The Iceman’s Last Meal Consisted of Fat, Wild Meat, and Cereals

Highlights

- The last meal of the Iceman, a European Copper Age mummy, was reconstructed.
- Our multipronged approach deciphers the meal composition and food processing.
- His high-fat diet was supplemented with wild meat and cereals.

Authors

Frank Maixner, Dmitrij Turaev, Amaury Cazenave-Gassiot, ..., Thomas Rattei, Rudolf Grimm, Albert Zink

Correspondence

frank.maixner@eurac.edu (F.M.), albert.zink@eurac.edu (A.Z.)

In Brief

Maixner et al. report the dietary reconstruction of the Iceman’s last meal using a combined multi-omics approach. The stomach content analysis of the 5,300-year-old glacier mummy shows that the Iceman’s diet preceding his death was a mix of carbohydrates, proteins, and lipids, well adjusted to the energetic requirements of his high-altitude trekking.
The Iceman’s Last Meal Consisted of Fat, Wild Meat, and Cereals

Frank Maixner,1,25,27,* Dmitrij Turaev,2,25 Amarya Cazenave-Gassiot,3,4,26 Marek Janko,5,6,25 Ben Krause-Kyora,7 Michael R. Hoopmann,8 Ulrike Kusebauch,9 Mark Sartain,8 Gea Guerriero,9 Niall O’Sullivan,1 Matthew Teasdale,10 Giovanna Cipollini,1 Alice Paladin,1 Valeria Mattiacci,11 Marco Samadelli,11 Umberto Tecchiati,11 Andreas Putzer,12 Mine Palazoglu,13 John Meissen,13 Sandra Lösch,13 Philipp Rausch,15 John F. Baines,1,6,25 Bum Jin Kim,16 Hyun-Joo An,16 Paul Göstner,17 Eduard Egarter-Vigl,18 Peter Malferttheiner,19 Andreas Keller,20 Robert W. Stark,5,6 Markus Wenk,3,4 David Bishop,21 Daniel G. Bradley,10 Oliver Fiehn,13 Lars Engstrand,22 Robert L. Moritz,7 Philip Doble,21 Andre Frankel,7 Almut Nebel,7 Klaus Oeggl,23 Thomas Rattei,2,26 Rudolf Grimm,24,26 and Albert Zink1,26,*

1Eurac Research - Institute for Mummy Studies, Viale Druso 1, 39100 Bolzano, Italy
2CUBE - Division of Computational Systems Biology, Department of Microbiology and Ecosystem Science, University of Vienna, Althanstrasse 14, 1090 Vienna, Austria
3SLING, Life Sciences Institute, National University of Singapore, Singapore
4Department of Biochemistry, National University of Singapore, Singapore
5Institute of Materials Science, Physics of Surfaces, Technische Universität Darmstadt, 64287 Darmstadt, Germany
6Center of Smart Interfaces, Technische Universität Darmstadt, 64287 Darmstadt, Germany
7Institute of Clinical Molecular Biology, Kiel University, 24105 Kiel, Germany
8Institute for Systems Biology, 401 Terry Avenue North, Seattle, WA 98109, USA
9Environmental Research and Innovation (ERIN), Luxembourg Institute of Science and Technology (LIST), Esch/Alzette, Luxembourg
10Smurfit Institute of Genetics, University of Dublin, Trinity College, Dublin 2, Ireland
11Responsabile del Laboratorio di Archeozoologia della Soprintendenza Provinciale ai Beni culturali di Bolzano – Alto Adige, Ufficio Beni archeologica, 39100 Bolzano, Italy
12South Tyrol Museum of Archaeology, Museumsstrasse 43, 39100 Bolzano, Italy
13Department of Molecular and Cellular Biology & Genome Center, University of California, Davis, Davis, CA, USA
14Department of Physical Anthropology, Institute of Forensic Medicine, University of Bern, Sulgenauweg 40, 3007 Bern, Switzerland
15Max Planck Institute for Evolutionary Biology, August-Thienemann-Strasse 2, 38410 Plön, Germany
16Cancer Research Institute & Graduate School of Analytical Science and Technology, Chungnam National University, Daejeon, Korea
17Department of Radiodiagnostics, Central Hospital Bolzano, Bolzano, Italy
18Scuola Superiore Sanitaria Provinciale “Claudiana,” Via Lorenz Böhlter 13, 39100 Bolzano, Italy
19Department of Gastroenterology, Hepatology, and Infectious Diseases, Otto-von-Guericke University, Leipziger Strasse 44, 39120 Magdeburg, Germany
20Chair for Clinical Bioinformatics, Saarland University, Medical Faculty, Saarbrücken, Germany
21Elemental Bio-imaging Facility, University of Technology Sydney, Broadway, New South Wales, 2007, Australia
22Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, 141 86 Stockholm, Sweden
23Institute of Botany, Sternwartestrasse 15, University of Innsbruck, 6020 Innsbruck, Austria
24Agilent Technologies, 5301 Stevens Creek Blvd, Santa Clara, CA 95054, USA
25These authors contributed equally
26These authors contributed equally
27Lead Contact
*Correspondence: frank.maixner@eurac.edu (F.M.), albert.zink@eurac.edu (A.Z.)
https://doi.org/10.1016/j.cub.2018.05.067

SUMMARY

The history of humankind is marked by the constant adoption of new dietary habits affecting human physiology, metabolism, and even the development of nutrition-related disorders. Despite clear archaeological evidence for the shift from hunter-gatherer lifestyle to agriculture in Neolithic Europe [1], very little information exists on the daily dietary habits of our ancestors. By undertaking a complementary -omics approach combined with microscopy, we analyzed the stomach content of the Iceman, a 5,300-year-old European glacier mummy [2, 3]. He seems to have had a remarkably high proportion of fat in his diet, supplemented with fresh or dried wild meat, cereals, and traces of toxic bracken. Our multi-pronged approach provides unprecedented analytical depth, deciphering the nutritional habit, meal composition, and food-processing methods of this Copper Age individual.

RESULTS AND DISCUSSION

Human evolution is closely linked to dietary changes and the development of new food-processing techniques. This is clearly observed in the transition from hunter-gatherer lifestyle to agriculture, which gave rise to cultivation of crops, animal husbandry, and permanent settlements [1]. The more stable availability of food boosted the growth of Neolithic European populations [4, 5]. However, changes in diet had drawbacks
for health, such as increased rates of caries [6]. Added to this, permanent large settlements combined with the adoption of agriculture promoted the spread of density-dependent infectious diseases [7]. Considering the impact of dietary habits on human evolution and health, the reconstruction of past human subsistence appears to be vital to our understanding of past societies. Stable carbon (\textsuperscript{13}C/\textsuperscript{12}C) and nitrogen (\textsuperscript{15}N/\textsuperscript{14}N) isotope analyses of bone collagen have been widely used to study the dietary adaptations of Neolithic populations [8], lipid analysis on pottery residues dated the onset of milk use in Neolithic Europe [9], and numerous archaeological studies describe the occurrences of wild and domesticated animal and plant remains as indirect measures of past diets [10]. All these studies provide insights into major dietary changes during the Neolithic period. However, specific details on past diets such as the exact meal composition or food processing are missing. Knowledge of daily dietary habits of our ancestors is pertinent as food type, intake quantity, and food-processing techniques influence human metabolism and health.

A recent radiological re-examination of the Iceman, a 5,300-year-old European natural ice mummy, identified his completely filled stomach (Figure 1A) [2]. The well-preserved stomach content still contains ancient endogenous biomolecules as demonstrated from the complete reconstruction of the Iceman’s \textit{Helicobacter pylori} genome [11]. Previous isotopic analysis (\textsuperscript{15}N/\textsuperscript{14}N) of the Iceman’s hair suggested first a vegetarian lifestyle [12, 13] which was later, after careful re-examination of the data, changed to omnivorous diet [14]. Further analyses of lower intestinal tract samples of the Iceman confirmed that he was omnivorous, with a diet consisting of both wild animal and plant material. Among the plant remains, there were cereals, pollen grains of hop-hornbeam, and fragments of bracken and mosses [14–20].

The study material included 11 gastrointestinal (GI) content biopsies of the stomach and small and large intestines (Figure 1A; Data S1). Using microscopy and a combined multi-omics approach targeting different biomolecules (ancient DNA, proteins, metabolites, and lipids) (see STAR Methods), we determined the exact composition of the Iceman’s diet preceding his death and present evidence how people during the Copper Age processed food.

Figure 1. The Iceman’s Gastrointestinal Tract
(A) Gastrointestinal (GI) tract preservation and content texture. The radiographic image shows the completely filled stomach (asterisk) and the intestinal loops of the lower GI tract (arrows). Content samples of the stomach (left, asterisk) and of two different sites in the lower GI tract (middle, right) that were re-hydrated in phosphate buffer are shown below the radiographic image.
(B) Animal muscle fibers detected in the stomach content using light microscopy. Scale bar, 50 \( \mu \)m. The black box contains a zoomed-in view of one muscle fiber (scale bar, 20 \( \mu \)M).
(C) Plant tissue detected in the stomach content using light microscopy. Scale bar, 50 \( \mu \)m. See also Figure S1 for additional plant and animal remains detected in the Iceman intestinal contents.
display a hydrophobic “fatty-like” character (Figure 1A). The most prevalent tissue types in the stomach content samples are animal muscle fibers arranged mostly in bundles (Figures 1B and S1) and plant fragments identified as bran and glumes belonging to the Triticum/Secale type (Figures 1C and S1). The striated muscle fiber structures are characteristic for cardiac and skeletal muscle tissue (Figure S1B) [21]. Further microscopic analysis of the plant micro-remains identified cereal pollen from Triticum type as well as airborne arboreal pollen and an abundance of spores and sporangia from the bracken fern Pteridium aquilinum (Figure S1J).

Metabolite, glycan, stable isotope, and elemental analyses confirm the omnivorous character of the Iceman’s last meal (Data S2). Metabolic compounds indicate the presence of ruminant fat or dairy products (phytanic acid) and support the existence of whole-grain cereals (azealic acid) in the Iceman’s diet. In addition, gamma-terpinene that occurs in coriander oil, and skeletal muscle tissue (Figure S1B) [21]. Further microscopic analysis of the plant micro-remains identified cereal pollen from the Triticum-type as well as airborne arboreal pollen and an abundance of spores and sporangia from the bracken fern Pteridium aquilinum (Figure S1J).

Metabolite, glycan, stable isotope, and elemental analyses confirm the omnivorous character of the Iceman’s last meal (Data S2). Metabolic compounds indicate the presence of ruminant fat or dairy products (phytanic acid) and support the existence of whole-grain cereals (azealic acid) in the Iceman’s diet. In addition, gamma-terpinene that occurs in coriander oil, and skeletal muscle tissue (Figure S1B) [21]. Further microscopic analysis of the plant micro-remains identified cereal pollen from the Triticum-type as well as airborne arboreal pollen and an abundance of spores and sporangia from the bracken fern Pteridium aquilinum (Figure S1J).

Importantly, the presence of ibex and red deer in the stomach content samples was independently confirmed in the Molecular Population Genetics Laboratory, Trinity College, Dublin, by sequencing of a mammalian mitochondrial capture (Data S1). Moreover, PCR-based analysis targeting animal- and plant-DNA barcodes and further assignment of the shotgun data against the DNA
Barcode of Life Data dataset (www.boldsystems.org) support the first metagenomic results on a phylogenetic higher hierarchical level (genus, family) (Figure S4).

Our analysis of the ancient DNA identified the main components in the Iceman’s meal. However, the highly degraded state of this biomolecule excluded any further quantification of certain foods.

Using multistep solubilization and fractionation proteomics, we identified 167 animal and plant proteins in the stomach metaproteome, of which 13 were taxonomically unambiguously assigned to the Triticinae, 6 to the Caprinae, and 1 protein to the Cervinae subfamily (Figure 2C; Data S2). The proteomic hits not only confirm the previous metagenomic results but also provide important information on the food source. The detected Triticinae plant proteins are primarily expressed in the endosperm (Globulin 3A, Triticin) [23] and pericarp (Oxalate oxidase 2) [24] of wheat seeds. The Caprinae Titin-like protein is part of the sarcomere apparatus in muscle tissue [25]. Thus, we have combined genomic and proteomic evidence that the Iceman’s last meal contained whole seeds of the T. monococcum/T. urartu-like wheat and muscle fibers of ibex. However, due to the lack of diagnostic proteins, we cannot determine which tissues of the red deer were ingested by the Iceman.

Approximately 46% ± 19% (w/w) of the stomach content is fat residues. Comparative lipid analysis of the Iceman’s stomach content to contemporary wild ruminant muscle and adipose tissue, and to goat dairy products (milk and cheese), using reverse-phase liquid chromatography separation and quadrupole time-of-flight (QToF) tandem mass spectrometry analysis (RP-LC/MSMS) identified triacylglycerols (TAGs) as a major component of the stomach content, with molecular compositions consistent with ruminant muscle/adipose fat (Figure 3A). The TAGs identified in the Iceman’s stomach contain between 44 and 56 total carbon atoms in the fatty acyl chains and resemble the TAG profile of contemporary muscle and adipose tissue. The high level (up to 20%) of odd numbered fatty acyl chains is consistent with TAG profiles from ruminant animals (Data S2) [27]. Short-chain TAG species characteristic of dairy products are absent in the Iceman TAG profile. However, the high level of saturated bonds in the TAGs of the Iceman’s stomach compared to the fresh tissues indicates an already ongoing preferential degradation of unsaturated fatty acids in the ancient specimen (Figure 3B) [28]. Therefore, we cannot fully exclude that short-chain dairy-specific lipid species were degraded, although the identification of di- and poly-unsaturated TAG indicates a still high level of preservation. Furthermore, we have histological evidence for the presence of adipose tissue in the Iceman’s stomach content (Figure S1C). Further comparison of the dispersion factor with the average carbon number (M) of each detected TAG in the analyzed samples points to the ibex as potential source of the...
adipose tissue (Figure 3C). Thus, the ibex served the Iceman as food source for both meat and fat.

Charcoal particles detected in a previous study in the Iceman’s lower intestine content suggested the involvement of fire in the food processing [16]. We experimentally tested the effect of heat and drying on contemporary animal muscle tissue and compared their structural and chemical composition with the muscle fibers detected in the Iceman’s stomach by using atomic force microscopy (AFM) and Raman spectroscopy. High magnification AFM images revealed striated fiber structures in both ancient and fresh or dried contemporary muscle fibers while structural changes of the fibers were observed when the samples were thermally treated (Figure 4A). After frying or cooking, the regular patterns in the meat fibers disappeared, indicating the denaturation of their protein compounds. Complementary confocal Raman spectroscopy measurements support the AFM results and indicate heat-related conformational changes of the molecular composition of the meat fibers (Figure 4B). The Raman spectra of the ancient muscle fibers largely matched the spectra taken from fresh and dried contemporary meat fibers. After heat treatment, the Raman band at about 1,093 cm\(^{-1}\) becomes weaker, and bands at around 1,240 cm\(^{-1}\) appear that are indicative of heat-induced changes in the secondary structure of the myofibrils [29]. Thus, both the AFM and Raman spectroscopy results suggest that the Iceman consumed either fresh or dried wild meat. Besides, a slow drying or smoking of the meat over the fire would explain the charcoal particles detected previously in the lower intestine content.

Our forensic multi-omics study provides important insights into the general life and nutritional habits of a Copper Age individual in the Alpine area. The microscopic and molecular data presented support the presence of three major components in the Iceman’s last meal: fat and game meat from ibex and red deer supplemented with cereals from einkorn. Intriguing is the pervasive presence of bracken particles. Despite of its toxicity, bracken is still consumed today by different indigenous people [30]. However, it is also possible that fern leaves, as it was previously suggested for the moss Neckera complanata [20], were used to wrap the food and the spores and sporangia entered the ingesta unintentionally. Unexpectedly, there is a high proportion of fat in the diet. The distribution of triglycerides and their constituting fatty acids is consistent with the consumption of ibex muscle and adipose tissues. The extreme alpine environment in which the Iceman lived and where he have been found (3,210 m above sea level) is particularly challenging for the human physiology and requires optimal nutrient supply to avoid rapid starvation and energy loss [31]. Therefore, the Iceman seemed to have been fully aware that fat displays an excellent energy source. On the other hand, the intake of animal adipose tissue fat has a strong correlation with increased risk of coronary artery disease [32]. A high saturated fats diet raises cholesterol levels in the blood, which in turn can lead to atherosclerosis. Importantly, computed tomography scans of the Iceman showed major calcifications in arteries and the aorta indicating an already advanced atherosclerotic disease state [33]. Both his high-fat diet and his genetic predisposition for cardiovascular disease [34] could have significantly contributed to the development of the arterial calcifications. Finally, we could show that the Iceman either consumed fresh or dried meat. Drying meat by smoking or in the open air are simple but highly effective methods for meat preservation that would have allowed the Iceman to store meat long term on journeys or in periods of food scarcity. In summary, the Iceman’s last meal was a well-balanced mix of carbohydrates, proteins, and lipids, perfectly adjusted to the energetic requirements of his high-altitude trekking.
STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- CONTACT FOR REAGENT AND RESOURCE SHARING
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - The Iceman
- METHOD DETAILS
  - Sampling
  - Microscopic analysis
  - Metabolite analysis
  - Glycan analysis
  - Stable isotope analysis
  - Elemental analysis of the Iceman stomach content
  - DNA extraction, library preparation and Illumina sequencing
  - Bioinformatic analysis of the Illumina datasets
  - Animal mitochondrial genome enrichment in the Iceman's stomach content
  - Polymerase chain reaction (PCR)-based analyses
  - Proteomic analysis of the Iceman stomach content
  - Lipid analysis of the Iceman stomach content
  - Atomic force microscopy (AFM) and Raman spectroscopy
- QUANTIFICATION AND STATISTICAL ANALYSIS
- DATA AND SOFTWARE AVAILABILITY
- ADDITIONAL RESOURCES

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and two data files and can be found with this article online at https://doi.org/10.1016/j.cub.2018.05.067.

ACKNOWLEDGMENTS

We acknowledge the following funding sources: Provincia Autonoma di Bolzano, grant legge 14 agreement n. 1/40.3, 23.11.2012 (F.M., N.O., G.C., A. Paladin, M. Samadelli, A.Z.), the DFG Graduate School Human Development in Landscapes at Kiel University (B.K.-K., J.F.B., A.F., A.N.), Ludwig Erhard and his team from the Provincia autonoma di Bolzano - Alto Adige, Ripartizione Foresti are highly acknowledged for providing the modern wild animal samples for this study. M.T. and V.M. were supported by European Research Council Investigator Grant 295729-CodeX to D.G.B. The National Institutes of Health from the National Institute of General Medical Sciences under Grant Nos. R01 GM087221 (R.L.M.), S10 RR027584 (R.L.M.), and the 2P50 GM078547/Center for Systems Biology (R.L.M.). The authors thank the Department of Innovation, Research and University of the Autonomous Province of Bozen/Bolzano for covering the open access publications costs. Gaia Brusco is highly acknowledged for the artwork.

AUTHOR CONTRIBUTIONS

F.M. and A.Z. conceived the investigation. F.M., D.T., A.C.-G., M.J., M.R.H., R.L.M., A.F., A.K., M.W., R.W.S., O.F., P.D., T.R., R.G., and A.Z. designed the experiments. P.M., P.G., L.E., E.E.-V., M. Samadelli, F.M., and A.Z. were involved in the sampling campaign. F.M., A.C.-G., M.J., B.K.-K., M.R.H., U.K., M. Sartain, N.O., M.T., G.C., V.M., M.P., J.M., S.L., P.R., B.J.K., H.-J.A., and D.B. conducted the experiments. F.M., D.T., A.C.-G., M.J., M.R.H., N.O., M.T., S.L., J.F.B., B.J.K., H.-J.A., and K.O. performed analyses. F.M. wrote the manuscript with contributions from D.T., A.C.-G., M.J., M.R.H., A. Paladin, A. Putzer, G.G., U.T., A.N., D.G.B., K.O., R.G., T.R., and A.Z.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: April 5, 2018
Revised: May 15, 2018
Accepted: May 23, 2018
Published: July 12, 2018

REFERENCES

1. Fowler, C., Harding, J., and Hofmann, D. (2015). Defining the ‘Neolithic in Europe’: Diverse and Contemporaneous Communities, c. 6500-2500 BC. In The Oxford Handbook of Neolithic Europe, C. Fowler, J. Harding, and D. Hofman, eds. (United Kingdom: Oxford University press).
2. Ostner, P., Perner, P., Bonattie, G., Graefen, A., and Zink, A.R. (2011). New radiological insights into the life and death of the Tyrolean Iceman. J. Arch. Sci. 38, 3425–3431.
3. Spindler, K. (1994). The Man in the Ice (Harmony Books).
4. Shennan, S., Downey, S.S., Timpson, A., Edinborough, K., Collede, S., Kerig, T., Manning, K., and Thomas, M.G. (2013). Regional population collapse followed initial agriculture booms in mid-Holocene Europe. Nat. Commun. 4, 2486.
5. Richards, M.P. (2002). A brief review of the archaeological evidence for Palaeolithic and Neolithic subsistence. Eur J Clin Nutr 56, 1270–1278.
6. Nicklischn, N., Gansmeier, R., Siebert, A., Friederich, S., Mellauer, H., and Att, K.W. (2016). Holes in teeth - Dental caries in Neolithic and Early Bronze Age populations in Central Germany. Ann. Anat. 203, 90–99.
7. Diamond, J. (2002). Evolution, consequences and future of plant and animal domestication. Nature 418, 700–707.
8. Richards, M.P., Schulting, R.J., and Hedges, R.E. (2003). Archaeology: sharp shift in diet at onset of Neolithic. Nature 425, 366.
9. Evershed, R.P., Payne, S., Sherratt, A.G., Copley, M.S., Coolidge, J., Urem-Kotsu, D., Kotsakis, K., Ozoglan, M., Ozoglan, A.E., Nieuwenhuysie, O., et al. (2008). Earliest date for milk use in the Near East and southeastern Europe linked to cattle herding. Nature 455, 528–531.
10. Fowler, C., Harding, J., and Hofman, D. (2015). Movement of Plants, Animals, Ideas and People. In The Oxford Handbook of Neolithic Europe, C. Fowler, J. Harding, and D. Hofman, eds. (United Kingdom: Oxford University press).
11. Maximen, F., Krause-Kyora, B., Turave, D., Herbig, A., Hoopmann, M.R., Hallows, J.L., Kusebauch, U., Vgl, E.E., Malferther, P., Meirgrud, F., et al. (2016). The 5300-year-old Helicobacter pylori genome of the Iceman. Science 351, 162–165.
12. Macko, S.A., Engel, M.H., Andrusievich, V., Lubec, G., O’Connell, T.C., and Hedges, R.E. (1999). Documenting the diet in ancient human populations through stable isotope analysis of hair. Philos Trans R Soc Lond B Biol Sci 354, 65–75.
13. Macko, S.A., Lubec, G., Teschler-Nicola, M., Andrusievich, V., and Engel, M.H. (1999). The Ice Man’s diet as reflected by the stable nitrogen and carbon isotopic composition of his hair. FASEB J. 13, 559–562.
14. Dickson, J.H., Oeggli, K., Holden, T.G., Handlei, L.L., O’Connell, T.C., and Preston, T. (2000). The omnivorous Tyrolean Iceman: colonic contents (meat, cereals, pollen, moss and whipworm) and stable isotope analyses. Philos. Trans. R. Soc. Lond. B Biol. Sci. 355, 1843–1849.
15. Oeggli, K. (2000). The Diet of the Iceman. In The Iceman and his natural environment, S. Bortenschlager and K. Oeggl, eds. (Vienna: Springer), pp. 89–115.
16. Oeggli, K., Koffer, W., Schmidl, A., Dickson, J.H., Egarter-Vigl, E., and Gaber, O. (2007). The reconstruction of the last itinerary of “Ötzi”, the
and the genetic history of the Neolithic Tyrolean Iceman. Sci. Rep. 6, 18932.

57. Janko, M., Stark, R.W., and Zink, A. (2012). Preservation of 5300 year old red blood cells in the Iceman. J. R. Soc. Interface 9, 2581–2590.

58. Maixner, F., Overath, T., Linke, D., Janko, M., Guerriero, G., van den Berg, B.H., Stade, B., Leidinger, P., Backes, C., Jaremek, M., et al. (2013). Paleoproteomic study of the Iceman’s brain tissue. Cell. Mol. Life Sci. 70, 3709–3722.

59. Keller, A., Kreis, S., Leidinger, P., Maixner, F., Ludwig, N., Backes, C., Galata, V., Guerriero, G., Fehlmann, T., Franke, A., et al. (2017). miRNAs in ancient tissue specimens of the Tyrolean Iceman. Mol. Biol. Evol. 34, 793–801.

60. O’Sullivan, N.J., Teasdale, M.D., Mattiangelii, V., Maixner, F., Pinhasi, R., Bradley, D.G., and Zink, A. (2016). A whole mitochondria analysis of the Tyrolean Iceman’s leather provides insights into the animal sources of Copper Age clothing. Sci. Rep. 6, 31279.

61. Makristathis, A., Schwarzmeier, J., Mader, R.M., Varmuza, K., Simonitsch, A., Fry, B. (2006). Stable Isotope Ecology (New York: Springer-Verlag).

62. Schoeninger, M.J., and DeNiro, M.J. (1984). Nitrogen and carbon isotopic composition of bone collagen from marine and terrestrial animals. Geochim. Cosmochim. Acta 48, 625–639.

63. Tang, J.N., Zeng, Z.G., Wang, H.N., Yang, T., Zhang, P.J., Li, Y.L., Zhang, A.Y., Fan, W.Q., Zhang, Y., Yang, X., et al. (2008). An effective method for isolation of DNA from pig faeces and comparison of five different methods. J. Microbiol. Methods 75, 432–436.

64. Lee, E.J., Makarewicz, C., Renneberg, R., Harder, M., Krause-Kyora, B., Müller, S., Ostritz, S., Fehren-Schmitz, L., Schreiber, S., Müller, J., et al. (2012). Emerging genetic patterns of the Neolithic European: perspectives from a late Neolithic Bell Beaker burial site in Germany. Am. J. Phys. Anthropol. 148, 571–579.

65. Kircher, M., Sawyer, S., and Meyer, M. (2012). Double indexing overcomes inaccuracies in multiplex sequencing on the Illumina platform. Nucleic Acids Res. 40, 63.

66. Meyer, M., and Kircher, M. (2010). Illumina sequencing library preparation for highly multiplexed target capture and sequencing. Cold Spring Harb Protoc 2010, pdb.prot5448.

67. Coordinators, N.R.; NCBI Resource Coordinators (2017). Database Resources of the National Center for Biotechnology Information. Nucleic Acids Res. 45 (D1), D12–D17.

68. Andrew, S. (2011). FastQ Screen. Available online at. http://www.bioinformatics.babraham.ac.uk/projects/fastq_screen/.

69. Guindon, S., and Gascuel, O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52, 696–704.

70. Rohland, N., and Hofreiter, M. (2007). Comparison and optimization of ancient DNA extraction. Biotechniques 42, 343–352.

71. Gamba, C., Jones, E.R., Teasdale, M.D., McLaughlin, R.L., Gonzalez-Fortes, G., Mattiangelii, V., Domboroczki, L., Kóvari, I., Pap, I., Anders, A., et al. (2014). Genome flux and stasis in a five millennium transect of European prehistory. Nat. Commun. 5, 5257.

72. Ermini, L., Olivieri, C., Rizzi, E., Corti, G., Bonnal, R., Soares, P., Luciani, S., Marota, I., De Bellis, G., Richards, M.B., and Rollo, F. (2008). Complete mitochondrial genome sequence of the Tyrolean Iceman. Curr. Biol. 18, 1687–1693.

73. Berry, D., Ben Mahfoudh, K., Wagner, M., and Loy, A. (2011). Barcoded primers used in multiplex amplicon pyrosequencing bias amplification. Appl. Environ. Microbiol. 77, 7846–7849.

74. Der, J.P., Barker, M.S., Wickett, N.J., dePamphilis, C.W., and Wolf, P.G. (2011). De novo characterization of the gametophyte transcriptome in bracken fern, Pteridium aquilinum. BMC Genomics 12, 98.

75. Bligh, E.G., and Dyer, W.J. (1959). A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 1911–1917.

76. Garnier, N., Rolando, C., Htte, J.M., and Tokarski, C. (2009). Analysis of archaeological triacylglycerols by high resolution nanoESI, FT-ICR MS and IRMPD MS/MS: Application to 5th century BC–4th century AD oil lamps from Olbia (Ukraine). Int. J. Mass. Spectrom. 284, 47–56.

77. Folch, J., Lees, M., and Sloane Stanley, G.H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. 226, 497–509.

78. Krank, J., Murphy, R.C., Barkley, R.M., Duchoslav, E., and McAnoy, A. (2007). Qualitative analysis and quantitative assessment of changes in neutral glycerol lipid molecular species within cells. Methods Enzymol. 432, 1–20.

79. Vizzanio, J.A., Csordas, A., del-Toro, N., Dianes, J.A., Gries, J., Lavidas, I., Mayer, G., Perez-Riverol, Y., Resinger, F., Ternent, T., et al. (2016). 2016 update of the PRIDE database and its related tools. Nucleic Acids Res. 44 (D1), D447–D455.
## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Biological Samples** | | |
| Iceman gastrointestinal tract content samples and tissues biopsies | [11] | N/A |
| **Chemicals, Peptides, and Recombinant Proteins** | | |
| EDTA disodium salt dihydrate | Carl Roth | Cat#8043.2 |
| Chloroform | SIGMA-ALDRICH | Cat#C2432 |
| Chloroform | Fisher Chemical | Cat#C297-4 |
| Chloroform -isoamylation (24:1, v/v) | SIGMA-ALDRICH | Cat#C0549 |
| Methanol | Carl Roth | Cat#4627.1 |
| Methanol | Fisher Chemical | Cat#A456-4 |
| Acetonitrile | Fisher Chemical | Cat#A955-4 |
| Isopropanol | Fisher Chemical | Cat#A461-4 |
| Cetyltrimethylammonium bromide CTAB | Carl Roth | Cat#9161.3 |
| Polyvinylpyrrolidone-K10 | SIGMA-ALDRICH | Cat#PVP10-100G |
| Tris-HCl | Carl Roth | Cat#9090.2 |
| Acrylamide | Carl Roth | Cat#0189.2 |
| Ammonium peroxydisulphate APS | Carl Roth | Cat#9592.2 |
| TEMED | Carl Roth | Cat#2367.3 |
| Sodium acetate | Carl Roth | Cat#6773.1 |
| Guanidine hydrochloride | Carl Roth | Cat#6069.2 |
| Beta-Mercaptoethanol | Carl Roth | Cat#4227.3 |
| BSA (20 mg/ml) | New England Biolabs | Cat#B9000S |
| Bst polymerase, large fragment (8 U/μl) | New England Biolabs | Cat#M0275S |
| dNTPs (2.5mM each) | Thermo Fisher Scientific | Cat#R72501 |
| T4 DNA ligase (5 U/μl) | Thermo Fisher Scientific | Cat#EL0014 |
| **Critical Commercial Assays** | | |
| Min Elute PCR Purification Kit | QUIAGEN | Cat#28006 |
| Amplitaq Gold mastermix 360 | Thermo Fisher Scientific | Cat#4398881 |
| NEBNext End Repair Module | New England Biolabs | Cat#E6050L |
| Accuprime Pfx Supermix | Thermo Fisher Scientific | Cat#12344040 |
| **Deposited Data** | | |
| Iceman gastrointestinal tract content shotgun datasets | ENA: ERP012908 |
| Iceman stomach content enriched dataset | This Study | ENA: PRJEB26465 |
| Iceman stomach content amplicon datasets | This Study | ENA: PRJEB26365 |
| Iceman stomach content proteomics data | This Study | PRIDE: PXD009565 |
| **Oligonucleotides** | | |
| 12419F CAAACTGGGATTAGATCC | This Study | Thermo Fisher Scientific |
| 12564R YRGAACAGGCCTCTCCTTAG | This Study | Thermo Fisher Scientific |
| CBF GCCTACGCAAATCTTCTGATC | [35] | Thermo Fisher Scientific |
| CBR CTGGCCTCAAATCTGATGAG | [35] | Thermo Fisher Scientific |
| trnL-g GGGCAATCCTGAGCCAA | [36] | Thermo Fisher Scientific |
| trnL-h CCATTGAGTCTCTGCACCTATC | [36] | Thermo Fisher Scientific |
| rbcL19 AGATTTCCGCGAGCCACTGCAGGCCCCTGCTTTC | [37] | Thermo Fisher Scientific |
| rbcLZ1 ATGTACCACAAACAGAGACTAAAGAAGGT | [37] | Thermo Fisher Scientific |

(Continued on next page)
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Frank Maixner (frank.maixner@eurac.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

The Iceman

The Iceman is one of the oldest human mummies ever found in human history [52]. With his body perfectly preserved throughout millennia in an Alpine glacier (3,210 m above sea level) and his secrets unlocked and shown for the first time to humanity by the melting of ice in 1991, it has been and still is a subject of great interest, fascination and speculations, since the veil of mystery enveloping the circumstances of his death has not yet fallen [3]. The Iceman was murdered when he was 40-50 years old by an arrow that lacerated the left subclavian artery, likely leading to a rapid, deadly hemorrhagic shock [53, 54]. It is not only his historical age and the bounty of prehistoric belongings that make him extremely valuable for scientists, but also how he was preserved for about 53 centuries. The Iceman is indeed an “ice mummy,” so-called for the retention of humidity in his cells [55]. The body tissues contain well preserved biomolecules such as lipids, proteins, and nucleic acids. This feature makes him extremely valuable for modern scientific investigations [11, 34, 56–61]. Initially, the Iceman’s stomach was not found radiologically and was generally assumed

---

**Continued**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Imaging Software NIS elements F 3.00 | Nikon | N/A |
| ChromaTOF software version 4.2 | LECO | N/A |
| FastQC | Babraham Bioinformatics | http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ |
| SeqPrep | N/A | https://github.com/jstjohn/SeqPrep |
| RAPSearch2 | [38] | http://rapsearch2.sourceforge.net/ |
| Krona | [39] | https://github.com/marbl/Krona |
| MEGAN6 | [40] | https://ab.inf.uni-tuebingen.de/software/megan6 |
| Burrows-Wheeler Aligner (BWA) | [41] | http://bio-bwa.sourceforge.net/ |
| blastn | [42] | N/A |
| FastQ Screen | Babraham Bioinformatics | http://www.bioinformatics.babraham.ac.uk/projects/fastq_screen/ |
| SAMtools | [43] | http://samtools.sourceforge.net/ |
| Picard tools | N/A | http://broadinstitute.github.io/picard/ |
| ANGSD | [44] | http://www.popgen.dk/angsd/index.php/ANGSD |
| MAFFT | [45] | https://mafft.cbrc.jp/alignment/server/ |
| ARB software package | [46] | http://www.arb-home.de/ |
| AdapterRemoval | [47] | https://github.com/MikkelSchubert/adapterremoval |
| DeDup | N/A | https://github.com/apeltzer/DeDup |
| mapDamage | [21] | https://ginolhac.github.io/mapDamage/ |
| GATK | [48] | https://software.broadinstitute.org/gatk/ |
| mothur | [49] | https://www.mothur.org/ |
| Trans-Proteomic Pipeline (TPP) software tool suite | [50] | http://tools.proteomecenter.org/wiki/index.php?title=Software:TPP |
| MassHunter Qualitative Analysis Software Version B.05.01 | Agilent | https://www.agilent.com/en/products/software-informatics/masshunter-suite/masshunter/masshunter-software |

| Other |
|-------|
| BOLD system database | [51] | http://www.boldsystems.org/ |
| Additional metadata for the Illumina shotgun datasets | [11] | http://cube.univie.ac.at/supplements/iceman |
METHOD DETAILS

Sampling
The sampling of the Iceman stomach content took place under sterile conditions at an ambient temperature of 4°C in the Iceman’s conservation chamber at the Archaeological Museum of Bolzano, Italy. The samples were immediately stored at −20°C in the ancient DNA laboratory of the Eurac Research - Institute for Mummy Studies. Furthermore, we had access to previously sampled gastrointestinal tract contents and to an Iceman muscle and lung tissue sample, which we used in addition to the extraction blanks as a negative control for our molecular analyses. See Data S1 for additional details to the samples used in this study.

Microscopic analysis
Small pieces of the stomach content (0.5cmx0.5cm) were processed for microscopy in the aDNA laboratory of the Eurac Research - Institute for Mummy Studies, Bolzano. After rehydration in 15mL solution consisting of 5 parts glycerol and 5 parts 4% formaldehyde for 48 h, the samples were fixed for 24 h in 4% formaldehyde and finally stored in 1x phosphate buffered saline solution at 4°C. For the microscopic analysis, small tissue pieces have been embedded with an aqueous mounting medium on a glass slide. In parallel, some fixed content samples were embedded on a cryostat in 10 μm thick sections (Leica, CM1950). The cryosections were histochemically counterstained with the lipid stain Sudan III [62]. The images were recorded with a CCD camera (Nikon DS-Fi1) mounted on a light microscope (Nikon Eclipse E600), by using the imaging software NIS elements F 3.00. Additionally, ancient animal meat fibers detected in the stomach content were sent to the Center of Smart Interfaces at the TU Darmstadt, Germany, where the coarse topography of meat fibers was analyzed by laser scanning microscope (LSM) images (VK 8710, Keyence, Osaka, Japan).

Metabolite analysis
Primary metabolism was assessed using GC-TOF MS. 2 mg fresh weight of stomach contents were extracted using 1 mL of degassed and pre-cooled (−20°C) acetonitrile/isopropanol/water solvent at the volume ratio 3:3:2. After centrifugation, the supernatant was removed and evaporated to complete dryness. A clean-up step with 500 μL acetonitrile/water (1:1) removed membrane lipids and triglycerides and after centrifugation. To the dried extracts, C8-C30 fatty acid methyl esters were added as internal standards. Samples were derivatized by 10 μL methoxyamine hydrochloride in pyridine (20 mg/mL) at 30°C for 90 min, followed by the addition of 90 μL N-methyl-N-trimethylsilyl trifluoroacetamide at 37°C for 30 min. 0.5 μL of sample was injected at 50°C (ramped to 250°C by 12°C/s and held for 3 min) in splitless mode in a Gerstel cold injection system with multi-baffled glass liners. Metabolites were separated using an Agilent 6890 GC system (Santa Clara, CA, USA) equipped with a Rtx5Sil-MS column (30 m length x 0.25 mm i.d.) with a 0.25 μm 95% dimethyl/ 5% diphenyl polysiloxane film and additional 10 m integrated guard column (Restek, Bellefonte, PA, USA). Chromatography was performed at 1 mL/min helium from 50°C to 330°C with 20°C/min. Mass spectra were acquired on a Leco Pegasus IV time of flight mass spectrometer (St. Joseph, MI, USA) with electron ionization at 70 eV at 250°C. Masses were detected between m/z 85-500 at 17 spectra/s and 1800 V detector voltage. ChromaTOF software version 4.2 was used for data pre-processing including baseline subtraction and automatic mass spectral deconvolution and peak detection. The resulting chromatograms were further processed using the metabolomics BinBase database and spectra were further manually investigated by the Fiehnlib libraries and the NIST12 mass spectral library. Please refer for the list of metabolites identified in the Iceman stomach content to Data S2_Metabolomics.

Glycan analysis
Glycan analysis for the Iceman stomach content was performed at Asia-Pacific Glycomics Reference Site (AGRS) in Daejeon, Republic of Korea. Two stomach content samples of 0.021 g and 0.023 g, respectively were ground with 300 μL 70% EtOH into tissue grinder. High molecular weight materials including glycoproteins were enriched from ground samples by mini dialysis unit (10,000 MWCO). Supernatant was transferred to micro-centrifuge tube and denatured by mixture of NH4HCO3 and DTT (95°C, 2min). N-glycans were enzymatically released from denatured glycoproteins by PNGase F (37°C, overnight) and these were further purified and enriched using solid phase extraction with a porous carbon cartridge. N-glycans released from the Iceman stomach content were comprehensively characterized by nano LC PGC-chip/Q-TOF MS and tandem MS (Agilent 6530). Glycan candidates were selected from the mass-to-charge values extracted in raw LC/MS data based on monosaccharide combinations using hexose, N-acetyl hexoamine, fucose, N-acetyl neuraminic acid, and xylose. N-glycan compositions were further confirmed and determined by compositional analysis using tandem MS. In order to determine relative amounts of glycans, individual glycan compound was integrated using the ion count corresponding to individual peak and then normalized by total ion counts to obtain the relative abundance of each glycan. Additionally, isomers derived from PGC-LC analysis were combined in a single composition prior to quantitative analysis. Data S2_Glycomics lists all identified glycan structures.
Stable isotope analysis

The preparation of the stomach content (EURAC ID 1054) for the stable isotope analysis was conducted in the laboratory at the Department of Physical Anthropology, Institute of Forensic Medicine at University of Bern. Therefore, a sample of 1g was dissolved in ddH2O and freeze-dried for 47h. After homogenization by a ball triturator (Retsch), approx. 5.5mg of the lyophilized stomach content was weighed into tin capsules and shipped to IRMS at Isolab GmbH, Schweitenkirchen, Germany for measurement. Stable isotope ratios of carbon (13C/12C), nitrogen (15N/14N) and sulfur (34S/32S) were analyzed by isotope ratio mass spectrometry and the mean of six measurements was calculated to avoid measurement errors due to potential inhomogeneous material. All data are presented in δ-notation in per mil (‰) according to international standards for carbon (Vienna Pee Dee Belemnite, VPDB), nitrogen (Ambient Inhaleble Reservoir, AIR) and sulfur (Canyon Diablo Troilite, CDT) [63–65]. An internal standard was used for determination of the analytical error. It amounted to ± 0.1‰ for δ13C, ± 0.2‰ for δ15N and ± 0.3‰ for δ34S. For details for the stable isotope values of the Iceman stomach content please refer to the Data S2_ Stable isotope analysis.

Elemental analysis of the Iceman stomach content

An Agilent Technologies 7500cx inductively coupled plasma-mass spectrometer (ICP-MS) (Agilent Technologies, Mulgrave, Australia) was used with sample introduction via a micromist concentric nebuliser (Glass Expansion, West Melbourne, Australia) and a Scott type double pass spray chamber cooled to 2°C. The sample solution and the spray chamber waste were carried with the aid of a peristaltic pump. ICP-MS extraction lens conditions were selected to maximize the sensitivity of a 1% HNO3:HCl solution containing 1ng ml-1 of Li, Co, Y, Ce and Ti. Helium was added into the octopole reaction cell to reduce interferences. Calibration curves were constructed and the results analyzed using Agilent Technologies MassHunter software. A certified calibration standard containing Li, Be, B, Na, Mg, Al, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Sr, Mo, Ag, Cd, Sb, Ba, La, Eu, Ho, Yb, Ti, Pb, Bi, Th, U and P as well as a Hg standard was obtained from Choice Analytical, Thornleigh, Australia. Calibration curves were constructed from seven standards at 0, 1, 10, 50, 100, 500, and 1000 ng/mL. Baseline nitric acid (HNO3) hydrochloric acid (HCl) and hydrogen peroxide (H2O2) were purchased from Choice Analytical, Thornleigh, Australia. Two stomach content samples of 0.05915 g and 0.0279 g were each digested in 2 mL HNO3, 1 mL HCl and 1 mL H2O2 in a Milestone MLS 1200 microwave digester (Milestone, Sorisole, Italy), and diluted to approximately 50 g (accurately measured on an analytical balance) before analysis. A digestion blank was subtracted from each sample. The average concentrations of the elements detected in the Iceman’s stomach is listed in the Data S2_Elemental analysis.

DNA extraction, library preparation and Illumina sequencing

The datasets used for the metagenomic dietary analysis have been published in our previous study on the Iceman’s Helicobacter pylori genome. In the following sections, all major molecular steps will be briefly outlined once more. For methodological details, however, please refer to the publication of Maixner et al. [11]. The molecular analyses were conducted at the ancient DNA Laboratory of the Eurac Research - Institute for Mummy Studies, Bolzano, Italy, and in the ancient DNA facility of the Institute of Clinical Molecular Biology, Kiel University, Germany. Sample preparation and DNA extraction was performed in pre-PCR areas dedicated to ancient DNA procedures. DNA was extracted from the gastrointestinal tract samples (250 mg) and from lung tissue (50 mg) using a chloroform-based DNA extraction method according to the protocol of Tang et al. [66]. DNA extraction from the muscle tissue (100 mg) was performed with a magnetic-bead based technology using the Biorobot®-EZ1 (QIAGEN, Hilden, Germany), following previously described procedures [67]. Negative controls from all experimental steps were included to monitor external contamination. Library preparation and sequencing were performed at the Institute of Clinical Molecular Biology, Kiel University, Germany. Libraries for the sequencing runs were generated with a modified protocol for Illumina multiplex sequencing [88, 69]. The sequencing was carried out on the Illumina HiSeq 2000 and 2500 platform by 2 × 101 cycles using the HiSeq v3 chemistry and the manufacturer’s protocol for multiplex sequencing. For details to the metagenomic shotgun datasets please refer to Data S1_Illumina shotgun datasets.

Bioinformatic analysis of the Illumina datasets

The metagenomic shotgun datasets were subjected to a bioinformatic pipeline to identify the major animal and plant dietary composition of the Iceman’s last meals. The paired-end Illumina reads (101 bp length) were first quality-checked using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and then subjected to adaptor removal and read merging using SeqPrep (https://github.com/jstjohn/SeqPrep) (“SeqPrep -f forward-read-fastqfile -r reverse-read-fastqfile –1 forward-read-output-fastqfile –2 reverse-read-output-fastqfile –L 15 –A10 “AGATCGGAAGAGCACACGTCTGAA” -B “AGATCGGAAGAGCGTCG TGTAGGG” –s merged-fastq-file). Approximately 60%–90% of reads were merged (see Data S1_Illumina shotgun datasets). To obtain a first overview of the taxonomic composition of selected samples from the different gastrointestinal tract contents and of the muscle tissue control sample, we performed a sequence similarity search of the metagenomic reads using RAPSearch2 [38] and the complete NCBI-nr database [70]. A stringent minimum bitscore filter (min bitscore 70) was applied to reduce random hits and the results were taxonomically assigned using MEGAN6 [40] with the lowest common ancestor (LCA) of hits within 10% of the bitscore of the best hits and a minimum number of 100 reads that a taxon must obtain. Finally, the data was visualized using the Krona metagenomic visualization tool [39] providing a first taxonomic overview on the bacterial and eukaryotic reads. In the subsequent step, we focused on the eukaryotic reads aiming at identifying highly abundant animal and plant species possibly resembling the Iceman’s diet. We first aligned all metagenomic reads against all currently available full mitochondrial and chloroplast genomes of the NCBI database [70] using BWA [41] with default parameters. Subsequently, to obtain a taxonomic overview of the mapped reads and to identify and filter for the most prevalent taxonomic groups in the samples, we performed a sequence similarity search using
blastn [42] with default parameters and the complete NCBI-nt database as reference database [70]. Blast results were taxonomically assigned using MEGAN6 [40] with a Min percent value of 0.01 for the reads mapped against the mitochondrial genomes and with a Min percent value of 0.5 for the reads mapped against the chloroplast genomes (see Figure S2).

To further confirm our previous taxonomic assignments and to understand if the extracted reads are of ancient origin or if they display modern contamination, we reconstructed the plastid genomes of selected animal and plant species and performed phylogenetic assignments and DNA damage pattern analysis. Initially, for the plastid genome reconstruction, we extracted all reads of all intestinal content shotgun datasets (see Data S1_ Illumina shotgun datasets, both UDG-treated and non-UDG treated) that were previously taxonomically assigned to the four dominant animal and plant (sub)families Caprinae Cervinae, Dennstaedtiaceae and Triticeae (see Data S1_ Mapping metagenomic reads). Next, we selected for the alignment of each (sub)family reads the modern reference sequence based on the dominant species detected in the previous taxonomic assignment: the mitochondrial genome of Capra ibex (NC_020623) for the Caprinae reads, the mitochondrial genome of Cervus elaphus (AB245427) for the Cervinae reads, and the chloroplast genome of Pteridium aquilinum subsp. aquilinum (HM535629) for the Dennstaedtiaceae reads. However, the Triticeae reads have been only taxonomically assigned down to the genus Triticum. Thus, to obtain an appropriate modern reference for the alignment, we mapped the Triticeae reads against selected chloroplast genomes of domesticated and wild species in the genus Triticum using BWA [41] (with default parameters) implemented in the program FastQ Screen [71]. Thereby obtained FastQ Screen results indicate that the Triticeae reads preferentially map to both Triticum monococcum (KC912690) and Triticum urartu (KC912693) having the most specific reads to these two Triticum species (see Data S1_ FastQScreen Triticeae reads). The previously extracted reads (both UDG-treated and non-UDG treated) of the four dominant animal and plant (sub)families Caprinae Cervinae, Dennstaedtiaceae, and Triticeae were then re-mapped against the respective reference plastid genomes using BWA [41] and increased the mapping sensitivity parameters (-1 000, -o 1, -n 0.03). The obtained mapping statistics are summarized in the (see Data S1_ Mapping metagenomic reads). Resulting SAM files were filtered for a minimum mapping quality of 30, converted to BAM files, sorted, and indexed with SAMtools [43]. Duplicates were removed using MarkDuplicates from Picard tools (version 115, http://broadinstitute.github.io/picard/). A consensus FASTA sequence was generated for each sample using the ANGSD tool [44] and making a majority consensus call from overlapping reads (with the arguments _doFasta2 _doCounts1).

The reconstructed plastid genome sequences were subjected to comparative sequence and phylogenetic assignment. At first, a comparative plastid genome dataset was created. Nucleic acid sequences of modern plastid genomes that are closely related to the consensus sequences were retrieved from the public database using the BLAST search tool NCBI tblastn [42]. A multiple alignment was obtained by using the MAFFT multiple sequence alignment program [45]. The automatically inferred alignment was manually refined by using the sequence editor implemented in the ARB software package [46]. Phylogenetic analyses were performed by applying the maximum-likelihood method [PhyML [72] with the JTT substitution model]. For all datasets a stringent filter approach was applied to include into the phylogenetic assignment only position present in all sequences (please refer to Figure S3 for details to the number of informative position used).

To assess the nucleotide misincorporation patterns along the DNA fragments, we performed a mapDamage analysis [22] using all non-UDG treated reads of the Iceman intestine contents mapped against the above mentioned reference genomes. Mapping and filtering has been done as described for the preparation of the consensus sequences for the phylogenetic analysis (see Data S1). Thereby generated SAM files and the corresponding reads mapped against the reference genomes were used for the mapDamage analysis (see Figure S3D).

One limitation of the previous applied taxonomic assignment approach is that the currently still limited availability of full plant and animal plastid genomes. Especially the chloroplast genomes deposited in the NCBI database represent only a small percentage of the possible plant diets present in the European Alps during the Iceman’s time. Therefore, we decided to perform a comparative sequence analysis of selected shotgun read datasets against the DNA barcodes deposited in the BOLD system database (http://www.boldsystems.org/) [51]. The DNA barcodes deposited at the BOLD database comprise fragments of the cytochrome c oxidase I (COI) gene, the ribulose bisphosphate carboxylase (rbcL) gene and the maturase K (matK) gene and the number of deposited species by far exceeds the number of plastid genomes in the NCBI database. The comparative analyses were performed against the COI fragments deposited in the groups Mammalia, Actinopterygii, Astacidae, Nematoda, and Platyhelminthes and against the rbcL and matK fragments in the groups Magnoliophyta, Pinophyta, Bryophyta, and Pteridophyta. First, we performed a sequence similarity search with the reads of selected shotgun datasets using blastn [42] with default parameters against the DNA barcodes extracted from the BOLD database. In the analysis four Iceman intestinal content datasets (B0626, B0621, C1824, B0625) were included, together with the Iceman muscle tissue (C0004) and Extraction Blank (B0629) as control datasets. All hits from the previous step with minimum bitscore 70, min length 15, 80% alignment length, 95% identity were further subjected to a second sequence similarity search using blastn [42] with default parameters and the complete NCBI-nt database as reference database. Blast results were taxonomically assigned using MEGAN6 [40] with a Min percent value of 0.01 (see Figure S4A).

Animal mitochondrial genome enrichment in the Iceman’s stomach content

Ancient DNA extraction and library preparation were conducted in the dedicated ancient DNA laboratory at EURAC research, Institute for Mummy Studies, Bolzano. The DNA was extracted from stomach contents using in-solution silica based protocol for ancient DNA [73]. The extracted DNA was prepared for Illumina sequencing by blunt-end repair and ligation of universal Illumina adapters (31). The library was shipped at −20°C to Trinity College Dublin and Illumina barcoded at the 5’ end there [74]. A custom RNA capture produced from reference domesticate mitochondrial genomes by MYcroarray (Microarray, 5632 Plymouth Road, Ann Arbor, MI.
Polymerase chain reaction (PCR)-based analyses
To further confirm our metagenomic findings in the Iceman intestinal content shotgun datasets we additionally performed a PCR-based analysis with selected DNA extracts targeting small DNA fragments of animal and plant barcodes (see Key Resources Table). To assess the animal diet of the Iceman, we amplified the fragments of two different mitochondrial loci, the 12S rRNA gene (12S rRNA) and the cytochrome B gene (cytB). For the cytB gene we applied a previously published PCR assay specifically designed to target different animal species [35]. In parallel, this assay excludes the amplification of human background DNA. Furthermore, we designed for this study a second PCR assay targeting a smaller fragment of the 12S rRNA gene of different animal species, including the human 12S rRNA gene variant. To further investigate via PCR the plant diet of the Iceman we amplified in the intestinal content DNA extracts for this study a second PCR assay targeting a smaller fragment of the 12S rRNA gene variant. To further investigate via PCR the plant diet of the Iceman we amplified in the intestinal content DNA extracts associated with ancient DNA [22] (see Figure S3D), showing that on 10% of fragments had deamination lesions at the 5’ end of fragments. To investigate variants in the mapped mitochondrial data, GATK UnifiedCaller created vcf. files from the bam files and variants were quantified with MultiVcfAnalyser, genotypes with a quality below 30 were filtered [48]. To further reduce the potentially confounding data from cross mapping or misincorporation of deaminated positions, only SNPs with a consensus above 80% were considered with respect to the overlapping reads (see Data S1_Variants enriched reads). This filtering strategy effectively removed confounding SNPs as demonstrated by the exact match to the Iceman’s mitochondrial haplotype defining SNPs [75].

Proteomic analysis of the Iceman stomach content
We studied the proteome of three distinct stomach content samples (1051L, 1051LP & 1054LP) by liquid chromatography-mass spectrometry (LC-MS) proteomics using Orbitrap and QExactive technologies. For methodological details to the sample preparation, the LC-MS approach and the mass spectrometry data analysis please refer to the publication of Maixner et al. [11]. Briefly, each stomach content sample was solubilized, tryptic digest and factionated using 1D SDS-PAGE or OffgGEL isoelectric focusing (OGE) and then analyzed by high-mass accuracy Orbitrap or QExactive mass spectrometry instruments interfaced with nanospray liquid chromatography. Peptide identities were determined using the Trans-Proteomic Pipeline (TPP) software tool suite to define proteins contained in the stomach content samples [50].

The protein database used in the comparative analysis contained amino acid sequences constructed from multiple species. 75,649 protein sequences were to Homo sapiens (UniProt reference proteome, release 2012_10 ftp.uniprot.org/pub/databases/uniprot/previous_releases/release-2012_10/relnotes.txt) supplemented with sequences translated directly from the Iceman’s genome [34, 58]. Additionally, 936 and 1,278 Cervus elaphus protein sequences were obtained from NCBI and UniProt, respectively (obtained November 17, 2015). 30,155 Capra hircus protein sequences were obtained from NCBI (October 19, 2015) as the closest surrogate for the ibex proteome. The 1,011 available Triticum monococcum protein sequences from UniProt were supplemented with 95,051 Triticum aestivum sequences from UniProt (release 2015_11) to provide a complete proteome representing Triticaceae. 28,356 open reading frames of at least 30 amino acids were obtained from a previously published Pteridium aquilinum transcriptome.
were added. Samples were homogenized using a benchtop homogenizer, and the homogenizer probe was rinsed with an extra extract. The bottom organic phases were recovered and transferred to clean tubes, and the solvent was dried under nitrogen gas. Dried lipid extracts were kept at -80°C until LC/MS analysis for which they were re-suspended in 500 µL chloroform/methanol 1/2 v/v. The homogenizing rotor was carefully rinsed between each sample. A tube containing methanol only was used as negative control.

LC/MS: Chromatography conditions were optimized to enable efficient separation of triacylglycerols (TAGs) by reverse phase separation. LC/MS experiments were undertaken using an Agilent 1200 series HPLC-Chip system connected to Agilent 6540 Q-ToF. An Agilent C18 chip was used (75µm i.d. x 43 mm length, 5µm particle size, 300A pore size) with a binary solvent system at a flow rate of 0.3µL/min. Solvent A was acetonitrile/water 40/60 v/v containing 5mM ammonium acetate and 0.1% acetic acid, and solvent B was isopropanol/acetonitrile 90/10 v/v containing 5mM ammonium acetate and 0.1% acetic acid. The gradient was as follows: 0%B for 1.5min, increase to 50%B in 2.0min, increase to 100% B in 18min, stay at 100% B for 10min, decrease to 0%B in 0.1min, stay at 0%B for 3.4min, total runtime: 35min. The Agilent 6540 Q-ToF spectrometer was operated in positive ion mode; electrospray voltage was set to 1450 V (Vcap), temperature 300°C, drying gas 4L/min, fragmentor voltage 150 V. The instrument was operated with MS acquisition rate of 1 spectra/sec. Injection volume was 1 to 2 µL. To monitor sample LC carryover and cross-contamination, solvent blanks were run between samples.

Data Analyses: LC/MS data files were processed with the MassHunter Qualitative Analysis Software Version B.05.01. The data were manually curated to identify triglyceride ions ([M+NH₄]⁺ adducts) based on retention time and accurate mass measurement with mass accuracy < 5ppm. Intensities of the [M+NH₄]⁺ adduct mono-isotopic peaks were used for calculating the relative abundance of triglyceride molecular species. TAG have been successfully used in the identification of food remains in an archaeological context [26, 79]. Therefore, we decided to compare the relative abundance of TAG between the Iceman’s stomach samples and with contemporary animal samples (See Data S2_Modern comparative samples). Following the genomics results, C. ibex and C. elaphus meat samples were obtained from local hunters. In addition, and in view of the interest regarding the question of dairy consumption in early humans, samples of C. hircus milk and cheese were also sourced. To better compare TAG distribution between samples, Mirabaud et al. [26] made use of two parameters: the average carbon number (M) and the distribution factor (DF), which can be calculated by the following formulae:

\[
M = \frac{\sum (P_i C_i)}{\sum P_i} \\
DF = \sqrt{\frac{(C_i - M)^2 C_i}{\sum P_i}}
\]
where $P_i$ is the relative abundance of a given TAG (in percentage of total TAG) and $C_i$ is the total number of carbon atoms in the fatty acyl chains.

**Seattle analytical method**

**Sample Preparation:** Portions of two Iceman samples and four fresh male tissue samples were dissected with a razor on an aluminum-foil covered block on top of dry ice, and the weights were noted: 1) Iceman Lipid accumulation 5: 73 mg, 2) Iceman Lipid-protein mix 5: 70 mg, 3) 0065206-19 adipose: 182 mg, 4) 0065251-10 adipose: 135 mg, 5) 0065251-29 skin (full thickness): 209 mg, 6) 0065206-09 skin (full thickness): 184 mg. The tissue pieces were transferred to homogenization tubes containing 2.38 mm metal beads, and 1 mL methanol. To serve as negative controls, two tubes containing methanol only were carried through each step of sample preparation. Following bead beating, BCA protein estimation was performed on the soluble portion of each lysate. The fresh tissue samples each contained between 500-2000 µg of soluble protein, and, surprisingly, each of the two Iceman samples contained roughly 500 µg soluble protein. The lysates were transferred to silanized 13x100 glass vials, and total lipids were extracted with chloroform/methanol using a modified “Folch” extraction protocol [80]. The combined aqueous upper phase and interphase (containing protein precipitates) from each sample were dried and processed by multi-dimensional fractionation for proteomics analyses in Seattle (see above). The lower organic phases were dried under nitrogen and stored at −30°C. The visual appearance of the dried lipid extracts differed remarkably: Iceman extracts appeared as a dry brown powder, and the fresh tissue extracts appeared as a viscous yellow liquid. For lipid LC/MS, 8 mL chloroform/methanol (2:1 v/v) was added to each extract, and 5 µL was used for injection. The extract was also diluted 100-fold and 5 µL was again used for injection. Additionally, neutral lipids were obtained from the total lipid extract by solid-phase extraction (SPE) following a previously published method [81]. Briefly, 100 µL of the total lipid extract was evaporated under N2 gas, re-dissolved in 1 mL isooctane/ethanol acetate (75/25 v/v), sonicated, and then loaded onto 100 mg Biotage Isolute XL silica cartridges. Neutral lipids were collected from the flow-through, and further washed and collected from the column with isooctane/ethanol acetate (75/25 v/v). Eluents were evaporated under nitrogen gas, dissolved in 1 mL chloroform/methanol (2:1 v/v), and 5 µL was used for injection. Additionally, a 10-fold dilution was also used for injection.

**LC/MS:** Chromatography conditions were optimized to enable efficient separation of triacylglycerols (TAGs). An Agilent 1290 Infinity LC with a 2.1 mm (i.d.) x 100 mm, 3.5 µm Agilent ZORBAX Eclipse XDB-C18 Narrow Bore Rapid Resolution column heated to 30°C was used with a binary solvent system and a flow rate of 500 µL/min. The system was equilibrated with 80% of solvent A (5 mM ammonium formate in methanol/water (99:1 v/v)) and 20% solvent B (5 mM ammonium formate in isopropanol/water (99:1 v/v)). A 5 µL injection volume of the lipid extract (in 2:1 chloroform/methanol) was applied to the column, followed by a 30.0-min linear gradient to 20% solvent A/80% solvent B, and held for 2.5 min. An Agilent 6530 Q-ToF equipped with an Agilent JetStream ESI source was used for accurate mass analysis of the LC eluent. Positive ion mass spectra data were acquired in 2 GHz Extended Dynamic Range mode at a rate of 1.02 spectra/s, and data was collected as profiled spectra over a mass range of 150 to 1500 m/z. For MS/MS analyses, a fixed collision energy of 40 V was used for fragmentation. To monitor sample LC carryover and cross-contamination, several solvent blanks were run between samples, and sample order was considered carefully.

**Data Analyses:** LC/MS data files were processed with the MassHunter Qualitative Analysis Software version B.03.01. Molecular features (MFs) were extracted from the raw MS1 data using the Molecular Feature Extraction (MFE) algorithm with a peak filter of ≥ 10,000 counts. The resulting MFs were then identified with MassHunter by searching a custom database containing 690 TAG ions corresponding to [M+Na]+ and [M+NH4]+ adducts, and 99 [M+Na]+ cholesterol ester ions, using a ± 5 ppm search window. For quantitative purposes, lipid groups were compared using the [M+NH4]+ molecular ion height. See Data S2 for a summary of the lipid analysis results.

**Atomic force microscopy (AFM) and Raman spectroscopy**

Meat fiber assemblies within the Iceman food bolus samples were identified with an inverted optical microscope (Axiovert 135, Zeiss, Oberkochen, Germany). Subsequently, the coarse topography of the putative meat fibers was analyzed by laser scanning microscope (LSM) images (VK 8710, Keyence, Osaka, Japan) while high-resolution images of defined sample areas were taken with a NanoWizard-II AFM (JPK Instruments, Berlin, Germany). The AFM was operated in the intermittent contact mode and silicon cantilevers (BS Tap 300, Budget Sensors, Redding, USA) with nominal spring constants of 40 N/m, resonance frequencies of 300 kHz, and tip radii of 10 nm were used. All images were taken at lab conditions (22°C and about 45% relative humidity).

To assess the molecular composition of the samples, Raman spectra were taken with a confocal Raman microscope (WITec alpha 300 R, WITec GmbH, Ulm, Germany). The Raman system was operated with a laser (frequency doubled Nd:YAG-laser with an excitation wavelength of 532 nm) at 1.0 mW laser power, to avoid sample damage (photodegradation). The backscattered light from the sample was detected with a vacuum sealed, high-sensitivity, back-illuminated CCD camera cooled to −56°C. The spectrometer was equipped with a 600 g/mm grating enabling a spectral resolution of approximately 3 cm⁻¹ per CCD-pixel. Single spectra of each sample are average spectra of 10 accumulations with up to 180 s of integration time. Due to the confocal setup of the microscope and the objectives used, Raman spectra were collected from a sample area with approximately 400 nm diameter and a focal depth of about 1 µm.
QUANTIFICATION AND STATISTICAL ANALYSIS

Phylogenetic analyses were performed by applying the maximum-likelihood method [PhyML [58] with the JTT substitution model] implemented in the ARB software package [46]. For all datasets a stringent filter approach was applied to include into the phylogenetic assignment only position present in all sequences.

DATA AND SOFTWARE AVAILABILITY

The Illumina datasets are available from the European Nucleotide Archive (ENA) under accession number ENA: ERP012908. The enriched Illumina dataset has been deposited in ENA under the accession number ENA: PRJEB26465. The 454 amplicon datasets has been deposited in ENA under the accession number ENA: PRJEB26365. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository [82] with the dataset identifier PXD009565.

ADDITIONAL RESOURCES

Additional metadata for the Illumina shotgun datasets are provided under the following link: http://cube.univie.ac.at/supplements/iceman.