htm)

3.2.2. BCL2 protein family

The B-cell leukemia/lymphoma-2 family proteins are encoded by BCL2 gene. The BCL2 protein family members all share sequence and structural similarity in their BCL2 homology (BH) domains. The BCL2 proteins have an important role in cell apoptosis. They can be divided into two subgroups according to their function: pro- and anti-apoptotic proteins. Apoptosis or programmed cell death can be triggered by cellular stress at the mitochondria (the intrinsic pathway). This pathway is responsible for the release of cytochrome c from the mitochondrial intermembrane space into cytosol, eventuating the formation of apoptosome and caspase-9 activation. The BCL2 proteins have important role in the regulation of this step (Ottina et al., 2012).

BCL2A1, B-cell lymphoma related protein A1 (Figure 2) has anti-apoptotic function during B-cell maturation. Overexpression of BCL2A1 indicates the presence of tumorous mutation including leukemia (Nagy et al., 2003), lymphoma (Mahadevan et al., 2005), stomach (Choi et al., 1995), breast cancer (Beverly et al., 2009), melanoma (Riker et al., 2008) and oral squamous cell carcinoma (Saleh et al., 2010). Increased level of BCL2A1 could be a possible reason for chemotherapeutic drug resistance. BCL2A1 proteins suppress apoptosis upon toxic insults and prevent cell death during chemotherapy. (Yajima et al., 2009)

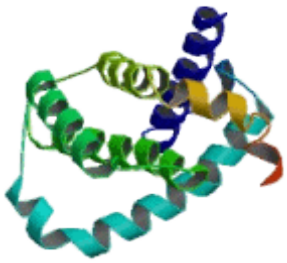


Figure 2: 3D structure of BCL2A1 protein (Length: 175 amino acids; 20.3 kDa) (http://www.uscnk.com/directory/BCL2-Related-Protein-A1(BCL2A1)-80252.htm)

3.2.3. DJ1 protein

DJ1 protein (Figure 3) also known, as Parkinson disease (autosomal recessive, early onset) 7, PARK7 protein is encoded by DJ1 gene. DJ1 protein participates in transcriptional regulation, antioxidative stress reaction and in chaperone, protease and mitochondrial regulation. DJ1 has also a protecting role against oxidative stress induced cell death. The protein is located in the cytoplasm, nucleus and in the mitochondria of cells. DJ1 is secreted in almost all cells as well as in cancer cells and tissues. Significant level of DJ1 have been detected in squamous cell (Zhu et al., 2010), pancreatic (Tian et al., 2008), lung (Merikallio et al., 2012), renal cell carcinomas (Sitaram et al., 2009) and leukemia (Liu et al., 2008).

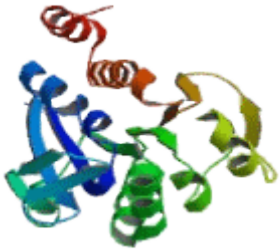


Figure 3: 3D structure of DJ1 protein (Length: 187 amino acids; 19.9 kDa) (http://www.uscnk.com/directory/Parkinson-Disease-7-Protein(PARK7)-80059.htm)

3.2.4. DLC1 protein

DLC1, deleted in liver cancer gene (Figure 4) encodes a Rho GTPase-activating protein (RhoGAP) DLC1, identified as a potent tumor suppressor (Kim et al., 2003; Plaumann et al., 2003; Yuan et al., 2004). DLC1 protein oncosuppressive effect is connected to its RhoGAP activity. The RhoGAP domain of DLC1 protein is able to enhance the GTPase activity of the RhoGTPase proteins promoting the hydrolysis of their bound GTP to GDP, rendering these proteins inactive. The different small GTPase members of RhoGAP family have a significant role in cell growth, morphogenesis, cell motility, cytokinesis, transformation and metastasis. These processes also involve oncogenic transformations and cancer progression (Guan et al., 2008; Kim et al., 2007). The down-regulation of DLC1 gene expression results the activation of Rho GTPases, the key mediators of oncogenesis (Peng et al., 2013; Qin et al., 2013). Reactivation of DLC1 gene in tumorous cells eventuate suppression in the proliferation and migration of cancer cells leading to apoptosis in vitro (Ullmannova-Benson et al., 2009). The down-regulation of DLC1 protein was found in melanoma (Sjoestroem et al., 2014), hepatocellular carcinoma (Wolosz et al., 2014) and in lung cancer (Kim et al., 2013) as well.

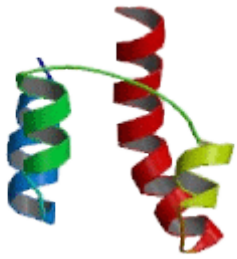


Figure 4: 3D structure of DLC1 protein (Length: 1091 amino acids; 122.7 kDa) (http://www.uscnk.com/directory/Deleted-In-Liver-Cancer-(DLC1)-6500.htm)

3.2.5. Heat shock proteins

Heat shock proteins (Hsps) were first discovered when proteins were disordered by heat shock, oxidative stress or other protein-damaging events (Lindquist and Craig, 1988). They have a key role in the protection of malignant cells from spontaneous or therapy generated apoptosis. The elevated level of Hsps in various cancer types (Atkins et al., 2005; Kang et al., 2013; Kim et al., 2011; Li et al., 2012; Marinova et al., 2013) indicates the importance of these proteins in tumor progression and results chemoresistance. Hsps could shift the balance from denaturated state of protein toward functional protein. Hsps are molecular chaperons, which are able to modify the structures and interactions of other proteins. Hsp27, 70 90, 110 are the most common expressed proteins after induced stress (Ciocca et al., 2005).

Figure 5: 3D structure of Heat-shock protein beta-6
(Length: 160 amino acids; 17.1 kDa)
(http://www.uscnk.com/directory/Heat-Shock-Protein-B6-Alpha-Crystallin-Related(HSPB6)-5358.htm)

3.2.6. S100 protein family

The S100 family contains 20 small acidic proteins containing calcium-binding EF-hand motifs. EF-hand motifs consist of two helixes (E and F) joined by a loop, this loop is responsible for the binding of Ca^{2+}. One of the EF hands is on the C-terminal portion the other one is located at the N-terminus (specific to this protein family). S100 proteins show different cell-specific and tissue-specific patterns, which result their distinct functions in specific tissues or cell types. The members of this family take part in a variety of biochemical activities such as protein phosphorylation, enzyme activities, Ca^{2+} homeostasis, inflammation, cell proliferation and differentiation. S100 family proteins have significant role in tumorigenesis and metastasis. Some members of the family act as tumor promoters and others act as tumor suppressors (Salama et al., 2008).

Calgizzarin or S100A11 protein (Figure 6) has an important biological role in cell proliferation and differentiation. According to previous studies Calgizzarin is involved in tumor development and immortalization. Overexpression of Calgizzarin is seen in many cancers including colorectal (F Lam et al., 2010; Tanaka et al, 1995; Wang et al, 2008), gastric (Li et al., 2011; Mori et al., 2004; Oue et al., 2004), pancreatic (Chen et al., 2009; Ji et al., 2014; Ohuchida et al., 2006; Xiao et al., 2012), breast (Cross et al., 2005; Liu et al., 2010; McKiernan et al., 2011), prostate (Rehman et al., 2004), lung (Hao et al. 2012; Tian et al., 2007), bladder (Yao et al., 2007) and squamous cell carcinoma (Roesch-Ely et al., 2007; Wang et al, 2013).

Figure 6: 3D structure of Calgizzarin (Length: 105 amino acids; 11.7 kDa) (http://www.uscnk.com/directory/S100-calcium-binding-protein-A11(S100A11-S100C)-0568.htm)

3.2.7. Vimentin

Vimentin or VIM (Figure 7) is a notable member of the intermediate filament family of proteins; it is expressed in normal mesenchymal cells. VIM is responsible for maintenance of cellular integrity and provides resistance against stress. Furthermore VIM has been identified as a marker for several tumorigenic events related epithelial-mesenchymal transition (EMT). Elevated level of VIM is associated with numerous epithelial cancers, including prostate cancer (Rodrigues et al., 2011; Singh et al.; 2003; Wei et al., 2008), gastrointestinal tumors (Hou et al., 2012; Noguchi et al., 2002), tumors of the central nervous system (Satelli and Li, 2011), breast cancer (Bocca et al., 2014; Deng et al., 2013; Lehtinen et al., 2013), malignant melanoma (Fink-Puches and Smolle, 1993; Hendrix et al., 1996) and lung cancer (Feng et al., 2012; Giarnieri et al., 2013; Tauler et al, 2010). Also because of its overexpression in different tumorous cells, participation in the growth, invasion and migration of the tumorous cells, VIM can be used as a target to deliver therapeutic agents to the tumor site (Zamay et al, 2014).

Figure 7: 3D structure of VIM protein (Length: 466 amino acids; 53.6 kDa) (http://www.uscnk.com/directory/Vimentin(VIM)-1040.htm)

3.3. Ancient evidence of osteosarcoma and tuberculosis

Osteosarcoma is the most common primary bone tumor characterized by the production of osteoid matrix from malignant cells derived from progenitor cells in the osteoblast lineage. It typically occurs in the long bones of the near metaphyseal growth plates of children and young adolescents (Beckingsale and Gerrand, 2010). These mesenchymal tumor related mutations escape from the cells leading to excessive proliferation without differentiation from normal osteoblastic cells during the cell cycle. This highly aggressive neoplasm frequently metastasizes in the lungs. After an initial diagnosis, patients usually receive standard treatment such as neoadjuvant chemotherapy and surgical resection.

Osteosarcoma has a poor prognosis because metastases are usually developed prior to clinical diagnosis. Despite intensive research for new therapies, the survival rate for patients with advanced disease still remains low.

The earliest known case affected a male Celt (ca. 800–600 BC) from Switzerland with a possible osteosarcoma or chondrosarcoma (Brothwell, 1967). A possible osteosarcoma of the pelvis has been noted in a young individual from Ancient Egypt, dating to about 250 AD (Ruffer and Willmore, 1914), and a well-documented case of osteosarcoma, with the typical radiographic “sunburst” pattern, has been reported in the femur of a native Peruvian dating to 800 BP (Aufderheide et al., 1997). Additional cases of osteosarcoma have been observed in a young female femur from the prehistoric population of Oahu in Hawaii (Suzuki, 1987), and in a zygomatic bone from the French Middle Ages (Dastugue, 1965) in a case of 17th century mandible from West Virginia (Kelln et al.1967). Possible osteosarcomas have been detected in a young male from the Saxon necropolis of Standlake, England (Brothwell, 1981) and in medieval skulls from the Czech Republic (Strouhal et al., 1996) and France (Blondiaux, 1986). Probable cranial hemangiosarcoma has been documented in an elderly female from Italy, 3rd Century BC (Capasso et al., 1992) and in a humerus from Peru, 12–14th Centuries AD (Aufderheide et al., 1997) and a possible Ewing’s sarcoma in a juvenile skull from Bronze Age of Tartaren, Spain (Campillo, 1976). Only a few cases of neoplasms have been documented in Central and South American mummies, for example a rhabdomyosarcoma (4–7th Centuries AD) in 2 children from Chile (Gerszten and Allison, 1991).

Tuberculosis is a chronic infectious disease, caused by bacteria of the *Mycobacterium tuberculosis* complex (MTC), including *Mycobacterium tuberculosis* and *Mycobacterium bovis* being the most common cause of illness in humans. Tuberculosis, one of the oldest recorded human afflictions, is still one of the most deadly and common major infectious disease today, despite the worldwide use of a live attenuated vaccine and several antibiotics (Kinsella et al., 2003; Mathema et al., 2006; Smith, 2003; Stone et al., 2009). The most common symptoms are chronic cough with blood-tinged sputum, fever and weight loss (phthisis). Tuberculosis could infect the lymph nodes in the neck, armpits and groin forming large, swollen lumps known as scrofula. The infection is spread through the air with coughing and sneezing. It infects approximately one-third of the world's population, although the vast majority of these potential cases are in Africa or Asia.

Mycobacterium tuberculosis complex could be very old. The first evidence of the existence of *Mycobacterium* infection is represented by an extinct bison dated 17,000 years BC (Rotschild et al., 2001). The excavation of human skeletal remains can confirm evidence of infection as a proportion of chronic cases will have bony lesions. The most characteristic parts of the skeleton involved are the spine, hip and knee joints, the hands and the feet, but any bone can involved. Typical lesions are destructive with some bone regeneration during healing. This may result in collapse of vertebral bodies causing angulation of the spine (kyphosis), ankyloses of joints and cavity formation in any bone. The presence of the disease was detected in an 5400-year-old Predynastic Egyptian human skeleton (Crubezy et al., 1998) and in ancient remains from the end of Predinastic time, from around 3000 BC (Zink et al., 2001). Further, precolumbian presence of the disease was found in Chile (Arriaza et al., 1995). European evidences of tuberculosis are from Dorset, England (400-230 BC) (Mays and Taylor, 2003) and from Lithuania (Faerman and Jankauskas, 2000) both from the Iron Age. Researchers have found the medieval presence of tuberculosis in England (Mays et al., 2001; Taylor et al., 1999) and in Hungary as well (Haas et al., 2000a).

3.4. Biomarker investigation of ancient remains

Recent advances in bioanalytical techniques especially in modern mass spectrometry (MS) have made the possibility to obtain sequence information from subpicomolar quantities of fragmented ancient proteins and peptides (Asara et al., 2007; Boros-Major et al., 2011; Buckley et al., 2008a, 2008b, 2009, 2010; Cappellini et al., 2012; Nielsen-Marsh et al., 2005, 2009; Orlando et al., 2013; Ostrom et al., 2006).

Certain proteins, which are characteristic for tumorous disease can be extracted from the diseased cells, were presumably transported earlier by the blood and absorbed to the bone hydroxyapatite (Schultz et al., 2007). Diagnosis of cancer as well as osteogenic sarcomas from ancient human skeletal remains is not an easy task by using classic morphological methods. Therefore a biomolecular approach to diagnosis in addition to osteological examination can be beneficial (Schultz et al., 2007). Recently, proteomic profiling of human tumors has provided a better understanding of the molecular pathogenesis of neoplastic diseases and has identified novel biomarkers for early diagnosis. SELDI-TOF-MS (Surface Enhanced Laser Desorption/Ionization Time of Flight Mass Spectrometry) and protein microarray high throughput analysis enable to detect biomarkers from the serum samples of osteosarcoma patients (Li et al., 2009). Based on the MALDI-TOF MS analysis of clinical benign bone tumor and malignant osteosarcoma several biomarkers are up- or down-regulated (Li et al., 2010). Also MALDI-TOF MS analysis of human osteosarcoma MG-63 cells showed the alterations of some genes and proteins (Zhao et al., 2006).

Mycobacterium tuberculosis has the property to disseminate in the body via the blood and lymphatic systems and replicate in bones, leaving characteristic lesions in it. These facts make the paleopathological/paleoepidemiological research of tuberculosis particularly timely. Unfortunately, there are several biases of the classical osteoarcheological studies of MTC, such as the differentiation between the taphonomic and paleopathologic conditions of the examined series, the problematic of the diagnostic criteria (Palfi and Molnar, 2009).

A biomolecular approach to diagnosis is probably more reliable than gross osteological examination of archaeological skeletal remains. The survive of pathological biomarkers in ancient skeletal remains is a major prerequisite for any molecular analysis and is thus essential for the pathological examination of archaeological bones. The use of DNA techniques to detect pathogenic agents in archaeological remains has exponentially increased recently. Numerous papers include detection of aDNA for tuberculosis (Crubézy et al., 2006; Donoghue et al., 2005; 2009; 2010; Gernaey et al., 2001; Haas et al., 1999; Hershkovitz et al., 2008; Taylor et al., 2007), leprosy (Haas et al., 2000b; Montiel et al., 2003; Rafi et al., 1994; Taylor et al., 2000; 2009), malaria (Sallares and Gomzi, 2001; Taylor et al., 1997), plague (Drancourt et al., 1998; Raoult et al., 2000) and syphilis (Kolman et al., 1999). However, many of these studies have been criticised and doubts have been cast authenticity of their results (Kolman and Tuross, 2000; Montiel et al., 2003; Wilbur et al., 2009).

However the analysis of *M. tuberculosis* is more reliable investigation method compared to the analysis of other possible biomarker for tuberculosis such as mycolic acid (Mark et al., 2010). Mycolic acids are long-chain fatty acids (up to 90 carbon atoms); these molecules are components of the mycobacterial cell envelope. In case of mycolic acids, clinical protocols and standards cannot directly be used for paleopathological samples (Minnikin et al., 2010) due to the chemical and biochemical processes e.g. amidation, carbamidomethylation, ester formation, fragmentation etc. taking place during hundreds of years. Another identification problem is that mycolic acid standards (Sigma-Aldrich) are not reproducible and do not correlate always with the authentic material (Mark et al., 2011). Only in cases where the mycolate profiles closely mirror those of modern standards of mycobacterial disease can be diagnosed with high confidence level.

3.5. Identification of proteins by mass spectrometry

3.5.1. Proteomics

The term „proteome” originates from the words protein and genome. It includes all of the proteins encoded by the genome in an organism. The genome of an organism is always static contrarily to its proteome, which is changing dynamic to one cell type to another and keeps changing even at different activity and development stages of the same cell type.

Proteomics deals with the identification, quantity, structure, function, localization, modifications and interaction of proteins. Proteins are major constituents of muscle, cartilage and bones. Proteins have various crucial functions, some of them serve as a catalyst for several biochemical reactions (e.g. enzymes), act as a transmitter (e.g. neurotransmitters), regulate cell reproduction, influence the growth and development of various tissues (e.g. trophic factors), participate in the transport of vital molecules (e.g. haemoglobin) or defend the body against disease (e.g. antibodies). Missing or defective proteins are the cause of many diseases and therefore serve as diagnostic indicators (biomarkers) and can help in drug development. The change in the proteome reflects also the differential activity of genes dependent on the cell type to express the protein needed for the particular function.

Furthermore, the protein profile of the cell can vary depending on the different modification of the protein such as acetylation, phosphorylation and glycosylation. These posttranslational modifications alter the functions of proteins.

Unequalled sensitivity, detection limits, speed and diversity of its applications made mass spectrometry an excellent tool for proteomic investigations.

3.5.2. Protein separation by gel electrophoresis

SDS-PAGE is the most commonly practised gel electrophoresis technique used for proteins. Electrophoresis is based on the migration of charged proteins in an electric field. The speed of migration depends on the charge, shape and size of the protein. Electrophoretic separations are generally carried out in gels made up of chemically inert, polymerization product of acrylamide. The polyacrylamide gel acts as a molecular sieve, the proteins migrate in proportion to their charge-to-mass ratio (Figure 8).

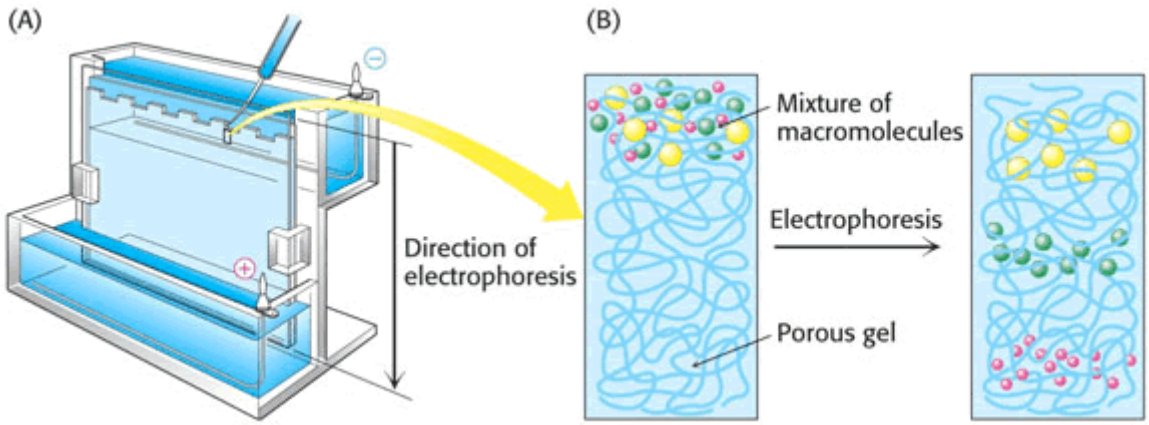


Figure 8: Sample loading (A) and the separation of macromolecules in gel electrophoresis (B) (http://elte.prompt.hu/sites/default/files/tananyagok/practical_biochemistry/ch07s03.html)

The proteins are first dissolved in SDS (sodium dodecyl sulphate) solution. SDS is an anionic detergent that disrupts the noncovalent interactions in native proteins. SDS divides hydrogen bonds, blocks hydrophobic interactions and substantially unfolds the protein molecules, eliminating the secondary and tertiary structures. SDS gives a complex with the denaturated protein, the complex is negatively charged. The net negative charge depends on the mass of the protein, because one molecule of SDS is connected to two amino acid residues. The negative charge of the complex is much greater than the charge on the native protein; this

native charge is thus rendered insignificant. DTT (dithiothreitol) is also added to the protein sample to reduce disulphide bonds between cysteine residues.

The protein samples are loaded into the gel pockets using Hamilton syringe. The electrical current in an electrophoresis cell is mainly transmitted by the ions supplied by buffer compounds; proteins contain only a small portion of current carrying ions. Moreover the buffers maintain the desired pH, and provide a medium for heat dissipation.

After electrophoresis, the proteins in the gel are visualized by adding a dye such as Coomassie Brilliant Blue. The detection limit of this staining method is 0.1 µg (~2 pmol). The mobility of the polypeptide chains is linearly proportional to the logarithm of their mass. The position of an unidentified protein is compared with the positions of proteins of known molecular weight migrate in the gel (molecular weight standard). For the digestion of the excised gel bands a proteolytic enzyme such as trypsin is used. Trypsin cleaves after arginine and lysine residues (Olsen et al., 2004) except if they are followed by proline.

3.5.3. General construction of a mass spectrometer

The basic functions of a mass spectrometer are: the production of gas-phase ions from sample molecules (in the ion source), the separation of the ions according to their mass-to-charge ratio (m/z) (in the analyser) and the detection of the separated ions. Mass spectrometers consist of three main parts: an ion source, a mass analyser and a detector (Figure 9). The instrument requires high vacuum (10^{-4}-10^{-7} mbar) provided by a roughing and a turbomolecular pump in two steps. The ions produced in the ion source have to go through the mass spectrometer without hitting air molecules, because collisions could neutralize the charge of the ions or change the path of the ions leading to the loss of sensitivity and reproducibility.

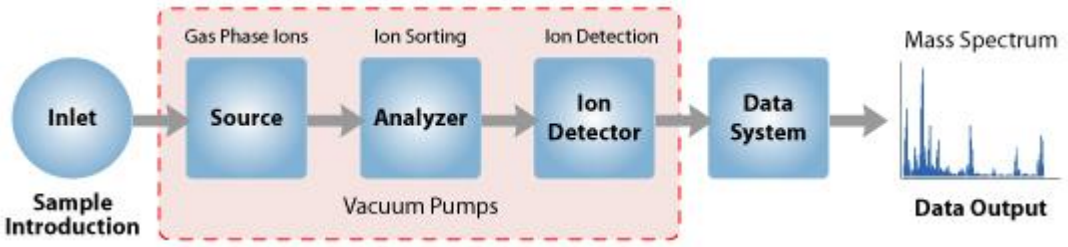


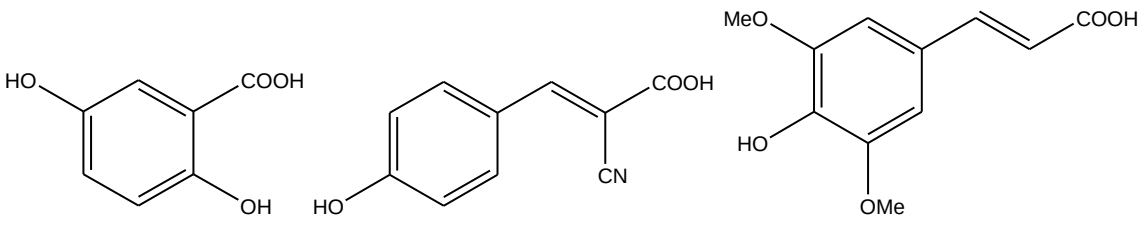
Figure 9: Schematic representation of a mass spectrometer. (http://www.premierbiosoft.com/tech_notes/mass-spectrometry.html)

For the ionization of gas phase analyte ions two methods are used. Positive ionization mode means generally the addition of an H^+ ion; negative ionization mode is achieved mostly by the removal of an H^+ ion. Proteins and peptides are usually analysed in positive ionization mode, because the NH_2 group of the proteins readily accepts H^+ ion. The most commonly used soft ionization methods are electrospray ionization (ESI) and Matrix Assisted Laser Desorption/Ionization (MALDI). The most often used analysers are time-of-flight (TOF), ion trap (IT) and quadrupole-time-of-flight (Q-TOF) analysers. The ion intensity at different m/z values is recorded normally at an electron multiplier type detector.

3.5.4. Matrix Assisted Laser Desorption/Ionization (MALDI)

Matrix Assisted Laser Desorption/Ionization (MALDI) is an ideal tool for the analysis of high molecular weight, non-volatile biopolymers, especially proteins and peptides. MALDI soft ionization method was developed at the University of Munster, Germany by Franz Hillenkamp and Michael Karas (Hillenkamp et al., 1990; Karas et al., 1987; Karas and Hillenkamp, 1988) simultaneously with Koichi Tanaka, Shimadzu Research Laboratories, Kyoto, Japan (Tanaka et al., 1988). While Tanaka used finely dispersed metal powder in a glycerol matrix for ionization upon irradiation by a pulsed UV laser, Hillenkamp worked with organic matrix to facilitate ionization and desorption of the sample for the same purpose.

MALDI matrices are aromatic, UV light (energy) absorbing molecules dissolved in a volatile and acidic solution. The matrix plays a key role in the absorption of the laser light energy and causing the analyte to vaporize. It also serves as a proton donor or acceptor ionizing the analyte either in positive or in negative ionization mode. The matrix is usually mixed with the sample on a target plate; this is the widely used dried droplet method. The solvent is evaporated before the sample is introduced into high vacuum by laser ablation. The most popular, frequently used matrices in proteomics are: α-cyano-4-hydroxycinnamic acid (CHCA), 2,5 dihydroxybenzoic acid (DHB) and sinapinic acid (SA) (Figure 10).



A) B) C)

Figure 10: Widespread used MALDI matrices: A) DHB, B) CHCA, C) SA

MALDI MS instrument generally equipped with time-of-flight (TOF) analyser, first published in 1946 by Stephens (Stephens, 1946). The pulse of nitrogen laser (=337nm) is focused onto the sample-matrix spot initiating ionization and desorption/ablation. Due to the extremely short laser shots the analyte avoids thermal degradation, longer irradiation cause only the heating of the bulk material. The ions formed are accelerated to the same kinetic energy. In a field-free drift tube they are separated in time according to their different m/z values, lighter ions reach the detector earlier than the heavier ones. The detector is usually a microchannel plate (MCP) detector which converts the ions to electrons; the whole process is based on the multiplication of electrons via secondary emission in order to increase the signal intensity of the detected ions. Single-shot spectra show a low signal-to-noise ratio, therefore 500-1000 single-shot spectra are usually accumulated to produce the final spectrum. MALDI has a large tolerance to contamination by salts, buffers and detergents (Chen et al., 1998; Stump et al., 2002).

Typical MALDI spectra include mainly the monocharged molecular species by protonation in positive ionization mode, deprotonated compounds are usually detected in negative ionization mode. The instrumental setup where the ions are travelling on a straight line from the point of their creation to the detector is called linear TOF. The resolution is limited because laser-desorbed ions possess large initial kinetic energy which is superimposed to their kinetic energy during the acceleration process. Higher acceleration voltage results higher resolution.

The reflectron/reflector has been developed by Mamyrin and coworkers in St. Petersburg (Mamyrin et al., 1973). The reflectron acts as an ion mirror that allows of the ions to penetrate the reflectron until they reach zero kinetic energy. A reflecton generally consists of a retarding electric field located behind the field-free region opposed to the ion source (Figure 11). The kinetic energy of the leaving ions remains unaffected; the time-of-flight depends only on the m/z of the ion, how deep the ion penetrates into the reflecting field. The more energetic ions will fly deeper into the electric field, therefore, have a longer flight path. The correction in initial kinetic energy provides higher resolution. MALDI TOF/TOF tandem mass spectrometers apply linear TOF as the first mass analyser and a reflectron TOF as the second mass analyser.

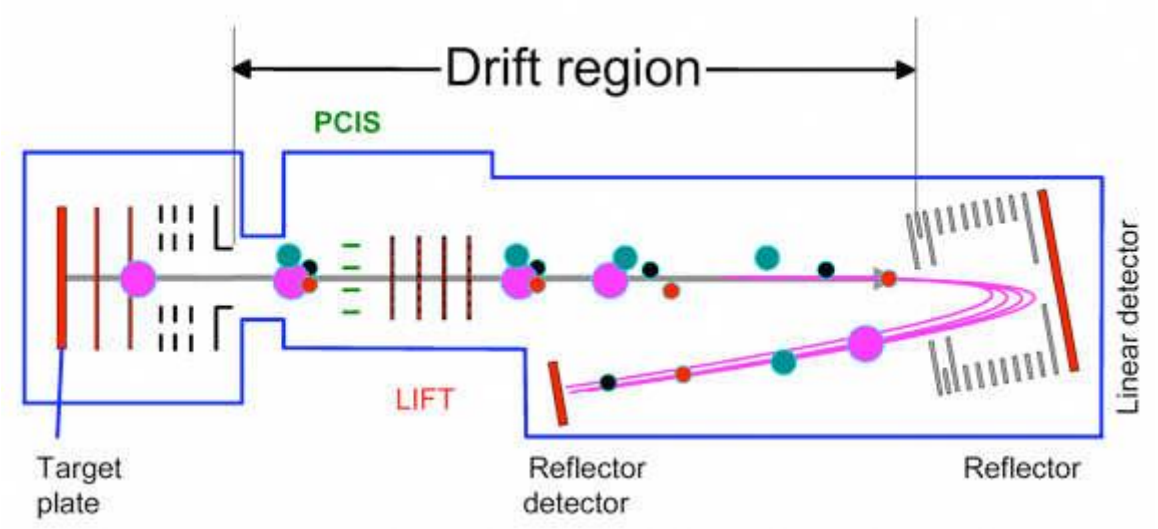


Figure 11: Construction of an MALDI TOF/TOF MS instrument. (http://www.giga.ulg.ac.be/jcms/cdu_15169/maldi-tof/tof-bruker-ultraflex-ii-tof/tof-april-2005)

The process of peptide mass fingerprinting (PMF) means the separation of proteins typically with 1-or 2D gel electrophoresis, enzymatic in-gel digestion (using e.g. trypsin) and the analysis of samples generally with the aim of an MALDI TOF/TOF MS instrument. Protein identification by means of peptide profile is performed by correlating experimental MS spectra with theoretical spectra using database search approach, such as SEQUEST (Eng et al., 1994) or Mascot search engine (Perkins et al., 1999). The most commonly used databases are the National Center for Biotechnology Information's (NCBI) (Pruitt et al., 2005) and Swiss-Prot (Boeckmann et al., 2003). The list of matches is ranked according to the scoring scheme; the best scoring peptide match has the highest likelihood of being correct. Mascot reports a match as significant if it has a match with a less than 5% chance of being a random hit ($p<0.05$). The chance of getting a random hit is proportional to the number of sequences being searched. One way to increase significance of a hit is to reduce the number of sequences being searched by restricting the search to a specific group of species (taxonomy).The disadvantage of this sensitive and quick protein identification process is the useless in the analysis of more complex protein mixtures.

MALDI TOF/TOF MS post source decay (PSD) is fragmentation process includes laser-induced dissociation (LID) and collision–induced dissociation (CID). In case of LID no gas is added to the collision cell, only variation of the laser fluence is employed to trigger the dissociation processes (Campbell et al., 2007; Macht et al., 2004). The parent ion fragments after reacceleration in the LIFT cell but prior to it reaches the entrance of the reflectron. Analyte ions have enough energy from photoactivation and molecular collisions during the desorption process (metastable ions) but they fragment only in the mass analyzer. The precursor ion and the precursor fragments are separated by their kinetic energy. It is used for obtaining sequence information (Spengler et al., 1991).

Collision–induced dissociation (CID) is the most frequently used fragmentation method in MALDI TOF/TOF MS due to the unimolecular bond dissociations (McLuckey, 1992; Shukla and Futrell, 2000; Suckau et al., 2003). During CID, a collision is induced between the selected precursor ion and the inert argon gas molecules in the collision cell. This process is followed by the decomposition of the precursor ion. The fragments will be separated by their mass / kinetic energy (Figure 12). CID is also suitable for gaining structural, sequence informations.

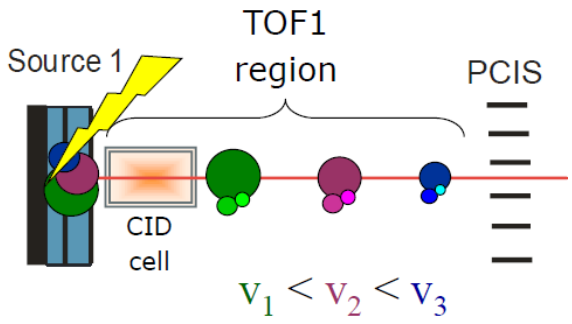


Figure 12: CID fragmentation process. (Bruker Daltonics)

High energy CID results mainly b-, y-ions (Figure 13), immonium ions and ions from neutral loss of ammonia and water from the b-, y-ions.

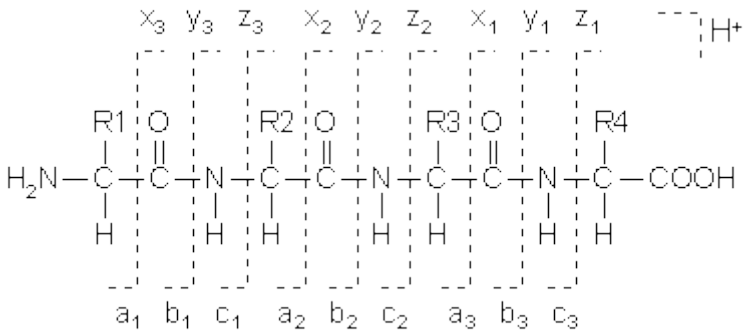


Figure 13: Nomenclature for peptide sequencing.
(http://www.matrixscience.com/help/fragmentation_help.html)

The bottom up MS/MS based protein identification method has become widely adopted (Figure 14). It is based on the digestion of proteins into peptides and the determination of their sequence using tandem mass spectrometry (MS/MS).The fragmentation pattern encoded by the MS/MS spectrum allows more precise identification of the amino acid sequence of the peptide that produced it. Sequence coverage is calculated by dividing the number of amino acids observed by the protein amino acid length. MS/MS identification is often used for protein mixtures. The MS/MS data acquisition process consists of two stages. The first stage involves reading all peptide ions that are introduced into the instrument at any given time (MS spectrum). At the second stage, selected peptide ions (often referred to as “precursor” or “parent” ions) are fragmented into smaller pieces (fragment ions). The resulting MS/MS spectra are compared with the theoretical MS/MS spectra from a protein database (NCBI, Swiss Prot) for identification.

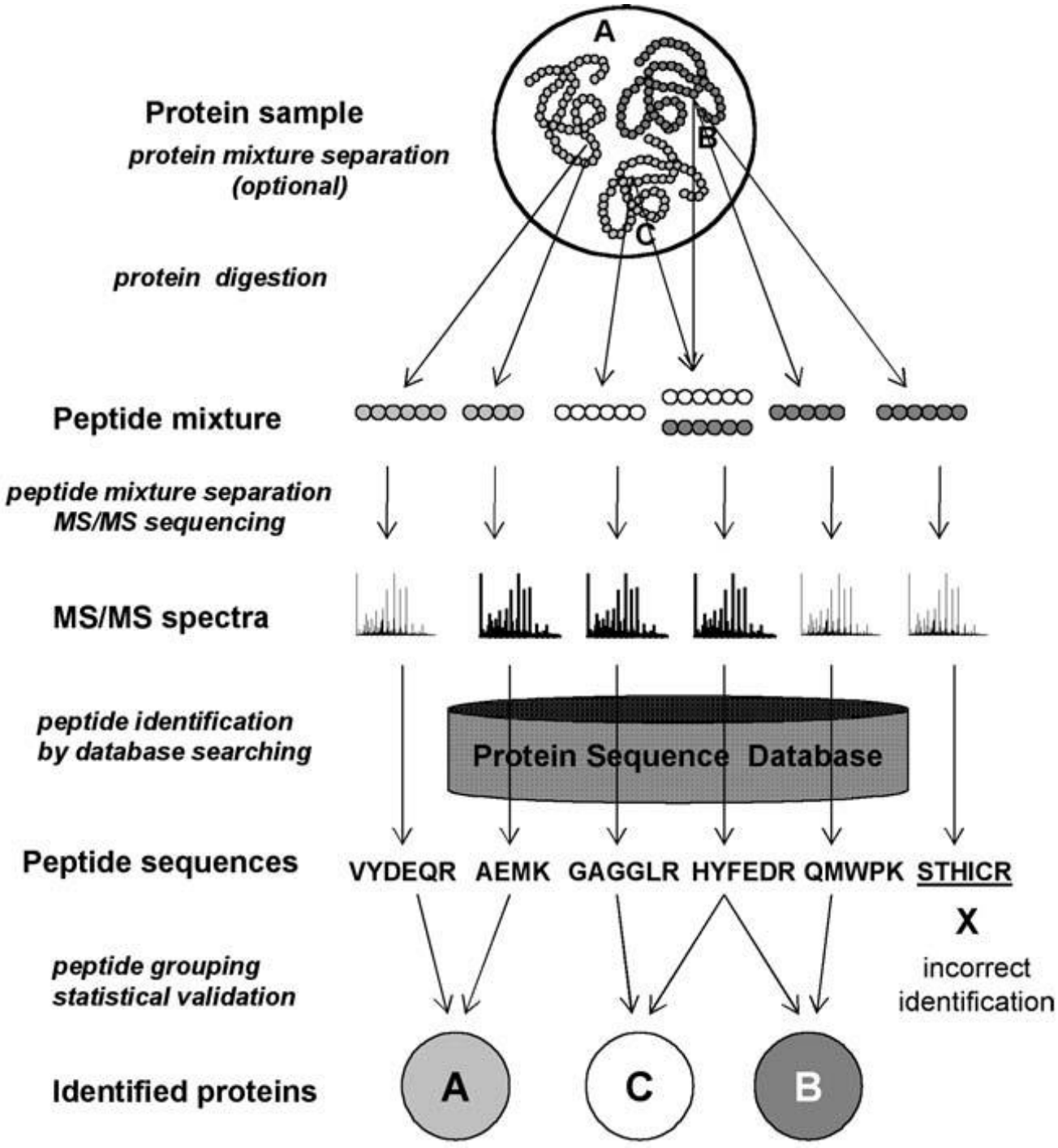


Figure 14: Steps of protein identification based on tandem mass spectrometry. (Matthiesen R., Mass Spectrometry Data Analysis in Proteomics)

4. Aims of the study

Biomarker discovery has become a crucial field of proteomics. Saliva has previously been analysed for identifying possible tumor markers by our research group. Our interest shifts towards the identification of protein-type disease biomarkers in ancient bone samples. MALDI TOF/TOF MS-based targeted peptide analysis method is cheap, non-invasive, easily automatable and high-throughput method. We investigated two different ancient diseases in archaeological human bone samples. One of them was tuberculosis, the most frequent infectious disease worldwide, caused by *Mycobacterium tuberculosis*. On the other hand osteosarcoma, a tumorous mutation was examined using mass spectrometry.

The aim of the study was:

- The extraction of ancient proteins derived from osteosarcoma and tuberculoid bone samples developing an optimized workflow.
- The separation of the extracted proteins based on their mass applying SDS gel electrophoresis.
- The mass spectrometric (MALDI TOF/TOF MS) analysis of the tryptic digested differently expressed bands (excised from the gel) compared to the control (healthy) samples.
- The identification of malignant bone tumor related molecular biomarkers and mycobacterial proteins in archaeological human skeletal remains utilizing Mascot and ProteinScape server database search engines.
- The statistical analysis of osteosarcoma, pathological (tuberculotic) and non-pathological control sample cohorts.

5. Materials and Methods

5.1 Archaeological bone samples

5.1.1. Osteosarcoma archaeological bone sample

The fragmented skeleton of a 25–35-year-old female with osteogenic sarcoma has been excavated at the Late Roman archaeological site of Szombathely (Savaria) - Szent Marton street 53, Hungary (municipal location code 6583/1) grave 186 (Figure 15).

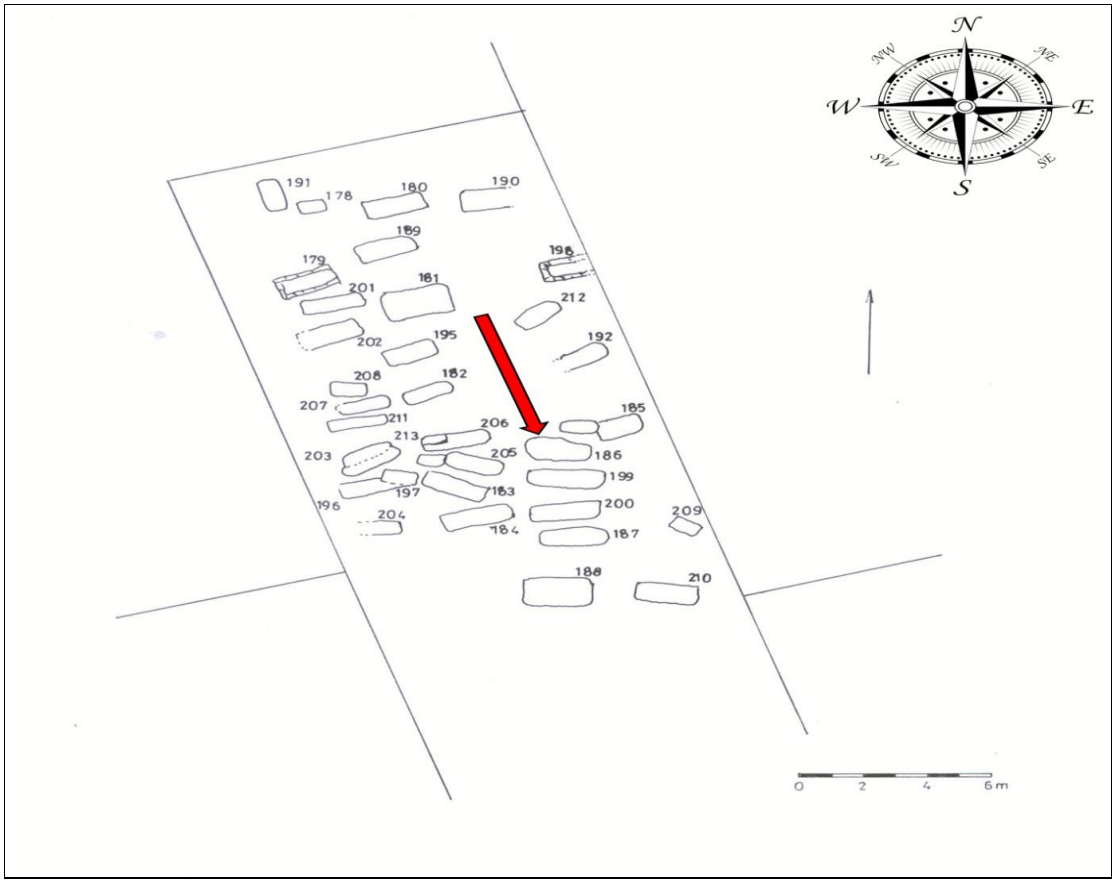


Figure 15: The line drawing map of the archaeological site. At this site we uncovered 33 graves from the late Roman period, arranged in four rows, very close to each other. Grave 186 (indicated with a red arrow) is located in the south-eastern section of the site, at 270-90°. The upper perimeter dimensions of the grave were 218 by 126cm, the base 185 by 120cm, with a depth of 48cm.

The analyzed bone sample was collected from the cortical region of the right humerus (Figure 16). The surface of the tumor was rough because of the irregular bone proliferation and also because of the destruction of the cortical region. The structure of the bone was typically expanded in the areas affected by the tumor.

Figure 16: The paleoanthropological sample chosen for mass spectrometric analysis. A) and B) Anterior and posterior views of the analyzed right humerus with osteogenic sarcoma. C) X-ray radiograph of the measured human remain. The red ellipse shows the location of the sampling.

The X-ray study demonstrates the medullary involvement, with mixed osteolytic and osteoblastic areas. Tumor infiltration of the cortex is also apperant as irregular rarefication and lytic lesions.

The anthropological (determination of sex and age, macroscopic and morphological examinations) and paleopathological investigations (X-ray radiography) were carried out based on Knussmann (Knussmann, 1988) and Jozsa (Jozsa, 2006). A humerus from a non-cancerous skeletal remain from this cemetery (grave 199, adult female) was used for the investigations as a control sample. For further statistical validation we used our previous proteomic results of some non-pathological and *M. tuberculosis* infected bone samples (Table 1 and 2).

Location	**Grave**	**Age**	**Sex**	**Period**
Mohacs	-	Adult	Male	Recent (forensic)
Bataszek	-	Adult	Male	Recent (forensic)
Bataszek	-	Senium	Male	Recent (forensic)
Mohacs	-	Adult	Female	Recent (forensic)
Pecs	-	Adult	Female	Recent (forensic)
Hodmezovasarhely-	16	Senium	Female	Neolithic
Mezokovesd Patakrajaro	4/a	Adult	Male	Chalcolithic
Kiskundorozsma	15	Adult	Female	Bronze Age
Algyo Barakktabor	4	Mature	Male	Scythian
Szegvar Oromdulo	918	Adult	Female	Sarmatian
Kishomok	89	Mature	Female	Gepids
Szegvar Oromdulo	740	Adult	Female	Early Avar
Szekkutas Kapolnadulo	30	Adult	Female	Late Avar
Kiskundorozsma	100	Senium	Male	Hungarian Conquest
Esztergalyhorvath	284	Adult	Female	Hungarian Conquest
Kecskemet Torokfaj	65	Mature	Female	Arpad Age, X-XIth AD
Szegvar Oromdulo	617	Mature	Female	Arpad Age, XIth AD
Derekegyhaza Ibolyas	13	Senium	Male	XI-XIIth AD
Csengele Bogarhat	57	Adult	Male	XIIIth AD
Kecskemet Ferences	350	Adult	Female	XIV-XVth AD

Table 1: The analyzed healthy control bone samples.

Location	Grave	Age	Sex	Period
Sukosd-Sagod	19	Adult	Female	VII-VIIIth AD
Sukosd-Sagod	19	Adult	Female	VII-VIIIth AD
Bacsalmas-	39	Mature	Male	XVIIth AD
Bacsalmas-	39	Mature	Male	XVIIth AD
Belmegyer-Csomoki	65	Mature	Female	VIIIth AD
Belmegyer-Csomoki	65	Mature	Female	VIIIth AD
Csongrad-Elles	183	Mature	Male	XI-XIIIth AD
Csongrad-Elles	183	Mature	Male	XI-XIIIth AD
Csongrad-Elles	183	Mature	Male	XI-XIIIth AD
Csongrad-Elles	183	Mature	Male	XI-XIIIth AD
Csongrad-Felgyo	205	Adult	Female	VIIIth AD
Csongrad-Felgyo	205	Adult	Female	VIIIth AD

Table 2: The measured pathological control (*Mycobacterium tuberculosis* infected) archaeological bone samples.

5.1.2. Tuberculoid archaeological bone samples

Various *Mycobacterium tuberculosis* infected and morphologically “healthy” human skeletal remains have been analyzed from different archaeological periods. The measured paleopathological bone samples are presented in Table 3. The human bone remains were provided by the Department of Anthropology, University of Szeged, Hungary (Marcsik and Palfi, 1993; Molnar and Palfi, 1994; Palfi, 1991).

No.	Location	Grave	Age	Sex	Anatomy	Date
1	Bélmegyer-Csömöki domb	65	30-40	Female	vertebra	700-800 AD
2	Csongrád-Ellés	183	40-45	Male	vertebra	1000-1200 AD
3	Bácsalmás-Homokbánya	39	40-50	Male	vertebra	1500-1600 AD

Table 3: Anthropological data of the measured *M. tuberculosis* infected archaeological skeletal remains.

The paleopathological remains were investigated for DNA of *Mycobacterium tuberculosis* complex (except Csongrád-Ellés) and the test was positive in all cases (Haas et al., 2000a; Maczel, 2003; Molnar et al., 2005). Furthermore, the presence of mycolic acids as lipid biomarkers for mycobacterial infection was described previously from these archaeological bone samples as well (Mark, 2007; Mark et al., 2010). The presence of mycolic acids cannot be detected in the control samples.

5.2. Extraction of ancient proteins

The sample preparation, separation and mass spectrometric analysis is of a vital importance for the quality of the results. In our previous study we developed an optimized workflow for proteomic analysis of ancient proteins (Boros-Major et al., 2011) (Figure 17). The bone fragments were washed with phosphate buffer saline (PBS) and distilled water to remove contaminants. Bone powder was ground by hand using pestle and an agate mortar, the particle size was ~0.2 mm. Next, 100 mg of crude bone powder was decalcified with 1.00 ml of 0.5 M EDTA (pH=8.0), the pellet was resuspended with 100 µl of 6 M guanidine-HCl in 0.1 M Tris (pH=7.5) at room temperature. The extraction of the proteins was carried out by continuous shaking at 4°C for 8 hours with the presence of protease inhibitor cocktail (Sigma Aldrich Kft., Budapest, Hungary).

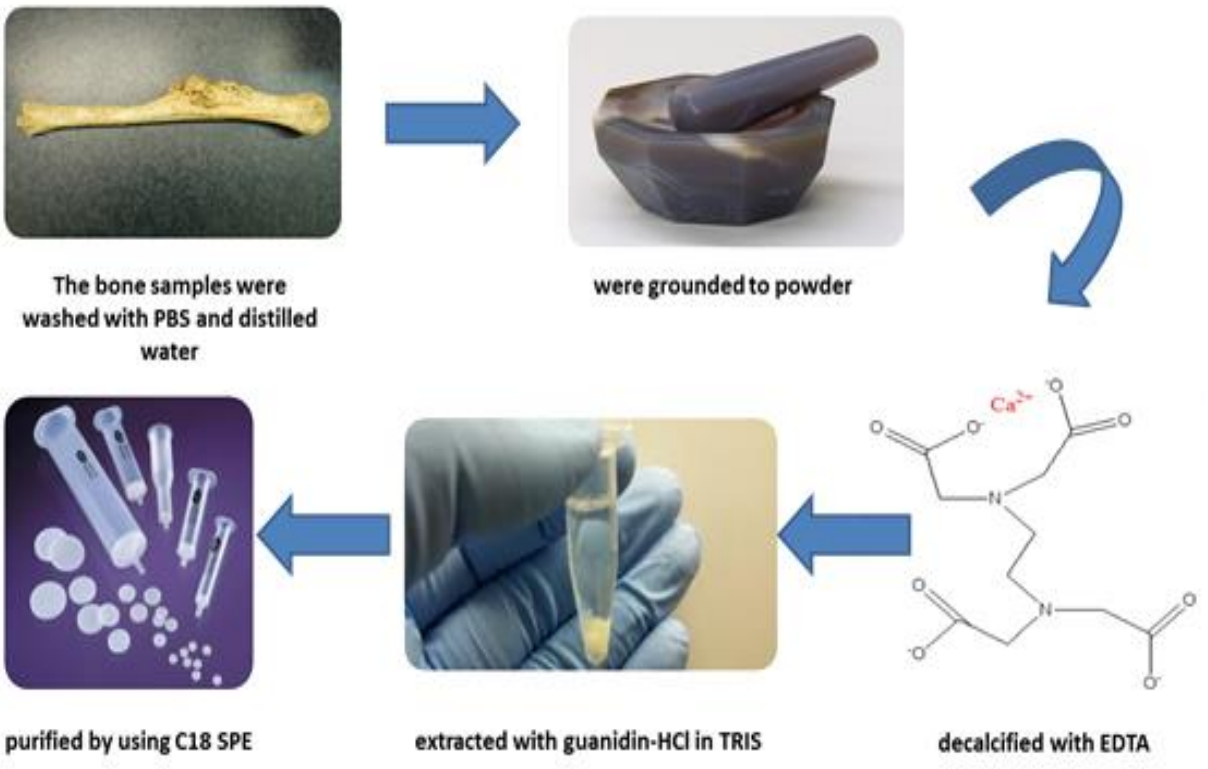


Figure 17: The steps of ancient protein extraction.

The protein extract was purified by using C18 solid phase extraction (SPE) cartridge. For this purification step a homemade octadecylsilane modified silica-based stationary phase was used with average particle size of 5 μm and pore size of 120 Å. The stationary phase was activated with an aqueous 0.1% TFA solution, the loaded protein extract was washed with 100 μl of 2% acetonitrile in 0.1% TFA three times. The proteins were eluted by 50 μl of 50% acetonitrile in 0.1% TFA solution.

The solution was lyophilized to powder and stored at -86°C until further processing. The bone sample with tumorous lesion was measured in eight technical replicates.

5.3. SDS-PAGE gel electrophoresis and enzymatic digestion

The lyophilized protein extract of the archaeological sample was dissolved in 100 µL of 20 mM Tris/HCl buffer, pH 7.4 containing 3 mM EDTA, 5 mM betamercaptoethanol and 1% sodium dodecylsulphate (SDS). After the addition of 1% bromphenolblue, the samples were boiled for 2 minutes and clarified by centrifuging (8000 g for 2 min).

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 12% gel by Laemmli's method. A low molecular weight calibration kit (Pharmacia) was used for estimation of the molecular weight. To increase the quality of the separation and visibility of the bands the gel was run at 4ºC. Gels were stained with Coomassie Brilliant Blue R-250 and destained with a solution containing 5% (v/v) acetic acid and 16% (v/v) methanol.

The bands of the overexpressed proteins (compared to the healthy archaeological samples) were excised from the gel with a razorblade, were cut into three pieces and placed in Eppendorf tubes, and destained by washing three times for 10 min in 200 µL of 50% (v/v) acetonitrile solution containing 50 mM NH_4HCO_3. The disulphide bonds of cysteines were then reduced by 50 µL of 20 mM dithiotreitol (DTT) in 100 mM NH_4HCO_3 and acetonitrile 5% for 1 h at 55°C. The alkylation of the cysteine groups was carried out in 50 µL of 20 mM iodoacetamide solution. The gel pieces were dehydrated at room temperature by a Speed Vac Concentrator (Speed Vac Plus, SC100A, Savant) and covered with 10 µL of modified trypsin (Promega, Madison, WI, sequencing grade) (0.04 mg / mL) in Tris buffer (2.5 mM, pH 8.5) and left at 37°C overnight. Trypsin as a serine protease, cleaves peptide chains after lysine or arginine (except they are followed by proline).

The digestion were stopped with 15 µL aqueous solution of acetonitrile and formic acid (49/50/1 v/v/v). The digested samples were incubated for 60 minutes at room temperature. In the last step the peptide solutions were lyophilized. The steps of in-gel digestion are shown in Figure 18.

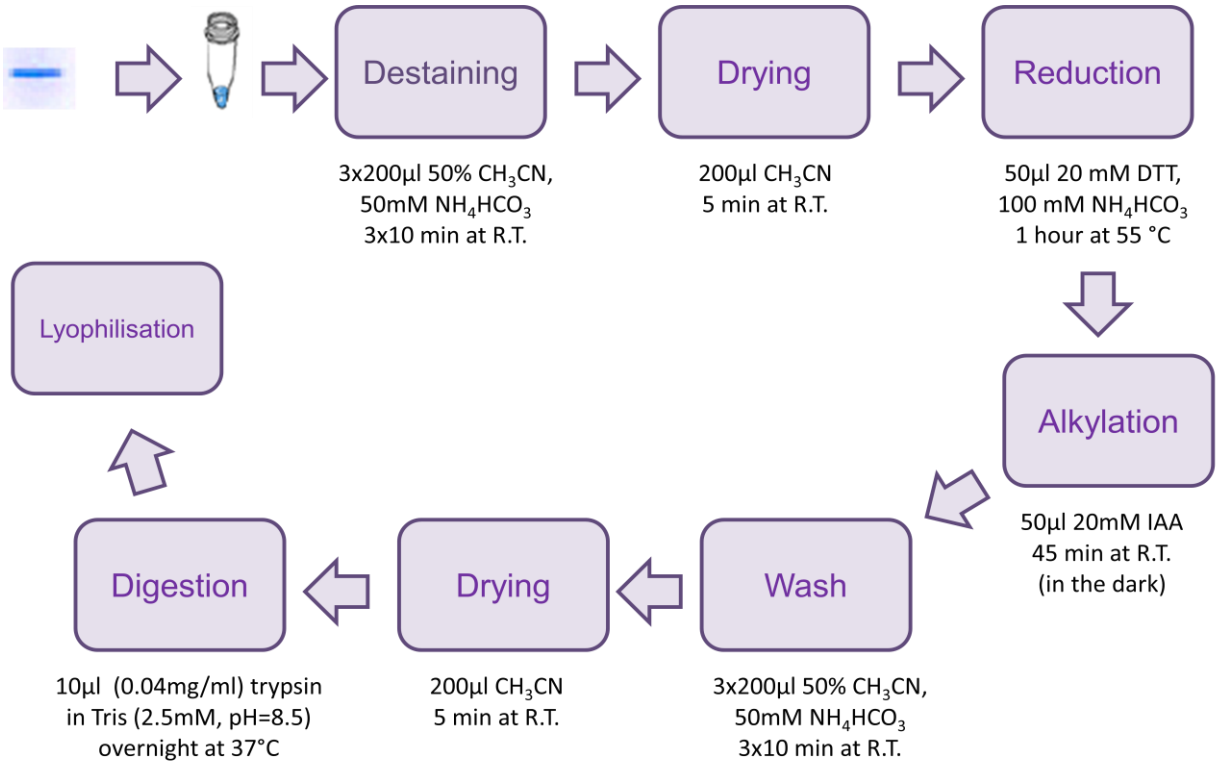


Figure 18: Flow diagram of in-gel digestion.

5.4. MALDI TOF/TOF MS-based identification of the ancient proteins

After lyophilization the samples were redissolved in 0.1% trifluoroacetic acid (TFA). The aqueous solutions of the lyophilized protein digests were concentrated and desalted by using C18 ZipTip SPE pipette tips (Millipore Kft, Budapest, Hungary). Then the purified peptides were eluted directly onto the target plate (MTP 384 massive target T, Bruker Daltonics, Bremen, Germany) by using of 3 µL of a saturated matrix solution, prepared fresh every day by dissolving α-cyano-4-hydroxycinnamic acid (CHCA) in acetonitrile/ 0.1% TFA (1/2, v/v). The mass spectrometer used in this work was an Autoflex II TOF/TOF (Bruker Daltonics, Bremen, Germany) operated in reflectron mode for peptide mass fingerprinting (PMF) or in LIFT mode for LID (laser induced decay) and CID (collision induced decay) fragmentation. The accelerating voltage was set to 20.00 kV. The instrument uses a 337 nm nitrogen laser (model MNL-205MC, Lasertechnik Berlin GmbH., Berlin, Germany). External calibration was performed in each case using Bruker Peptide Calibration Standard (#206195 Peptide Calibration Standard, Bruker Daltonics, Bremen, Germany). Peptide masses were acquired in the range of m/z 700 to m/z 5000. Each spectrum was produced by accumulating data from at least 500 consecutive laser shots. The FlexControl 2.4 software (Bruker Daltonics, Bremen, Germany) was used to control the instrument and FlexAnalysis 2.4 software (Bruker Daltonics, Bremen, Germany) was used for spectra evaluation. Singly charged monoisotopic peptide masses were searched against Swiss-Prot and NCBInr databases (last accessed: 11/19/2012) by utilizing the MASCOT database search engine (version 2.2) (www.matrixscience.com, Matrix Science Ltd., London, UK), Bruker BioTools 3.0 software (Bruker Daltonics, Bremen, Germany) and Bruker ProteinScape server 2.1 (Bruker Daltonics, Bremen, Germany). Maximum one missed tryptic cleavage was considered, and the mass tolerance for monoisotopic peptide masses was set to 80 ppm carbamidomethylation was set as global modification while methionine oxidation was set as variable modification. Additionally LID and CID fragmentation of the matched peptides were carried out for MALDI TOF/TOF MS to provide further evidence for the presence of the identified proteins.

5.5. Statistical analysis

Mass spectrometric techniques play an emerging role in biomarker research due to their high mass accuracy, sensitivity, and speed of detection. The ClinProTools software builds on mass spectrometric results and allows researchers to directly evaluate peptide/protein profiles derived from different clinical samples. The ClinProTools software provides highly sophisticated bioinformatics tools, multivariate statistics enables the analysis and discovery of hidden and complex peak patterns leading to class prediction.

Statistical analysis was carried out only to demonstrate the predictive value of the identified biomarkers the mass spectrometric results were statistically evaluated by ClinProTools 2.2 (Bruker Daltonics, Bremen, Germany) clustering software. Multiple spectra of the analyzed bone samples from different sample cohorts, such as osteosarcoma, non-pathological and pathological (tuberculotic) control samples were distinguished together. Recalibration, spectral alignment, peak normalization, peak detection and peak area calculation of spectra were carried out automatically by ClinProTools. A logistic regression model was performed to identify the significant predictive peaks on the basis of the normalized peak areas. Wilcoxon signed-rank test was used for non-parametric statistical analysis of the different sample cohorts.

The Wilcoxon rank-sum test is a non-parametric alternative to the paired Student's t-test. The null hypothesis tested is that a sample is symmetrically distributed around a specified center. Scores exactly equal to the central point are excluded and the absolute values of the deviations from the central point of the remaining scores are ranked such that the smallest deviation has a rank of 1. Tied scores are assigned for a mean rank. The sums for the ranks of scores with positive and negative deviations from the central point are then calculated separately. A value S is defined as the smaller of these two rank sums. S is then compared to a table of all possible distributions of ranks to calculate p, the statistical probability of attaining S from a population of scores that is symmetrically distributed around the central point. As the number of used scores, n, increases, the distribution of all possible ranks S tends towards the z-distribution, so for an n of greater than 10 this distribution is used to calculate p. This test assumes that the compared sample sets originate at least from a common distribution.

6. Results and Discussion

6.1. Osteogenic Sarcoma

This work partly focused on the identification of possible protein biomarkers of osteogenic sarcoma from a 2000-year-old anthropological sample. The proteins were separated by 1D gel electrophoresis (Figure 19) and the interested bands were enzymatically digested.

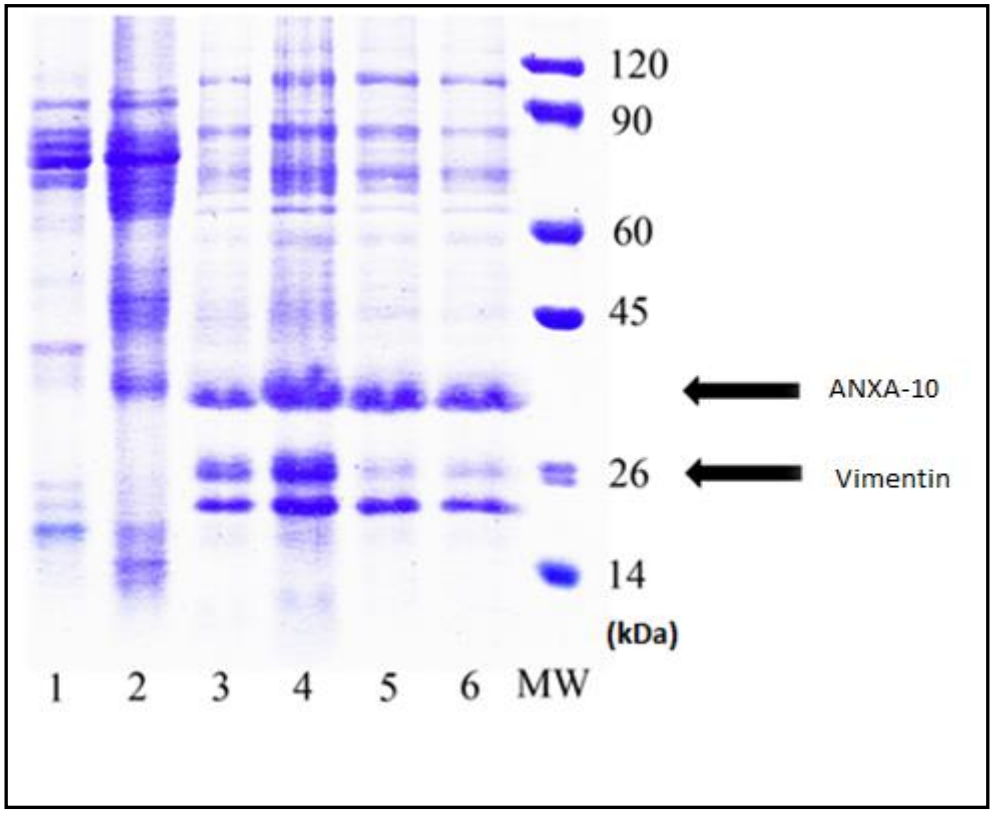


Figure 19: Characteristic 1D SDS PAGE electrophoretogram of healthy control and the pathological bone samples. Lanes 1 and 2 are healthy control samples from Hodmezovasarhely-Gorzsa and Szegvar-Oromdulo, lanes 3–6 are osteosarcoma samples. Arrows show the bands of Annexin A10 (37.3 kDa) and Vimentin (26.8 kDa). The parameters of the separation are mentioned in the text.

The tryptic peptides were analyzed by MALDI TOF/TOF MS and the identification of the resulted proteins was carried out using a PMF or MS/MS search. Based on our results several known, previously published osteosarcoma or tumor related proteins and gene products were detected from the ancient pathological bone sample (Table 4).

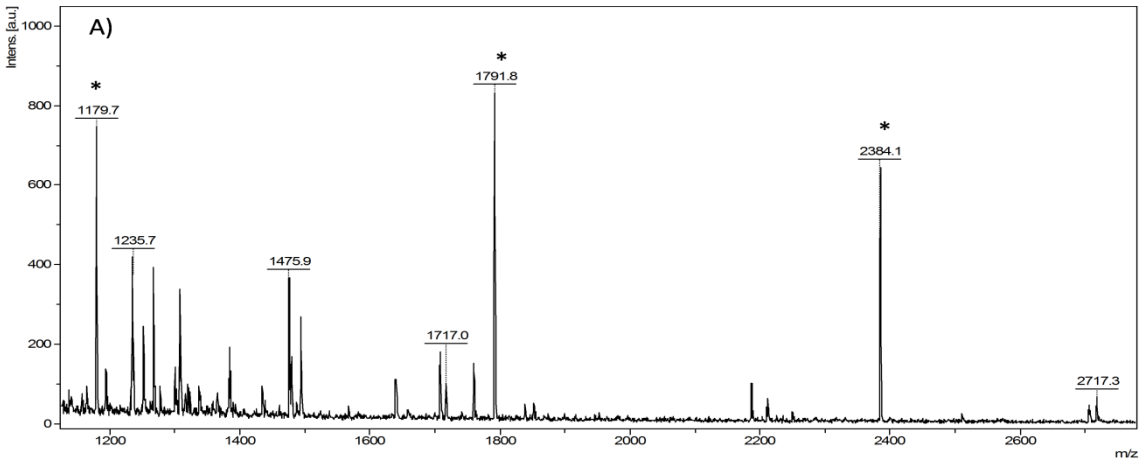
Accession	Name	MW [kDa]	Peptides	SC [%]
AK1A1_HUMAN	Alcohol dehydrogenase (NADP+)	36,5	5	14,2
gi|225939	aldehyde reductase	36,3	5	14,2
gi|48762937	annexin A10	37,3	8	27,2
gi|62087532	arginine/serine-rich splicing factor 6 variant	31,8	6	27,6
ARI5B_HUMAN	AT-rich interactive domain-containing protein 5B	132,2	11	13,0
gi|33878074	BAT2 protein	17,1	5	29,9
gi|49456879	BCL2A1	20,3	7	44,0
VMDL3_HUMAN	Bestrophin-4	76,1	7	14,5
gi|882391	bone morphogenic protein type II receptor	59,9	6	13,2
S10AB_HUMAN	Calgizzarin (S100 calcium-binding protein A11)	11,7	4	43,8
CAN7_HUMAN	Calpain-7	92,6	7	12,3
K1C10_HUMAN	Cytokeratin 10	59,5	14	23,1
gi|33188433	deleted in liver cancer 1 isoform 1	170,5	13	7,9
gi|28704113	DHX8 protein	138,7	13	13,8
G59435	DLC-1	122,7	10	10,9
DNL3_HUMAN	DNA ligase III	102,6	10	16,3
TDT_HUMAN	DNA nucleotidylexotransferase	58,4	9	22,2
MP2K6_HUMAN	Dual specificity mitogen-activated protein kinase kinase 6	37,5	7	23,4
DTNA_HUMAN	Dystrobrevin alpha	83,9	6	10,5
gi|15010856	galectin-12 isoform d	30,0	5	30,5
GCC2_HUMAN	GRIP and coiled-coil domain-containing protein 2	184,5	18	12,3
gi|40555827	heat shock factor protein 2 isoform c	27,0	7	23,9
HSPB6_HUMAN	Heat-shock protein beta-6	17,1	5	35,0
CAC10772	Immunoglobulin heavy chain variable region	12,4	5	65,5
gi|17318569	keratin 1	66,0	15	25,8
Q8N175_HUMAN	Keratin 10	58,8	14	23,5
gi|31559819	keratin 25C	49,8	8	17,2
gi|47132620	keratin 2a	65,4	9	16,4
CAA82315	keratin 9	62,1	11	16,9
A44861	keratin, 67K type II epidermal	65,8	9	16,3
K1C9_HUMAN	Keratin, type I cytoskeletal 9	61,9	11	20,7
AAP97338	Methyl-CpG-binding domain protein 4	60,9	13	16,3
NEBL_HUMAN	Nebulette	116,4	14	16,7
NRAP_HUMAN	Nebulin-related-anchoring protein	197,0	24	14,9
gi|4506335	parvalbumin	12,1	8	56,4
PRVA_HUMAN	Parvalbumin alpha	11,9	8	56,9
gi|39653323	PHD finger protein 20-like 1 isoform 1	47,7	7	15,8
gi|39653321	PHD finger protein 20-like 1 isoform 3	16,6	6	42,0
gi|31873386	phospholipase C	39,7	7	30,7
gi|346323	phosphoprotein phosphatase (EC 3.1.3.16) X catalytic chain	35,1	6	26,1
PDIP2_HUMAN	Polymerase delta-interacting protein 2	42,0	7	22,0
CAF00150	Proteasome subunit beta type 3	16,6	5	34,9
gi|565647	proteasome subunit HsC10-II	22,9	5	25,4
PARK7_HUMAN	Protein DJ-1 (Oncogene DJ1)	19,9	5	32,3
gi|55859594	PTAR1 protein	32,4	7	30,1
gi|4960030	Rab GDP dissociation inhibitor beta	41,0	6	22,0
gi|39841018	RAB GTPase activating protein 1-like	92,5	8	10,3
gi|537327	receptor tyrosine kinase	18,5	6	30,0
gi|2665850	rheumatoid factor RF-ET7	10,9	6	74,5
RHG07_HUMAN	Rho GTPase-activating protein 7	122,7	10	10,9
gi|41152086	serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 6	42,6	9	29,3
gi|38382764	SET-binding protein isoform b	26,4	7	25,6
gi|46250431	Transcription factor NRF	77,7	11	20,4
gi|62088924	Transducin-like enhancer of split 3 splice variant 1 variant	22,9	6	37,1
gi|37747855	Transferrin	77,0	10	21,9
gi|4507659	translocated promoter region (to activated MET oncogene)	265,4	24	12,0
gi|4827050	ubiquitin specific protease 14	56,0	8	20,6
gi|71774197	ubiquitin specific protease 47	147,1	10	13,4
gi|34532272	unnamed protein product (homolog of CCDC144A protein)	56,3	12	21,9
gi|21754902	unnamed protein product (homolog of Zinc finger protein 781)	17,8	7	48,7
gi|57471648	vimentin	26,8	8	38,2
WBP4_HUMAN	WW domain-binding protein 4	42,5	7	15,7
ZN224_HUMAN	Zinc finger protein 224	82,2	10	19,4
gi|74355161	Zinc finger protein 624	85,6	11	22,5

Table 4: The identified up-regulated proteins from 2000-year-old osteogenic sarcoma. (Mascot score > 50)

The identified annexins (ANXs) are calcium and phospholipid binding proteins, they play a crucial role in the exocytic and endocytic transport, regulation of cell growth, proliferation and apoptosis. It is well known, that the increased level of ANXs (ANXA-10) indicates tumor progression (Kim et al., 2010; Shimizu et al, 2012). The B-cell lymphoma 2-related protein A1 (BCL2A1) is a member of BCL2 proteins. BCL2A1 is responsible for the separation of pro-apoptotic BCL2 proteins. BCL2A1 shows an elevated level in case of different cancer types such as leukemia and lymphoma, also connected with autoimmunity and therapy resistance of different tumors (Bolden et al., 2013; Ottina et al. 2012; Vogler, 2012; Willimott and Wagner, 2012). Calgizzarin (S100A11) belongs to the calcium binding proteins S100 family, involved in cell growth, motility and differentiation. Calgizzarin has been correlated with tumor progression and metastasis (Melle et al., 2006; Rehman et al., 2004) DLC1 is a known tumor suppressor, acting through Rho GTPase-activating protein (RhoGAP), which is involved in the proliferation and migration of tumor cells, induces apoptosis in vitro (Kim et al., 2009; Yang et al.,2011; Zhang et al., 2009). Heat-shock protein's (HSP's) expression increases in case of thermal, physiological or other stress factors allowing the cells to survive lethal conditions. HSP's play key role in the apoptotic and cell death process (inhibition of caspase activation). Elevated level of HSP beta-6 was found in clinical samples of patients who suffered from osteosarcoma (Folio et al., 2009; Suehara et al., 2012). DJ-1 protein is a mitogen-dependent oncogene involved in ras-related signal transduction pathway. Overexpression of DJ-1 indicates tumorous mutation (Niforou et al., 2008; Tian et al., 2008). The RhoGAP family proteins play an important role in regulating cell migration, cell morphology and cytoskeletal organization. Down regulation of RhoGAP proteins decrease the tumor suppressive effect (Li et al. 2009). Transferrin is a member of iron-binding blood plasma glycoproteins, which is responsible for the regulation of the free iron content in the blood. Elevated level of transferrin is correlated with tumorous diseases such as osteosarcoma (Li et al., 2010; Suehara et al., 2012). The expression of a cytoskeletal intermediate filament protein vimentin (VIM) was also shown to increase in case of osteosarcoma (Flores et al., 2012; Kang et al., 2006; Li et al., 2010; Suehara et al., 2012) VIM is considered to be a tumor biomarker, as it is promoting the metastatic spread of the tumor cells. In this study, some keratins were identified as well. The origin and the importance of these proteins are not well known; probably the identified keratins are from recent or contemporary

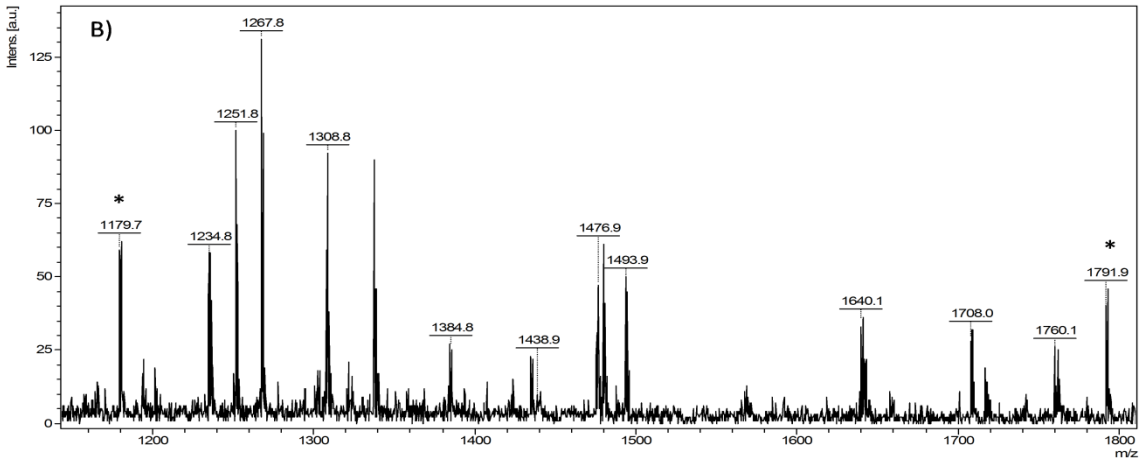
contaminations. However, the up-regulation of cytokeratins has been published in U2OS osteosarcoma specific cell line (Niforou et al., 2008).

Representative mass spectra and the list of the identified tryptic peptides of annexin A10 and vimentin are showed on Figure 20. The results show characteristic peaks of the identified biomarkers, however some keratin peaks are present on the spectra. This pollution is common from ancient bone tissue, but various types of keratins are up-regulated in different tumors as well.



Position	Molecular mass (Da)	Sequence	MS/MS	Modification
81-103	2717.24	DVMAGLMYPPPLYDAHELWHAMK		
193-202	1194.69	TMLQMILCNK	+	
193-202	1251.68	TMLQMILCNK		CAM(C)
193-202	1267.72	TMLQMILCNK		CAM(C)+ OX(M)
212-227	1760.66	QEFQNISGQDMVDAIN		
233-243	1234.78	VDAINECYDGY		
293-302	1235.60	YGKSLFHDIR	+	
296-310	1791.73	SLFHDIRNFASGHYK		
311-324	1716.95	KALLAICAGDAEDY	+	
313-324	1475.87	LLAICAGDAEDY	+	

Figure 20: Representative mass spectra and the list of the identified tryptic peptides of two identified tumor biomarkers. A) Annexin A10, B) Vimentin. Some keratin contamination has been detected in the sample, the tryptic peptides of keratin were used as internal calibration standards and the peaks are marked with asterisk.



Position	Molecular mass (Da)	Sequence	MS/MS	Modification
3-14	1639.91	NCFRLQEEMLQR	+	CAM(C)+ OX(M)
15-25	1323.66	EEAENTLQSFR		
89-100	1393.71	DVRQQYESVAAK		
111-122	1308.64	SKFADLSEAANR	+	
209-220	1439.79	MALDIEIATYRK		OX (M)
220-228	1060.63	KLLEGEESR		

Figure 20 (continued)

Based on our statistical analysis the different sample cohorts such as osteosarcoma, healthy control and tuberculotic control could be distinguished, using two peptide peaks (m/z 1180, 1385) derived from the investigated osteogenic bone sample. The spectral profile of the samples with osteosarcoma is significantly different as the other two group's profiles (Figure 21).

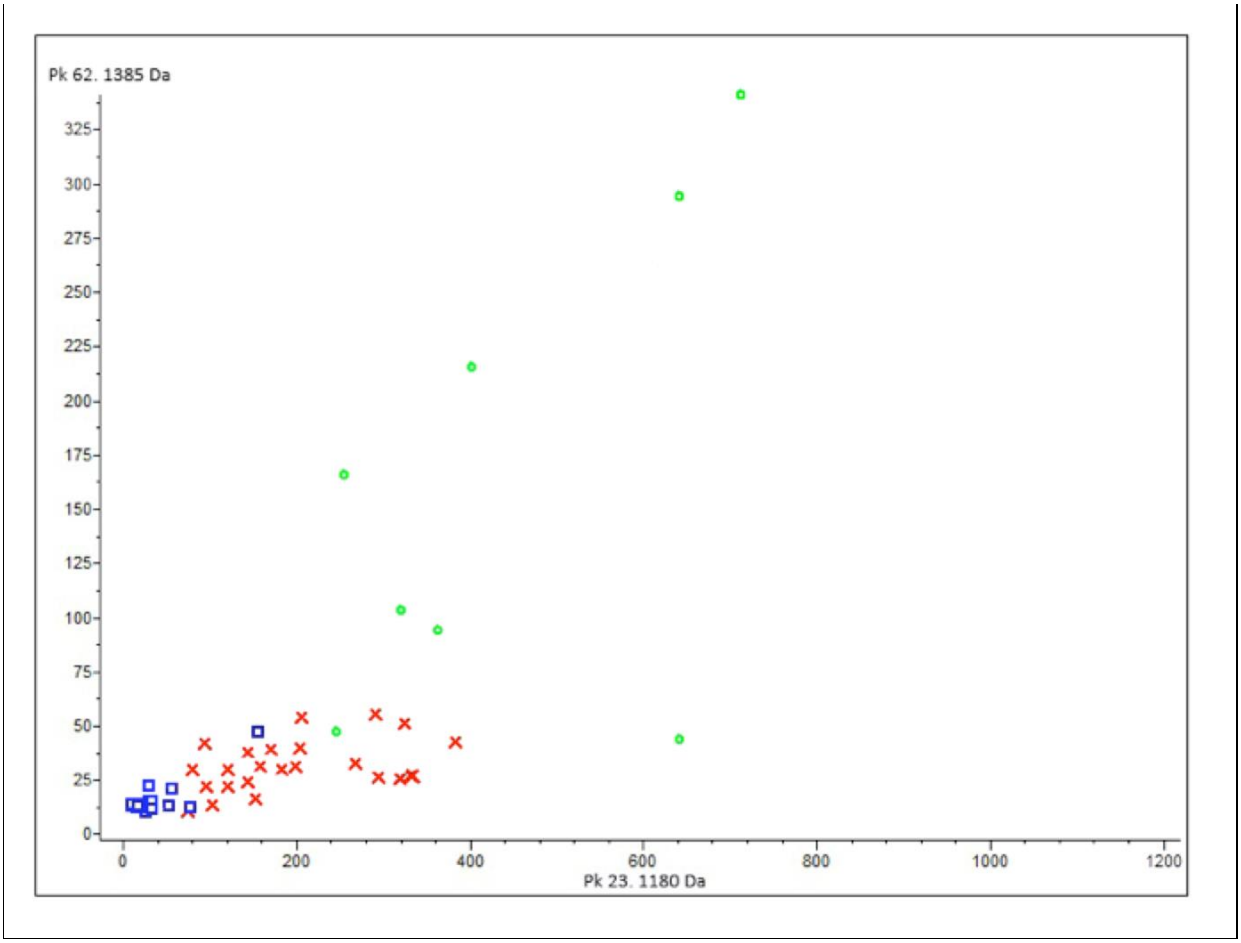


Figure 21: ClinProTools-based Wilcoxon non-parametric statistical test of different sample cohorts. Cluster analysis from sample sets of the osteosarcoma (green), healthy control sample (red) and tuberculotic control sample (blue) groups using the peptide peaks with m/z 1180 and 1385. The x-and y-axes correspond to the relative intensities of the peptide peaks.

6.2. Tuberculosis

Further target of our interest was the identification of ancient mycobacterial proteins. The protein bands were excised and digested with trypsin, and the resulting peptides were analyzed by MALDI TOF/TOF MS for protein determination. The identifications of *Mycobacterium* specific proteins using peptide mass fingerprint (PMF) analysis and direct sequencing of the tryptic peptides were successful in all investigated tuberculosis infected archaeological bone samples.

The proteomic results from the oldest investigated bone sample (Bélmegyer-Csömöki domb) have been showed in Table 5.

Protein ID	Accession no.	Theoretical MW (Da)	Mascot score	Peptides matched	Sequence coverage (%)
Adenylate kinase (*M. tuberculosis*)	gi|15607873	20113	263	14	65
Hypothetical protein (*M. tuberculosis*)	gi|254232569	50448	71	8	15
LysR family transcriptional regulator (*M. tuberculosis*)	gi|15607518	33616	68	8	29
Putative helicase (*M. tuberculosis*)	gi|260187090	111734	113	19	25
Translation initiation factor IF-2 (*M. tuberculosis*)	gi|215431780	54012	95	10	32

Table 5: Proteomic parameters of the identified mycobacterial proteins of the archaeological skeletal remain from Bélmegyer-Csömöki domb grave 65. Experimental conditions were mentioned in the text.

In this sample numerous mycobacterial proteins were identified such as adenylate kinase, LysR family transcriptional regulator protein, putative helicase, translation initiation factor IF-2 protein and a hypothetical protein. A characteristic mass spectrum has been displayed in Figure 22.

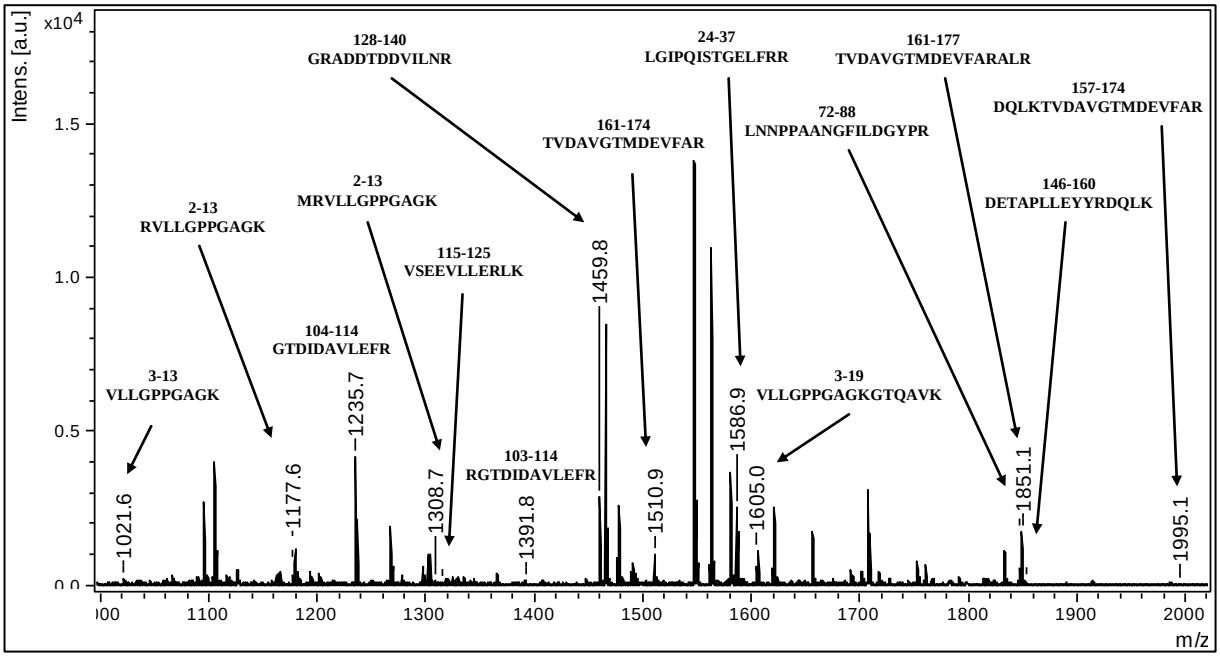


Figure 22: Mass spectra of skeletal remain from Bélmegyer-Csömöki domb grave 65.

The *Mycobacterium tuberculosis* related proteomic results of the other two archaeological sites (Csongrád-Ellés and Bácsalmás-Homokbánya) have been summarized in Table 6 and 7.

In these cases several microbial enzymes (catalase-peroxidase-peroxinitritase-T katG, dehydrogenase/reductase, fumarate reductase flavoprotein subunit, glycosyl transferase, oxidoreductase, peptide sythetase nrp) as well as mycobacterial regulatory and hypothetical proteins were identified.

Protein ID	Accession no.	Theoretical MW (Da)	Mascot score	Peptides matched	Sequence coverage (%)
Catalase-peroxidase-peroxinitritase-T katG (*M. tuberculosis*)	gi|219557862	51220	62	8	24
Fumarate reductase flavoprotein subunit (*M. tuberculosis*)	gi|15608690	63723	163	18	34
Hypothetical protein (*M. tuberculosis*)	gi|15843166	37267	85	10	28
Oxidoreductase (*M. tuberculosis*)	gi|15610689	36808	86	10	28
Peptide synthetase nrp (*M. tuberculosis*)	gi|254233496	269241	122	23	11

Table 6: Proteomic parameters of the identified mycobacterial proteins of the archaeological skeletal remain from Csongrád-Ellés grave 183. Experimental conditions were mentioned in the text.

Protein ID	Accession no.	Theoretical MW (Da)	Mascot score	Peptides matched	Sequence coverage (%)
Dehydrogenase/reductase (*M. tuberculosis*)	gi|218751933	32749	101	10	40
Fumarate reductase flavoprotein subunit (*M. tuberculosis*)	gi|15608690	63723	113	15	30
Glycosyl transferase (*M. tuberculosis*)	gi|218754716	20715	91	9	47
Hypothetical protein (*M. tuberculosis*)	gi|215428650	36324	93	12	31
Regulatory protein (*M. sp. JLS*)	gi|126436787	28284	119	8	43

Table 7: Proteomic parameters of the identified mycobacterial proteins of the archaeological skeletal remain from Bácsalmás-Homokbánya grave 39. Experimental conditions were mentioned in the text.

Tuberculoid bone samples represent different eras and different regions of Hungary and that serves as an explanation for the difference between the protein profiles.

In the case of the morphologically “healthy” control samples various types of human keratins, collagens were detected, but *M. tuberculosis* proteins cannot be identified.

The suggested proteomic approach by using tandem mass spectrometric sequencing of the peptides can be feasible for species identification as previously described in zoological samples (Buckley et. al., 2008a, 2009). All identified bacterial proteins are from human pathogen Mycobacterium species and consequently, the contamination with other e.g. soil living mycobacteria can be easily controlled and eliminated by using their different amino acid sequences. Namely, the taxa specific distinguishing of the referred and partly sequenced mycobacterial proteins can be easily carried out by using their exact amino acid sequences and the homology search of the proteins and peptides (e.g. NCBI BLAST).

7. Conclusions

- Osteosarcoma and tuberculosis-related proteins were identified from archeological bone samples using MALDI TOF/TOF MS for the first time.
- One of our goals was to find potential tumor biomarkers for ancient osteosarcoma using healthy bone samples as a control. Using an appropriate extraction method, developed by our research group, followed by SDS PAGE, tryptic digestion and MALDI TOF/TOF MS analysis, we were able to identify several proteins that were tightly connected to tumorous mutations. The overexpression of annexin A10 protein, BCL-2-like protein, calgizzarin, HSP beta-6, RhoGAP-activating protein 7, transferrin, and vimentin among others referred to healthy samples may indicate the presence of tumor in bones.
- In this study, we demonstrated that the peptide profile of the samples with osteosarcoma is statistically unique and it could be distinguished from other sample cohorts such as non-pathological (healthy) and pathological (tuberculotic).
- On the basis of our results the proteomic analyses could indicate the presence of osteosarcoma in bone tissues. Our findings showed that the well known, osteosarcoma-related clinical protein biomarkers are detectable in the investigated 2000-year-old tumorous skeletal remain. In the future, additional comparative proteomic investigations are needed for the further early stage biomarker discovery of ancient primary bone cancer.
- As further target of our investigations several mycobacterial proteins have been extracted and identified by MALDI TOF/TOF MS from archaeological human skeletal remains also for the first time.

- Our results suggest that the protein extraction, purification and identification from ancient skeletal remains can be carried out not only for the most abundant bone proteins, but also at the subproteome level. The identification of *Mycobacterium tuberculosis* proteins as molecular biomarkers avoids the recent problems of PCR-based mycobacterial aDNA analysis. The presented MS-based proteomic method provides species specific results, accordingly the different human pathogens (e.g. *M. tuberculosis, M. bovis, M. leprae*) can be distinguish together with this technique, furthermore the negative effect of the possible recent and contemporary contaminations can be minimized.

The present studies showed, that the MS-based protein analysis of the ancient proteins is a powerful technique for paleopathological examinations. It is a well known fact that the evolution of *M. tuberculosis* as well as *M. leprae* is unclarified. This complex problem can be feasible by using proteomics and genomics of the recent and ancient strains. Moreover, the paleoproteomic analysis can be an independent, robust, high-throughput technique for confirmation of the presents of human pathogen *Mycobacterium* species.

Our proteomic results could enhance the diagnosis of osteogenic tumors in ancient human skeletal remains. Furthermore the identification and sequencing of ancient mycobacterial proteins with further biomolecular techniques (aDNA, determination of lipid biomarkers) have the potential to expand our understanding of ancient epidemiology and evolution of these pathogens.

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10. List of Publications

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1. Bona A, Papai Z, Maasz G, Toth GA, Jambor E, Schmidt J, et al. Mass spectrometric identification of ancient proteins as potential molecular biomarkers for a 2000-year-old osteogenic sarcoma. PLoS One. 2014;9(1):e87215.
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2. Boros-Major A, **Bona A**, Lovasz G, Molnar E, Marcsik A, et al. New perspectives in biomolecular paleopathology of ancient tuberculosis: a proteomic approach. J. Archaeol. Sci. 2011;38:197–201.
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Budán Ferenc, Szabó István, Jámbor Éva, **Bóna Ágnes**, Váczy Alexandra, Maász Gábor, Ohmacht Róbert, Kiss István, Márk László, Tényi Tamás: A skizofrénia biomarkereinek kimutatása és azonosítása tömegspektrometriával új lehetőséget nyithat a megelőzésben. Magyar Epidemiológia 7: p. 69. (2010)

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Total impact factor: 13.205

Total citations: 39

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