Bone diagenesis and its implication for disease diagnosis: the relevance of bone microstructure analysis for the study of past human remains

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Abstract

When bone is exposed to the burial environment it may experience structural changes induced by multiple agents. The study of postmortem alterations is important to differentiate decomposition phenomena from normal physiological processes or pathological lesions, as well as to assess bone tissue quality. Microscopy is of great utility to evaluate the integrity of bone microstructure and it provides significant data on long-term bone decomposition. A total of 18 human bone sections (eight archeological and ten retrieved from an identified skeletal collection) were selected for analysis under plane light and polarized light. The aim of this exploratory study was to analyze the impact of diagenesis and taphonomy on the bone microstructure, as well as on the differential diagnosis of pathological conditions. The results showed that the microscopic approach to bone tissues contributed materially as an aid in the detailed description of the main diagenetic changes observed. It showed that gross inspection does not provide a realistic assessment of bone tissue preservation, which can impact in the characterization of lesions present and subsequent disease diagnosis. Therefore, researchers should continue to consider the application of histological techniques if the aim is to comprehend tissue integrity and its association with decomposition or disease.

Keywords: Burial environment, taphonomy, microscopy, bone tissue preservation, paleopathology, differential diagnosis.

Introduction

The human skeleton encapsulates many features of an individual's life history. The recovery and analysis of skeletal remains greatly contributes to the understanding of past human societies, since bones and teeth are valuable archives of paleoecological and paleoenvironmental information (Tütken & Vennermann, 2011). Nevertheless, during burial, bone undergoes a number of structural changes that may affect the quality of the data retrieved at the isotopic, molecular, biochemical and structural level (Hedges, 2002). In the endeavour to understand the mechanisms that affects skeletal preservation, taphonomy and diagenesis are powerful disrupting forces to consider (Stodder, 2008), since they determine whether a bone will decay and disappear from the earth, or be preserved throughout archeological and geological time (Turner-Walker, 2008). The term taphonomy was coined by the Russian paleontologist Efremov (1940) and focuses on the "… study of a process in the upshot of which the organisms pass out of the different parts of the biosphere and, being fossilized, become part of the lithosphere" (Efremov, 1940). The term diagenesis, deriving from earth science, has been used to describe the biological, chemical and physical interchanges that occur between the body and the burial environment, which may end up in the complete destruction of the remains or to its lithification (Grupe, 2007).

Hard tissue (i.e., bone and teeth) diagenesis is a complex and site-specific process (Hedges et al., 1995). It is dependent of different external features of the burial environment such as temperature, the presence of oxygen, soil composition (e.g., PH, flora and fauna), soil pressure and its drainage capabilities, groundwater chemistry and hydrological flow, microbial attack and particle transport (e.g., Grupe & Dreses-Werringloer, 1993; Hedges & Millard, 1995; Hedges et al., 1995; Nielsen-Marsh et al., 2000; Hedges, 2002; Reiche et al., 2003; Grupe, 2007; Turner-Walker, 2008; Turner-

Walker & Jans, 2008). All these factors, usually entangled, are responsible for the chemical destruction of the protein and mineral components of the bone, as well as its bioerosion (Nielsen-Marsh et al., 2000; Collins et al., 2002; Grupe, 2007); that is, the microbial alteration of bone caused by bacteria, cyanobacteria and fungi (reviewed by Jans, 2008).

The degradation of the organic component of the bone may occur through chemical hydrolysis or microbial attack (Collins et al., 2002; Hedges, 2002). While the rate of collagen hydrolysis depends upon time, temperature and soil PH, the microbial bioerosion may start soon after death (Collins et al., 2002). For instance, Jans et al. (2004) found that bones from complete burials are more often eroded, due to bacterial attack, than fragmented bones resulting from dismemberment or butchering (e.g., faunal remains). This is indicative that bacterial degradation of bone may be related to putrefaction, and with early stages of body decomposition. After collagen degradation there may be an increase in bone porosity which facilitates microbial attacks, and subsequent apatite dissolution and/or recrystallisation, especially in particular environmental conditions (e.g., presence of groundwater) (Turner-Walker & Parry, 1995; Nielsen-Marsh & Hedges, 1999; Nielsen-Marsh et al., 2000; Grupe, 2007). An early chemical dissolution of bone apatite may also expose bone to microbial attack followed by protein degradation (Collins et al., 2002).

Of the four diagenetic parameters defined by Hedges et al. (1995) to measure bone destruction (i.e. crystallinity changes, porosity, and protein content), the histological study of bone integrity, simply or combined with other parameters, has been frequently used, not only to assess diagenetic changes (e.g., Hackett, 1981; Garland, 1987; Maat, 1993; Hedges & Millard, 1995; Hedges et al., 1995; Nielsen-Marsh & Hedges, 1999; Pfeiffer & Varney, 2000; Jackes et al., 2001; Turner-Walker &

Syversen, 2002; Jans et al., 2004; Guarino et al., 2006; Turner-Walker & Jans, 2008; Abdel-Maksound, 2010; Hollund et al., 2012; Turner-Walker, 2012; Hollund et al., 2013), but also as a pre-screening tool to infer the degree of biomolecular (e.g., Hagelberg et al., 1991; Cipollaro et al., 1998; Zink et al., 2005), isotopic (e.g., Schoeninger et al., 1989; Balzer et al., 1997; Maurer et al., 2014) or paleopathological preservation (e.g., Stout, 1978; Bell & Jones, 1991).

Microscopy is a valuable tool, not only to the study and differentiation of diseases from past populations (i.e., within the discipline of paleopathology), but also to ascertain the impact of diagenetic factors on the architecture of normal and pathological bone. Moreover, it is useful to infer the role that bone abnormalities might play in the progression of diagenesis (Bianco & Ascenzi, 1993). Under polarized light, for example, the observation of "Maltese crosses" [an alternate pattern of light and dark bands (Schultz, 1997) that forms due to the presence of crystallized bone mineral oriented by the organization of the collagen fibers (Schoeninger et al., 1989)] is considered a good indicator of bone birefringence and of well-preserved lamellae in Haversian structures (von Hunnius et al., 2006). Furthermore, histology also allows differentiation between pathological lesions and pseudopathologies (Grupe & Dreses-Werringloer, 1993), which substantially reduces the amount of bias in the differential diagnosis of diseases. Even when bone is incomplete and shows taphonomic changes at the macroscopic level, a considerable amount of information concerning the underlying pathology can be gathered from histological analysis (Uytterschaut, 1993; Bell & Piper, 2000). For instance, in the study of two possible cases of Paget's disease, Bell & Jones (1991) showed that the bone fragment that was considered poorly preserved macroscopically, had extensive areas unaffected by diagenesis, whereas the piece

regarded as in excellent state of macroscopic preservation, had profound diagenetic changes at the microscopic level.

Regarding the impact that diagenesis has on bone tissue histology and disease diagnosis, the aims of this exploratory study were: (1) to identify and describe diagenetic changes observed at microscopic level within archeological and identified skeletal bone samples (henceforward referred to as "identified samples"; and (2) to discuss the implications of those changes in the differential diagnosis of bone lesions of infectious, traumatic, neoplastic and nonspecific origin, especially those involving periosteal new bone formation. This will be conducted via a comparative analysis of the macroscopic and microscopic bone tissue samples collected.

Material and Methods

A total of 18 bone samples were collected for analysis. Eight bone samples were collected from male and females individuals (one nonadult and seven adults) belonging to three Portuguese archeological assemblages. These were: the cemetery of Constância village (C) (14^{th}-19^{th} centuries) (n=3); the cemetery of the hospital of the Ordem do Carmo (HOC) in Porto (19^{th} century) (n=2), and the cemetery of the Royal Hospital of All Saints (RHAS) in Lisbon (18^{th} century) (n=3) (Table 1). The HOC cemetery was located in the city of Porto in the North of Portugal (Figure 1). The two remaining assemblages were geographically situated in the Center/South of Portugal. The C and RHAS necropolis shared common environmental features, and were both located in the vicinity of the Tagus River. The RHAS necropolis was situated in the urban network of the city of Lisbon close to the Tagus River estuary. The C necropolis was also located in the margins of the Tagus River but occupied an inland location in relation to the Atlantic Ocean. Age at death and sex diagnosis were performed using standard

morphological assessment methods such as those described in Buikstra & Ubelaker (1994), Bruzek (2002) and White & Folken (2005). For comparison, ten bone samples were retrieved from eight individuals, one nonadult (male) and seven adults (three females and four males) from the Lisbon Human Identified Skeletal Collection (LHISC) housed at the Bocage Museum/National Museum of Natural History in Lisbon, Portugal (Cardoso, 2005, 2006). The skeletons that form this collection started to be amassed in the beginning of the 1980's from modern cemeteries of the city of Lisbon. They correspond to human remains that were classified as abandoned or neglected by their relatives according to cemetery legislation (Cardoso, 2005, 2006). Age at death, sex and cause of death of these individuals are known, as the LHISC is an identified collection in which biographical data from the individuals is documented. For skeleton numbers 1196 (female, 75 y.o.) and 1534-A (male, 2 y.o.), permission was granted to take two samples from each of these individuals (rib and radius and rib and fibula, respectively).

The criterion used for sampling was based on the type and distribution of the bone lesions observed (i.e., periosteal reactions) and respective differential diagnosis (in the case of the archeological samples), or cause of death (in the case of the identified samples). Alongside these primary sample criteria, efforts were made to select specimens with a similar profile of age at death, sex, and skeletal anatomical provenance (Table 2).

Bone samples were cut using a handsaw. In most cases, slides were made from transverse sections of long bones and ribs. Thin sections of five samples, PF SK1492 (archeological sample), SK135, SK1412, SK198 and SK1534-A (identified samples) were made from half cross-sections of femora, tibia and fibulae, respectively. Samples were cleaned in multiple sonic baths, first in tap water and then in ethyl alcohol 95%. After cleaning, samples were embedded in epoxy resin (the embedding solution was

prepared by adding hardener to the epoxy resin following a 5:1 dilution ratio) and left to cure overnight.

The samples were epoxied to glass slides and cut transversely using a low-speed Isomet saw. Later, sections were polished on both sides to the desired thickness (0.1mm) using graded abrasive sandpapers and an abrasive slurry of aluminum oxidate to reduce scratch marks. During the last stage of the grinding process (after flipping the sample to the final glass slide using an epoxy-hardener mounting solution), a microscope was used to evaluate the thickness of the samples. No histochemical staining techniques were used. The specimens were examined under plane light and polarized light using a high resolution microscope with an incorporated digital camera - Nikon Eclipse 80i®. Previously to cover slipping, samples were dehydrated in ethyl alcohol 95% and immersed in xylene under vacuum. The sample preparation ended with the cover slipping using a small amount of mounting medium.

For each specimen the following parameters were considered (Table 3 and 4):

- Macroscopic bone changes – visual examination, following the descriptions of Buikstra and Ubelaker (1994);
- Microscopic bone changes using the categories described in Garland (1987), Hackett (1981) and Jans (2008);
- Presence or absence of bone birefringence, using the proposal of Jans (2005);
- Oxford Histological index (OHI), following the proposal described in Hedges et al. (1995).

Results

The results of the macroscopic and histological study of bone preservation and its impact on disease diagnosis, OHI, and bone birefringence are described in Table 5.

Of the samples analyzed microscopically (n=18), nine showed the presence of diagenetic changes, which were particularly evident in the archeological samples. Accordingly, of the eight bone samples collected from an archeological context, only three showed a partially intact microstructure with minor areas of bone destruction. In the remaining five samples, the identification of the main bone tissue units (osteons) and evidence of disease was compromised. It is important to emphasize that all archeological samples exhibited some form of postmortem change. In contrast, only one identified sample showed diagenetic alterations (a right fibula from the nonadult individual SK1534A). The remaining nine samples, all identified, presented an intact histological appearance composed of Haversian systems, interstitial lamellae, areas of bone resorption (Howship's lacunae) and empty osteocyte lacunae (Figure 2). The distribution of the bone samples in relation to the index of histological preservation (OHI) clearly illustrates these observations (Figure 3). Four archeological bone samples have OHI values of "0", which indicates the absence of bone features apart from Haversian canals, while two samples have OHI of "1" and "3", respectively. In comparison, nine identified samples had approximately 95% of their bone microstructure intact. All identified samples showed some degree of bone birefringence under polarized light that ranged from reduced (n=1) to high (n=9). The archeological samples were mainly characterized by low bone birefringence, which was detected in 50% of the specimens analyzed (Figure 4). In addition, six archeological samples exhibited destructive changes that ranged from generalized to focal destruction, in which some form of microscopic focal destruction (MFD), mainly at the cortical level, were detected. Microbial tunneling, microcracks and black and blue staining spots were also observed.

Regardless of the good macroscopic preservation, considerable diagenetic bone changes were observed in the specimens from Constância (C) and the Royal Hospital of All-Saints (RHAL) necropolis (Figure 5). In fact, one of the major results was the contradiction found between the gross appearance of the bone samples, and the diagenetic changes observed at microscopic level. This was seen, for example, in the rib sample collected from the nonadult SG22 SK4 that showed bone lesions compatible with tuberculosis (Figures 6a-6b). Macroscopically, the bone was intact without major taphonomic changes. At the microscopic level, however, a very distinct picture characterized by destruction of the bone tissue and reduced birefringence was observed. In this sample, no intact Haversian systems, interstitial bone, osteocyte lacunae were seen. Instead, the cortical bone exhibited a cloudy and amorphous appearance with erratic Haversian canals. In the pathological domain, a newly built bone deposit with a ruffled appearance and attached to the underlying cortex by pedestals was seen on the visceral surface; however, it was impossible to determine the arrangement of the collagen fibers. Multiple opaque/black spots and a blue/grey discoloration were seen in the new bone formation.

A case of differential preservation of the bone tissue was recorded in a subperiosteal ossified hematoma sampled from the left femur of an adult male (PF SK1492) (Figures 7a-7e). In the area affected by the hematoma, no preserved system of lamellae was seen around the vascular canals. Rather, the bodies of the osteons appeared to be formed by small fragments. These changes affected equally the inner and the outermost layers of the ossifying hematoma leading to lamellae and linear longitudinal MFD. Black inclusion randomly distributed and a blue discoloration was also observed at periosteal level. In contrast with the outmost layers, the bone cortex

showed a well-preserved and mature architecture composed of multiples rows of osteons with high bone birefringence.

The best preserved archeological samples were those retrieved from the hospital of the Ordem do Carmo (HOC), Porto. A left tibia sample with non-specific periosteal reaction and collected from an adult male (Porto UE6451-65) illustrates this observation (Figures 8a-8d). The histological examination showed cortical areas with good bone birefringence. Well-preserved osteons, Howship's lacunae, enlarged osteon canals, primary vascular canals and osteocyte lacunae were also seen. Two distinct bone layers were identified at the periosteal level. However, the presence of postmortem changes made it impossible to ascertain their microstructure. Other diagenetic changes observed were fragmented Haversian systems, micro-cracks, and lamellate and linear longitudinal MFD.

The presence of considerable diagenetic changes prevented the identification, in the archeological samples, of new bone deposits or other features of pathological origin. Such are the cases of a sample collected from a fibula of an adult female (PF 1310), which exhibited bone lesions compatible with a case of acquired syphilis; and the case of severe lesions found on the visceral surface of ribs of two individuals from the Constância necropolis – SG22 SK4 (nonadult, Figures 6a-6b) and SG25/26 SK2 (young adult female). In the latter cases both lesions were diagnosed as being associated with tuberculosis. In the case of acquired syphilis it was impossible to identify two histological features (i.e., grenzstreifen and sinous lacunae - see Table 5 for a complete description) described in the literature as being associated with the progression of the disease. Furthermore, the widespread destruction observed at both cortical and periosteal level did not enable confirmation of the presence of Polsters. For the case of a rib with macroscopic lesions described as "metastatic lesions" (SG19 SK7) it was

possible to observe resorption spaces, both at the periosteal and endosteal surfaces, but not the microstructure of newly built bone (Figures 9a-9b).

When compared to the archeological sample, the identified samples exhibited a well-preserved bone microstructure. At the same time, the deposits of periosteal new bone, of pathological etiology, showed a microstructure free of diagenetic changes (Figures 10a-10b). An absence of bioerosion alterations was also recorded. Even in those samples with macroscopic damage (SK1227, SK1534A – right rib, and SK1196), a normal histological appearance was recorded. The only exception was noticed on a fibula sample retrieved from a two year old male (SK1534-A) who died from pneumonia (Figures 11a-11c). In this case, and despite the macroscopic preservation of the bone, focal destruction affecting the cortex and periosteal new bone formation was recorded. In contrast, a rib sample collected in the same individual showed a well-preserved bone microstructure (Figures 12a-12c).

Discussion

Aside from the current study, only one other investigation has addressed bone tissue microstructure destruction in Portuguese archeological remains (i.e., Jackes et al., 2001). This current study has added another dimension in assessing bone microstructure preservation using plane and polarized light in the analysis using both archeological and identified samples. Furthermore, bone preservation assessment explored lesions of infectious, traumatic, neoplastic and nonspecific origin.

Several intrinsic factors, such as the age-at-death, sex and the presence of pathological bone conditions at the time of the death may predispose bone to diagenetic changes (Pinhasi & Bourbou, 2008). For example, skeletons of nonadults are expected to be prone to postmortem damage due to the poor mineralization of immature bone

(Mays, 1998). Also, skeletons from older individuals which have a decrease in their bone mass density associated with aging or caused by metabolic conditions (i.e. osteoporosis) are more likely affected by diagenetic changes (Mays, 1998). It is also important to note that bone preservation varies within the skeleton, according to type of bone, which affects the representativeness of each bone piece (Mays, 1998, Pinhasi & Bourbou, 2008, Jackes, 2011). For example, long bones are frequently portrayed as having a low rate of decay due to their rich composition of dense, cortical bone, when compared with other skeletal elements primarily composed of cancellous bone (Mays, 2008). Skeletal diseases that manifest through excessive bone resorption or by low mineralization may weaken the bone tissue architecture, facilitating the action of diagenetic factors. Accordingly, bone is more susceptible to decomposition and postmortem damage in osteolytic processes (e.g., metabolic and neoplastic diseases) than in osteoblastic processes, which are characterized by new bone formation (Pinhasi & Bourbou, 2008).

In the current study, no particular pattern emerged linking diagenetic changes with the sex and age group of the individuals or with the type of bone sampled. Only the destructive changes observed in the fibula sample retrieved from the SK1534-A individual may be associated with the individual's young age (2.y.o). But this does not exclude other disrupting processes since the rib sample collected from the same individual did not show any postmortem changes. The presence and type of bone lesions seems also to have had little impact in the progression of the diagenetic changes. For instance, in the rib specimens from Constância similar destructive changes were noticed in lesions exhibiting a predominance of periosteal new bone formation (e.g. SG22 SK4) or bone resorption (e.g. SG19 SK7). The only exception was noticed in PF SK1492 a femur sample presenting an ossified hematoma. In this case, the signs of

bioerosion were mostly seen at the periosteal level in the area affected by the ossified hematoma. The cortical tissue appeared unaffected. According to Jans et al. (2004), this pattern of microbial attack is more frequent when bone is invaded by soil bacteria. In spite of the bioerosion observed, the distinguishable annular structure of the osteons seems to suggest that at the time of the death, the subperiosteal haematoma was remodeled.

Although no major relationships emerged linking bone lesions and diagenesis, the presence of bone changes has affected the histological characterization of certain bone lesions, impacting on the differential diagnosis, especially in the archeological samples. This was particularly visible during the analysis of the fibula sample retrieved from the PF 1310 individual that showed macroscopic lesions compatible with a case of acquired syphilis or in the characterization of the SG19 SK7 rib lesions linked with a case of metastatic carcinoma. The presence of bioerosion in the SK1534-A fibula also prevented the complete analysis of the newly built bone and cortical tissue architectures. Nevertheless, this was the only case among identified samples. In fact, one important observation was the relatively good preservation of the identified samples when compared with those retrieved from archeological contexts, which may be explain by the reduced burial interval. According to the biographical information available, five of the individuals studied (SK's 1196, 1227, 1383, 1412, and 1534) were incorporated in the collection in the year 1993, which provides a minimum interval of inhumation ranging from 21 to 46 years since the time of death (Table 1). Regarding the effects of bioerosion, Jans (2008) states that extreme circumstances, such as high temperatures and the presence of bactericidal chemicals near the body (e.g. copper, mercury) may inhibit the bacterial degradation of bone that starts early after death. Unfortunately, no information concerning soil temperature or specific body treatments is available to

corroborate this scenario. The absence of major diagenetic changes in the identified samples seems, however, to support the assumption that microbial attack may not be as immediate as described in the literature (Hedges, 2002).

In addition with an extended burial interval, certain conditions of the burial environment may also have contributed to the differential preservation observed among archeological samples.

Burial practices that include the use of coffins tend to foster diagenesis (Pfeiffer, 2000). Surprisingly, the bone samples with a better preservation were those collected among individuals buried in coffins (i.e., HOC). One aspect that might have favored the bone preservation of the Porto individuals was the presence of cooper artifacts in the wooden coffins. It is known from the literature that the presence of copper artifacts tends to yield well-preserved bone (Pfeiffer, 2000). Another "contradictory" result was found when soil acidity is taken into account. In the literature, a low soil pH is described as increasing the rate of bone dissolution and destruction (Nielsen-Marsh et al., 2000). In the Porto region, the soil is usually acid due to the presence of granite. This environmental parameter seemed, however, to have had a minor impact, since the Porto samples were those exhibiting some of the better preservation among the archeological contexts.

In nature, water is an important mediator of almost all chemical reactions that may affect the preservation of skeletal remains (Hedges and Millard, 1995; Turner-Walker, 2008). Garland (1987) noted that groundwater can enter bones by diffusion, disrupting the internal protein-mineral bond and hydrolysing the protein components of bone. After protein hydrolysis the mineral component can be removed by the percolating groundwater (Garland, 1987). In burial places in which repeated cycles of wetting and drying occur, there will be a tendency to successive losses of calcium and

phosphorous from the bone matrix, leading to poorly preserved skeletal material (Turner-Walker, 2008). Well-drained soils (e.g. sands) that allow for the continuous flow of water through the bones and lack of saturation also have a deleterious effect upon the bone microstructure (Hedges and Millard, 1995). These last two factors (water and type of soil) may eventually explain the poor of histological preservation of the archeological samples from the Constância necropolis. The necropolis of Constância (14-19th centuries) was located in the vicinity of the Tagus River, in an area frequently affected by seasonal flooding. Moreover, the soil is mostly formed of fine-grained and permeable sands which may also have contributed to the mineral and organic degradation of bones. The environmental conditions of the RHAS necropolis do not vary much from those of the Constância necropolis; nevertheless, a better preservation was found.

As pointed out early, diagenetic changes vary across and within environments and depend on the interaction of several biological, chemical and physical factors (Hedges and Millard, 1995; Hedges et al., 1995). The differential preservation recorded between sites clearly reflects this reality. For some samples (i.e., C and HRAS), the environmental conditions of the burial place, such as the presence of groundwater, probably had a major impact on the bone tissue preservation. Regarding the Porto and the identified samples, a more recent chronology may have favored bone preservation. For all samples, the biological traits of the individuals have contributed little to bone tissue diagenesis. Contrariwise, the presence of diagenetic changes has prevented the histological characterization of certain bone lesions, affecting the difficult task of disease differentiation. As in previous investigations (e.g., Bell & Jones, 1991; Guarino et al., 2006), this study has confirmed that the gross preservation of bones may not find correspondence at the microscopic level. This has serious implications in the concepts

and definition of bone preservation, and their use to categorize human remains recovered from archeological and even modern contexts (Garland, 1987).

Concluding remarks

In this study, the application of histological techniques proved to be fundamental to assess bone preservation. Furthermore, it showed that visual inspection is not a good measure of bone tissue quality. This finding was particularly true in the analysis of bone samples retrieved from archeological contexts. All archeological samples (14^{th}-19^{th} centuries) exhibited some form of postmortem modification and an absence or low bone birefringence under cross polarized light, while the samples collected from the identified skeletons (20^{th} century) were exceptionally well-preserved. As a consequence, the identification and description of the main components of the bone tissue (Haversian systems, interstitial bone, osteocyte lacunae and endosteal and periosteal lamellae) or evidence of pathological lesions was found to be unattainable in most of the archeological specimens. This lack of bone preservation may be explained by long periods of inhumation or by particular conditions of the burial environment, such as the presence of groundwater. This investigation testifies to the importance and utility of the use of microscopy in past populations studies, especially those that aim to interpret disease based on the macro- and microscopic appearance of bone. Further studies incorporating large bone samples from other Portuguese sites with similar conditions may better clarify the role of the inhumation interval in the decay of bone tissue, especially when pathological lesions are present.

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