



# Mitogenomic diversity and stable isotopes provide insights into the maternal genetic history, mobility patterns, and diet of early medieval individuals from the Eastern Italian Alps

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## Abstract

The Eastern Italian Alps (South Tyrol) is a connection area between continental Italy and the northern Alps. Various local factors, such as the heterogeneous environment, complex historical events, and different mobility patterns, may have influenced the genetic makeup of early medieval alpine groups. However, no ancient genetic data from these groups are currently available. This study provides a first picture of the ancient mitochondrial DNA (mtDNA) diversity of alpine groups from four locations in South Tyrol (Adige, Isarco, Venosta, and Merano). In total, 94 ancient mitogenomes of individuals (dated from 400 to 1100AD) were reconstructed by shotgun sequencing and a mtDNA capture approach. Moreover, stable isotope ratios ( $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ ,  $\delta^{34}\text{S}$ ) were analyzed in a subset of 32 individuals. The results indicate different mtDNA haplogroup distributions among the alpine locations and the presence of rare lineages besides a possible maternal relatedness between individuals buried in the same and in diverse archaeological contexts. The study also shows differences in the genetic and mobility patterns ( $\delta^{34}\text{S}$ ) between individuals from the central and north-eastern parts (Adige, Merano, Isarco) and those from the north-western part of South Tyrol (Venosta). These results suggest genetic exchanges with allochthonous people in the first group probably linked to high mobility and to geomorphological, historical, and socio-cultural factors. Comparisons extended to present-day alpine populations also suggested maternal genetic continuity in this alpine area. Finally, stable isotope ( $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ ,  $\delta^{34}\text{S}$ ) data provided further support for regional differences in the diet of past alpine groups possibly linked to altitude and/or social status.

**Keywords** Ancient mitochondrial DNA · Carbon · Nitrogen · Sulfur · Migration period

## Introduction

Ancient DNA studies have highlighted key stages of the genetic history of European populations by showing major changes and turnover of the genetic lineages over time (Brandt et al. 2013; Lazaridis et al. 2014; Gamba et al. 2014; Margaryan et al. 2017; Mathieson et al. 2018; Furtwängler

et al. 2020). These studies have been conducted mostly on a macroregional scale and have mainly regarded ancient individuals that were geographically and temporally dispersed. Therefore, there have been very few ancient genetic studies carried out on a micro-geographic scale in Europe (e.g., Mittnik et al. 2019) that can highlight groups or individuals with particular genetic traits that may elude large-scale studies. Moreover, studies focused on ancient genetic diversity from Italy remain limited (Amorim et al. 2018; Antonio et al. 2019; Marcus et al. 2020; Saupe et al. 2021; Posth et al. 2021), especially from the Italian alpine area (Ermini et al. 2008; Keller et al. 2012; Fu et al. 2016) which has been an important connection between Mediterranean and the northern Alps since Prehistory.

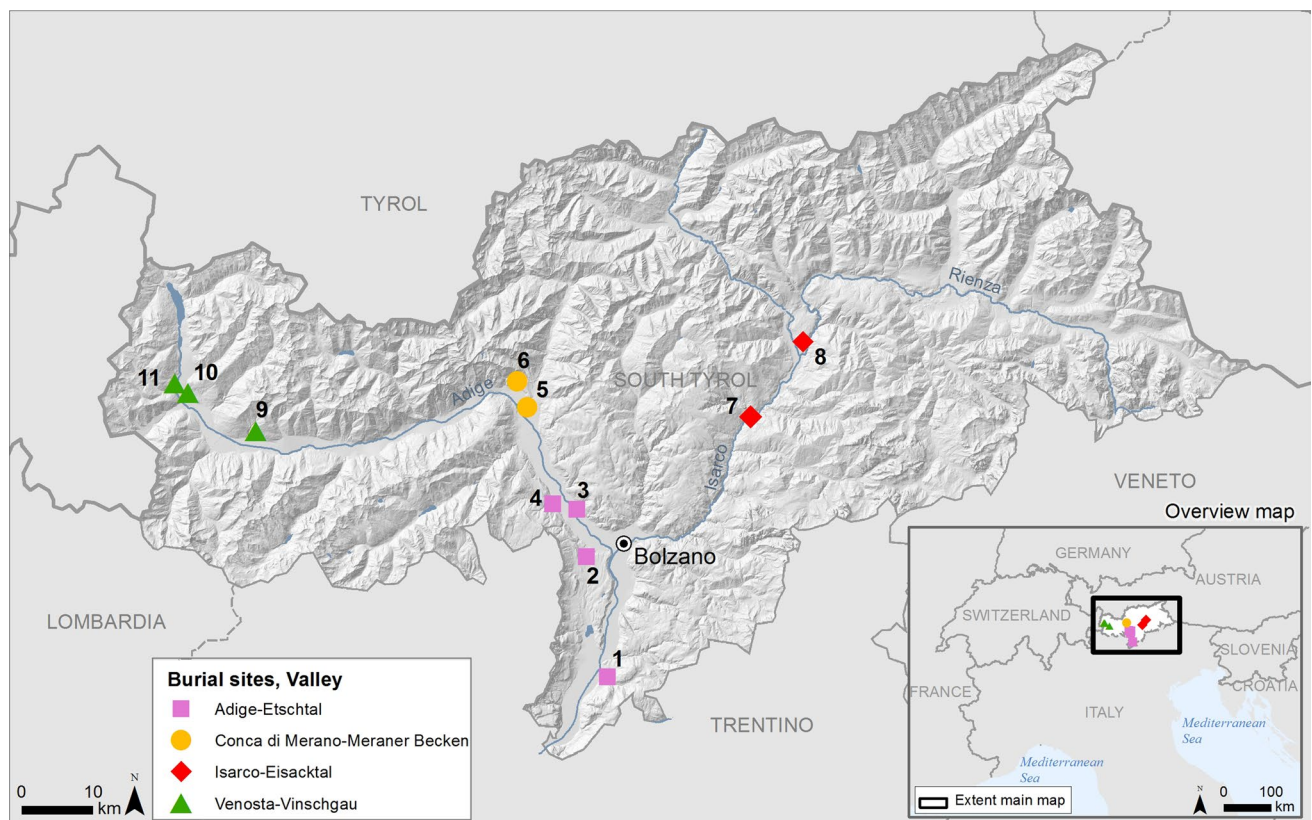
This study focused on early medieval individuals from a region in the Eastern Italian Alps (South Tyrol). Located on the southern slopes of the Alps, South Tyrol (Fig. 1) has a heterogeneous environment with various geographic zones

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**Fig. 1** Geographic distribution of the ancient samples from South Tyrol analyzed in this study. Violet square = Adige Valley. Sites: (1) Montagna Pinzano (Montan, Pinzon), (2) Appiano San Paolo Castelvecchio (Eppan, St. Paul Altenburg), (3) Terlano (Terlan), (4) Nalles Gebreid (Nals Gebreid). Yellow circle = Merano Basin. Sites: (5) Maia Bassa (Untermais), (6) Castel Tirolo (Schloss Tirol). Red rhombus = Isarco Valley. Sites: (7) Sabiona (Säben), (8) Bressanone

Elvas Necropoli 17 (Brixen, Elvas). Green triangle = Venosta Valley. Sites: (9) Tanas (St. Peter's path), (10) Malles Maso Pauli (Mals, Paulihof), (11) Malles Burgusio St. Stefano (Mals, St. Stephan ob Burgeis). See Table S11 for more details on the number of individuals analyzed for locations and archaeological sites. The map was created using the « ESRI ARC GIS Desktop» (<https://desktop.arcgis.com/en/>)

and altitudes (from ~200 in the Adige Valley to ~3900 m above sea level in the Venosta Valley) (Winckler 2012). Its complex territory is characterized mainly by mountain areas crossed by mountain passes (e.g., Brennero, Resia) but also by wide passageways (valley floors), features which may have influenced human mobility. Additionally, starting from the Roman Period (approx. from the first century BC to the fourth century AD in this area) and during the Early Middle Ages, this alpine territory was characterized by a complex communication network with connections not only to the Adriatic coast and the Po Valley, but also to central Europe along the Danube (Pesavento Mattioli 2000; Marzatico and Migliario 2011), thus favoring population movements, trading, and cultural exchanges.

At that time, the communities living in this area were involved in complex historical events. Indeed, after a long period of political stability and socio-cultural homogeneity of alpine groups under the Romans (“Romanization” process, Buchi 2000), local archaeological evidence suggests that from the sixth century AD, the societies changed.

Various cultural-political groups (e.g., Langobards, Baiuvars, Franks, Alemannic) contended the territory, marking the decline of the Roman political organization (Giostra and Lusuardi Siena 2004; Haas-Gebhard 2004; Heitmeier 2005; Cavada 2016). Thus, a cultural “Germanization” process by allochthonous people (*barbari*) of local Romanized groups took place (Albertoni 2005; Gasparri and La Rocca 2013), which involved cultural aspects, such as funerary customs, indicating the coexistence in the territory of possible different cultural groups (Supplementary Information).

So far, only stable isotope data ( $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ , and  $\delta^{34}\text{S}$ ) from early medieval alpine individuals are available (Paladin et al. 2020). The study clearly showed local-scale differences in stable isotope ratios among groups from different valleys in South Tyrol. The results indicated diverse subsistence strategies with a mixed nutritional basis of C3/C4 plants (e.g., wheat and millet) with a higher intake of C4 plants at lower altitudes (Adige Valley), and a primarily terrestrial diet (high  $\delta^{15}\text{N}$  ratios) with individuals living at higher altitudes showing greater access to animal protein (e.g., meat,

dairy products). Furthermore, the study reported different mobility patterns with greater mobility (higher  $\delta^{34}\text{S}$  ratios) in groups living at lower altitudes, although nutritional and geological factors (i.e., drinking of water enriched in sulfur, freshwater fish) may also have influenced this pattern (Paladin et al. 2020).

The present study intends to provide a first picture of the mitochondrial genetic diversity (mtDNA) of early medieval individuals from the Eastern Italian Alps. More specifically, our questions were as follows: (i) Are the groups from various locations in South Tyrol which showed differences in funerary culture, mobility pattern, and subsistence strategies also genetically differentiated? (ii) Are there differences in the genetic relationships between the various alpine groups and other European medieval individuals? In addition, we explored the genetic links between the ancient alpine groups and present-day populations, including those within the same region and beyond. To do that, the complete ancient mtDNA of 94 individuals mostly dated to the Early Middle Ages (400–1100 AD) (Table S11 and Table SM1A) were reconstructed by using both shotgun sequencing and a capture approach. The specimens were recovered from 11 closely located archaeological sites, which were distributed in three main valleys (Adige, Isarco, and Venosta) and in one basin (Merano) in South Tyrol (Fig. 1 and SI).

In order to gain further insights into the mobility patterns and diet of these alpine groups, a subset of 32 individuals underwent stable isotope analyses ( $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ , and  $\delta^{34}\text{S}$ ) and the results were combined with already available data (Paladin et al. 2020) for a total of 72 individuals.

## Materials and methods

### Genetic investigation

#### Sample processing and sequencing

In order to evaluate the skeletal representativity and state of preservation of the human remains as well as to estimate the individual sex and age at death (for methods, see Paladin et al. (2020), the human remains were anthropologically investigated at the Anthropology Laboratory of the Institute for Mummy Studies of Eurac Research in Bolzano.

Due to the uncertainty or lack of radiocarbon dating of human remains from two sites (Burgusio St. Stefano and Sabiona), six samples were analyzed for  $^{14}\text{C}$  measurements using an external service (Curt-Engelhorn-Zentrum Archäometrie gGmbH D6, Mannheim) (see Table SM1B and Supplementary Information for more details).

A total of 104 *pars petrosa* (PP) were selected for genetic investigation. After photographic documentation,

around ~ 150 mg of powdered bone was collected from the inner part of the PP by using a drill (Pinhasi et al. 2015) in a dedicated pre-PCR area of the ancient DNA laboratory in Bolzano and following all the strict rules required for ancient DNA analyses. Particular attention was paid during this sampling procedure in order to preserve the PP as much as possible, which was isolated or still attached to the skull, in order to make the samples available for further possible scientific investigations.

DNA samples were extracted using a purification method based on silica columns (Gamba et al. 2014) and Paired-End (PE) genomic libraries were constructed (Meyer and Kircher 2010), and sent to an external company (Macrogen) for shotgun sequencing (100 bp PE HiSeq2500 and 150 bp HiSeq-X systems, Illumina). Almost half of the samples (50), which showed less than 94% of mtDNA positions covered after bioinformatic analyses, were further enriched for the mitochondrial DNA using myBaits Mito, Global Panel (Arbor Biosciences), and the enriched libraries were sequenced by using the above-mentioned Illumina platforms.

The recovery, sampling, and all the analyses performed in this study on the human and faunal remains have been authorized by the competent authority (*13.2 Ufficio Beni archeologici, Provincia Autonoma di Bolzano-Alto Adige*).

### Bioinformatic and statistical analyses

Paired-end reads were trimmed and merged by PEAR (Zhang et al. 2014) if they overlapped by at least 25 bp and with a minimum length of the assembled sequences of 25. The QualityFilterFastQ.py script (Kircher 2012) was applied to eliminate reads with 5 bases below the quality threshold of 15. The reads were then aligned to the Genome Reference Consortium Human Build 37 (GRCh37/hg19) and to the revised Cambridge Reference Sequence (rCRS) (Andrews et al. 1999) with BWA (Li and Durbin 2010) with minimum mapping quality set to 25. Duplicates were removed by using Dedup (Peltzer et al. 2016). Damage patterns among the ancient reads were tracked and quantified (e.g., fragmentation and misincorporation patterns) by using mapDamage (Jónsson et al. 2013). Contamination estimates as well as mtDNA consensus sequences were inferred by a probabilistic iterative method (Schmutzi, Renaud et al. 2015). Fasta files were obtained from the “sample\_final\_endo.log” file reconstructed by Schmutzi, using log2fasta resulted in mitogenomes where the reported alleles were covered at least 3 times with base quality (PHRED) and average mapping quality  $\geq 30$ . Positions that did not satisfy these criteria were reported as missing positions (see Table SM1).

Haplogroup assignment was carried out by using Haplogrep 2.4.0 (Kloss-Brandstätter et al. 2011) and further checked by using Haplofind (Vianello et al. 2013), both

based on the phylogenetic tree of global human mitochondrial DNA variation (version 17) (van Oven and Kayser 2009). Polymorphic sites were checked through visual inspection (particular attention was paid to mutations that have been identified as global, missing, or private; Table SM1A).

For the comparative analyses, we focused on mitogenomes from ancient samples from Europe dated from ~400 to 1150 AD, except for one from the Roman period from southern Italy (1–400 AD; Emery et al. 2018) (no data are available on alpine or northern Italian samples dated to the Roman Period) (Table SM4). Moreover, we used only groups reported in the original publications with sample sizes  $\geq 7$ . Depending on availability in the literature, we used either Fasta files or raw data (fastq or bam files). The latter were then processed to extract mtDNA reads following the pipeline used in this study and described above. Thus, we excluded mtDNA Fasta sequences with too many uncertain positions in order to estimate reliable *Fst* values. Additionally, whole genome data from 16 present-day populations were retrieved from Batini et al. (2017). Finally, we used data from the hypervariable region 1 of the mtDNA (HVRI, from position 16,033 to 16,383) from 19 present-day alpine populations (see Table SM5 for more details and references).

Haplotype diversity and pairwise genetic distances (*Fst*, Reynolds' linearized distance) (Reynolds et al. 1983) were estimated using the Arlequin software (v.3.5.2.2) (Excoffier and Lischer 2010) and statistical significance of *Fst* values was estimated using 10,000 permutations. A multidimensional-scaling (MDS) plot of pairwise *Fst* values was computed with the *cmdscale* function and plotted with *ggplot* in R 4.0.3 (R Core Team 2021; Kassambara and Mundt, 2016). A phylogenetic network of the alpine haplogroup H was constructed using the median Joining method implemented in the Network program (v.10.1) (<https://www.fluxus-engineering.com>) (Bandelt et al. 1999) using the default parameters  $\mathcal{E} = 0$ . Forty-six early medieval alpine individuals were used, with the exclusion of sample 2736 due to uncertainty in the mutation at position 3010, which defines haplogroup H1 (see Table SM1).

## Isotope investigation

### Analytical methods and statistical analyses

Osteological human samples (from the cranium or femur, as a second choice) from a subset of 32 early medieval individuals and 14 faunal bone samples were collected for carbon ( $^{13}\text{C}/^{12}\text{C}$ ,  $\delta^{13}\text{C}$ ), nitrogen ( $^{15}\text{N}/^{14}\text{N}$ ,  $\delta^{15}\text{N}$ ),

and sulfur ( $^{34}\text{S}/^{32}\text{S}$ ,  $\delta^{34}\text{S}$ ) stable isotope analyses, at the Anthropology Laboratory of Eurac Research of Bolzano (Italy). Good-quality data from this study were combined with data from Paladin et al. (2020), reaching a total of 72 human (out of the 94 analyzed for mtDNA) and 43 faunal samples, which were used for the statistical analyses. Both human and faunal samples from the sites of Maia Bassa (Merano Basin) and Sabiona (Isarco Valley) were analyzed for the first time in this study (Table SM1B and Table SM9).

The faunal samples from different species were collected, after anatomical and taxonomical identification according to Schmid (1972) and Barone (1986) at the Archaeological Office of the Autonomous Province of Bolzano. These were analyzed to reconstruct the local trophic levels among humans and the different faunal species. The  $\delta^{34}\text{S}$  mean and standard deviation (SD) of the domesticated animals grouped per valley were used as local baselines.

The bone collagen extractions were performed at the laboratory of the Institute of Forensic Medicine of the University of Bern (Switzerland), following the standard laboratory protocol for acid–base extractions based on the studies of Longin (1971) and Ambrose (1990). More details are reported in Paladin et al. (2020). The  $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ , and  $\delta^{34}\text{S}$  analyses were measured by isotope ratio mass spectrometry (IRMS), at Isolab GmbH of Schweitenkirchen in Germany. The mean result of three measurements was calculated and used for the analyses. Results are reported in  $\delta$ -notation in units of *per mil* (‰) with reference to the international standards (VPDB for carbon, AIR for nitrogen and V-CDT for sulfur) (Schoeninger and DeNiro 1984; Fry 2006). In addition, the laboratory internal standard STD R (collagen from cowhide, EU project TRACE) was reported in this work. For  $\delta^{13}\text{C}$ , the analytical error was less than  $\pm 0.1\text{‰}$ , for  $\delta^{15}\text{N} \pm 0.2\text{‰}$ , and for  $\delta^{34}\text{S} \pm 0.3\text{‰}$ . The samples were of good quality when %C was in the range of 30–47%, %N in the range of 11–17.3%, and %S in the range of 0.15–0.35% (Ambrose 1990, 1993; Van Klinken 1999; Nehlich and Richards 2009). The analyses were completed by using IBM® SPSS® Statistics 25 to test the data distribution (Shapiro–Wilk test) and to detect the extreme (values with more/less than  $\times 3$  the interquartile range,  $3 \times \text{IQR}$ ) outliers, according to the locations, to be then excluded from the statistical tests. To determine significant differences between groups, the parametric tests (normal distributions) such as one-way ANOVA with a post hoc Tukey test and the non-parametric Kruskal–Wallis test with pairwise comparisons (non-normal distributions) were applied. A *p* value below 0.05 was considered significant.

## Results

### Genetic investigation

#### Data quality and authentication of aDNA

Genomic libraries constructed using DNA extracts from *pars petrosa* samples (104 individuals) were tested for their content of endogenous human DNA using shotgun sequencing. After bioinformatic analyses of the paired-end DNA sequencing reads, seven samples were excluded due to their low content of endogenous human DNA. Of the remaining individuals (97), almost half of the samples (47) showed sufficient coverage (approximately 7- to 55-fold) of the mitogenome. The other 50 samples were further enriched for mtDNA by using a hybridization capture approach. This resulted in average coverage values from 11- to 3721-fold (Table SM1A). The mtDNA sequence reads of all individuals were highly fragmented and showed characteristic postmortem damage patterns for ancient DNA (Table SM1A) (Orlando et al. 2015). Low contamination rates from modern human DNA (average values from 0 to 4%) were estimated for most of the samples (Renaud et al. 2015), except for three individuals (average from 7 to 16%) that were therefore excluded from the subsequent analyses. Consensus sequences for the remaining 94 samples were reconstructed as described in detail in the “Materials and methods” section.

#### Mitogenome diversity in the early medieval eastern Alps

We successfully reconstructed the mitogenome of 94 early medieval samples from South Tyrol, which comprise 86 different haplotypes (Table SM1A). Haplotype diversity in the four ancient alpine groups varied from  $1.000 \pm 0.024$  in the Merano Basin to  $0.978 \pm 0.054$  in the Adige Valley with same intermediate values in the other two locations,  $0.996 \pm 0.006$  and  $0.996 \pm 0.013$  in the Isarco and Venosta, respectively. Present-day population from South Tyrol also show a similar haplotype diversity of  $0.995 \pm 0.0044$  (Table SM2).

Identical haplotypes were found within the same location and archaeological site (haplotypes from 2 to 5; Table SM3) or among different locations and archaeological contexts (haplotypes 1 and 6). Moreover, one more possible shared haplotype was detected in the Isarco Valley (haplotype 7), although two positions cannot be determined based on the quality criteria used in this study and remain uncertain.

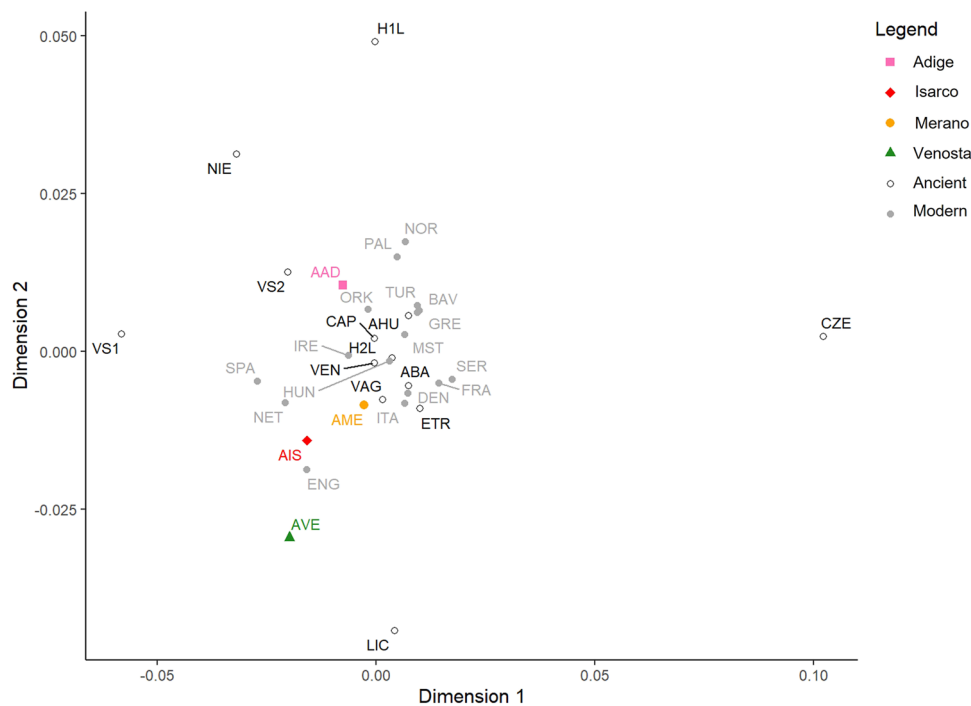
Mitochondrial haplogroups were assigned to all samples (average rank of 0.94; Table SM1A). Overall, a high

occurrence of the macro haplogroup H, which accounts for half of the total alpine mitogenomes (50%), was found with differences among groups from different locations since its frequency ranges from 20% (Adige) to 62.5% (Venosta) and it showed high diversity with several different H-sub haplogroups (Fig. SI9). Other frequent haplogroups are U (14.9%), T (9.6%), J and K (7.5%), HV (3.2%), and V (2.1%), while more rare lineages (~ 1%) are I, M, R, N, and W. Some of these lineages were found only in one location such as Adige Valley W (10%), Merano Basin M (7%), Isarco Valley R (2%) and V (4%), and, lastly, Venosta I and N (4%) (Figs. SI7 and SI8). When compared to extended datasets of ancient mitogenomes (AmtDB database, from Ehler et al. (2019), update 2021–10–13; Dataset\_v50.0\_1240K from David Reich Lab), some sub-lineages of haplogroup H (H11a5, H31, H75, H80) but also lineages of different haplogroups (HV4a2b, K1c1a, U8a1a1a1, and M1a3) present in the Alps have not been found in other specimens, while other lineages (e.g., H11a2a2, H39, T2b1, T2b24, T2k, J1c3c, K1b1c) were found only in few other ancient samples.

#### Comparison with other early medieval groups and present-day populations

To visualize the genetic relationships between our samples and other ancient and present-day populations, we performed a multidimensional-scaling (MDS) plot of the pairwise genetic distances (Fst values) (Fig. 2). Early medieval alpine groups from Adige Valley (AAD), Merano Basin (AME), and to a lesser extent Isarco Valley (AIS) fall into a main cluster together with most of the other ancient groups from Italy (ETR, VAG, VEN), Bavaria (ABA), Hungary (AHU, H2L), and Romania (CAP) with the exclusion of five outliers: the Alemannic from Niederstotzingen (NIE), Longobards from north Italy (LIC), Visigoth from Iberia (VS1), and finally two Longobard groups from Hungary and the Czech Republic (H1L and CZE, respectively), and all present-day populations used for the comparison. On the other hand, early medieval alpine group from the Venosta Valley (AVE) is separated from this main cluster (Fig. 2). Fst values estimated for the entire dataset ranged from 0 to 0.160. The highest Fst value was observed among the CZE and VS1, but it should be taken with some caution given the small sample size of these groups (Table SM4). Notably, the Adige Valley (AAD) has one of the higher Fst values estimated for the whole dataset with the nearby Venosta Valley (AVE) (Fst=0.023), followed only by the outliers from Collegno, Longobards (LIC) (Fst=0.041) and the Czech Republic (CZE) (Fst=0.084; Table SM6 and Table SM7).

The genetic relationship among ancient groups from the Alps and present-day populations was further



**Fig. 2** Multidimensional-scaling (MDS) plot of  $F_{st}$  pairwise genetic distances in the alpine samples divided according to location and in another 13 ancient groups from Europe and 16 present-day populations. Symbols and colors: ancient samples from present study: violet square=Adige Valley, yellow circle=Merano Basin, red rhombus=Isarco Valley, green triangle=Venosta Valley. Ancient groups from published data: open circles. Present-day populations: gray circle. Abbreviations, ancient: AAD=Adige; AIS=Isarco; AME=Merano; AVE=Venosta; CZE=Czech Republic; NIE=Niederstotzingen;

ABA=Bavarians; AHU=Hungary; H1L=Hegyko; H2L=Szólád; LIC=Collegno; CAP=Capidava; VS1 and VS2=Visigoths; ETR=Early Medieval Central Italy; VEN=Venosa. Modern: MST=Modern South Tyrol; DEN=Danish; ENG=English; FRA=French; BAV=Bavaria; GRE=Greeks; HUN=Hungary; IRE=Irish; ITA=Italians; NET=Netherlands; NOR=Norwegians; ORK=Orkney; PAL=Palestinians; SER=Serbs; SPA=Spanish; TUR=Turks (refer to Table SM4 for more details)

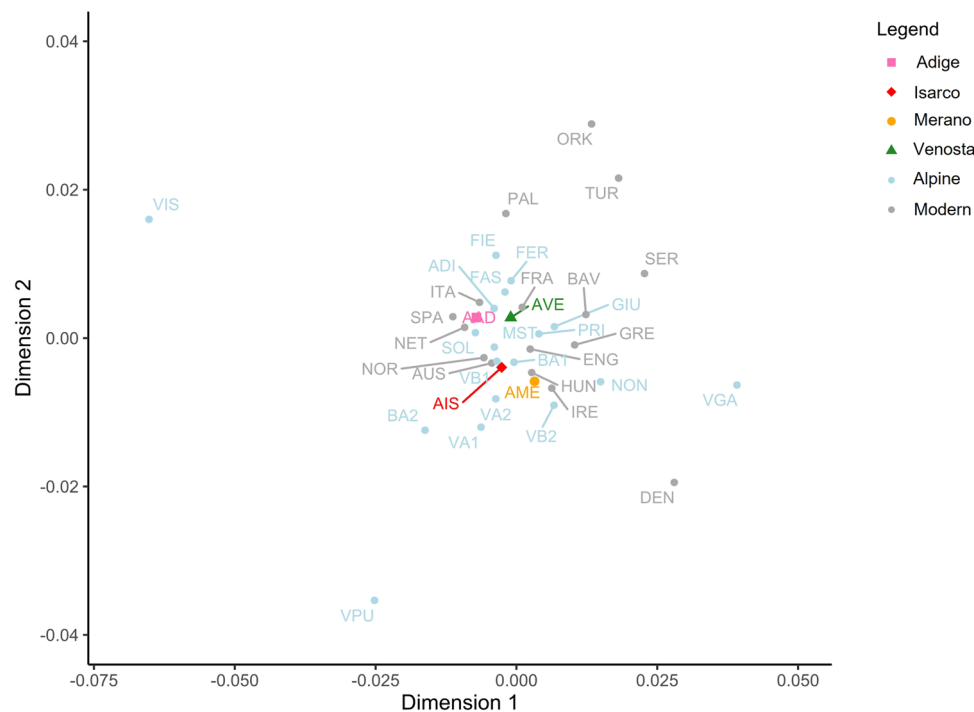
investigated using available low-resolution mtDNA data (hypervariable region 1, HVR1) from populations from the same alpine territory. The dataset includes present-day populations from three of the four locations considered in this study (Adige, Isarco, and Venosta Valleys) and other alpine populations from the surrounding area (Table SM5). The MDS plot of the  $F_{st}$  genetic distances (Table SM8 and Fig. 3) shows a principal cluster composed of early medieval alpine groups from all four locations and most of present-day populations including the alpine ones with the exception of modern Isarco Valley (VIS) and other two contemporary populations from South Tyrol (from Pusteria Valley and Ladins from Gardena Valley), which behave as outliers in the plot ( $F_{st}$  values in the total dataset from 0 to 0.103). Indeed,  $F_{st}$  values estimated among the present-day population of Isarco and the early medieval group from the same valley are high ( $F_{st}=0.042$ ) if compared to low  $F_{st}$  values estimated among ancient and modern populations from the other alpine locations ( $F_{st}$  values ranged from 0 to 0.010) (Table SM8).

## Stable isotope ratio analysis

### Data quality and stable isotope ratios of the early medieval alpine groups

All the human samples (32) analyzed in the present study yield good-quality collagen for  $\delta^{13}C$ ,  $\delta^{15}N$ , and  $\delta^{34}S$  stable isotope ratios as well as for most of the faunal remains (13/14) from the same archaeological sites and with the same chronology (Table SM1B and Table SM9). The descriptive statistics of both human and faunal remains are presented in Table SM10, while the trophic level shifts obtained between faunal and human  $\delta^{13}C$  and  $\delta^{15}N$  ratios are presented in Table SM11.

The plot in Fig. 4 compares the human and faunal  $\delta^{13}C$  and  $\delta^{15}N$  stable isotope ratios. In terms of human data, the carbon values showed that the group of Adige Valley had statistically significant enriched  $\delta^{13}C$  values (mean  $-17.44 \pm 0.84\text{‰}$ ) compared to the other locations (Merano Basin  $-18.55 \pm 1.25\text{‰}$ , Isarco  $-19.04 \pm 0.65\text{‰}$ , and Venosta  $-19.19 \pm 0.55\text{‰}$ ) (Table SM10). Extreme outliers for  $\delta^{13}C$  were detected in both



**Fig. 3** Multidimensional-scaling (MDS) plot of *Fst* pairwise genetic distances (mtDNA hypervariable region-1 from positions 16,033 to 16,383) among early medieval alpine groups and 35 present-day populations, including 19 from the Eastern Italian Alps. Symbols and colors. Ancient samples from present study: violet square=Adige Valley, yellow circle=Merano Basin, red rhombus=Isarco Valley, green triangle=Venosta Valley. Present-day populations from the alpine region: light blue dot; other present-day populations: gray dot. Abbreviations: AAD=Ancient Adige; ADI=Modern Adige; AIS=Ancient Isarco; AME=Ancient Merano; AVE=Ancient Venosta; AUS=Austria; BA1=Badia, Ladin; BA2=Badia, Ladin;

FAS=Fassa, Ladin; FER=Fersina; FIE=Fiemme; GIU=Giudicarie; MST=Modern South Tyrol; NON=non-valley; PRI=Primiero Valley; SOL=Sole Valley; VA1=Upper Venosta Valley 1; VA2=Upper Venosta Valley 2; VB1=Lower Venosta 1; VB2=Lower Venosta 2; VGA=Gardena Valley, Ladin; VIS=Isarco Valley; VPU=Pusteria Valley; DEN=Danish; ENG=English; FRA=French; BAV=Bavaria; GRE=Greeks; HUN=Hungary; IRE=Irish; ITA=Italians; NET=Netherlands; NOR=Norwegians; ORK=Orkney; PAL=Palestinians; SER=Serbs; SPA=Spanish; TUR=Turks (refer to Table SM5 for more details)

Isarco (BEN2b, BEN28, BEN45) and Venosta (TA4) Valleys (Fig. 4; Table SM1B).

Regarding the nitrogen values, the group of Venosta Valley exhibited the most enriched  $\delta^{15}\text{N}$  mean value ( $+10.62 \pm 0.71\text{‰}$ ), followed by Isarco Valley ( $+10.36 \pm 1.11\text{‰}$ ), compared to the groups of the other locations (Adige  $+10.01 \pm 0.58\text{‰}$  and Merano Basin  $+9.72 \pm 0.84\text{‰}$ ). However, the Kruskal–Wallis test confirmed significant differences only for nitrogen values between Venosta and Merano ( $p=0.001$ ) (Table SM12). Extreme outliers can be observed for individuals from the Merano Basin (TCT10 and TCT27 from Castel Tirol) and the Isarco Valley (BEN45 from Elvas Bressanone; Fig. 4, Table SM1B).

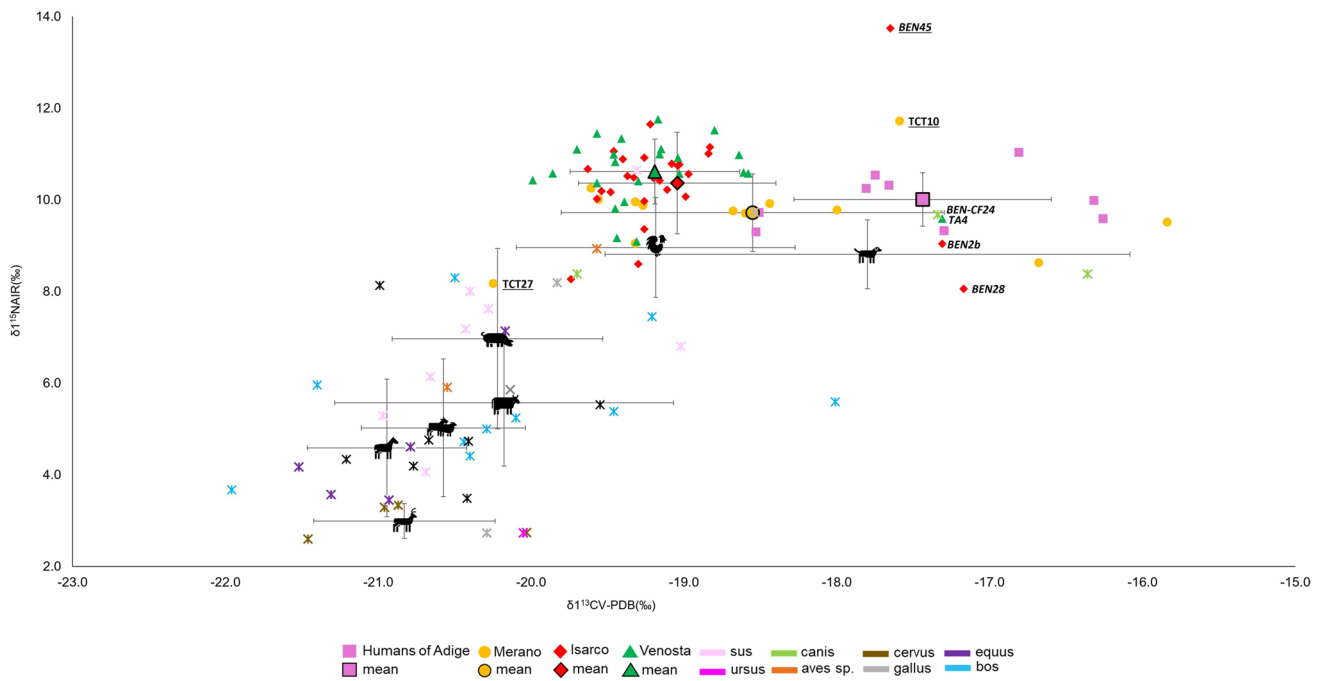
Figure 5 displays the mean  $\delta^{34}\text{S}$  values of the humans from the different locations in relation to the general  $\delta^{34}\text{S}$  faunal baseline ( $n=43$ , mean  $+6.65 \pm 2.14\text{‰}$ ) and to the baseline per location ( $n=8$  Adige:  $+7.49 \pm 3.27\text{‰}$ ;  $n=7$  Merano Basin:  $+5.22 \pm 0.99\text{‰}$ ;  $n=9$  Isarco  $+5.91 \pm 1.38\text{‰}$ ; and  $n=6$  Venosta  $+6.82 \pm 1.37\text{‰}$ ). The plot shows statistically supported differences (Table SM12) among the various

locations in South Tyrol. Most significant are the differences between the groups of the Adige (highest  $\delta^{34}\text{S}$  variation, mean  $+8.67 \pm 2.28\text{‰}$ ) and Venosta Valley (lowest  $\delta^{34}\text{S}$  variation, mean  $+5.65 \pm 0.63\text{‰}$ ). Additionally, the plot indicates that the sulfur values of some individuals from the Merano Basin (TCT27, TCT-US186, Castel Tirol), Isarco Valley (BEN11 and BEN2b, BEN29 and BEN45 Elvas Bressanone; SK74 Sabiona), and Venosta Valley (BSS-US158 and MHP2, from Burgusio St. Stefano and Malles Maso Pauli, respectively) differed from the local faunal baseline.

## Discussion

### Mitochondrial DNA diversity patterns in early medieval individuals from the Eastern Italian Alps

Overall, the 94 early medieval alpine samples show high variation in terms of number of different haplotypes (~92%).



**Fig. 4**  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  ratios of human ( $N=72$ ) and faunal samples ( $N=43$ ), including average values and standard deviations. The extreme outliers are indicated (italics = outliers for  $\delta^{13}\text{C}$ , underlined = outliers for  $\delta^{15}\text{N}$ ). The animals' symbols are from PowerPoint

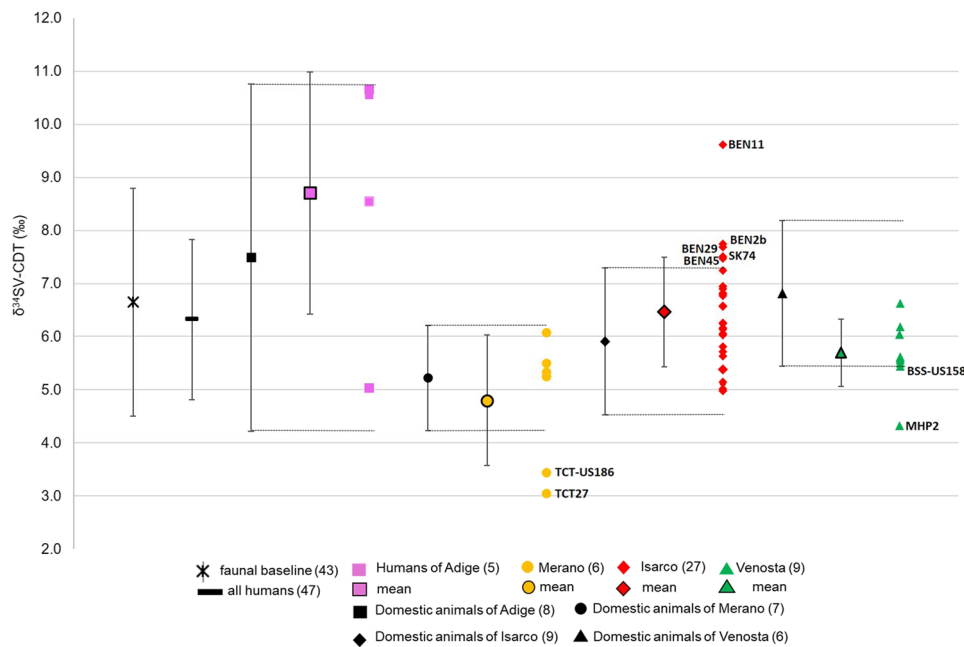
The haplotype diversity in the four ancient alpine groups is also high, with no significant differences among groups and similar to the haplotype diversity found in present-day population from South Tyrol.

The haplotype identity (Table SM3) of individuals buried within the same archaeological sites suggested possible close maternal relatedness such as the case of those from the site of Sabiona (Isarco) (SK63 and SK100; SK212 and SK95; SK162 and SK78). Interestingly, most of these individuals were buried with grave goods attributed to a possible common cultural Romanized group (Bierbrauer et al. 2015). The other two adults buried in the same site of Appiano, S. Paolo in the Adige Valley (AP-AL4 and AP-AL5), were found in two single graves located next to each other. Moreover, a close genetic relationship of the two adults from Burgusio St. Stefano is likely, as both were buried in the same grave (Supplementary Information) (Reuß 2016). But, matching haplotypes from different archaeological sites and locations are more difficult to interpret.

The early medieval alpine individuals analyzed in this study also show a high heterogeneity of the mtDNA haplogroups with several rare sub-lineages. According to database searches of whole ancient mtDNA, eight lineages (H11a5, H31, H75 and H80, HV4a2b, K1c1a, U8a1a1a1, and M1a3a) are virtually restricted to the eastern Italian Alps while the majority of haplogroups are widespread in other parts of Europe and the Middle East. A different distribution of

lineages in the alpine territory emerged such as in the case of haplogroup H which is present with a peak in the north-western part of the territory with a frequency similar to that observed in another early medieval group from north Italy (Lombards from Collegno, ~61%; Vai et al. 2019). Interpopulation analyses ( $F_{st}$  genetic distances) further highlighted maternal genetic differentiation among specimens from different locations from South Tyrol. Indeed, differences were observed between early medieval individuals from the central and north-eastern part of South Tyrol (Adige Valley and Merano Basin, Isarco Valley) and those from the valley situated in the north-western part of the territory (Venosta Valley). The most pronounced genetic differences were detected among specimens buried in the archaeological sites of the Adige and Venosta Valleys located at the two extreme altitudes (~335 m above sea level and on average ~1240 m above sea level, respectively). Moreover, the comparison extended to other specimens from Europe from around the same time period evidenced a maternal genetic affinity between early medieval alpine groups from the central and north-eastern area of South Tyrol and early medieval Europeans of various origins and cultures but the same was not evidenced for individuals from the Venosta Valley. These results suggested possible genetic exchange with allochthonous people especially for individuals from the central and north-eastern part of South Tyrol (Adige Valley and Merano Basin, Isarco Valley). Particularly for the





**Fig. 5**  $\delta^{34}\text{S}$  ratios of all human samples grouped per locations and  $\delta^{34}\text{S}$  baselines based on faunal samples (dashed lines), including average values and standard deviations. Numbers in brackets represent the sample size

Adige Valley, this is to be expected given its strategic position and the presence of the Adige River, which has always provided communication routes that have favored population movements, cultural exchange, and trading (Lanzinger et al. 2001). Moreover, regarding the Isarco Valley, especially starting from the second century AD onwards, it became the most important and best maintained road of the complex communication routes present in the alpine region which date back to Roman times (Pesavento Mattioli 2000). On the other hand, while in the first century AD, the Venosta Valley was crossed by one of the fundamental axes of this road system (*Via Claudia Augusta* inaugurated in 46 AD), this route lost its importance at a later date, leading to a certain isolation of this valley compared to the others. These circumstances may have favored mobility and genetic exchange more in the central and north-east of South Tyrol than in the Venosta Valley in the north-west. Additionally, even if the allochthonous and the Roman grave goods are often difficult to distinguish (Dal Ri and Rizzi 1995; La Rocca 2004, 2009; Albertoni 2005; Gasparri and La Rocca 2013), the material culture points toward cultural admixture, especially in the Isarco Valley (Sabiona and Bressanone Elvas). A few more funerary goods have also been retrieved in the other locations (Supplementary Information). However, also the effect of the genetic drift which could be particularly strong in small and isolated populations such as those from the mountainous areas may explain the genetic differentiation of

individuals from the Venosta Valley compared to those from the other alpine locations and from Europe. An additional outcome of the study suggests possible matrilineal genetic continuity in the territory of the Eastern Italian Alps, similar to what has been observed in other areas of Italy (e.g., Posth et al. 2021). In fact, comparisons with present-day populations from Europe (whole mtDNA data) and from the same alpine territory and valleys (Adige, Isarco, and Venosta; HVR-1 data) indicate broad genetic affinity between most early medieval alpine groups and modern populations (e.g., ancient and modern Venosta,  $F_{st} = 0.004\text{--}0.005$ ). An exception might be the Isarco Valley ( $F_{st} = 0.04$ ), as the population living in this area today is highly different genetically from the people who were buried in this valley during the Early Middle Ages suggesting maternal discontinuity in this area. A significantly high mtDNA genetic differentiation of modern-day populations from this location compared to the other alpine populations has previously been described (Pichler et al. 2006) and could be explained by the effect of genetic drift. Finally, it should be noted that the early medieval group from Venosta shows a genetic similarity to modern populations especially when the comparison is extended to present-day alpine populations, suggesting a closer genetic affinity to these populations. However, more mitogenomes from modern alpine populations would be necessary to better investigate the genetic relationship between early medieval and modern Venosta.

## Dietary and mobility variations of the early medieval alpine groups

The  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  stable isotopes reflect the plant and animal protein content in the diet (e.g., Schwarcz and Schoeninger 1991; Ambrose 1993; Van Klinken et al. 2002; Lösch et al. 2006). Additionally, the analysis of  $\delta^{34}\text{S}$  helps identify proportions of aquatic (marine/freshwater fish) and terrestrial diets and, as it reflects the local geology, it also aims in the reconstruction of human mobility patterns (Richards et al. 2003; Vika 2009; Nehlich et al. 2010; Nehlich 2015).

Differences in the dietary habits of the early medieval individuals from the Eastern Italian Alps were confirmed and extended in the current study.

Indeed, the group of the Adige Valley had a diet with a greater intake of  $\text{C}_4$  plants, most likely linked to the low altitude (mean altitude 348 m.a.s.l.), and therefore a suitable habitat for  $\text{C}_4$  crop cultivation (e.g., millet and sorghum), which require an abundant water supply and warmer temperatures to grow (Ehleringer et al. 1991, 2002). A  $\text{C}_4$  plant consumption has already been described in early medieval northern Italy (e.g., Paladin 2021; Reitsema and Vercellotti 2012; Castiglioni and Rottoli 2013; Iacumin et al. 2014; Laffranchi et al. 2020; Marinato 2017), in Germany (Wahl et al. 2014), and in Hungary (Amorim et al. 2018). Differently, a subsistence strategy mainly based on crop cultivations of terrestrial  $\text{C}_3$  plants (e.g., wheat) was found for the alpine individuals buried in the Merano Basin and Isarco and Venosta Valleys similarly to other European early medieval groups from northern Italy (Collegno; Amorim et al. 2018), Hungary (Amorim et al. 2018), and Austria (North Tyrol; McGlynn 2007). Additionally, the nitrogen stable isotope ratio of the individuals of the Merano Basin indicates a diet that was poor in animal protein and/or a possible consumption of N-fixating plants, such as pulses that lead to lower nitrogen ratios (Bogaard et al., 2013; Lösch et al. 2014; Siebke et al. 2020). On the other hand, in the group of the Isarco Valley,  $\delta^{15}\text{N}$  ratios suggest different subsistence strategies having an economy predominantly based on meat production and dairy products, similar to the group from the Venosta Valley, which also had most likely greater access to animal proteins. The greater consumption of animal proteins in the diet could also be indicative of high social status (e.g., Le Huray and Schutkowski 2005; Reitsema and Vercellotti 2012), and this would also be suggested by the rich burials with grave goods found particularly in some of the cemeteries present in the Isarco Valley (Supplementary Information). However, it cannot be excluded that the enriched nitrogen values were also due to other factors, including the manuring effect that increases the amount of nitrogen in the soil and in plants. Some individuals differ from these general patterns; indeed, the outliers for  $\delta^{13}\text{C}$  in Isarco (BEN2b, BEN28) and Venosta (TA4) had a stronger  $\text{C}_4$  intake in their diet compared to the

other individuals buried in the same valleys. This could suggest diverse dietary habits in terms of plant consumption, but also a different origin from the burial site (i.e., from lower altitudes). Other outliers for  $\delta^{15}\text{N}$  in the Merano Basin and Isarco Valley include two subadults (TCT10 and BEN45), which display the highest nitrogen values likely due to a detectable signal of breastfeeding (e.g., Dupras and Tocheri 2007; Bourbou et al. 2011; Beaumont et al., 2013; Stantits et al. 2019). Additionally, one adult female (TCT27) had the lowest  $\delta^{15}\text{N}$  value suggesting a possible diet with almost no or very little intake of animal proteins or it might be that the isotopic signature was influenced by the pathologies (e.g., Reitsema 2013) that afflicted this individual (Paladin 2021).

The present study also extended our understanding of the mobility patterns (based on  $\delta^{34}\text{S}$  ratios) involving the investigated alpine groups. Again, the most significant differences were observed between the groups located at the lower and highest altitudes (Adige and Venosta, respectively). In fact, we confirmed that the group of the Adige Valley is distinguished by the greatest  $\delta^{34}\text{S}$  variation in comparison to the other locations, followed by Isarco and Merano, which could be explained by a high mobility. In contrast, the group of the Venosta Valley shows the lowest mobility signal. This pattern is very similar to what we observe at a genetic level since Adige and Venosta are the most differentiated (one of the highest  $F_{st}$  value among these two alpine locations) and Venosta is different to most of the other groups considered for comparison.

The results additionally show several outliers (Fig. 5), especially from the Isarco Valley, having enriched (Isarco Valley: BEN2b  $\Delta 1.84\text{‰}$ , BEN11  $\Delta 3.70\text{‰}$ , BEN29  $\Delta 1.77\text{‰}$ , BEN45  $\Delta 1.56\text{‰}$ ; SK74  $\Delta 1.60\text{‰}$ ) or depleted (Merano Basin: TCT27  $\Delta 2.17\text{‰}$ , TCT-US186  $\Delta 1.78\text{‰}$ ; Venosta Valley: MHP2:  $\Delta 2.50\text{‰}$ , BSS-US158:  $\Delta 1.38\text{‰}$ )  $\delta^{34}\text{S}$  values compared to the local faunal baseline, indicating the potentially non-local origin of these individuals with respect to their place of burial.

The distribution of mtDNA haplogroups in these possibly non-local individuals may provide further information and suggest a complex maternal genetic origin for some of them. For instance, the infant (BSS-US158, Venosta) carried the rare lineage H39 which it shares with one other early medieval individual from Hungary (Pannonia; Vai et al. 2019) and which is present today especially in Finland and Sweden (e.g., Finnilä et al. 2001; Moilanen et al. 2003). Another interesting case regards the adult (possibly female) BEN28 (Isarco) that carried the rare H1f haplogroup (plus mutation T16093C), which indicates a possible maternal genetic connection with ancient Scandinavia (one Viking from Sweden; Margaryan et al. 2020) and present-day populations from Finland (e.g., Kiiskilä et al. 2019). Intriguingly, also a different nutritional basis in terms of plant consumption (stronger  $\text{C}_4$  intake) was observed for this individual compared to those from the same alpine location.

## Conclusion

The mitochondrial DNA and  $\delta^{34}\text{S}$  analyses highlighted regional differences in terms of genetic relationships with allochthonous groups and mobility patterns among individuals from the central and north-eastern part of South Tyrol (Adige Valley, Merano Basin, and Isarco Valley) and those from the north-western part of the territory (Venosta Valley). More pronounced were those among ancient alpine individuals buried at the lowest (Adige, ~335 mamsl) and the highest (Venosta, on average ~1240 mamsl) altitudes, indicating that this factor, and the potentially greater isolation of the Venosta, may have played an important role in the genetic structure and mobility of these alpine groups. The differences observed could also be related to the geomorphology of the territory and to the road system present since Roman Times, which may have favored human mobility and genetic exchanges with allochthonous people more in the first (north-east) than in the second (north-west) group, particularly in the Isarco Valley as also indicated by cultural materials. Furthermore, the study suggested genetic continuity among early medieval groups and present-day populations in this alpine area but with some interesting exceptions (Isarco Valley).

Finally, different subsistence strategies were also detected (stable isotopes of  $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ ) indicating a diverse exploitation of the territory in the various areas of South Tyrol, especially linked to environmental factors (e.g., altitudes) and/or differences in the social status. The differences mainly concern the group from the Adige Valley (consumption of  $\text{C}_4$  plants) compared to groups from all other alpine locations ( $\text{C}_3$  plants) and those from Venosta and Isarco Valleys which displayed the most enriched  $\delta^{15}\text{N}$  values linked to a higher consumption of animal proteins.

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**Author contribution** V.C. and A.P. designed the research together with F.M. and A.Z.; A.P. conducted the anthropological analyses and collected the bone samples; V.C. and S.Z. performed the genetic experiments; V.C. carried out the bioinformatic and the statistical analyses of the molecular data together with M.C.; A.P. performed the data quality and the statistical analyses of the stable isotope data and S.L. supervised the analysis of the isotope experiments; A.P. and E.C. curated the archaeological and historical aspects. V.C. in collaboration with A.P., F.M., and A.Z. wrote the paper. All authors have reviewed and approved the final manuscript.

**Data availability** The BAM files with the reads mapped to the Revised Cambridge Reference Sequence (rCRS) of the Human Mitochondrial DNA generated during the current study are available at the European Nucleotide Archive (ENA) with the accession number PRJEB43507.

## Declarations

**Competing interests** The authors declare no competing interests.

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