



TOMASZ KOZŁOWSKI, BEATA STEPAŃCZAK, LAURIE J. REITSEMA, GRZEGORZ OSIPOWICZ,
KRZYSZTOF SZOSTEK, TOMASZ PŁOSZAJ, KRZYSTYNA JĘDRYCHOWSKA-DAŃSKA,
JACEK PAWLYTA, CZESŁAWA PALUSZKIEWICZ, HENRYK W. WITAS

OSTEOLOGICAL, CHEMICAL AND GENETIC ANALYSES OF THE HUMAN SKELETON FROM A NEOLITHIC SITE REPRESENTING THE GLOBULAR AMPHORA CULTURE (KOWAL, KUYAVIA REGION, POLAND)

ABSTRACT: In 2007 a ceremonial complex representing the Globular Amphora Culture was discovered in Kowal (the Kuyavia region, Poland). Radiocarbon dating demonstrated that the human remains associated with the complex are of similar antiquity, i.e., 4.105 ± 0.035 conv. and 3.990 ± 0.050 conv. Kyr. After calibration, this suggests a period between 2850 and 2570 BC (68.2% likelihood), or more specifically, 2870 to 2500 BC (95.4% likelihood). Morphological data indicate that the skeleton belonged to a male who died at 27–35 years of age. The unusual morphology of his hard palate suggests this individual may have had a speech disorder. Stable oxygen isotope values of the individual's teeth are above the locally established oxygen isotope range of precipitation, but due to sample limitations we cannot conclusively say whether the individual is of non-local origin. Stable carbon and nitrogen isotope ratios were analyzed to reconstruct the diet of the studied individual, and show a terrestrial-based diet. Through ancient DNA (aDNA) analysis, the mtDNA haplogroup K2a* and lactose intolerance as evidenced by homozygous LCT-13910C allele were identified. These aDNA results are the first sequences reported for an individual representing the Globular Amphora Culture, enriching the still modest pool of human genetic data from the Neolithic.

KEY WORDS: Globular Amphora Culture – Neolithic – Human burial – Skeleton – Stable isotopes – aDNA

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INTRODUCTION

Modern comprehensive bioarcheological analysis of skeletal remains relies on the cooperation of scientists representing various fields including archeologists, anthropologists, physicists, chemists and molecular biologists (Haak *et al.* 2005, 2008, Linderholm 2008, Roche *et al.* 2010). Rapid development of research techniques based on stable isotopes of oxygen, carbon and nitrogen and the analysis of ancient DNA enables a more complete evaluation of the general biological condition of an individual. In contemporary anthropological and archeological research, the analyses of skeletal material increasingly are conducted in cooperation among researchers working in various fields of science (Roberts *et al.* 2013, Vercellotti *et al.* 2008, Zvelebil, Weber 2013). Through collaborative anthropological research, reconstruction of diet through stable carbon and nitrogen isotope analysis, life history including geographic origins through stable oxygen isotope analysis, and frequency of certain genetic traits and possible ancestry through ancient DNA analysis can be discerned (Evans *et al.* 2006, Fornander *et al.* 2008, White *et al.* 1998, Witas *et al.* 2006, 2007, 2010, Wright, Schwarcz 1999, Zawicki, Witas 2008).

In this study, we explore the living conditions and lifestyle of an individual representing the Globular Amphora Culture, whose tomb was found at the Kowal 14 site in Kuyavia (Central Poland). First, an anatomical/anthropological analysis was undertaken and a complete archeological description of the cultural and environmental background was prepared. Next, stable oxygen isotope ratios in tooth phosphate as well as stable carbon and nitrogen isotope ratios in rib collagen and carbonate were determined. Finally, ancient DNA (aDNA) from buccal teeth was extracted to reveal select sequences of nuclear (*MCM6* alleles) and mitochondrial (HVR) genomes.

ARCHEOLOGICAL BACKGROUND

Globular Amphora Culture

At the end of the early and the beginning of the Late Eneolithic, Central Europe saw a transformation from settled populations practicing farming/husbandry to mobile shepherd communities. Archeologically speaking, the two processes correspond to the emergence of the Globular Amphora Culture (GAC) first and the Corded Ware Culture later (Kozłowski 1998, 1999). The origins of the Globular Amphora Culture are complex.

Its formative stage took place along the European Lowlands from Mecklenburg (Germany) to Kuyavia (Poland). It is believed that the culture came into being in late 4th millennium BC as a result of interactions between the Funnel Beaker Culture (TRB) and hunter-gatherers inhabiting the middle Oder and low Elbe catchment area (Kaczanowska 2005, Kozłowski 1999, Wiślański 1979). In its heyday, the territory of the GAC covered an area similar to TRB, but did not reach the size of Lower Saxony or today's Netherlands or Denmark. In the east, it extended over Podlachia and today's Ukraine – Volhynia along Dniester, as far as Kiev (Wiślański 1979). In Poland, traces of the GAC are visible, especially in the Polish Lowlands, Silesia and part of Lesser Poland (Kaczanowska 2005, Kozłowski 1999, Wiślański 1979).

The GAC population in Poland settled on the richest soil (Kuyavia, the vicinity of Ślęza, the Lublin region, the vicinity of Solawa and Kraków/Sandomierz loess), which, in juxtaposition with data from sources including paleofaunal and paleofloral remains, indicates that its economy was farm- and husbandry-based (mainly cattle and pigs). It seems that the culture was first to domesticate the horse; this assessment is supported by the graves of horses discovered, for example, in Potyry near Płońsk (Wiślański 1969).

The site of Kowal

The site of Kowal is located at the edge of the Kuyavian moraine plateau (where the Kuyavian Plain merges with the Płock Basin) at the altitude of approximately 90 m above sea level (*Figure 1*). It covers the edge and slope of a small valley exposed to the north, northeast and east. The region does not contain any larger water bodies, although there are two small springs 150 m to the northwest. These are well-exposed against the surrounding landscape and might have had a major impact on the extent of pre-historic settlement in this region. The surface of the site is covered with powdery brown soil, and the geological base contains clays of different weight, occasionally interspersed with clay sand. Certain areas of the site are very rocky. In total, an area of 7041 m² was studied.

Overall, 348 items of different function and chronology were identified. Of particular interest are Neolithic objects, including a ceremonial complex of the Globular Amphora Culture. This complex comprised a megalith, a human tomb, and an animal burial site accompanied by a pyre.

Originally the megalith was partly submerged in the ground, and covered an area of approximately 2023 m².

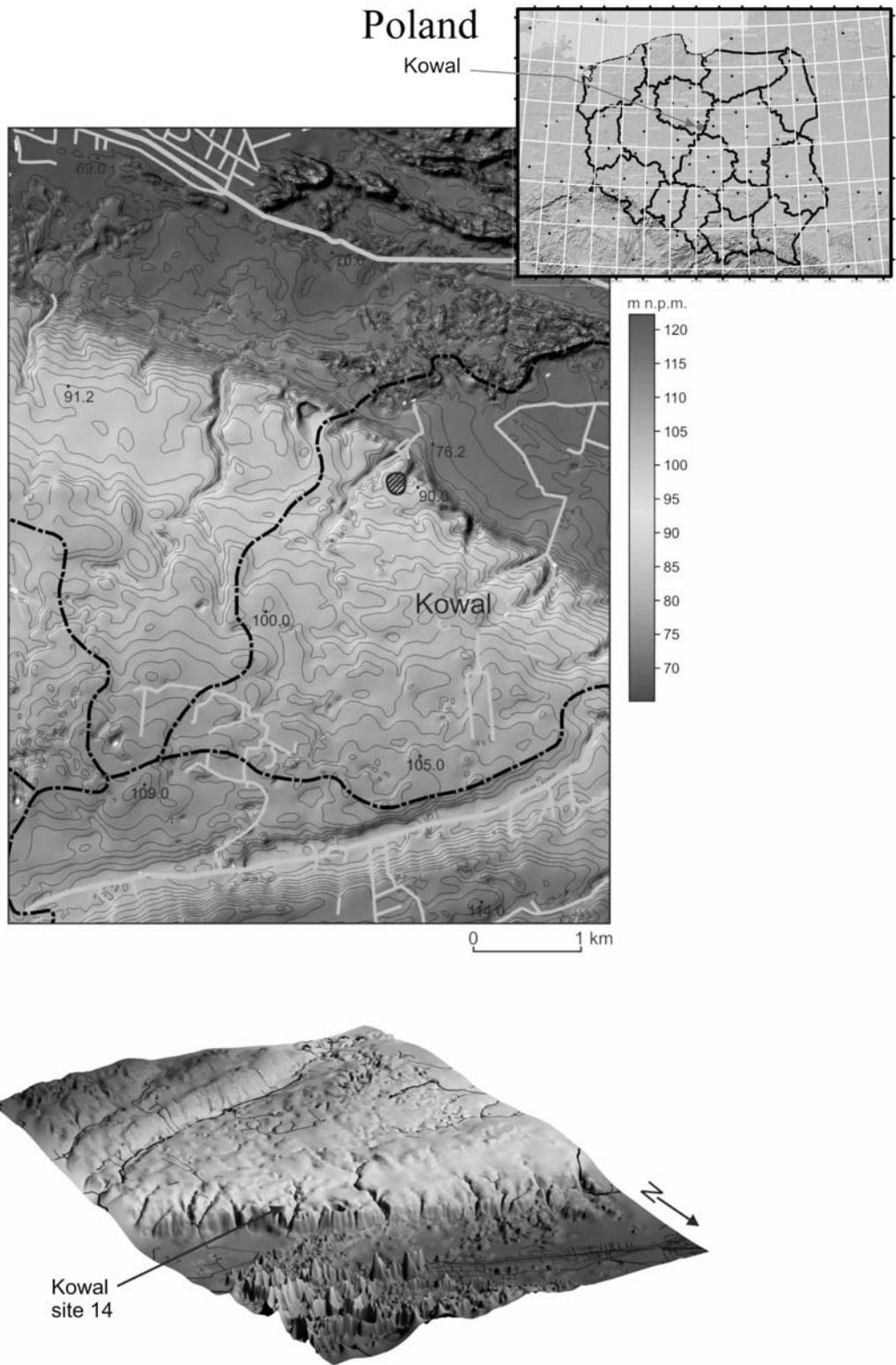


FIGURE 1. Location and topographic plan of site 14 area in Kowal. Drawing by T. Górzyński and P. Weckwerth.

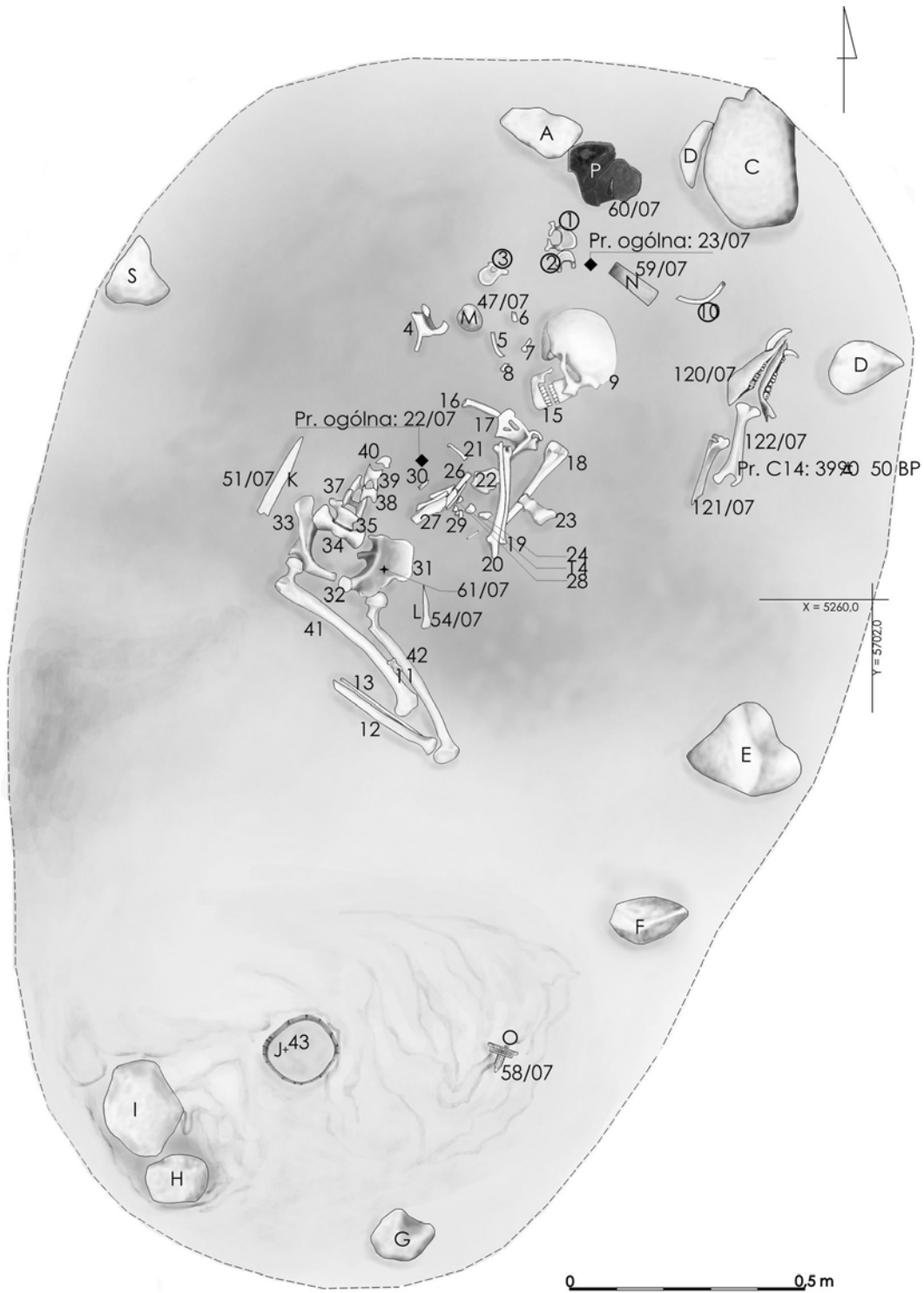


FIGURE 2. Human tomb of the Globular Amphora Culture, site 14, Kowal district. Drawing by A. Balonis, computer processed by A. Górzyńska.

Its thickness was about 1.5 m, with the upper parts made of stones of diameters greater than 0.5 m and weights probably approaching 300 kg in some cases. Some of those stones bore marks of having been processed.

A ceremonial complex with an animal burial accompanied by a pyre was discovered west of the megalith. The structure had been partly destroyed both by contemporary and by pre-historic human activities (i.e., ploughing). It was unearthed as a rectangular pit

(430×160 cm) aligned along the east/west axis, with a slight deviation of the western section to the north.

The human tomb was located in the immediate vicinity of the megalith. When discovered, the tomb pit was a 367×160 cm oval, 80 cm deep. Its bottom section was limited by stonework made of pebbles, 10–20 cm in diameter, placed every 20–60 cm, which delineated a 250×160 cm oval. In its centre lay a well-preserved skeleton (*Figure 2*).



FIGURE 3. Human skeleton *in situ* and an idea of the body's arrangement in the tomb. Photo Archive of the Institute of Archaeology at the NCU, drawing by T. Kozłowski.

The human skeleton at Kowal

The cadaver had been laid on his left side, along the NE (head)/SW (legs) axis, his head pointing to the east. The bones from the human skeleton were discovered mostly in the anatomical position. The way in which the bones were arranged suggests that the limbs had been forcefully pressed against the body and bent at large joints (i.e., elbow, hip and knee). The upper limbs had been pressed against the torso and presumably crossed on the chest. A plausible interpretation of this arrangement is that the body had been tied up (e.g., wrapped in a fabric or tied with a rope) (Figure 3).

The tomb furnishing was quite elaborate, and included three ceramic vessels, a lavishly decorated T-shaped badge, a tool made from wild boar's tusk, several bone objects (a spindle-shaped blade, a piercing pin, a chisel and a piece of an unspecified smoothed tool), a striped flint axe, a chocolate flint chip, a flake core, a stone grinder, and an ochre lump. In the northeast section of the structure, around 25 cm from the skull, a pig mandible and shank bones were found.

The first sample for radiocarbon dating, collected from the human bones, was dated to 4105 ± 35 conv. BP (Poz-21912). After calibration, this suggests a period between 2850 to 2570 BC (68.2% likelihood), and 2870 to 2500 BC (95.4% likelihood). The second sample was collected from a long bone of the pig. The dating was 3990 ± 50 conv. BP (Poz-21910). After calibration: 2850–2580 BC (68.2% likelihood), and 2870–2840 BC (95.4% likelihood).

APPLYING ISOTOPIC ANALYSES AT THE KOWAL SITE

To obtain as much information as possible from the isolated human skeleton at Kowal, stable oxygen, carbon and nitrogen isotope analyses were undertaken. Oxygen is incorporated into enamel and dentine via ingested food and water. Stable oxygen isotope analysis of human and animal remains is used primarily for studying climate changes which took place in the past (Hedges *et al.* 2004, Stephan 2000) and the origin and migration routes of individuals and entire populations (Budd *et al.* 2004, Dupras, Schwarcz 2001, Fricke *et al.* 1995, Knudson, Torres-Rouff 2009, Müller *et al.* 2003, Price *et al.* 2010, White *et al.* 1998, White *et al.* 2004). The oxygen isotope ratio analysis of various bone sections enables us to retrospectively reconstruct the dynamics of individuals' movements in their lifetime. This is possible because of the fact that during an individual's life there is both sustained and

spontaneous bone remodeling (Pate 1994, Streeter *et al.* 2010), and stable oxygen ratios in phosphates and carbonates of the bone apatite vary with the oxygen isotope signatures of the sources of drinking water in the area inhabited by a given individual during life (Daux *et al.* 2005, Daux *et al.* 2008, Dupras, Schwarcz 2001, Luz *et al.* 1984). Considering the duration of the long bone development (approximately 10 years or more), isotope analyses of long bones allow us to reconstruct an individual's activity over the last decade or so of their life. Stable isotope signatures from teeth, however, remain unchanged after they have been completely mineralized, thus reflecting living conditions (diet and/or geographic point of origin) of an individual as a child and/or young adult, depending on which tooth is analyzed. By examining teeth selected on the basis of budding, mineralization and eruption times, it is possible to trace nutritional habits and mobility of a given individual at various stages of their life (Hillson 1996, White, Folkens 2005). Isotopic analyses of single skeletons can be carried out using a larger number of samples from the same individual, as this is a method for a more complete reconstruction of a particular human being's life history (Prowse *et al.* 2007, Roberts *et al.* 2013, White *et al.* 2004).

The analysis of stable oxygen isotope ratios is also used in weaning studies (Katzenberg 2000, Wright, Schwarcz 1999, White *et al.* 2004). Isotope fractionation of oxygen during mother's milk synthesis results in breast-fed children having a 2–3‰ higher oxygen isotope ratio in their tissues than their mothers, who drink water. Switching the child's diet over to solid or mixed food causes a drop in stable oxygen isotope concentrations as a consequence of the elemental reconstruction, until these concentrations reach levels typical of adults (Wright, Schwarcz 1999).

Stable carbon and nitrogen isotope ratios from bone collagen and carbonate are widely used in anthropology to reconstruct diet. The use of stable carbon and nitrogen isotope analysis to paleodietary studies derives from the fact that isotopic ratios of different types of food are preserved in the tissue chemistry of consumers (for a recent review, see Schoeninger 2011). Stable carbon isotope ratios provide information about local ecosystems, distinguishing between terrestrial versus marine niches and between consumption of plants adapted for temperate (C_3 plants) versus hot, dry environments (C_4 plants). Examples of C_3 plants are vegetables, wheat, and barley; examples of C_4 plants are millet and maize. Stable nitrogen isotope ratios are positively correlated with an organism's trophic position in the local foodweb and distinguish between herbivores,

omnivores, and carnivores. The relationship between nitrogen isotope ratios and trophic position can be complicated by variations in climate, soil chemistry and consumer physiology (Ambrose 1991, Britton *et al.* 2008, Fuller *et al.* 2005, Hobson *et al.* 1993).

METHODS

Estimation of age at death, sex and body height

Age of the individual was estimated on the basis of changes on the surface of the pubic symphysis and *facies auricularis* of the *os coxae* (Buikstra, Ubelaker 1994, White, Folkens 2005). Sex was determined primarily on the basis of pelvic form, i.e., greater sciatic notch and subpubic morphology, supplemented with cranial features (Acsádi, Nemeskéri 1970, Bruzek, Murail 2006, Buikstra, Ubelaker 1994). Intra vitam body height was reconstructed on the basis of long bones (humerus, radius, femur and tibia) and stature estimation equations proposed by Ruff *et al.* (2012).

Stable oxygen isotope analysis

Analysis of stable oxygen isotope ratios was carried out on phosphate from a piece of femur, and the enamel and dentine of selected permanent mandibular teeth (second incisor, first premolar, first molar). Out of the three potential components of the apatite containing oxygen atoms (PO_4^{3-} , CO_3^{2-} and OH^-), the phosphate groups are the longest lasting ingredient of the bone mineral and the post mortem substitution of oxygen caused by diagenesis occurs less frequently in phosphate groups. Accordingly, the proportions of stable oxygen isotopes were established in isolated bone and dental phosphates (Brady *et al.* 2008, Kohn *et al.* 1999).

Each tooth was sectioned and enamel separated from dentine. In the case of M1, dentin from around the crown was taken, I2 was represented by enamel and dentin from the whole root, and for P1 just the enamel was analyzed.

Phosphate $\delta^{18}\text{O}$ measurements of selected teeth and bones were taken with the broadest interpretative spectrum of ontogenetic development in mind. Mineralization of the analysed teeth typically spans approximately birth to 10 years of age. The mineralization of enamel in the case of the second permanent incisor takes place from age 4 months to 4 years, while formation of dentin from this tooth occurs from about age 4 to 10 years. Dentin from the first premolar forms between age 2 to 6 years, and crown dentin from the first molar between age 0 and 3 years (Reid, Dean 2006, White, Folkens 2005).

Formation and mineralization of teeth occurs sequentially, starting with crown nodules (or incisal edges) to the tip of the root (Reid, Dean 2006). In the case of enamel, after mineralization, the given layer is no longer subject to remodeling and isotopic reconstruction. Unlike enamel, dentine can remodel and develop secondary dentine, although these processes only minimally affect isotopic values from childhood (Balasse 2003, Reid, Dean 2006). Therefore, the $\delta^{18}\text{O}$ values from a given tooth are taken to represent that tooth's particular mineralization period.

The pig bone found beside the human skeleton was used as a reference material representing the local stable oxygen isotope signature. It was subjected to the same analytical procedures as samples collected from the human skeleton.

Silver phosphate (Ag_3PO_4) was obtained from ground bones, enamel and dentine, according to the technique recommended by O'Neil *et al.* (1994). The stable oxygen isotope ratio analyses were carried out in the Department of Radioisotopes, Institute of Physics, Silesian University of Technology – GADAM Centre on an IsoPrime mass spectrometer coupled to a EuroVector elemental analyzer. The result for each sample was presented as a δ deviation relative to the NIST 120c reference according to the formula:

$$\delta = (\text{R}_{\text{sample}} - \text{R}_{\text{standard}}) / \text{R}_{\text{standard}} \times 1000.$$

The results were standardized in relation to the original international reference, SMOW (Standard Mean Ocean Water) (Stephan 2000).

Stable carbon and nitrogen isotopes analysis

Both collagen and carbonate from the same rib were sampled from the individual at Kowal. These mineralized tissues record slightly different dietary information. Bone collagen is formed from amino acids, many of which are obtained from dietary protein (Ambrose, Norr 1993, Tieszen, Fagre 1993). Carbonate in bone apatite, a carbon-bearing inorganic fraction of bone, is formed primarily from the major energy source in diet, carbohydrates, via dissolved bicarbonates in blood (Krueger, Sullivan 1984). Therefore, collagen and carbonate provide complementary information about dietary protein and dietary energy, and are best used together in diet reconstructions (Kellner, Schoeninger 2007). In bone, which remodels slowly throughout life, these dietary signatures represents a time-average of well over 10 years (Hedges *et al.* 2007).

A small piece of rib was divided into two fractions and ground by hand in a mortar. The collagen fraction was prepared according to protocol described in

Richards and Hedges (1999). The apatite fraction was prepared according to protocol described in Reitsema and Crews (2011). Collagen was analyzed on a Costech Elemental Analyzer coupled to a Finnigan Delta IV Plus stable isotope ratio mass spectrometer under continuous flow using a CONFLO III interface in the Stable Isotope Biogeochemistry Laboratory at The Ohio State University. In the same laboratory apatite was acidified under vacuum with 100% ortho-phosphoric acid and analyzed using an automated Carbonate Kiel device coupled to the mass spectrometer. Using the same equation used for stable oxygen isotope values, stable carbon and nitrogen isotope values were reported in relation to the standards VPDB (Vienna Pee Dee Belemnite) and AIR (ambient inhalable reservoir), respectively.

Assessing sample preservation

Bone is susceptible to diagenetic alteration (Wright, Schwarcz 1996). To assess the reliability of isotopic values from carbonate and phosphate, Fourier transform infrared spectroscopy was conducted to compare the mineral composition of the archeological materials to that of modern and well-preserved archeological bones. The utility of this method is described elsewhere in greater detail (Garvie-Lok *et al.* 2004, Wright, Schwarcz 1996). The bone, enamel and dentine crystallinity index was defined on the basis of the formula $CI = (A_{605} + A_{565}) / A_{595}$, where A stands for absorbency peak values at the

wavelengths of 605 and 565 cm^{-1} respectively, characteristic of apatite phosphate groups, and narrowings between them – 595 cm^{-1} (Wright, Schwarcz 1996). Next, based on the ratio of the absorbance peak heights at the wavelengths of 1415 and 1035 cm^{-1} , characteristic respectively of carbonate and phosphate groups ($\text{CO}_3 / \text{PO}_4 = A_{1415} / A_{1035}$), the degree of contamination of bone apatite by geological carbonates was estimated. Collagen diagenesis was assessed using the carbon/nitrogen (C:N) atomic ratio, and carbon and nitrogen content in collagen (%C and %N) (Ambrose 1990).

Ancient DNA analysis

DNA was isolated individually from two molars and one premolar of the mandible. After the removal of the external surface, and before grinding in a cryogenic mill (SPEX 6750), the tooth was rinsed in sodium hypochlorite for 15 minutes followed by sterile distilled water and 70% ethanol for 30 minutes, and then irradiated under UV for 15 minutes on each side.

The aDNA analysis was conducted in a dedicated clean room equipped for work with ancient materials. After powdering, the material was divided into three parts (ca. 400 mg) and stored in sterile test tubes at -20°C .

Neolithic DNA was isolated after decalcification of tooth powder in a 0.5M EDTA for 48 hrs and hydrolytic degradation of proteins by K proteinase (10 μl of 10 mg/ml solution). Addition of *N*-phenacylthiazolium bromide (PTB) (50 μl of 0.1M solution) provided

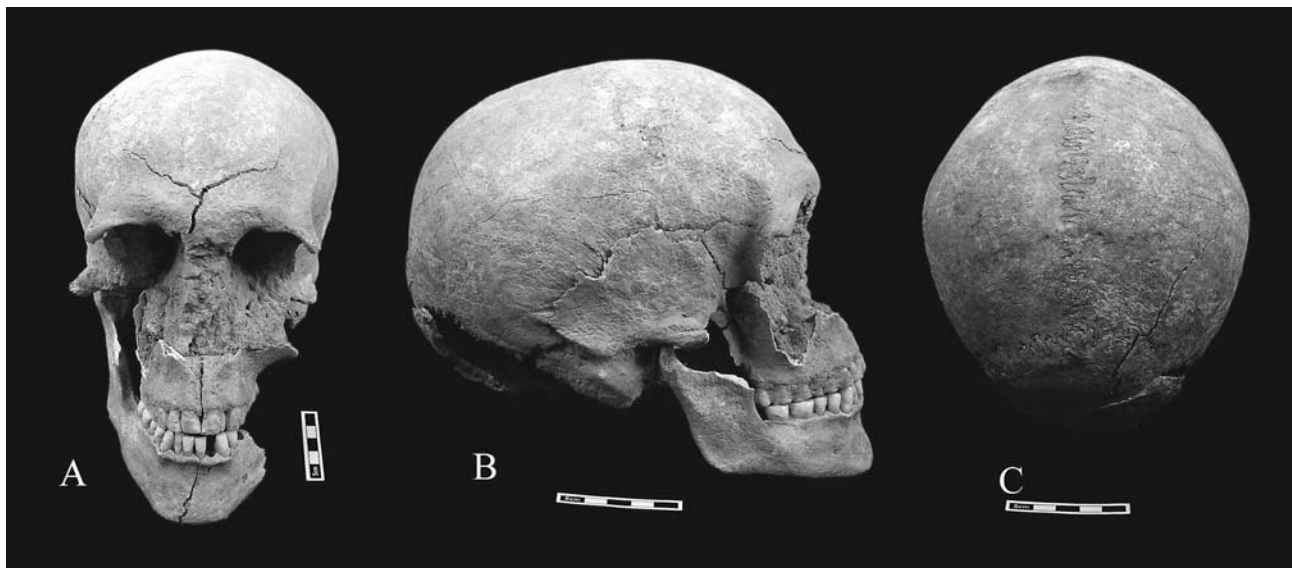


FIGURE 4. The skull from the Kowal as seen in three planes: A, frontal; B, lateral; C, posterior. Photo by T. Kozłowski.

elimination of intra and intermolecular crosslinks after incubation at 58°C for 2 hrs. After centrifugation (5000 rpm) DNA dissolved in a supernatant was isolated in semi-automated *MagNA Pure® Compact Nucleic Acid Purification System* (by Roche) according to the manufacturer's manual. Obtained DNA isolate was processed further no later than 24 hrs after the end of isolation procedure. During allele amplification (PCR) GeneAmp® PCR System 2700 and 9700 thermal cyclers (Applied Biosystems) were used, and polymerases AmpliTaq Gold® (Applied Biosystems), DNA DFS® (Bioron) and Taq DNA (Polgen) were applied. PCR primers were synthesized at the DNA Laboratory of Sequencing and Oligonucleotide Synthesis in the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences in Warsaw. The reaction products were identified in a 10% polyacrylamide gel stained with ethidium bromide or silver nitrate, and then sequenced.

Amplicons were sequenced after cleaning on Clean-Up columns (A&A Biotechnology). A BigDye® 3.1 kit (Applied Biosystems) was used for reactions with labeled nucleotides. Reaction mixture (20 µl) contained 4 µl of BigDye, 30 ng of each primer and 50–70 ng of matrix DNA. Reaction conditions: initial denaturation at 95°C for 5 minutes, then 36 cycles at 95°C for 30 s, at 60°C for 8 s and at 60°C for 4 minutes. PCR products were cleaned of labelled nucleotides on ExTerminator columns (A&A Biotechnology) and suspended in 20 µl of deionized formamide. Sequencing was performed on ABI Prism 310™ Genetic Analyzer (Applied Biosystems). Sequences were edited using BioEdit and MEGA 4: *Molecular Evolutionary Genetics Analysis*.

In addition to standard procedures applied to avoid contamination, i.e., multiple isolation and analysis of DNA from distinct teeth, the presence of exogenous molecules was screened in reagents and on disposables, as well as collagen content as a marker of macromolecule's preservation (> 2%) was estimated. The authenticity of the results was verified by comparing a haplogroup of the analyzed individual with those of all people involved in sample excavating, transporting and analysis procedure.

RESULTS AND DISCUSSION

Anthropological characteristics

The bones belonged to an individual who had died in adulthood. All long bones had completed their longitudinal growth. There are no traces of growth plates at the borderline between epiphyses and shafts. The

epiphyses are entirely fused. Cranial sutures from the ectocranial side are clearly visible, although quite "compressed". The sphenooccipital synchondrosis is fused. Changes on the surface of the pubic symphysis and facies auricularis of the os coxae are typical of persons deceased between 27 and 35 years of age.

Skull measurements are presented in *Table 1*. The skull is long (cranial index = 72.6) and characterized by average massiveness (*Figure 4*). This concerns, in particular, the frontal bone, where the region of glabella and supra-orbital margin is delicate. The mastoid processes of the temporal bones are large and massive. The occiput is averagely formed, albeit prominent. The frontal and parietal tubers are weakly visible. The mandible is very massive. Its

TABLE 1. Measurements of the skull of the Kowal individual.

Measurements	Value [mm]
Skull circumference	530?
g-op	190
eu-eu	138
ba-b	142?
n-b	114
o-l	100
b-l	118
o-ba	44.5
n-ns	53?
n-pr	71?
n-gn	124
n-ba	103?
pr-gn	54?
ba-ns	89?
ba-pr	93?
co-co	119
ft-ft	92
po-po	107
mast-mast	97
Orbit height	-/36?
ol-sta	45
enm-enm	33
Palate height	18
Mandible height	57
Mandible length	107
gn-go	82
id-gn	36
ml-ml	43

TABLE 2. Measurements of the postcranial bones of the Kowal individual. AP, anterior-posterior; ML, medial-lateral.

Measurements	Value [mm]	
	Left	Right
Scapula glenoid length	37?	
Scapula glenoid breadth	27	30
Clavicle midshaft max.	12	
Humerus length	297	301
Humerus head	46	46
Humerus midshaft AP		22
Humerus midshaft ML		23.5
Humerus distal condyle		55
Radius max length	229	226
Ulna max length	247	247
Femur length	410	413
Femur length (natural position)	409	410
Femur head	44	44
Femur sub. troch. AP	22	22
Femur sub. troch. ML	30	30
Femur midshaft AP	27	27.5
Femur midshaft ML	25	24.5
Patella length	40	
Patella breadth	41	
Tibia length	342	
Proximal tibia epiphysis breadth ML		73
Tibia shaft, nut. foramen AP	32	33
Tibia shaft, nut. foramen ML	21	21
Talus max. length		54
Talus articular length		36?

corpus is high, with a strongly prominent mental protuberance and rather broad angles. The preserved facial section is very narrow. Its bottom part seems quite "heavy" and massive. Despite the intermediate character of the frontal bone, the skull must have belonged to a male. As for morphological features, the pelvis has a typically male build as regards its general shape and size, the greater sciatic notch and the pubic region.

Measurements of postcranial bones include *Table 2*. Average intra vitam body height (*Table 3*) is 156.4 cm. It is very likely that his body height did not exceed 160 cm, conceivably ranging from 154.8 cm (calculated on the basis of femur) to 157.7 cm (calculated on the basis of tibia). This is in line with the estimated height variability range in Neolithic populations, characterized by short or short to average stature (Vancata 2000).

TABLE 3. Intra vitam body height of the Kowal individual reconstructed on the basis of selected long bone measurements using estimation equations by Ruff *et al.* (2012).

Bone	Stature estimation [cm]
Humerus	155.9
Radius	157.8
Femur	154.8
Tibia	157.7
Femur + Tibia	155.8
Average stature	156.4

Palaeopathological observations

The skeleton reveals a very interesting developmental anomaly of the facial skeleton (i.e., maxilla) and dentition. The roof of the palate is very high and abnormally deep (the so-called "gothic" or "narrow" palate). The dental arc is narrow, and the alveolar ridge is very high (*Figure 5*). The front teeth of the maxillae and the mandible are compressed and rotated (upper – I2, lower – I2 and C), being pushed towards the inside and the outside from the dental arc. Medial upper incisors are aligned markedly at an angle in relation to each other, forming a prominent top of the dental arc. This indicates that dentition, particularly front teeth in this individual, lacked space in the dental arc, which is the result of an impairment/inhibition of the latitudinal growth of maxillae bones (and perhaps all bones of the entire face). A deep carious lesion of the upper right M3 was also observed (*Figure 5:C*).

A visible oval defect (*Figure 6:H*), reminiscent of a trepanation-like hole, adjacent to the foramen magnum, proved to have been caused by rodents, whose teeth left bite marks on its endocranial edges. Degenerative changes in the form of small osteophytes are present in the vertebrae and peripheral joints.

Harris lines (HL) are visible in X-ray images of tibia (*Figure 7:HL*). They are adjacent to the distal end of the left tibia (the distal section of the right tibia was missing). No lines were observed in X-ray images above this part of the bone shaft. Any visible HLs are relatively faint. Some of them had probably already been partially resorbed. Their location, i.e., close the borderline between the epiphysis and the end of the shaft, thus their significant remoteness from the shaft centre, suggests that they had developed at the final stages of bone growth – late childhood (age 12–14 yrs.). No other phenotypic stress markers were noted in the skeleton. It may be concluded that the individual lived in relatively



FIGURE 5. A narrow and very high palate of the Kowal skull. Compression and rotation of front teeth. Deep caries (C) of right upper M3. Photo by T. Kozłowski.

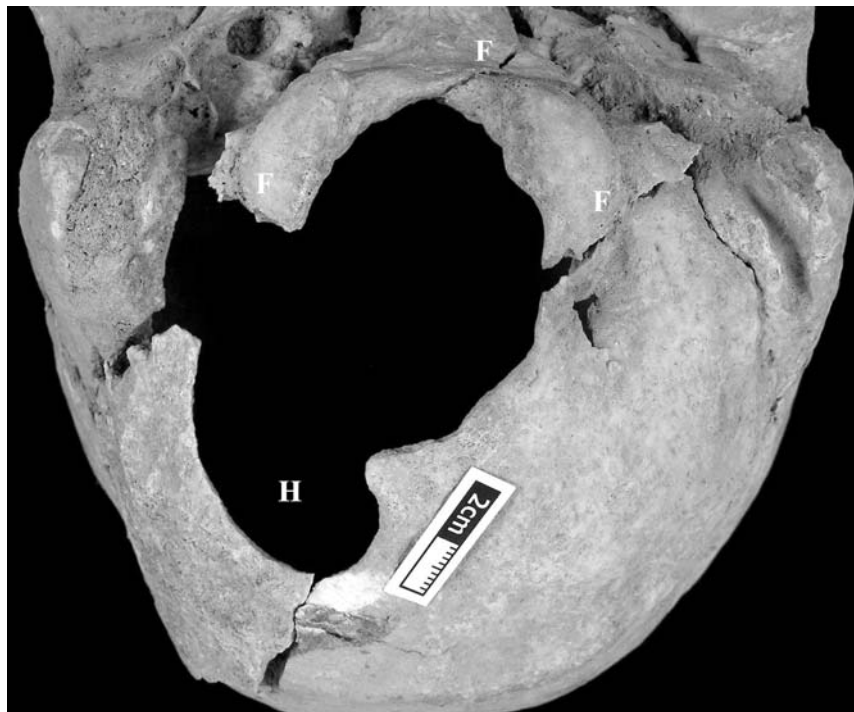


FIGURE 6. The skull in *norma basilaris*. Cranial base fractures (F) and an elliptic defect (H) made by rodent in the vicinity of *foramen magnum*. Photo by T. Kozłowski.



FIGURE 7. An X-ray picture of the distal end of right tibia. Harris lines (HL) are clearly manifested as linear areas of extensive saturation perpendicular to the long axis of the bone.

favourable conditions and that his health was rather good.

Stable isotopes analysis: diagenesis

Sample tests for diagenetic changes of the teeth did not reveal any significant post-mortem contamination in terms of CI or CO_3/PO_4 . Table 4 presents the FTIR and isotope results of the phosphate analyses. The crystallinity indices (CI) for the samples ranges from 2.81 to 3.3, and the carbon/phosphorus index (CO_3/PO_4) is contained within the 0.18–0.40 range, which indicates that bones and teeth did not undergo recrystallization (Thompson *et al.* 2009, Wright, Schwarcz 1996). FTIR spectra of the samples did not contain any additional peaks which could suggest any contamination.

FTIR analysis was also applied to bone apatite, and pre- and post-acid treatment subsamples were compared to study the effects of acid treatment. Prior to acid treatment, CI was 3.2 and CO_3/PO_4 was 0.36. After acid treatment, the CI rose to 3.5 and the CO_3/PO_4 ratio fell

TABLE 4. Results of the phosphate $\delta^{18}\text{O}$ analyses of samples collected from the individual and animal (pig) bone.

Sample	CI (FTIR)	CO_3/PO_4 (FTIR)	$\delta^{18}\text{O}_{\text{VSMOW}}$ (‰)
Enamel (lower I2)	3.19	0.25	25.23
Root dentine (lower I2)			23.74
Enamel (lower P1)	3.26	0.21	22.21
Crown dentine (lower M1)	3.12	0.26	24.95
Compact bone (femur)	3.03	0.29	20.86
Compact bone (pig)	2.81	0.40	21.65

to 0.29. Acid treatment appears to have successfully removed diagenetic carbonates from the sample, and the final measurements are consistent with other well-preserved archeological bones (Wright, Schwarcz, 1996). There is no shoulder or peak at wavenumber 1096 cm^{-1} , which is another indicator of good preservation.

Bone collagen quality was assessed using criteria described by Ambrose (1990). As measured during mass spectrometry, collagen was 13.8% nitrogen and 38.6% carbon for a C:N ratio of 3.3. These measurements are all within the range of well-preserved bone collagen.

Stable oxygen isotope ratios

Phosphate stable oxygen isotope ratios for samples collected from the skeleton are shown in Figure 8. $\delta^{18}\text{O}$ values from all tooth sections were plotted against a scale accounting for the age of tooth mineralisation and eruption, as well as the rate of bone remodelling. This is likely in part due to the fact that production of metabolic water in the body is a fractionating process that leaves the body water pool slightly enriched compared to drinking water and free water in foods (O'Grady *et al.* 2010). In the Polish territory the isotopic variability of oxygen in precipitation is on the order of -6 to -12% , a low range when considered in relation to global variation (IAEA 2001). This range translates to a bone phosphate range of $\delta^{18}\text{O}$ range of 14 to 19‰ (Daux *et al.* 2008). Results from Kowal are above this local precipitation range.

Because details about the specific isotopic baseline of the local environment are lacking and on the basis of single available animal (oxygen isotopic concentration was $\delta^{18}\text{O} = 21.65\%$) conclusions from $\delta^{18}\text{O}$ data must remain preliminary.

In the parts of the skeleton which are first to bud and mineralize, a similar $\delta^{18}\text{O}$ level was observed (dentine of first molar: $\delta^{18}\text{O} = 24.95\%$; enamel of second incisor:

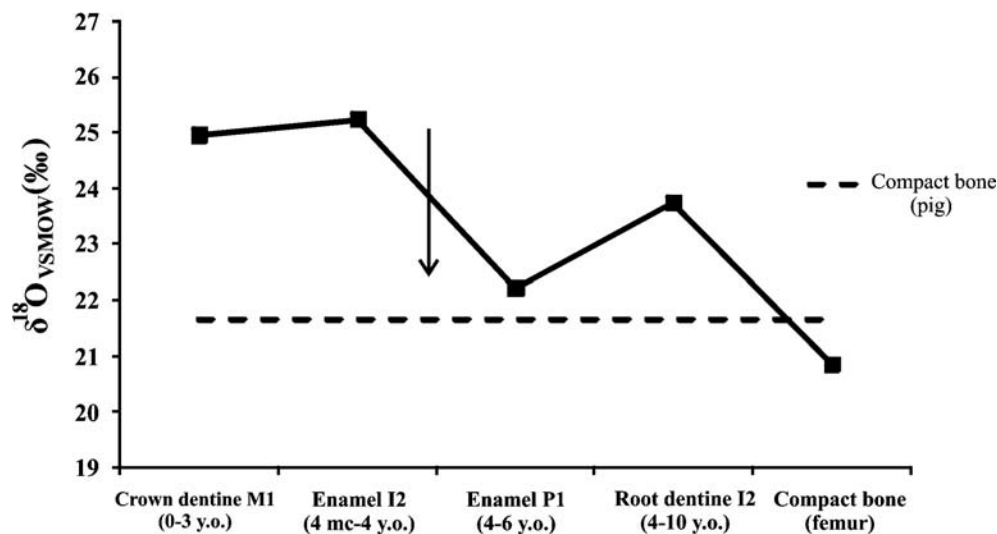


FIGURE 8. Changes in the level of $\delta^{18}\text{O}$ over the individual's life period.

$\delta^{18}\text{O} = 25.23\text{‰}$). For the remaining samples (the enamel of the first premolar, the dentine of the second incisor and the femur), a 1–3‰ decrease in the $\delta^{18}\text{O}$ level is noteworthy.

The period of formation of dentin originating from the crown of M1 and enamel I2 lies in the range between the 1st month and the 4th year of age. Thus, the isotopic results from their analysis cover quite a wide range of the early childhood.

Higher value of oxygen observed within these bone fragments (the dentin of M1 and the enamel of I2) relative to remaining analyzed tissues do not necessarily indicate that the studied individual might have assimilated isotopically different water in a non-local environment until the 4th year of age, compared to the later age. It could be result from natural and anthropogenic processes, such as evaporation and boiling, that can potentially raise drinking water $\delta^{18}\text{O}$ value above that of local precipitation. Thus, it is possible the individual did indeed spend his childhood in this or another isotopically similar region, but that these processes served to increase his tooth $\delta^{18}\text{O}$ values relative to his drinking water (Brettell *et al.* 2012). It is known that the Globular Amphora Culture people were characterized by a rather settled way of life, as indicated by the settlements that they built (Kaczanowski, Kozłowski 1998). It is rather unlikely that the individual had travelled long distances as a child, although this possibility cannot be excluded.

Note that the $\delta^{18}\text{O}$ level for the dentine of the second incisor is higher than the oxygen level in the enamel of

the first premolar, even though a full mineralisation of these dental tissues is completed almost at the same time (White, Folkens 2005). Although providing an explanation of this finding is difficult, such a result might be related to a different tooth mineralization and eruption model in the Neolithic period as opposed to today's model. It is assumed that the order and rate of tooth formation in children and teenagers have changed over the ages (Jerszyńska 2004, Kaczmarek 1995).

Stable carbon and nitrogen isotope ratios

The individual from Kowal exhibits a $\delta^{13}\text{C}_{\text{coll}}$ value of -20.3‰ and a $\delta^{15}\text{N}$ value of 9.9‰ . This suggests that terrestrial animals were the primary source of protein. Humans usually exhibit $\delta^{13}\text{C}_{\text{coll}}$ values approximately 1‰ higher and $\delta^{15}\text{N}$ values approximately 3–5‰ higher than the animals they consume (Drucker, Bocherens 2004, Schoeninger 1989). Thus, animals with $\delta^{13}\text{C}_{\text{coll}}$ values of approximately -21 to -22‰ and $\delta^{15}\text{N}$ values of approximately 5 to 7‰ formed the protein base of this individual's diet. Such values are reported for pigs, cows, and deer in Neolithic Germany (Dürrwächter *et al.* 2006). Bones of all these animals are found at Neolithic sites in Central Poland (Grygiel, Bogucki 1993). The relatively high $\delta^{15}\text{N}$ value from Kowal corroborates trace element data from Neolithic skeletons in Central Europe which indicated a diet high in animal protein (Szostek, Głab 2001).

Although some isotopic data are available for humans and other animals from Poland at other time periods (c.f. Reitsemá *et al.* 2010, 2013), no other isotopic data of

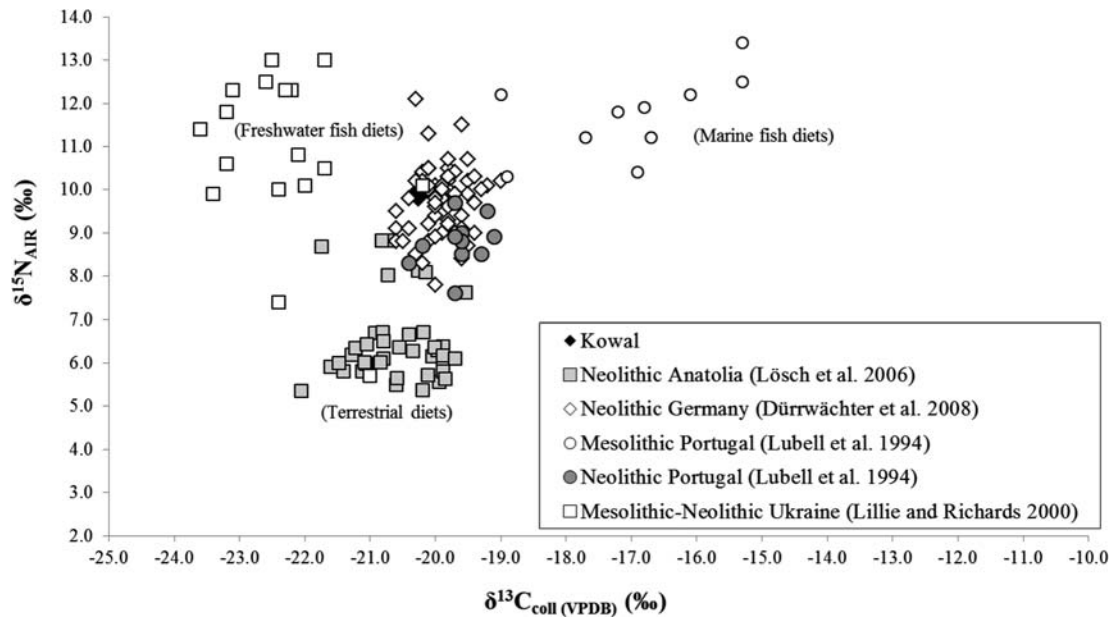


FIGURE 9. $\delta^{13}\text{C}_{\text{coll}}$ and $\delta^{15}\text{N}$ data from Kowal and comparative sites.

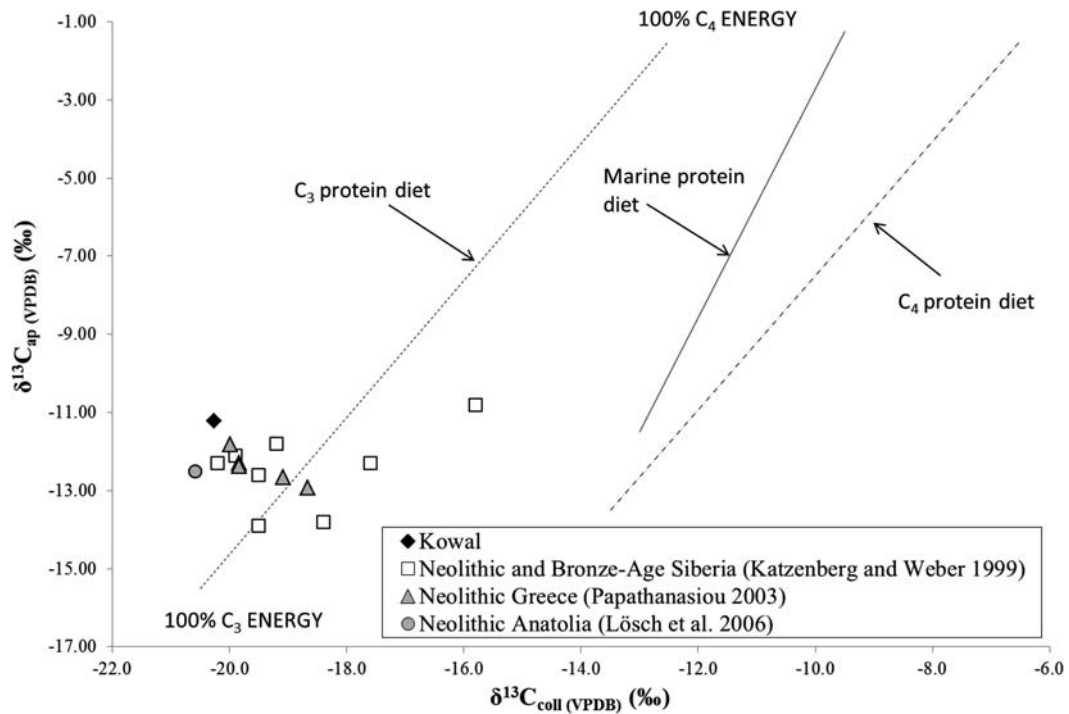


FIGURE 10. $\delta^{13}\text{C}_{\text{coll}}$ and $\delta^{13}\text{C}_{\text{ap}}$ data from Kowal and comparative sites shown in comparison to the regression lines developed by Kellner and Schoeninger (2007) and corrected for isotopic differences in Neolithic and modern CO_2 values. Regression lines are shown shifted by +1.5 permil to account for changes in atmospheric carbon values since pre-industrial times.

comparative populations are available from Neolithic Poland. Collagen isotope data from Kowal are shown in comparison to other Neolithic populations elsewhere in *Figure 9*. Collagen values from Kowal are most similar to a Neolithic sample from Germany which consumed terrestrial, C₃-based protein diets (Dürrwächter *et al.* 2006). There are clear differences between Kowal and populations who relied heavily on freshwater fish (Lillie, Richards 2000) and on marine fish (Lubell *et al.* 1994). Compared to a Neolithic Anatolian population that also had a terrestrial diet (Lösch *et al.* 2006), Kowal and the Neolithic German sample exhibit higher $\delta^{15}\text{N}$ values. This may be due greater consumption of animals, especially omnivore protein (such as pigs), supplemental amounts of freshwater fish, or consumption of plants grown on manured fields in the latter two samples.

The $\delta^{13}\text{C}_{\text{ap}}$ value from Kowal, which reflects total diet and not just protein, is -11.22% . This suggests a total diet based on C₃ plants but also including millet, a C₄ cereal cultivated in Poland during the Neolithic (Wasylikowa *et al.* 1991). Assuming respective C₃ and C₄ endpoints of -26% and -14% and a $+12\%$ diet-apatite space (Harrison, Katzenberg 2003, Lee-Thorp *et al.* 1989), the $\delta^{13}\text{C}_{\text{ap}}$ value from Kowal could indicate a diet containing as much as 25% millet. The $\delta^{13}\text{C}$ signatures from Kowal are shown in comparison to other Neolithic populations in *Figure 10*. With the exception of Kowal, in *Figure 10* data points represent populations, not individuals. The C₃, C₄ and marine protein lines pictured are from the model of Kellner and Schoeninger

(2007). The Kowal sample plots near other examples of terrestrial-based diets: Neolithic Greece (the two inland sites reported) (Papathanasiou 2003), Neolithic and Bronze Age Siberia (also inland sites) (Katzenberg, Weber 1999) and Neolithic Anatolia (Lösch *et al.* 2006). The fact that Kowal plots slightly higher than those samples reflects its relative enrichment in $\delta^{13}\text{C}_{\text{ap}}$, likely due to millet consumption. Kowal appears dissimilar from populations where marine and anadromous fish contributed to diet (Katzenberg, Weber 1999, Papathanasiou 2003).

Ancient DNA

Two sequences of mtDNA control region (HVRI and HVRII) and intron 13 of *MCM6* (LCT-13910C/T, lactase persistence) were analyzed (*Table 5*).

Chemical and physical characteristics like that of the Kowal archeological site limit structural changes of macromolecules, so we encountered no problems isolating mitochondrial and nuclear DNA.

Analysis of hypervariable region (HVR) sequence showed five differences in comparison with the reference sequence (CRS), at locations 16224, 16311 (within HVR I), 73, 146, 152 (within HVR II). Mutations found indicate that the individual belonged to K2a* haplogroup.

Sequencing PCR products of intron 13 of *MCM6* gene revealed the presence of LCT-13910T allele in the studied sample. Lactose intolerance of the analyzed individual was determined by the homozygotic variant.

TABLE 5. Conditions of identifying the analyzed alleles: primary structure of primers, PCR product length, primer annealing temperature.

Alleles	Primers	Length of PCR product	Annealing temperature	Method of identification
C/T ₋₁₃₉₁₀	5'GCGCTGGCAATACAGATAAGATA3' 5'AATGCAGGGCTCAAAGAACAA3'	111 pz	54°C	Sequencing
HVR I	5'CGTACATTACTGCCAGCC3' 5'TGGTATCCTAGTGGGTGAG3'	186 pz	56°C	Sequencing
	5'CACACATCAACTGCAACTCC 3' 5'TCAAGGGACCCCTATCTGAG 3'	168 pz		
HVR II	5' GCATTTGGTATTTTCGTCTGG 3' 5' TTGAACGTAGGTGCGATAAAT 3'	130 pz	58°C	Sequencing
	5'CGCAGTATCTGTCTTTGATTCC3' 5'CTGTGTGGAAAGTGGCTGTG3'	156 pz		

The LCT-13910C/C genotype is responsible for lactose intolerance, characteristic of the European Neolithic representatives, including LBK and Körös (8–7 Kyras ago) (Burger *et al.* 2007). The authors suggest that T allele of lactose tolerance was either absent or extremely rare.

It is believed that haplogroup K arose approximately 20.5 Kyras ago, when it evolved out of haplogroup U8 somewhere between Near East and Europe (Soares *et al.* 2009). According to the literature, haplogroup K was present in early European farmers (Bramanti *et al.* 2009, Haak *et al.* 2005). Currently, haplogroup K is found in 4–6% of the European population (Ruiz-Pesini *et al.* 2007), although in a few locations its frequency is quite high, such as in certain regions of France, e.g., Morbihan (ca. 15%) (Dubut *et al.* 2004). It is noteworthy that one of subclades of haplogroup K1a, i.e., K1a1b1a, not determined here, is typical of 45% of Ashkenazi Jews (Behar *et al.* 2006).

Discovery of the coexistence of various alleles in the area where technologies of European Neolithic cultures were being fixed stimulated genetic research and attempts to reproduce an undoubtedly complex initial mechanisms of European populations formation. It has been found that in the early period, 8–7 Kyras ago, allele LCT-13910C, associated with hunter-gatherer groups (Burger *et al.* 2007) was present in the oldest Neolithic population of the Central Europe (Bramanti *et al.* 2009, Haak *et al.* 2005) together with haplogroup K of Middle-Eastern origin (Soares *et al.* 2010). It is noteworthy that an individual with European haplogroup U4 or H1 living 4.2–4.8 Kyras ago on Gotland was LCT-13910C/T and could drink milk (Malmstrom *et al.* 2010).

CONCLUSION

A well-preserved human skeleton of a representative of the Globular Amphora Culture discovered at site 14 in Kowal supplied much valuable anthropological data on the life of an individual who belonged to the Neolithic population that lived at the part of today's Poland (Kuyavia) and, more generally, East-Central Europe. The investigated tomb is unique, considering the type of burial and the items found inside.

It is difficult to ascertain whether the individual is "local" or "non-local" in the absence of a larger faunal baseline and specific isotopic measurements of local precipitation. Oxygen isotopic investigations of human migration do not necessarily identify local individuals from non-local individuals definitively, and cannot

identify migrants from isotopically identical regions. In our study, the analysis of stable oxygen isotopes in tooth phosphate showed a significant drop in the $\delta^{18}\text{O}$ level between the enamel of the lower second incisor and the enamel of the first mandibular premolar, which corresponds to the age of 3–4 years. Despite the fact that $\delta^{18}\text{O}$ values of the analyzed samples are above the oxygen isotopic range of precipitation determined for the Polish territory, we cannot definitively assign a non-local origin to the individual.

Results of stable carbon and nitrogen isotope analyses indicate a diet based on C_3 terrestrial foods with perhaps some input from a C_4 plant, millet. Animal meat and/or produce were significant sources of dietary protein. These results support a mixed subsistence strategy of agriculture and animal husbandry.

The results presented here from aDNA analysis of the individual from Kowal are the first description of sequences isolated from remains of a representative of local Globular Amphora Culture, enriching the still modest pool of human genetic data from the Neolithic.

Conceivably, the isolated DNA belonged to a man whose ancestors originated from the LBK culture, who in turn arrived at the Hungarian Plain 7–8 Kyras ago, as suggested by the identified haplogroup K, one of the four haplogroups (besides T, N1a and W) characteristic for this population (Haak *et al.* 2010). Haplogroup K identified in the Kuyavia region suggests the direction of the gene flow, at least through maternal lineage and thus the origin of farming in Kuyavia.

The LCT-13910C allele encoding lactose intolerance found in the studied individual's DNA is characteristic for a native European population, and may suggest gene flow through the paternal lineage. However, according to some authors (Haak *et al.* 2010), allele LCT-13910T which is responsible for lactose tolerance came to Europe with representatives of the LBK people, and was selected much later. The Y chromosome haplogroup must be identified to investigate possible descent in the maternal lineage.

In order to determine whether the Neolithic male from Kuyavia was a stranger from far away or a native, further isotope assays would be necessary. In particular, strontium isotope analysis of tooth and bone mineral would help identify his origin. In addition, further verification of the aDNA results would require carrying out a population analysis, which is a challenging task, considering the limited availability of research material in the form of well-preserved bone remains. Although a variety of research techniques were applied to better understand the lifestyle and significance of the individual at Kowal, expanding our knowledge of our ancestors' life

strategies, it is impossible to reveal all their secrets. Even straightforward questions such as "who are you?" and "what was your life like?" are yet to be answered.

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Tomasz Kozłowski
Department of Anthropology
Nicolaus Copernicus University
Lwowska 1
87-100 Toruń
Poland
E-mail: kozlow@umk.pl

Beata Stepańczak
Department of Anthropology
Jagiellonian University
Gronostajowa 9
30-387 Kraków
Poland

Department of Anatomy
Jagiellonian University Medical College
Kopernika 12
31-034 Kraków
Poland
E-mail: b.stepanczak@uj.edu.pl

Krzysztof Szostek
Department of Anthropology
Jagiellonian University
Gronostajowa 9
30-387 Kraków
Poland
E-mail: szosy@wp.pl

Laurie J. Reitsema
Department of Anthropology
University of Georgia
250 Baldwin Hall
Athens, GA 30602-1619
USA
E-mail: reitsema@uga.edu

Grzegorz Osipowicz
Institute of Archaeology
Nicolaus Copernicus University
Szosa Bydgoska 44/48
87-100 Toruń
Poland
E-mail: grezegor@poczta.onet.pl

Tomasz Płoszaj
Krystyna Jędrychowska-Dańska
Henryk W. Witas
Department of Molecular Biology
Medical University of Łódź
Narutowicza 60
90-136 Łódź
Poland
E-mail: tomlodz@gmail.com
E-mail: kjd@vp.pl
E-mail: hhww111@gmail.com

Jacek Pawlyta
Department of Radioisotopes, Institute
of Physics
Silesian University of Technology
Krzywoustego 2
44-100 Gliwice
Poland
E-mail: jacek.pawlyta@polsl.pl

Czesława Paluszkiewicz
AGH University of Science
and Technology
Faculty of Materials Science and Ceramics
Mickiewicza 30
30-059 Kraków
Poland
E-mail: ucpalusz@cyf-kr.edu.pl

