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Variability of bone preservation in a confined environment: the case of the catacomb of Sts Peter and Marcellinus (Rome, Italy)

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Abstract

Most of the studies investigating the diagenetic trajectory of fossil bones focus on open-air sites and very little work have been published in confined environments such as catacombs. While the stable thermal history of catacombs should favor bone preservation, the accumulation of corpses over a short period of time could favor their destruction. The aim of this study is to describe the diagenetic trajectory of 128 human bone samples coming from six different burial chambers of the catacomb of Sts Peter and Marcellinus (SSPM, Rome, Italy). A multi-proxy approach was undertaken to provide an assessment of the molecular preservation as well as a direct record of the isotopic composition itself. Collagen yield, carbon and nitrogen abundances, C:N ratio, FT-IR based collagen and carbonate contents and crystallinity index, radiocarbon dating and stable isotope analysis of bone collagen and carbonate indicate that both the mineral and the organic fractions are impacted by diagenesis to various degrees, and that bones originating from the small burial chambers are more affected than those coming from the large ones. While some of the bones were strongly recrystallized, the impact of bone diagenesis on the stable isotope values of bone carbonate was limited. Comparison with contemporary sites from the Latium showed that conditions prevailing in catacombs seem overall to favor, rather than disadvantage bone preservation.

1. Introduction

Excavations conducted from 2004 to 2010 in the central area of the catacomb of Sts Peter and Marcellinus (hereafter the SSPM catacomb) located in the south-east of Rome revealed several mass graves dated to between the first and the third century AD based on radiocarbon and artefact (coins) dating (Castex et al., 2007; Castex et al., 2011). They contained tens to hundreds of articulated human skeletons laid together according to well-reasoned management (Blanchard et al., 2007; Castex et al., 2007; Castex et al., 2009; Castex

et al., 2011). A significant number of these individuals received a specific funerary treatment characterized, in particular, by the use of gypsum to partially or entirely cover the corpses (Castex and Blanchard, 2011; Castex et al., 2009; Devièse et al., 2010; Vanhove, 2006). A mortality crisis following an epidemic episode is thought to be at the origin of these mass graves suggesting that they have been used for a relatively limited period of time (Blanchard et al., 2007; Castex and Blanchard, 2011; Castex et al., 2009; Castex et al., 2011; Kacki et al., 2013). Unfortunately, the study of the biological identity (sex, age at death, stature, non-metric traits, pathological conditions, *etc.*) of the individuals was restricted due to the state of skeletal preservation (Blanchard et al., 2007; Castex and Blanchard, 2011; Castex et al., 2007; Castex et al., 2009). Many bones and teeth have lost their anatomical integrity even though the preservation quality differs strongly among burial chambers, different deposit levels within a chamber, and different skeletons within the same level (Blanchard et al., 2007; Castex and Blanchard, 2011; Castex et al., 2007; Castex et al., 2009). Stable isotope approaches offer invaluable assistance when the macroscopic anthropological observations are limited by the poor quality of skeletal preservation (*e.g.* Katzenberg et al., 2009; Oelze et al., 2012; Wright et al., 2010). Measurements of the stable isotope composition of the SSPM population would allow us to better understand their life history, provided that *in vivo* isotopic compositions are preserved.

Checking whether the original isotope compositions of biomineral fractions are not altered by diagenesis is an essential prerequisite to interpret isotope analyses (Bocherens et al., 2005; Bocherens et al., 2008; Brady et al., 2008; Shin and Hedges, 2012). Most of the studies investigating the diagenetic trajectory of fossil bones focus on open-air sites. To our knowledge, very little work has been published in confined environments. The advantage of such an approach was demonstrated by Bocherens et al. (2008) in Chauvet cave. In this Palaeolithic site, the authors showed that the preservation of bear bones exposed on the floor

of the cave varied widely probably due to spatial variation in local conditions. The Chauvet cave results cannot be directly translated to man-made cavities such as catacombs because the age and taphonomic contexts are quite different. Like in caves, the thermal history of catacombs is more stable than in open-air sites which should *a priori* favor their preservation. On the other hand, the accumulation of corpses over a short period of time in catacombs could have an effect on the soil pH and therefore favor the destruction of human bones. The aim of this study is to describe the diagenetic trajectory of the human bone samples from the SSPM catacomb in order to assess their preservation and compare it with the preservation observed in contemporary open-air sites from the region of Rome. A multi-proxy approach (measurement of classical collagen preservation indicators; Fourier-transform infrared spectroscopy (FT-IR) based collagen preservation indicators, carbonate content and crystallinity, ^{14}C measurements, and stable isotope analysis of the collagen and carbonate fractions) was undertaken to provide a record of the preservation of the molecules. Our results show that both the mineral and the organic fractions are affected by diagenesis to various degrees, and that bones originating from the small burial chambers are the most diagenetically altered. While some of the bones were strongly recrystallized, the stable isotope values of bone carbonate remained reliable. Comparison with contemporary sites from the Latium showed that conditions prevailing in catacombs seem overall to favor, rather than disadvantage bone preservation

2. Study area and material

A total of 128 individuals were selected for this study. The material comes from six of the seven burial chambers discovered in 2003 in a previously unexplored area (region X) of the SSPM catacomb (Blanchard et al., 2007; Castex et al., 2007) (Figure 1). These burial chambers are part of a vast funerary complex that covers approximately 3 ha with 4.5 km of

101 underground galleries (Guyon, 1987). Four of them (X78/T15, X80/T16, X81 and X82/T18)
102 are small in size (< 7m^2), while the remaining two (X83 and X84) are larger in size (> 9m^2)
103 (Castex et al., 2007; Castex et al., 2009). Different phases of multiple deposits separated by a
104 layer of sediment were identified in the small chambers (Blanchard et al., 2007; Castex et al.,
105 2011; Kacki et al., 2013; Sachau-Carcel et al., 2013). At least three phases were recognized in
106 X78/T15 (Pagni and Burdassi, 2004) whereas 9 and 12 phases were distinguished in X80/T16
107 and X82/T18, respectively (Blanchard et al., 2007; Castex et al., 2007). In contrast, no
108 stratum of soil was discovered between the skeletons in X81, X83 and X84 (Castex and
109 Blanchard, 2011; Castex et al., 2009; Castex et al., 2011).

110 For each individual, a fragment of long (*i.e.* humerus, radius, ulna, metacarpus, femur
111 and tibia), flat (*i.e.* cranial vault, rib and hip bone), irregular (*i.e.* mandible and maxilla) or
112 short (calcaneus) bone was sampled. Due to the poor skeletal representation of bone remains
113 of five individuals from the burial chamber X82/T18, several fragments of different skeletal
114 parts were selected in order to obtain enough material to achieve the required analyses
115 (hereafter named miscellaneous samples). Modern reference material required for FT-IR
116 analysis was composed of bones of modern ox, calf, horse and sheep in order to take into
117 account variation due to species and age at death.

118

119 **3. Methods**

120 3.1. Bone carbonate preparation and analysis

121 Between 30 and 36 mg of bone powder (grain size < 0.3 mm) was first soaked in 2 to
122 3% sodium hypochlorite at room temperature for 48 h to remove organic matter then rinsed
123 five times with distilled water. The remaining fraction was treated with 1 M acetic acid for 1 h
124 to remove exogenous carbonate then rinsed five times with distilled water. Sodium
125 hypochlorite and acetic acid solutions were renewed half-way through each process. Samples

were oven-dried for 18 h at 65°C following pretreatment. The purification process resulted in a weight loss comprising between 10 and 80%. Bone carbonate $\delta^{13}C$ and $\delta^{18}O$ values were measured at the Service de Spectrométrie de Masse Isotopique of the MNHN (SSMIM). Samples weighing from 580 to 630 mg were reacted for 360 s with 100% phosphoric acid at 70°C in individual vessels in an automated cryogenic distillation system (Kiel IV device), interfaced with a ThermoScientific Delta V Advantage isotope ratio mass spectrometer. The stable isotope results are reported in per mil (‰) relative to VPDB. Over the period of analysis, the analytical precision calculated based on repeated measurements of our internal carbonate standard (Marble LM) was 0.02‰ for $\delta^{13}C_{carb}$ and 0.05‰ for $\delta^{18}O_{carb}$ values.

3.2. Bone collagen extraction and analysis

Bone collagen extraction followed Longin's (1971) protocol modified by DeNiro and Epstein (1981) and Bocherens et al. (1988; 1991). Between 0.1 and 1.1 g of bone powder (grain size comprised between 0.3 and 0.7 mm) was soaked in 40 ml of 1 M hydrochloric acid for 20 min at room temperature then filtered for decalcification using 5 µm cellulose filters. The residue was soaked into 0.125 M sodium hydroxide for 20 h at room temperature to oxidize fulvic and humic acids then filtered. The gelatin was then solubilized in 0.01 M hydrochloric acid at 100°C for 17 h, and filtered again to remove impurities. The solubilized collagen was then freeze-dried for 48 to 72 h and extraction yields calculated. Stable isotope composition was measured at the environmental isotope laboratory at James Cook University's Advanced Analytical Centre (Cairns, Australia) using samples weighing between 200 and 400 µg. Carbon and nitrogen abundance and isotope composition ($\delta^{13}C_{col}$ and $\delta^{15}N_{col}$ values) were determined using a Costech Elemental Analyzer 4010 fitted with a zero-blank auto-sampler coupled via a ConFlo IV to a Thermo Scientific Delta V^{PLUS} using Continuous-Flow Isotope Ratio Mass Spectrometry. The $\delta^{13}C_{col}$ and $\delta^{15}N_{col}$ values are reported in per mil

151 (‰) deviation relative to VPDB and AIR, respectively. Over the period of analysis, the
152 carbon and nitrogen abundances were ±5% of the value for laboratory internal standards and
153 the analytical precision for $\delta^{13}C$ and $\delta^{15}N$ was better than ±0.1‰ and ±0.2‰, respectively.
154 Weight percent carbon and nitrogen were used to calculate atomic C:N ratios.
155

156 3.3. FT-IR analysis

157 Attenuated total reflexion mode (ATR) FT-IR analysis was performed on bone powder
158 (grain size < 0.3 mm) before and after pretreatment. Infrared spectra were collected by
159 accumulation of 64 scans with a spectral resolution of 2 cm^{-1} on a Bruker Vector 22 FT-IR
160 spectrometer equipped with a diamond-ATR accessory (Specac Golden Gate). Spectral data
161 analyses were performed using OPUS software (Bruker). Crystallinity was evaluated using
162 the Infrared Splitting Factor (IRSF) (Figure 2). The sum of the two ν_4PO_4 bands at 565 and
163 600 cm^{-1} were divided by the valley at 590 cm^{-1} following the protocol described by Weiner
164 (1990). Relative carbonate content was measured by the intensity ratio of the ν_3CO_3 band at
165 1415 cm^{-1} over the ν_4PO_4 band at 600 cm^{-1} (adapted from Wright and Schwarcz 1996).
166 Relative collagen content was measured by the intensity ratio of the Amide I band at 1660 cm^{-}
167 ^{1} over the ν_4PO_4 band at 600 cm^{-1} (adapted from Trueman et al., 2004). The precipitation of
168 secondary calcite was monitored by the presence of its specific band at 712 cm^{-1}.
169

170 3.4. Radiocarbon dating

171 The collagen and apatite fraction of five individuals from burial chambers X80/T16,
172 X82/T18 and X83 were radiocarbon dated. An aliquot of collagen (between 8 and 13 mg) was
173 combusted for 15 to 60 min at 800°C. Carbonate was purified following a modified version of
174 Krueger et al. (1991). About 2 g of bone powder (grain size < 0.1 mm) was first soaked in 2

175 to 3% sodium hypochlorite at room temperature for six days to oxidize the organic matter.
176 Bone powder was then rinsed several times with distilled water and dried for 3 h at 100°C. It
177 was then placed in 100 ml of 2 M acetic acid for 24 h at room temperature under weak
178 vacuum. The evolution of fine gas bubbles usually slows down drastically after 1 h, and stops
179 after 10+ h, suggesting that all secondary calcite as well as the smallest apatite crystals had
180 been dissolved. The acetic acid treatment resulted in weight losses between 40 and 80%. An
181 aliquot of purified bioapatite weighing between 220 and 280 mg was then reacted in
182 orthophosphoric acid for 12 min at 70°C under vacuum. CO_2 derived from the collagen and
183 apatite samples was cryogenically trapped and about 1 mg C was sealed in a glass tube for
184 radiocarbon dating. Graphitization and AMS measurement were conducted at Queen's
185 University's ^{14}Chrono Centre (Belfast, Northern Ireland).
186
187 **4. Bone diagenesis evaluation**
188 4.1. Macroscopic preservation
189 Bone samples were classified into three categories according to their macroscopic
190 characteristics as well as their strength and resistance when cutting: (1) "good preservation"
191 characterizes a solid and heavy bone difficult to cut and crush; (2) "poor preservation"
192 corresponds to a bone which is fragile, soft and easy to cut and crush; and finally (3) "medium
193 preservation" refers to a bone with intermediate characteristics (Figure 3).
194
195 4.2. Collagen quality
196 Bone collagen preservation was evaluated based on three criteria: bone collagen
197 extraction yield (wt%), carbon and nitrogen abundances in collagen (%), and atomic C:N ratio
198 (Table 1). Collagen concentration in modern bone is around 20.4 wt% (SD ±3.9 wt%)
199 (Bocherens et al., 1991), and values <1 wt% are usually considered as being unreliable

200 (Dobberstein et al., 2009; van Klinken, 1999). In modern bones, carbon and nitrogen
201 abundances range from 13 to 47% and from 4.8 to 17.3% respectively (Ambrose, 1990), and
202 values below or above these thresholds indicate alteration or contamination (Garvie-Lok,
203 2001; Iacumin et al., 1998; Reitsema, 2012, van Klinken, 1999). Average atomic C:N ratio in
204 modern bones is 3.2 to 3.3 (Ambrose, 1990; van Klinken, 1999), and vary between 2.9 and
205 3.6 (DeNiro, 1985). Atomic ratios below or above these thresholds indicate alteration or
206 contamination (Ambrose, 1990; DeNiro and Weiner, 1988; Grupe et al., 2001). Samples that
207 fail to pass any of these three criteria were considered to be poorly-preserved.
208
209 4.3. Infrared spectroscopy
210 Bone recrystallization promotes the formation of a more stable mineral phase
211 characterized by a higher crystallinity (*i.e.* crystal size and lattice perfection) and lower
212 carbonate content. These changes are related to both microbial activities (bioerosion) and
213 interaction of bone mineral with the burial environment (*i.e.* pH) that control dissolution-
214 recrystallization processes (Berna et al., 2004; Hedges and Millard, 1995; Smith et al., 2007).
215 Crystallinity and carbonate content can be evaluated by FT-IR using the IRSF and CO_3/PO_4
216 ratio, respectively. IRSF values vary between 2.5 and 3.3 for modern bones (Stiner et al.,
217 1995; Wright and Schwarcz, 1996; Nielsen-Marsh and Hedges, 2000; Berna et al., 2004;
218 Munro et al., 2007; Asscher et al., 2011) and usually reaches values between 3.4 and 4.5 for
219 archaeological samples (Weiner and Bar-Yosef, 1990; Stiner et al., 1995; Wright and
220 Schwarcz, 1996; Munro et al., 2007). Bones are usually considered altered for values above
221 3.8 to 4.0 (Shemesh, 1990; Stiner et al., 1995; Berna et al., 2004). In moderrn animal bones,
222 CO_3/PO_4 ratio is comprised between 0.7 and 1.0. This ratios decreases to less than 0.2 for
223 altered samples (Lebon, 2008). The fate of the mineral phase is mainly dependent on the
224 degradation of the organic phase (Collins et al., 2002; Lebon et al., 2011; Trueman et al.,

2008). FT-IR analyses offer also the ability to measure the relative collagen content of a bone by measuring the AmideI/PO_4 ratio. This ratio is comprised between 0.9 and 1.2 for modern bones and decreases as collagen decays. The AmideI/PO_4 and CO_3/PO_4 ratios are usually correlated (Lebon, 2008; Lebon et al., 2011).

4.4. Radiocarbon dating of bone collagen-carbonate pairs

The $^{14}C/^{12}C$ and $^{13}C/^{12}C$ ratios differ by several orders of magnitude. This results in a higher sensitivity of the $^{14}C/^{12}C$ ratio over the $^{13}C/^{12}C$ ratio to post-mortem isotope exchange. Therefore, ^{14}C is a useful proxy to assess the influence of diagenesis on the carbon stable isotope composition of carbonate in apatite (Shin and Hedges, 2012; Zazzo, in press). To quantify carbon isotope exchange between bone carbonate and dissolved inorganic carbon during diagenesis, we measured the radiocarbon age difference between the collagen and carbonate fraction ($^{14}C_{col\text{-}carb}$) from the same bone. If bone collagen passes the three tests outlined above, any deviation should be the result of carbon isotope exchange in the carbonate fraction. This difference can then be transformed in percent of carbon isotope exchange assuming that exchange takes place with modern carbon (Hedges et al., 1995a). This is probably a reasonable approximation as an extensive review of the literature and a recent ^{14}C dating program of several archaeological sites worldwide demonstrated that diagenetically altered apatites are always younger than the reference age for the site (Zazzo, in press; Zazzo and Saliège, 2011). Radiocarbon ages are presented as BP and fraction modern (F_m).

5. Results

5.1. Collagen preservation

Collagen preservation varies greatly between different samples (Table 2). Collagen yields differ according to bone location within the catacomb, the anatomical part category and

the macroscopic aspect (Figure 4). In total, 29 (23%) bone collagen samples do not pass at least one of the three tests defined (collagen yield, carbon and nitrogen abundances, and C:N ratio (Table 3). Within this group, all but two were classified as macroscopically poorly-preserved (Table 3). These samples all originate from three of the the four lowest levels of the chamber X80/T18 and from all the levels of the chamber X82/T18 (Table 3). They consist largely of long and flat bones (31 and 38%, respectively). Additionally, 46% of the flat bones and 100% of the miscellaneous samples have poorly-preserved collagen (Table 3). Macroscopic preservation was variable among the 99 (77%) bone collagen samples with reliable collagen (*e.g.* see Figure 5 for the variations of atomic carbon and nitrogen abundances). Poor scores were obtained for macroscopically poorly-preserved specimens coming from two small chambers (X80/T16 and X82/T18).

5.2. FT-IR

The modern animal reference samples have relatively low IRSF values (Mean = 3.4; SD ±0.1) and high CO_3/PO_4 ratios (Mean = 0.85; SD ±0.10), consistent with values previously published (Lebon et al., 2010). With the exception of three samples, all the archaeological human bone samples present higher IRSF values and lower CO_3/PO_4 ratios than modern animal reference samples (Figure 6). IRSF values measured on the archaeological samples before pretreatment range from 3.6 to 5.1 (Mean = 4.3; SD ±0.4), while CO_3/PO_4 ratios vary between 0.15 and 1.01 (Mean = 0.49; SD ±0.20). IRSF values are negatively correlated with bone CO_3/PO_4 ratios ($R^2 = 0.75$) (Figure 6). No trace of calcite was detected (detection limit 1.5 %) in bone samples before pretreatment. Amide I/PO_4 ratios vary from 0.07 to 0.86 (Mean = 0.35; SD ±0.25) and are lower for the archaeological bones than for the modern animal reference samples (Figure 7). Carbonate and collagen relative contents are positively correlated ($R^2 = 0.76$) (Figure 7). Small chamber samples are significantly

275 different from large chambers samples with respect to IRSF values, CO_3/PO_4 and Amide
276 I/PO_4 ratios (Mann-Whitney U tests; p-values < 0.03). Samples from small chambers have
277 lower relative carbonate content, lower relative collagen content, and higher IRSF values
278 compared to samples from larger chambers. Following chemical pretreatment of bone
279 carbonate, IRSF values increase (Mean = 4.6; SD ±0.3) and relative carbonate content
280 decrease (Mean = 0.28; SD ±0.10). CO_3/PO_4 ratios remain negatively correlated to IRSF
281 values ($R^2 = 0.59$) (Figure 8).
282
283 5.3. Stable isotope analysis
284 Bone collagen carbon and nitrogen isotope values were measured for all specimens
285 that yielded a collagen extract (Table 2). There was no significant difference in $\delta^{13}C_{col}$
286 between the well-preserved and the poorly-preserved collagen samples (Mann-Whitney U
287 test; p-value = 0.52). In contrast, the well- and poorly-preserved collagen samples have
288 significantly different $\delta^{15}N_{col}$ values (Mann-Whitney U test; p-value = 0.00). There was no
289 relationship between the $\delta^{13}C_{col}$ or $\delta^{15}N_{col}$ values and the collagen yield ($R^2 < 0.05$), the
290 atomic carbon and nitrogen abundances ($R^2 < 0.2$) or the atomic C:N ratios ($R^2 < 0.07$). There
291 was no significant difference between the isotope values and the burial chambers, the skeletal
292 part categories, or the macroscopic appearances when either one of the two groups or the
293 totality of the corpus is considered (Mann-Whitney U tests; p-values >> 0.05). We found one
294 exception, however. When samples with well-preserved collagen were considered (n = 99),
295 $\delta^{15}N_{col}$ values measured in macroscopically poorly- and well-preserved samples differed
296 significantly (Mann-Whitney U test; p-value = 0.04).
297 Bone carbonate carbon and oxygen stable isotope values were measured for the 128
298 individuals (Table 2). Bone $\delta^{18}O_{carb}$ values in X82/T18 differed significantly from bone
299 $\delta^{18}O_{carb}$ values in X80/T16 and X84 (Mann-Whitney U test; p-value = 0.01). When the totality

of the corpus was considered, $\delta^{13}C_{carb}$ values of irregular and flat bones, irregular and long bone, and long and miscellaneous bones differed significantly (Mann-Whitney U tests; p-values < 0.04). The same conclusion was reached (excepted for long vs. miscellaneous bones) when only samples with well-preserved collagen were considered (Mann-Whitney U tests; p-values < 0.04). Moreover, when the totality of the corpus was considered bone macroscopically identified as well-preserved had $\delta^{13}C_{carb}$ and $\delta^{18}O_{carb}$ values that differed significantly from those measured in medium and poorly-preserved bones, respectively (Mann-Whitney U tests; p-values =< 0.015). The samples which yielded well- (n = 99) and poorly-preserved (n = 29) collagen samples also had significantly different $\delta^{13}C_{carb}$ and $\delta^{18}O_{carb}$ values (Mann-Whitney U test; p-value = 0.01 and 0.02, respectively). There was no relationship between the $\delta^{13}C_{carb}$ or $\delta^{18}O_{carb}$ values and the IRSF values ($R^2 < 0.01$), the CO_3/PO_4 ratio ($R^2 < 0.01$) or the AmideI/PO_4 ratio ($R^2 < 0.01$) before or after bone carbonate pretreatment.

5.4. Radiocarbon dating

The $^{14}C_{col}$ ages range from 1779 ± 27 BP to 1965 ± 31 BP whereas the $^{14}C_{carb}$ ages range from 1690 ± 27 BP to 1848 ± 22 BP for the five individuals (Table 4). The $^{14}C_{col\text{-}carb}$ age differences vary between -1 ± 40 and 153 ± 46 ^{14}C yr (Table 4). Except for one individual in chamber X83 where the age measured in the two fractions are identical, the $^{14}C_{carb}$ age is always younger than the $^{14}C_{col}$ age. The $^{14}C_{col}$ ages are positively correlated with the $\delta^{13}C_{col}$ values ($R^2 = 0.95$), negatively correlated with the $\delta^{13}C_{carb}$ values ($R^2 = 0.68$) but are not correlated with the $\delta^{15}N_{col}$ values ($R^2 = 0.24$). The $^{14}C_{carb}$ ages are negatively correlated with the $\delta^{13}C_{carb}$ values ($R^2 = 0.86$) but are not correlated with the $\delta^{18}O_{carb}$ values ($R^2 = 0.01$). The $^{14}C_{col\text{-}carb}$ age difference is not correlated with the $\delta^{13}C_{col}$, $\delta^{13}C_{carb}$, or $\delta^{18}O_{carb}$ values ($R^2 < 0.42$), but is negatively correlated with bone $\delta^{15}N_{col}$ values ($R^2 = 0.88$). In terms of ^{14}C

activity, Fm values of bone collagen varies between 78.30 ± 0.31 and 80.13 ± 0.26 pMC while bone apatite is about 1% more active on average and varies between 79.45 ± 0.22 and 81.02 ± 0.27 pMC (Table 4).

6. Discussion

6.1. Diagenetic trajectory

The high IRSF values displayed by bone apatite from the SSPM catacomb, ranging between 3.6 and 5.1, are indicative of a moderate to high degree of recrystallization for these samples (Table 2, Figure 6). The presence of a PO_4 peak at 1093 cm^{-1}, and of shoulders at 3570 cm^{-1} and 630 cm^{-1} in the most severely altered samples argue for a partial recrystallization in hydroxylapatite. The inverse relationship between mineral crystallinity and carbonate content is typical of dissolution/recrystallization processes that take place following burial (Nielsen-Marsh and Hedges, 2000). Such an alteration of the mineral phase is frequently linked to the loss of the organic matter. This is confirmed by the correlation observed between collagen and carbonate relative contents (Figure 7). This relationship can be explained by the intimate association of the apatite crystals and collagen fibrils at the nanoscale in the bone structure. Mineral dissolution exposes the collagen to microbial or physico-chemical alterations while, in turn, the loss of the organic matter increases micro-porosities and interactions of bone minerals with surrounding water (Collins et al., 2002). The combination of collagen loss, decrease in carbonate content and increase in crystallinity could have been driven by collagen hydrolysis and/or microbial activity (Smith et al., 2007). Bacterial attacks generally result in a loss of the chemical integrity of collagen and a loss of collagen in bone (Balzer et al., 1997; Child, 1995; Grupe, 2001; Harbeck and Grupe, 2009) and begin quickly following burial (Hedges, 2002). Chemical hydrolysis of collagen is another, slower mechanism (Child, 1995; Smith et al., 2002). The lack of data regarding the

histological preservation and the porosity of samples does not allow us to distinguish between the two processes. However, archaeological evidence suggests that both could have occurred. The simultaneous or quasi-simultaneous decomposition of the high number of bodies in the mass graves from the SSPM catacomb could have been conducive to microorganism development. Moreover, the burial chambers from the SSPM catacomb were carved in a pozzolan (Funicielllo et al., 2008), which presents an alkaline pH ranging from 7.5 to 7.9 (measurements carried out by Zitelli (2013) on samples from ceiling of X82/T18 and X83). These results are consistent with the pH of environmental water recorded in other catacombs in Rome varying between 7.5 and 7.8 (Sánchez-Moral et al., 2005a). However, a much lower pH ranging from 5.9 to 6.4 has been found at the floor level of the X82/T18 and X83 chambers (Zitelli 2013). This decrease of the rock pH at the bottom of the stratigraphic sequences could be caused by the decomposition fluids (principally organic acids; Vass et al. (2002)) released from the corpses in putrefaction (Carter and Tibbett, 2008; Gill-King, 1997; Schotsmans et al., 2014). In addition, the gypsum used as part of the funerary practices inside these graves could have also contributed to the acidification of the burial environment. In environmental management, gypsum is frequently incorporated alone or with other amendments in alkaline soils in order to reduce soil pH (Carter, 1986; Courtney and Kirwan, 2012; Kordlaghari and Rowell, 2006; Wong et al., 2009). Bone apatite is known to have a low solubility in alkaline environments (pH > 7.5) while its solubility becomes relatively high in acidic environments (pH < 6.5) (Berna et al., 2004; Nielsen-Marsh and Hedges, 2000; Pate et al., 1989). The drop in pH of the burial environment in the chambers could have favored dissolution/recrystallization processes leading to the high IRSF values that we have measured. Finally, the high porosity of the pozzolan (Sánchez-Moral et al., 2005a; Sánchez-Moral et al., 2005b) could have facilitated water drainage. These particular conditions, associated with the absence of sediments between the different strata of corpses in certain burial chambers may

375 have limited the capacity of the burial environment to buffer water and increase interaction of
376 bone with groundwater (Nielsen-Marsh et al., 2007; Smith et al., 2002). These conditions
377 could then have accelerated the recrystallization of bone apatite and the subsequent alteration
378 of the collagen matrix.
379
380 6.2. Spatial heterogeneity of bone preservation
381 Bone preservation in the SSPM catacomb is highly variable. While some of the
382 archaeological samples present IRSF values and CO_3/PO_4 ratios very close to those observed
383 for the modern samples, most of them display higher IRSF values and lower CO_3/PO_4 ratios.
384 Collagen extraction yields are also variable (from 0 to 100%). Time, burial environment
385 (temperature, hydrogeology, soil pH, etc.) and taphonomic processes (inhumation type, filling
386 of the grave, anthropogenic or animal disturbance, etc.) are the three main parameters
387 controlling the state of preservation of bones in archaeological contexts (Collins et al., 2002;
388 Hedges, 2002). These different parameters interplay in a complex manner between and within
389 archaeological sites (Hedges et al., 1995b; Nielsen-Marsh and Hedges, 2000; Pestle and
390 Colvard, 2012). In the SSPM catacomb the organization and taphonomic evolution of the
391 deposition levels suggests massive accumulation of corpses (Castex et al., 2007; Castex et al.,
392 2009; Castex et al., 2011). Despite the fact that contemporary inhumation of 2000-3000
393 corpses *stricto senso* is unlikely given the dimensions of the burial chambers (Sachau-Carcel,
394 2012; Sachau-Carcel et al., 2013), sepulchral areas appear to have been used during a
395 relatively short period of time (Castex and Blanchard, 2011; Kacki et al., 2013). Therefore,
396 time cannot be invoked to account for the strong heterogeneity of the conservation of the
397 remains and other parameters need to be discussed.
398 The heterogeneity of bone preservation is not randomly distributed. With the
399 exception of the small chamber X81 in which bone samples were well-preserved, most of the

400 well-preserved samples come from the large chambers (X83 and X84) while the poorly-
401 preserved samples are found mostly in the small chambers (X80/T16 and X82/T18). In
402 chamber X78/T15, the limited sampling (n= 2) did not allow us to conclude on the quality of
403 collagen preservation in this room. This result is in keeping with anthropological observations
404 performed during the excavation although local variations can occur depending on the
405 disposition of the bones within a chamber (see below). Like in other great Roman catacombs,
406 the SSPM catacomb has a relatively stable climate throughout the year. Sánchez-Moral et al.
407 (2005a) reported a number of micro-environmental parameters in the catacombs of Domitilla
408 and St. Callistus that can easily be extrapolated to the SSPM catacomb given the similar
409 characteristics and geographical proximity (a few kilometers away) between these sepulchral
410 complexes. A high humidity close to saturation (≥ 97%) and a constant temperature of 15-
411 17°C were recorded in the two catacombs. Given the small size of the central sector of the
412 SSPM catacomb and the existence of large openings between chambers, differences in
413 microclimate between rooms are highly unlikely. During the excavation, visual inspection of
414 bones in the large chambers indicated that bones close to the chamber walls were more altered
415 than in the center (Castex et al., 2009). We propose that runoff against the walls and higher
416 humidity locally could explain this difference in preservation between the center and the
417 periphery of the large chambers. In the smaller chambers, all the bones remain relatively close
418 to the walls and would be equally affected by the water runoff. In the large chambers,
419 buffering conditions (constant humidity and temperature) would have favored exceptional
420 bone collagen preservation for bones situated further away from the walls. Due to its central
421 position, the small chamber X81 has several openings (Figure 1). This has probably restricted
422 runoff against the walls, which could explain the better preservation of the bones in this
423 chamber in general. Finally, trampling of the human remains by the grave diggers, families of
424 the deceased and/or pilgrims along the walls of the burial chambers, especially in the large

425 ones, could be another explanation. A circulation area along the west wall of the chamber
426 X83 has been identified causing the loss of the anatomical integrity of the bone in this sector
427 of the chamber (Castex et al., 2009).
428
429 6.3. Testing for the preservation of the isotopic composition of bone apatite using radiocarbon
430 dating
431 A crucial point for the use of bone apatite isotope values to infer diet and mobility
432 patterns of past populations is to establish whether bone recrystallization was associated with
433 carbon and oxygen isotope exchange. We found apatite to be slightly more ^{14}C active
434 (between 0 and 1.5 pMC) than collagen from the same individual. This is in keeping with
435 differences calculated for bones in archaeological sites ranging in age between 0 and 9.5 kyr
436 BP (Zazzo, in press). The positive $^{14}C_{col\text{-}carb}$ age difference together with the correlation
437 between $^{14}C_{carb}$ and $\delta^{13}C_{carb}$ values, indicate that carbon isotope exchange between bone
438 apatite and dissolved inorganic carbon took place with a younger source of carbon with higher
439 $\delta^{13}C$ values. The $^{14}C_{col}$ age offset can be used to calculate a percent contamination of the
440 carbonate fraction with modern carbon if this value represents the true age of an individual.
441 However, the positive correlation found between $\delta^{13}C_{col}$ and $^{14}C_{col}$ values suggests that the
442 individuals with the highest $\delta^{13}C$ values could have consumed marine foods as these usually
443 have more positive $\delta^{13}C$ values than terrestrial foods (Craig et al., 2013; Richards et al., 2005;
444 Schoeninger et al., 1983). The lack of correlation between the $\delta^{15}N_{col}$ values and the $^{14}C_{col}$
445 ages could be explained by the consumption of low trophic level fish (e.g., anchovies,
446 sardines, mackerel, *etc.*) or bivalves (e.g., oysters, cockles, clams, etc.) (Carlier et al., 2007;
447 Garvie-Lok, 2001). This result is coherent with the significant (ca. 15-20%) proportion of
448 marine food found in the diet of the victims of the 79 AD Vesuvius eruption at Herculaneum
449 (Craig et al., 2013). According to the equation in Figure 3A from Craig et al. (2013), $\delta^{13}C_{col}$

values measured for the five individuals could correspond to ^{14}C age excesses between 60 (for Individual US216/Mand1) and 133 (for Individual 64) ^{14}C yr. This would affect the collagen age more than the apatite age because collagen records the protein fraction of the diet, while apatite carbon records the whole diet, leading to an overestimation of the age differences between the two fractions. A large dependence on marine food in individual 64 could explain the large age difference (180 ± 40 ^{14}C yr) measured between the two individuals dated in the burial chamber X82/T18 (Table 4). This hypothesis is also supported by the fact that in chamber X80/T16, the lack of $^{14}C_{col}$ age difference between individuals 24 and 79 corresponds to identical $\delta^{13}C_{col}$ values for these specimens, suggesting a similar amount of marine food in their diet. For these reasons, the value of 1.5 pMC contamination must be considered as a maximum estimate.

The central area of the entire SSPM catacomb and numerous others roman catacombs (e.g. the Domitilla and St Callixtus catacombs) is carved through pozzolan, a tuff layer of the Villa Senni formation resulting from an eruptive event of the Colli Albani volcano (Funicielllo et al., 2008; Giordano, 2010). Bulk measurements on samples coming from the Domitilla and St Callixtus catacombs indicate that pozzolan can contain 1-5% calcite (Sánchez-Moral et al., 2005a; Sánchez-Moral et al., 2005b). To our knowledge, there is no published stable isotope data for this ignimbrite. However, $\delta^{13}C$ of carbonate in lavas and ejecta from the same volcanic complex have been measured and range from -19.8 to 3.5‰ (Fornaseri and Turi, 1969). If we use the highest $\delta^{13}C$ value as an estimate for the diagenetic endmember, a simple mass balance calculation taking into account the intra-individual differences in ^{14}C activity allows us to estimate a $\delta^{13}C$ values shift between 0.00‰ (SD ±0.07‰) and 0.27‰ (SD ±0.08‰) as a result of diagenesis. Therefore, the intake of exogenous carbon in bone carbonate is limited and does not modify its carbon isotope composition to any great extent. This is in keeping with the slightly acidic conditions in the

475 SSPM catacomb. As previously mentioned, the correction corresponds to a maximum
476 estimate. Thus, the isotopic composition of the carbonate fraction of bone can be considered
477 as reliable as that of the collagen fraction and can be used to reconstruct the life histories of
478 the individuals buried in the central area of the catacomb. This is beyond the scope of this
479 study and will be the focus of another article.

480 Finally, the significant carbon and oxygen isotope difference of apatite found between
481 samples whose collagen was well- and poorly-preserved is intriguing and deserves attention.
482 Samples which did not pass the collagen preservation tests had $\delta^{13}C_{carb}$ values 0.3‰ lower
483 and $\delta^{18}O_{carb}$ 0.3‰ higher on average than those with well-preserved collagen (Table 2). The
484 difference between the two groups, although small, was significant for $\delta^{13}C_{carb}$ and $\delta^{18}O_{carb}$
485 (Mann-Whitney U tests; p-values $<< 0.05$). Because of the negative correlation found
486 between $^{14}C_{carb}$ ages and $\delta^{13}C_{carb}$ values, lower $\delta^{13}C_{carb}$ are an unlikely result of a contaminant
487 with a more negative $\delta^{13}C$ value. We propose a different interpretation for the difference
488 found between the two groups. It is striking that the direction and magnitude of the isotopic
489 shifts that we observed are similar to those measured by Koch et al. (1997) between untreated
490 and acetic-acid treated modern enamel and bone samples. The two sub-groups of bones from
491 the SSPM mimic the untreated and treated samples from the Koch et al. (1997) experiment.
492 Moreover, bones with low collagen yields are also highly recrystallized and have lost part of
493 their structural carbonate. Carbonates in apatite are present in two different positions, A and
494 B, A-bonds being the weakest. We found OH- ions in some of the spectra, possibly
495 substituting for A-carbonates. As postulated by Koch et al. (1997) it is plausible to assume
496 that these two sites with different chemical bonds discriminate stable isotopes differently.
497 Since A/B type carbonate ratios are likely different in recrystallized and preserved apatites,
498 the slight isotopic difference measured at the population level could well reflect this

difference in isotope fractionation. This difference would then result from recrystallization, not isotope exchange.

6.4. Regional variability in bone preservation: catacombs vs. open-air sites

Rutgers et al. (2009) hypothesized that the microclimate of catacombs affects negatively bone collage preservation. To further investigate this issue, we compared bone collagen preservation in the catacombs and in contemporary open air sites from Italy (Figure 9). The choice of sites included in this comparison is dependent on the collagen extraction protocol used on each site. Some methods, such as ultra-filtration, are known to drastically reduce collagen extraction yields. Because of this possible methodological bias, our comparison only includes sites from central Italy – all located in Rome's region, also known as Latium - (Killgrove and Tykot, 2013; Prowse et al., 2004; Prowse, 2001; Prowse et al., 2007; Prowse et al., 2005; Rutgers et al., 2009), but excludes sites from southern Italy (Craig et al. 2009; Craig et al. 2013). With an average collagen extraction yield close to 50%, bones from the SSPM appear to be better preserved than most of the contemporary sites of the Latium. Comparison indicates that the quantity of extracted collagen is similar (50% or below) in the small chambers (with the exception of X81) but much higher (between 60-90% on average) in the large chambers. It is also interesting to note that the two sites presenting the highest (although highly variable) average extraction yields in the Latium are catacombs. Despite the limitations described above, the relatively stable climatic conditions prevailing in catacombs seem overall to have favored, rather than disadvantaged bone collagen preservation and therefore contradicts Rutgers et al. (2009)'s assumption. However, an opposite pattern seems to emerge if one looks at the mineral fraction of bone. The comparison of the crystallinity values calculated for the bone samples from the SSPM catacomb with those published for open air sites from different Roman Empire provinces clearly shows that

524 the mineral phase of bones is much more altered in the SSPM catacomb than in the other sites
525 (Figure 10). Although we are aware that the comparison of IRSF values reported in different
526 studies can be problematic due to variances in sample preparation protocols and measurement
527 methods (KBr vs. ATR mode), the IRSF values calculated for the SSPM catacomb are similar
528 to those observed for highly degraded bones (Berna et al., 2004; Lebon et al., 2010). Overall,
529 bones found in catacomb contexts seem to present *a Janus face*[1], showing both a well-
530 preserved organic and a poorly-preserved mineral phase. But as previously shown, this high
531 degree of recrystallization is not correlated with isotope exchange of the mineral phase with
532 the surrounding environment, possibly due to the relatively low pH reigning in the catacomb.
533 Based on this extensive study, we can conclude that it will be possible to examine traits of the
534 life history of the individuals buried in the catacomb based on the combined analysis of bone
535 collagen (when preserved) and apatite isotope values.
536

537 **7. Conclusion**

538 This study - the first focusing on diagenesis in a catacomb context – illustrates the
539 effect of post-depositional processes on bone preservation. Our multi-proxy approach showed
540 that extreme differences in preservation can be found between contemporary individuals
541 within a small area, highlighting the influence of localized variations on taphonomy. We
542 found that the heterogeneity of bone preservation was not randomly distributed in the SSPM
543 catacomb and that bone was better preserved in the large chambers than in the small ones.
544 This is in keeping with anthropological observations performed during the excavation and we
545 propose that the combination of acidic conditions, together with condensation of air humidity
546 and runoff against the walls could explain this differential preservation. Comparison with
547 contemporary sites from the Latium indicates the two sites presenting the highest (although

[1] named after the Roman deity Janus who had two faces, one looking forward, the other looking backward. By extension, this term refers to a deceitful person or an object having or containing contrasting characteristics.

highly variable) average extraction yields are catacombs, and that bone collagen in large chambers is much better preserved than in other open air contemporary sites. Therefore, the relatively stable climatic conditions prevailing in catacombs seem overall to have favored, rather than disadvantaged bone preservation.

Despite high rates of recrystallization observed for some of the bone samples, coherent radiocarbon ages measured in the carbonate and collagen fractions of several individuals demonstrates a lack of significant (>1%) isotopic exchange. We noticed a slight (0.3‰) difference in both $\delta^{13}C$ and $\delta^{18}O$ values between the well-preserved and the most severely recrystallized samples. This difference is similar in magnitude and direction with differences observed on modern samples pretreated with acetic acid and could be due to selective leaching of A vs. B-type carbonate in apatite during recrystallization. This shift is close to analytical precision and does not preclude reliable reconstructions of the individual history of these specimens based on stable isotope analysis of the carbonate fraction. Therefore, further dietary reconstructions can include all the individuals of the population, even those for which bone collagen is no longer preserved.

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Figure Captions

Figure 1: Plan of the burial chambers in the region X from the SSPM catacomb. After M. Ricciardi, modified.

Figure 2: FT-IR spectrum of a fresh bone showing the regions of interest as well as the IRSF calculation procedure.

Figure 3: Photographs showing examples of good (A), medium (B) and poor (C) preservation of the SSPM bones, based on their macroscopic characteristics

Figure 4: Collagen yield of the SSPM bone samples grouped by the burial chamber, skeletal part category and macroscopic appearance. Boxplots represent the 25th, 50th (median) and 75th percentiles in the distribution of scores. Whiskers indicate the variability outside the upper and lower quartiles. Outliers are identified by individual points. The shaded area corresponds to the collagen yield measured in modern bones (after Bocherens et al. 1991).

Figure 5: Carbon and nitrogen abundances of the SSPM bone samples. Samples which fail one of the collagen preservation criteria defined in Table 1 are considered poorly-preserved (closed symbols) while those passing all the preservation criteria are considered well-preserved (open symbols).

Figure 6: IRSF plotted against CO_3/PO_4 ratio of the SSPM bone samples. Modern bone samples are shown for reference.

Figure 7: CO_3/PO_4 ratio plotted against Amide1/ PO_4 ratio of the SSPM bone samples. Modern bone samples are shown for reference.

Figure 8: IRSF plotted against Amide1/ PO_4 ratio of the SSPM bone samples. Modern bone samples are shown for reference.

Figure 9: Inter-site comparison of the collagen extraction yields measured on contemporary human bone samples from the Latium. The Isola Sacra necropolis is located near Ostia and Portus Romae and dated from the 1st to 3rd c. AD (Prowse et al., 2004; Prowse, 2001; Prowse et al., 2007; Prowse et al., 2005). The ANAS cemetery is located between Ostia and Rome and dated from the 1st to 3rd c. AD (Prowse et al., 2004; Prowse, 2001). The necropolises of Casal Bertone and Castellaccio Europarco are located in Rome and dated from the 2nd to 3rd and from the 1st to 2nd c. AD, respectively (Killgrove, 2010; Killgrove and Tykot, 2013). The St Callixtus catacomb is located in Rome and dated from the 3rd to 5th c. AD (Rutgers et al., 2009). Boxplots represent the 25th, 50th (median) and 75th percentiles in the distribution of scores. Whiskers indicate the variability outside the upper and lower quartiles. Outliers are identified by individual points. The shaded area corresponds to the collagen yield measured in modern bones (after Bocherens et al. 1991). OAS stands for open air site and SS stands for subterranean site.

Figure 10: Inter-site comparison of the IRSF values measured on human bone apatite samples from the Roman Empire. The Kellis 2 cemetery is located near Mut in the Dakhleh Oasis in Egypt and dated from the 1st to 5th c. AD (Dupras, 1999; Dupras and Schwarcz, 2001). The Leptiminus cemeteries are located in the modern day town of Lamta in Tunisia and dated from the 2nd to 6th century AD (Keenleyside et al. 2009). The Poseidonia

necropolises are located near Capaccio-Paestum in Italy and dated from the 7th c. BC to the 2nd c. AD (Alfano et al. 2009). The Isola Sacra necropolis is located near Ostia and Portus Romae and dated from the 1st to 3rd c. AD (Prowse et al., 2004; Prowse, 2001; Prowse et al., 2007; Prowse et al., 2005). Boxplots represent the 25th, 50th (median) and 75th percentiles in the distribution of scores. Whiskers indicate the variability outside the upper and lower quartiles. Outliers are identified by individual points. The hatched area corresponds to the IRSF values found for modern bones. The shaded area corresponds to the samples mildly and highly recrystallized. OAS stands for open air site and SS for subterranean site.