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NEW PERSPECTIVES ON THE SELECTIVITY OF PLAGUE: PALEOEPIDEMIOLOGICAL ANALYSES ON FRAILTY, AGE AND SEX OF PLAGUE VICTIMS

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INTRODUCTION

Paleopathology studies diseases in individuals of the past. The lives of past people, both from prehistory and from recent history, can only be truly understood if we also consider the diseases they suffered from and their general health condition (Cook, 2015). Moreover, since diseases represent a form of stress, the knowledge of the prevalent pathologies of the past may help us to better understand evolutive processes, as well as the acting of today's diseases. Similar to paleopathology, paleoepidemiology is a recently born branch that also studies the evidence left by ancient diseases in skeletal remains. Its main objective, however, is to generate results that can be interpreted in the context of modern medicine and epidemiology, such as the increased risk of dying of different diseases associated with various pathological characteristics of bones and teeth (biomarkers) that can be found in human remains. The analyses of archaeological skeletons are thus compared with the investigations of contemporary or quasi-recent populations on the basis of clinical evidence, public health surveys and registers of deaths (Milner and Boldsen, 2017).

One of the diseases of the past that mainly influenced the history of the humankind and that still today represents a danger to health is the bubonic plague.

Plague is not only still present to date, in some endemic areas of the world, but represents a significant risk to health since it can be used as a bacteriological weapon (Riedel, 2005). Today there are antibiotic treatments that allow, if taken in time, to heal from the infection. Despite, many patients still die of bubonic plague every year. Understanding how the contagion, virulence and mortality of plague are influenced by the biological characteristics of its victims and their state of health is not a simple matter. This PhD-thesis aims to address questions on plague mortality through a paleopathological and paleoepidemiological analysis.

1.1   THE PLAGUE

Plague is an infectious disease caused by *Yersinia pestis*, a bacterium usually infecting small mammals and their fleas (Who, 2018). Fleas pass the disease on the animals they infest, typically small rodents. Humans can be contaminated by flea bites or by other infected vectors (e.g. lice (Dean et al., 2019)), by direct contact with infected materials from other people or by inhalation of infected droplets. After an incubation period of about 3-7 days, people infected with *Y. pestis* usually develop "flu-like" symptoms: fever, with a temperature of 40 °C (104 °F) or higher, chills, head and body pains, but also vomiting and nausea (Who, 2018).

Plague can take three different forms depending on the infection route: bubonic, septicemic and pneumonic. Bubonic plague is caused by the bite of an infected parasite. *Y. pestis* is injected by the

flea or another vector into the dermis, where it begins to replicate, then travels through the lymphatic system to the nearest lymph node, where it continues to replicate. The lymph node becomes inflamed and swollen, and it becomes painful, forming the so-called "bubo" (Fig. 1.1). In the advanced stages of the infection, the buboes can turn into open suppurated sores. It is commonly accepted that bubonic plague cannot be transmitted directly from human to human without a vector (Who, 2018). Bubonic plague is the most common form and the less virulent one: if not treated, its mortality rate is 40-60%. Septicemic plague occurs when the infection spreads through the bloodstream. Septicemic plague can be primary when the flea bite spreads the bacterium directly into a vessel, or by contact with infected materials through open wounds and cracks in the skin. Septicemic plague can also be secondary to bubonic plague: in advanced stages of the bubonic form, the bacterium may spread from the lymphatic system to the blood system. Individuals affected by this form of plague die from blood poisoning (Who, 2018).

Pneumonic plague is the most virulent and least common of all forms of plague. The pulmonary form is caused by spreading to the lungs in an advanced phase of bubonic plague (secondary pneumonic plague). A person with secondary pneumonic plague can transmit it directly to other humans through aerosolized infected droplets expelled by coughing or sneezing (primary pneumonic plague). If not treated in time, this form of plague has a mortality ratio close to 100% (Who, 2018).

Figure 1.1: A plague victim shows three physicians the bubo in his armpit. Picture found in a medical treatise on plague (Pestbuch); "Sick man in bed and three doctors," The College of Physicians of Philadelphia Digital Library, https://www.cppdigitallibrary.org/items/show/229

The plague has spread in Europe and the rest of the world multiple times, contributing to shaping the history of humanity. While biomolecular analysis has traced *Y. pestis* back to the Bronze Age and even the Neolithic (Rasmussen et al., 2015; Andrades Valtueña et al., 2017; Spyrou et al., 2018; Rascovan et al., 2019), the most important and historically acknowledged pandemics of plague are three: the first is the Justinian plague, dated to the 6th century. It started in Egypt, during the reign of the Byzantine emperor Justinian and it spread into Europe. Repeated cycles of plague epidemic have continued until 750 CE (Boire, 2014; Bramanti et al., 2016).

The second pandemic began in the 14th century with a large outbreak, the Black Death (1346-1353), that killed almost one-third of the European population of the time; it returned cyclically in Europe until the end of the 18th century (Boire, 2014; Bramanti et al., 2016).

The third pandemic originated in China at the end of the 18th century and spread in 1894 from Hong Kong, all around the world along the major commercial routes, reaching Europe in 1896 (Echenberg, 2010; Bramanti et al., 2019).

Only recently, it was established that it was the same bacterium, *Y. pestis*, the pathogen responsible for all the three pandemics, even if different strains where involved (Haensch et al., 2010; Bos et al., 2011, 2016, Spyrou et al., 2016, 2018; Namouchi et al., 2018).

Plague is still present nowadays, with thousands of cases reported every year worldwide: 38,310 cases from 1989 to 2003 (2845 deaths), and 3248 cases from 2010 to 2015 (584 deaths) were notified. There is a risk that plague will be transmitted to humans wherever the human population coexists with natural reservoirs of plague (Who, 2018), which can be defined as a place where the bacterium, an animal population with non-susceptible hosts, and a vector are present at the same time (Bramanti et al. 2016). Currently, the three countries most endemic to the plague are the Democratic Republic of Congo, Madagascar and Peru.

1.2 PLAGUE MORTALITY DEBATE

Whether plague was a "universal killer" or it was selective in its effects of mortality, is a question that historians, bioarchaeologists, paleoepidemiologists, and biomedical scientists often face. Historically, it is claimed that the plague made no distinction about the health status of the victims, their sex or age and this thesis is supported by many researchers (e.g. Signoli et al., 2007, 2009; Castex and Kacki, 2016; Kacki, 2016). Others argue that this was only true for the major pandemics of the 14th (Margerison and Knüsel, 2002) and 17th centuries (Alfani and Murphy, 2017), whereas other scholars claim that mortality had a social component, killing the poorest in the population (Slack, 1985; Carmichael, 1986; Cohn, 2010).

Recently, scholars have questioned the view of the indiscriminate killing of the Black Death and highlighted elements of selectivity for the victims' pre-existing health and age (DeWitte and Wood, 2008; DeWitte, 2010a, 2014; DeWitte and Hughes-Morey, 2012). Kacki S. (Kacki, 2016), anyway,

argues that in plague pits there were more healthy individuals than in attritional cemeteries, concluding that plague did not select its victims on the basis of their pre-existing health.

Regarding the possibility that plague selects its victims in relation to their sex, there are again divergent opinions: some works based on paleodemographic and anthropological data have highlighted a higher mortality in women than in men (e.g. (Guellil et al.-in preparation; Cervellati, 1986; Ell, 1989; Signoli et al., 2002; Frandsen, 2010); a historical study proposes a greater selection of male individuals (Hollingsworth and Hollingsworth, 1971). Others, anyway, agree in affirming that there was no selectivity for sex (e.g. (Alexander, 1980; Whittles and Didelot, 2016; Alfani and Murphy, 2017).

However, recent studies have seen a difference in plague mortality between men and women regarding their health status: De Witte (DeWitte, 2010b) observed that Black Death killed more men than women in England and that men showed more indicators of skeletal frailty due to pre-existing physiological stress. In other words, she proposed a lower risk of plague death for healthy men than for healthy women. A further study (Curtis and Roosen, 2017), this time on paleodemographic mortality data in Belgium from the period 1349-1450, revealed that during the Black Death (1349-51), as well as during subsequent plague epidemics until 1450, female mortality was higher than that in the years without plague epidemics.

The debate remains open, and there is not yet a confident answer to the question whether plague selected its victims. It is possible that the answer is not univocal, and that there are differences between pandemics, even if from the biomolecular point of view, *Y. pestis* has undergone few changes (Demeure et al., 2019).

With this PhD-thesis, we attempt to answer this question, using bioarchaeological and paleoepidemiological data and interpreting them from a biomedical point of view.

1.3   IMMUNITY TO INFECTION

Immunity in humans and most vertebrates consists of two main systems: the innate immune system and the adaptive immune system. The innate immune system provides an immediate, but non-specific, response against external stressors, such as lesions and infections; its main protagonists are neutrophils, monocytes and macrophages. The adaptive immune system, on the other hand, responds more slowly to attacks by pathogens, but its action is pathogen-specific, and it leaves an immunological memory that allows it to respond to subsequent attacks of the same pathogen with a shorter response time; the cells involved are the T and B lymphocytes (Pancer and Cooper, 2006).

Y. pestis is a gram-negative bacterium; it is genomically very similar to the enteric pathogen *Y. pseudotuberculosis*. A series of events of gene gain and loss, however, has led *Y. pestis* to differentiate from its antecessor, by acquiring plasmids with unique virulence factors that allowed it to infect fleas and to surpass the immune responses in host mammals, leading to rapid death of the latter, in the absence of adequate treatment (Demeure et al., 2019). Nevertheless, even before the

advent of adequate treatments (i.e. antibiotics), while the death toll was huge, people could recover from the infection of plague, at least in its bubonic form.

Immunity reacts differently to infectious diseases based on the biological features of the host (sex and age) and the health status pre-existent to the infection. In particular, the role of iron is essential in facing infectious disease.

1.3.1 Sex and Age differences in immunity

It is commonly thought that differences in infectious diseases between women and men may depend on different lifestyles, so that men, who are generally more out of the home, are more subjected to infection (Who, 2007). Recent studies have shown that the biological pathways leading to inflammation and immune activation are regulated by sex-related factors: sex hormones and genes encoded within sex chromosomes (vom Steeg and Klein, 2016). The nature and strength of immune responses, against the attack by pathogens, show specific differences between the two sexes: while women, compared to men, generally have a stronger immune response to infections, vaccinations and some malignancies, they also suffer more from inflammatory and autoimmune diseases (Ingersoll, 2017; Lotter and Altfeld, 2019). On chromosome X, there are genes connected to the immunological response, the presence of two X chromosomes in females allows an expression of the immune response that is both stronger and more versatile against infections (Fish, 2008; Libert et al., 2010; Klein, 2012; Schurz et al., 2019). Also the hormones play an essential role in immunity: estrogen has been shown to promote immune response while testosterone slows it down (Walker, 2011; Klein, 2012; vom Steeg and Klein, 2016); moreover, after menopause, with the subsequent decline of the estrogen levels, women are more susceptible to infectious disease (Lotter and Altfeld, 2019).

A recent study (Úbeda and Jansen, 2016) has proposed that some pathogens have evolved different sex-specific virulence, depending on the various transmission routes the two sexes can provide. In particular, some pathogens might have developed a less virulence towards females, to exploit the vertical transmission (the transmission from mothers to their children).

Likewise, age also affects the immune response to infections: innate immunity begins to develop during the first few months of life, but new-borns and infants are highly dependent on immunoglobulins the mothers share through breast milk (Joachim and Kobzik, 2018). Even after weaning, children younger than 4 or 5 years still do not have an experienced immune system, which can make them less susceptible to infectious diseases. After that age, there is a drastic drop in the susceptibility: prepubertal children show more competent immune responses than younger children and infants, but they have not yet acquired the immunological competence a pubescent and an adolescent have due to the increase in sex hormones. Nonetheless, it seems that prepubertal children are less susceptible to infections than adolescents. In puberty, in fact, energy goals change significantly, from the somatic maintenance typical of the prepubertal age, including immunity, to body growth, development of sexual characteristics and reproduction (Joachim and Kobzik, 2018).

Therefore, young children are the most susceptible to infectious disease and to die from it. Prepubertal children are less vulnerable than young children but also than adolescents, because of the shift from conservation of energies to the development of the sexual characteristics to the detriment of immunity. Nonetheless, once sexual maturity is reached the immunity is strengthened by the hormones, especially in the women. Finally, in older age, individuals are again more susceptible to infections, particularly women, due to the fall in hormones levels at menopause. Moreover, the elderlies are weakened by the burden of biological stress that they have built up during their lives, also called frailty. This makes them more susceptible to disease.

1.3.2 Frailty and pre-existing health

Environmental, genetic and lifestyle factors such as ageing, malnutrition and infections can increase individuals' susceptibility to disease (Au, 2001). Malnutrition, infections and ageing are physiological stressors that can affect the immune system defences and make the individuals more susceptible to infections. The load of physiological stress that an individual sustained in life is called frailty (Vaupel, 1988; Dent et al., 2016).

It is not clear how frailty increases the risk of negative consequences for health, but it has been seen in clinical researches that frail individuals have high levels of inflammation. This state of chronic inflammation can lead to a dysregulation of the immune system, both innate and adaptive (Li et al., 2011; Drew et al., 2017).

Moreover, pre-existing health conditions and disease can accelerate the virulence, enhance contagiousness and lethality of other pathogens (Thorburn, 2009; Singer, 2010). The frailest and those with pre-existing health problems have a weaker immune system in response to infections. It is, therefore, possible that also in the case of plague, the weakest will be more affected.

1.3.3 The Role of Iron

Iron is an essential trace element for almost all living organisms. In vertebrates, iron is required as a functional component of many proteins involved in diverse vital biochemical functions (i.e. transport of oxygen and energy production) (Ganz and Nemeth, 2015).

Iron levels in plasma and cells are strictly regulated to ensure the availability of essential functions and avoid the toxicity associated with iron excess (Parrow et al., 2013). Iron in the ferrous state (Fe^{2+}), as it is found inside the heme-molecule, is potentially toxic due to its ability to catalyse the production of reactive oxygen and nitrogen species (Graf et al., 1984), which can damage biological molecules, including DNA (Bergeron et al., 2010). Iron can be stored into the cells, mainly within macrophages, in the form of ferritin, a protein that can bind iron ions in the ferric form (Fe^{3+}). In this form, iron is not toxic.

Two primary regulators of iron homeostasis are hepcidin and ferroportin. Ferroportin is a protein that promotes the exchange of iron from cellular storages to plasma to make it available. Hepcidin, a hormone derived from the liver, blocks ferroportin, reducing the influx of iron from stores into the plasma and preventing further absorption of dietary iron from the intestine. When body iron levels

decrease, the production of hepcidin is reduced accordingly (at the same time the production of ferroportin is enhanced), a mechanism which allows the resumption of iron absorption and the increase of iron levels in the plasma (Ganz et al., 2008; Ganz and Nemeth, 2015; Soares and Hamza, 2016; Sangkhae and Nemeth, 2017).

Hepcidin is upregulated not only in case of iron overload in the plasma but also in case of an infection. In the course of infectious diseases, in fact, host and pathogen compete for iron; the availability of this metal can have a significant impact on both the virulence of the pathogen and on the immune defences of the host (Parrow et al., 2013; Nairz et al., 2014, 2017; Ganz and Nemeth, 2015). For this reason, hepcidin is produced during an infection, to deplete the plasma and therefore the pathogens of the necessary iron. While less circulating iron may be useful in response to extracellular pathogens, the increase of intracellular iron may facilitate some intracellular pathogens, which would thus have a large reserve of available iron (Parrow et al., 2013; Ganz and Nemeth, 2015; Nairz et al., 2017).

Some gram-negative bacteria, included *Y. pestis*, overcome the first immunity of the mammal host, since they are able to survive the attack of the macrophages that phagocyte them at the site of primary infection, and reproduce within them by exploiting the reserves of iron that the action of hepcidin has kept within them (Wang and Cherayil, 2009; Nairz et al., 2014). *Y. pestis*, travels within the macrophages through the lymphatic system, until they die, pouring the bacteria into it. At this point, the buboes in the lymph nodes may appear. Afterwards, the adaptive immune system reacts, and the individuals can recover in almost 50% of the cases. This mechanism is represented in a simplified form in Figure 1.2 (yellow arrows).

In the last years it has been suggested that iron deficiency anemia, generally detrimental to a good health and responsible for a slower immune response (McLean et al., 2009), could instead, in some cases, be beneficial in preventing infectious diseases (Wander et al., 2009; Hadley and DeCaro, 2015). Iron deficiency can have opposite effects on infectious diseases: it can decrease susceptibility to pathogens, limiting the availability of iron, but at the same time it can increase susceptibility by compromising the immunocompetence of cells (Wander et al., 2009; Hadley and DeCaro, 2015). Therefore, it has been suggested that a compromise between the two phenomena, a moderate iron deficiency, might effectively protect against acute infections and represent a nutritional adaptation to stress from infectious diseases, particularly endemic ones (Wander et al., 2009; Hadley and DeCaro, 2015).

Iron deficiency can be beneficial in case of infection from extracellular pathogens, i.e. malaria (Sazawal et al., 2006; Adam, 2016); but also intracellular ones, e.g. *Salmonella enterica*, are inhibited by a decrease of iron in the macrophages caused by an increased expression of ferroportin and a diminution of hepcidin in response to iron deficiency anemia (Wang and Cherayil, 2009). There are different kind of anemia and iron disorders that can influence susceptibility to infectious disease, depending on the proliferative behaviour (extracellular or intracellular) of the pathogen. In Table 1.1 are summarised different iron disorders and the effects on extracellular (serum) and intracellular iron levels (Pak et al., 2006; Cazzola and Malcovati, 2015; Katsarou and Pantopoulos, 2018).

Table 1.1. Iron-related disorders and the effects on extracellular and intracellular iron levels (Pak et al., 2006; Cazzola and Malcovati, 2015; Katsarou and Pantopoulos, 2018)

Iron disorders	Serum Iron	Tissue Iron	Hepcidin	Storage Iron (macrophages)	Hemoglobin
Hemochromatosis	↑	↑	↓	↓	NORMAL
Iron Deficiency Anemia	↓	↓	↓	↓	↓
Iron-Refractory Iron Deficiency Anemia (IRIDA)	↓	NORMAL	↑	↑	↓
Iron loading Anemias (Thalassemia, Sideroblastic anemia)	↑	↑ Or NORMAL	↓	↓	↓
Anemia of Chronic Disease (ACD)	↓	NORMAL	↑	↑	↓
Vitamin B12 Deficiency (pernicious anemia)	↑ Or NORMAL	↑ Or NORMAL	↓ Or NORMAL	↓ Or NORMAL	↓

Regarding *Y. pestis*, it has not yet been determined experimentally whether iron deficiency can be a protective factor, but a recent study has shown that a reduction in circulating iron, and an increase in storage iron, has the effect of slowing down the spread of the bacterium, thus leaving more chance for the immune system to respond to the infection (Zauberman et al., 2017). If the downregulation of hepcidin due to iron deficiency anemia is detrimental to *Y. pestis* proliferation, as it is for other Gram-negative bacteria, individuals who present a mild iron deficiency anemia could be protected by the infection; a simplified interpretation of this process is represented in Figure 1.2 (green arrows).
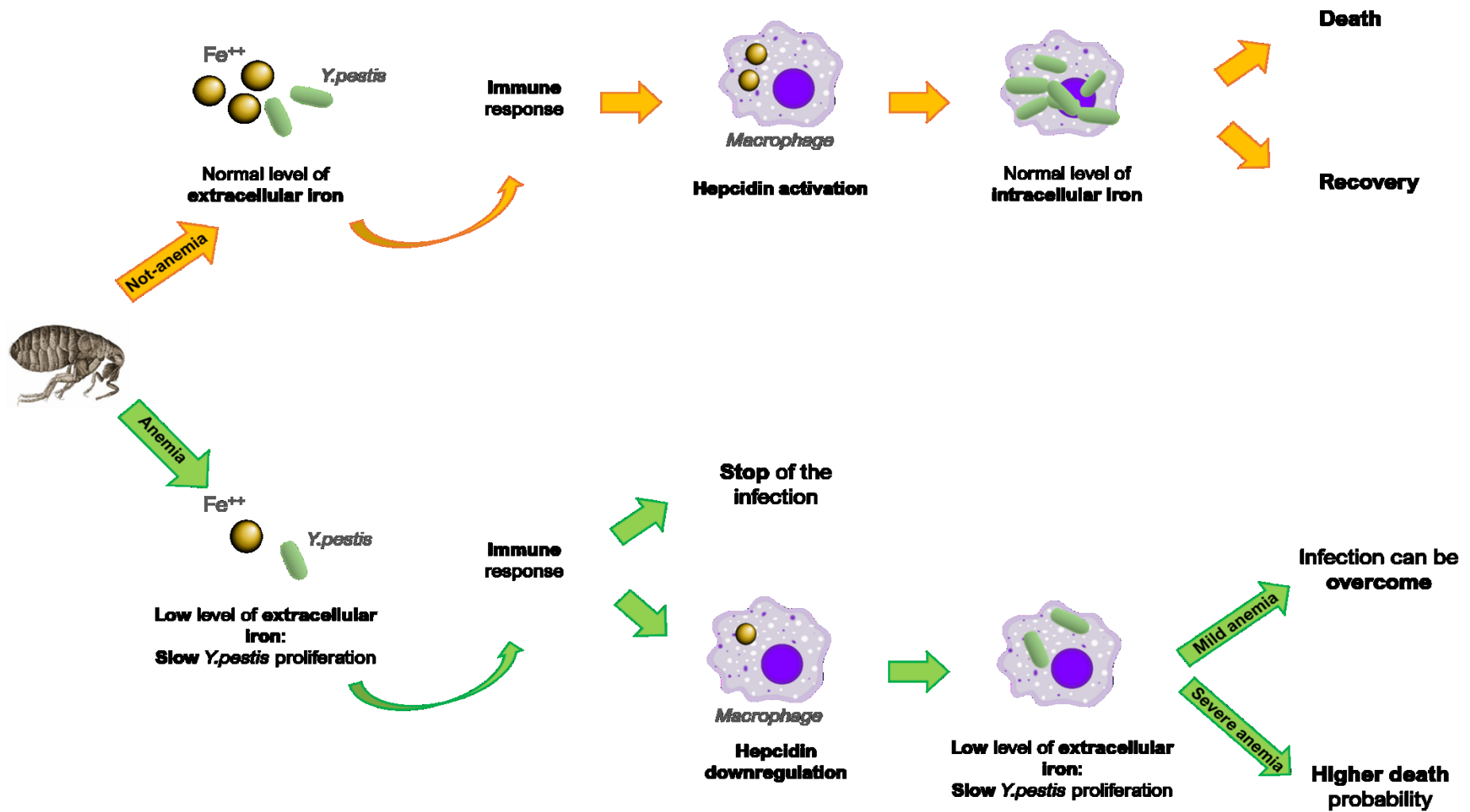
Figure 1.2: Simple representation of the infection by *Y. pestis* in an anemic (green arrows) and non-anemic (yellow arrows) individual.

1.4 IRON DEFICIENCY IN PALEOPATHOLOGY

If indeed iron deficiency anemia is protective in case of infection of plague, we should find less signs of anemia in their victims than in other assemblies of non-plague victims.

In skeletal remains, there are different methods to detect iron deficiency anemia.

The first one is the macroscopic analysis: anemia, in fact, causes a malfunction in the production of blood cells in haematopoietic centres, such as the skull and the epiphysis of humerus and femur, where a process of hypertrophy or hyperplasia takes place (increasing the size of cells or their number) and then the expansion of the trabecular tissue. This expansion of the trabecular tissue leads to partial resorption of the cortical tissue, in which small holes of various sizes and distribution are formed. These are mainly found on the external surface of the orbits (Cribra Orbitalia) and on the vault of the skull (Porotic Hyperostosis) (Ortner, 2003; Brickley, 2018; Rinaldo et al., 2019).

Initially, cribrotic manifestations on both the orbital surface and the cranial vault were considered caused by the same pathology, Porotic Hyperostosis (Angel, 1966; Facchini et al., 2004; Rivera and Mirazón Lahr, 2017). Yet, in the last years, a distinct etiology for the cribra on the orbital roofs and that on the cranial vault has been suggested, based on the independent manifestation of the two lesions (Wapler et al., 2004; Walker et al., 2009; Rothschild, 2012; Rivera and Mirazón Lahr, 2017). Both Cribra Orbitalia (CO) and Porotic Hyperostosis (PH) are considered a manifestation of anemia, either genetic (Angel, 1966) or acquired (Walker et al., 2009; Oxenham and Cavill, 2010; Rothschild, 2012; Mcilvaine, 2015), but the exact etiology could not yet be defined, nor is clear how severity and healing of the lesions are connected with iron levels in the body at the moment of the death.

The second method is the recovering and measurement of elemental iron in the skeleton. Different methods can be used to detect trace element iron in the bones: plasma mass spectrometry (e.g.(Hare et al., 2011; Kohn et al., 2013), Proton Induced X-ray Emission (PIXE) (e.g. (Williams and Siegele, 2014), X-ray fluorescence (XRF) (e.g. (Miculescu et al., 2012). The search for trace iron in the skeleton, however, is affected by the diagenesis, which can heavily change the level of iron, the less affected being the teeth (Lambert et al., 1985; López-Costas et al., 2016).

The third methodology is the analysis of the paleo-proteins: many plasmatic proteins have been detected in ancient bones and teeth, even transferrin, the protein involved in the transport of iron in the plasma (Thomas and Leaver, 1975; Mbuyi et al., 1982; Bona et al., 2014). In iron deficiency anemia, transferrin levels increase, while ferritin, the iron storage protein, decrease. Measuring the levels of transferrin recovered from an ancient skeleton could help in detecting possible conditions of iron deficiency anemia. Proteomic research, as well as genomic analysis, has recently often been applied to the study of ancient materials. Some of the potential advantages of proteomics lie in its ability to discriminate between the sources of molecules, rather than between the particular species

or individual. Besides, proteins are also considered capable of surviving longer than DNA (Buckley, 2018). Nonetheless, there are many limitations to this technique, and one is that there is not yet a routined methodology to quantify the proteins in ancient skeletal remains, an issue that strongly limited its use for our purposes.

Finally, there is the analysis of ancient DNA (aDNA). Various anemias and iron overload disease are known for having a genetic basis. In some cases, these pathologies or disorders could be beneficial or detrimental to the survival of the individual to the attack of *Y. pestis*. Table 1.2 shows the main genetic diseases that alter the homeostasis of iron (Camaschella and Silvestri, 2011; Silva and Faustino, 2015).

In case of the iron overloading disease hemochromatosis, different theories were proposed: some researchers sustained that the downregulation of hepcidin and parallel decrease of iron stored in macrophages may help the individuals who have hemochromatosis to survive plague infections (Moalem and Prince, 2007). On the other side, the case of the researcher with hereditary hemochromatosis (HH) who died from a non-virulent laboratory-acquired plague, suggested that the iron abundance in its serum was, instead, detrimental, and enhanced the bacterial virulence (Quenee et al., 2012).

Table 1.2: Main genetic diseases that alter the homeostasis of iron (Camaschella and Silvestri, 2011; Silva and Faustino, 2015).

Pathology	Affected Genes	Mode of transmission	Iron homeostasis disruption
HH type 1	HFE	Recessive	Iron overload
HH type 2	HAMP, HJV	Recessive	Iron overload
HH type 3	TFR2	Recessive	Iron overload
HH type 4	SLC40A1	Dominant	Iron overload
Hyperferritinemia	FTL	Dominant	Iron overload
Aceruloplasminemia	CP	Recessive	Iron overload
Atransferrinemia	TF	Recessive	Low transferrin, iron overload
IRIDA	TMPRSS6	Recessive	Low transferrin, high expression of hepcidin
X-linked sideroblastic anemia	ALAS2	X-linked recessive	Low hemoglobin, iron overload
Beta Thalassemia	HBB	Recessive	Low hemoglobin, iron overload

1.5   DISSERTATION STRUCTURE

This dissertation is divided into seven chapters. This chapter has provided an introduction about state of the art on the plague mortality debate, along with all the possible discriminant variables that can influence it. Additionally, we introduced the methods to assess iron deficiency anemia in human skeletal remains.

Chapter two will report about a review of many anthropological studies on plague victims published in different languages, along with an attempt to discern if any selection by plague is detectable from the published data.

Chapter three will deal with the two main biomarkers of iron deficiency anemia in skeletal remains, Porotic Hyperostosis and Cribra Orbitalia, and propose a new method to score them.

Chapter four will outline a new index of frailty for the analysis of human skeletal remains, while chapter five will present the application of this index to one sample of plague victims in comparison with another of non-plague victims. The analysis aimed to detect if any differences exist between the two skeletal series, regarding frailty and iron deficiency.

Chapter six will concern the epidemiological analysis of data about infection, death or recovery of plague patients from around the world, between 1813 and 1945. These data on plague provide information on the infection that cannot be generated by archaeological skeletons.

The final chapter will present the conclusions of this study and some answers to the research questions posed earlier. This last chapter ends with a discussion on the possibilities of future research.

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2

REVIEW OF ANTHROPOLOGICAL INVESTIGATIONS ON
PLAGUE VICTIMS

Plague epidemics, caused by the bacterium *Y. pestis*, have cyclically affected Europe for more than 1,400 years with devastating results, yet, plague still poses a threat in many countries around the world. From the skeletonised victims of past pandemics, we can obtain valuable biological information, age at death and sex, but also their previous health status, which can improve our understanding of paleoepidemiological determinants and lethal factors of plague. The following is a review of a large number of anthropological studies conducted over the last twenty years on the victims of the First and Second Plague Pandemics in Europe. We statistically elaborated the data collected to highlight if the disease was selective towards particular groups of people in the distinct populations based on their biological characteristics. We have also tried to discern whether the behaviour of the infection was similar in two different geographical regions, northern-central Europe versus the Mediterranean area.

With our analysis, we have highlighted the absence of a common trend of selectivity; the distribution of anthropological markers considered in the different populations is often uneven. However, the diverse criteria used by the existing studies for the data collection may have limited the potential of the statistical analyses.

2.1   INTRODUCTION

The first acknowledged plague pandemic broke out in Europe during the 6th-7th centuries, starting with the Justinian plague (541-542 CE). It spread from the Mediterranean Basin to Central Europe and UK (Bramanti et al., 2016).

The second time plague struck in Europe was during the 14th century. The epidemic, which is called the *Black Death*, lasted in the period 1346-1353 and caused high mortality in whole of Europe. Plague reached Italy on merchant ships from the East and spread northwest across Europe, where it claimed more than half of the population (Bramanti et al., 2016). After this big epidemic, plague struck in Europe periodically during the following centuries until the 19th century.

As the plague killed rapidly, and in high number, often cemeteries were not suited to collect the high number of victims, so that, throughout the acutest phase of an epidemic, bodies were buried in multiple graves and pits, or trenches (Namouchi et al., 2018), often outside of regular cemeteries to avoid further risks of contagion. Archaeologists have discovered and dug up many multiple burials in different regions of Europe. Biological anthropologists have subsequently investigated the human

remains recovered from the pits, which in some cases were confirmed to be plague victims based on historical records and/or molecular tests.

The goal of this review was to list the largest number of anthropological studies carried out on confirmed skeletonised plague victims of the past. We reported here the demographic composition of each archaeological site (sex and age at death) along with data on indicators of the general health status of the populations of plague victims (body dimensions and paleopathological lesions). Some anthropologists consider the skeletons retrieved from plague pits to more clearly reflect the general physical condition of the population of origin than skeletons from regular cemeteries (Prechel, 1996). This is because in regular cemeteries, a sample bias or a selection due to professional or familial reasons can occur, whereas plague victims are thought to have died without distinctions of class or age. Other anthropologists have challenged this view and proposed that plague killed the weakest in a population, i.e. children and elderly individuals, or persons with poor health status included that due to other infectious diseases (DeWitte and Wood, 2008). Most of the conclusions were anyway drawn on the outcomes of studies on single or few plague pits, in different regions of Europe. We tried here to observe whether there was a homogeneous trend for plague lethality by comparing and synthesising the results of several selected studies.

Unlike other infectious diseases like leprosy, tuberculosis, and syphilis that could be identified by a paleopathological inspection of the skeletons, plague does not leave any detectable lesion on the bones. Therefore, in the absence of precise historical records attesting that the pits contained plague victims, retrospective diagnosis of plague can only be made based on the retrieval of ancient DNA (aDNA) of the etiological agent, *Y. pestis* (Haensch et al., 2010; Bos et al., 2011, 2016a; Spyrou et al., 2016; Namouchi et al., 2018). Immunological tests were also occasionally used to seek proteins of the bacteria from teeth and bones (Pusch et al., 2004; Bianucci et al., 2007; Malou et al., 2012). Here, we have not singularly evaluated the methods employed for diagnosis; instead, we used the results of positive aDNA identifications, or a combination of immunological and historical records, to select studies that anthropologically analysed skeletons where plague is likely the cause of death.

2.2 MATERIALS

The study reviewed the data of 25 anthropological publications (Table 2.1 and 2.2) that respected the eligibility criteria listed below: 20 of the 25 papers (Table 2.1) provided information on sex and age of the individuals that composed the skeletal assembly; 11 publications of the 20 provided additional data on paleopathological lesions of the plague victims (Table 2.2). The paleodemographic analysis, therefore, compares data from 22 sites of plague in Europe, from the First Pandemic (6th-7th centuries) to the Black Death and subsequent epidemics of the Second Plague Pandemic (Table 2.1). The analysis of the health status of the plague victims, based on the data of the skeletal biomarkers of stress, has been performed on data from the skeletal assembly of 11 sites from both the First (only one site) and the Second Pandemic (Table 2.2).

2.3   METHODS

2.3.1   Search strategy and eligibility criteria

This review was carried out following the PRISMA guidelines (Moher et al., 2009).

All the publications until 2016, which provided information on sex, age and biomarkers of physiological stress of plague victims were systematically researched on online databases (e.g. PubMed and Google Scholar) and journals of physical anthropology (*American Journal of Physical Anthropology*, *Homo*, and *International Journal of Osteoarchaeology*). The following keywords were used for the search: '*Yersinia pestis*', 'plague', 'Black Death', 'plague pandemic' combined in turn with 'skeletal remains', 'skeleton', 'skeletal biomarkers', 'paleopathology', 'demography', 'sex', and 'age'.

Further, the search was extended to the reference lists of the retrieved studies, which included works published in German, Italian and French, and some published and digitalised books and PhD theses that contributed with information not provided by other publications. After eliminating duplicates, the records were screened: all the studies that were not on confirmed plague victims or did not give bio-anthropological information or were not conducted on human skeletal remains, but on historical sources, were excluded.

The selected literature, composed of 52 publications, is reported in the Supplementary Table S2.1. This Table contains all the published records of anthropological analysis of putative plague victims until 2016 as well as the anthropological information they provide. The 53 publication were then further selected based on the following criteria: the presence of at least ten individuals in the sample, the evidence that the individuals in the samples died from plague, as revealed by aDNA, historical or paleo-immunological analyses. A further criterium for selection concerned different publications on the same burial site. This selection was carried out by following definite selective criteria: papers with raw data were preferred to those only containing data in a graph, then we chose the one with the bigger sample, and ultimately, the more recent and more informative one (Fig.2.1).

The data on paleo-demography and health status, as inferred by skeletal markers of stress, were addressed separately (Table 1.1, 1.2).

2.3.2   Statistical analysis

To evaluate the difference in mortality for sex, age and biomarkers of stress among different sites, we used the χ^2 test. When the *p*-value was < 0.05, the results were considered significant.

Further, we tried to identify predictors for differences in the sex-ratio (Number of males / Number of females) among the indicators of health status. Only seven sites were considered, for which data were provided on Linear Enamel Hypoplasia (LEH), Cribra Orbitalia (CO) and Porotic Hyperostosis (PH), which represent the most recurrent skeletal markers of biological stress in our samples (Table 2.3, 2.4). All the selected mass graves are dated to the Second Plague Pandemic: Barcelona, Royal Mint, Hereford, Saint-Pierre de Dreux, Les Fedons, Puy St. Pierre and Maria Troon. Sex

determination cannot be confidently carried out in sub-adults; therefore, we considered only sex-frequencies of adult individuals in each population (Tables 2.3, 2.4). Several linear regression models were employed to test the association between sex-ratio (dependent variable) of individual sites and health indicators, period and latitude with a two-step model. In the first step, we tested which of the skeletal stress markers, used as an independent variable, could be considered the best predictor of frailty associated with the sex ratio among LEH, CO, and PH. In the second model, we used only one of the skeletal biomarkers previously tested (LEH) as the best proxy for frailty, and we examined how it was associated with the sex-ratio. We added the latitude of each archaeological site, used as a continuous variable. We further added the historical period, as a categorical variable: period 1-16th century (Les Fedons and Maria Troon), period 2-1630 (Puy St. Pierre) and period 3-the 14th century (St. Pierre de Dreux, Barcelona, the Royal Mint and Hereford). Period 3 was used as the reference period. This categorisation into periods is intended to reproduce the differences in plague selectivity observed by other scholars in different outbreaks. We also weighted the model to the sample size to reduce the bias. All statistical analyses were performed with STATISTICA for Windows (version 11.0, StatSoft, Tulsa, OK).

IDENTIFICATION

Records identified
through database
searching
(n= 163)

Additional records
identified through
other sources
(n= 27)

Records after removal of
duplicates
(n =170)

SCREENING

Records
screened
(n = 170)

Records
excluded
(n = 118)

ELIGIBILITY

Full-text papers
assessed for eligibility
(n =52)
(Table S2.1)

Full-text articles excluded with
reasons
(n =27)
- Less than 10 individuals in
  the sample
- Absence of mass graves
- No proof of plague
- Duplicate of studies on the
  same burial sites

INCLUDED

Studies included in
review
(n =25)

Paleodemography:
(n= 20 out of 25)
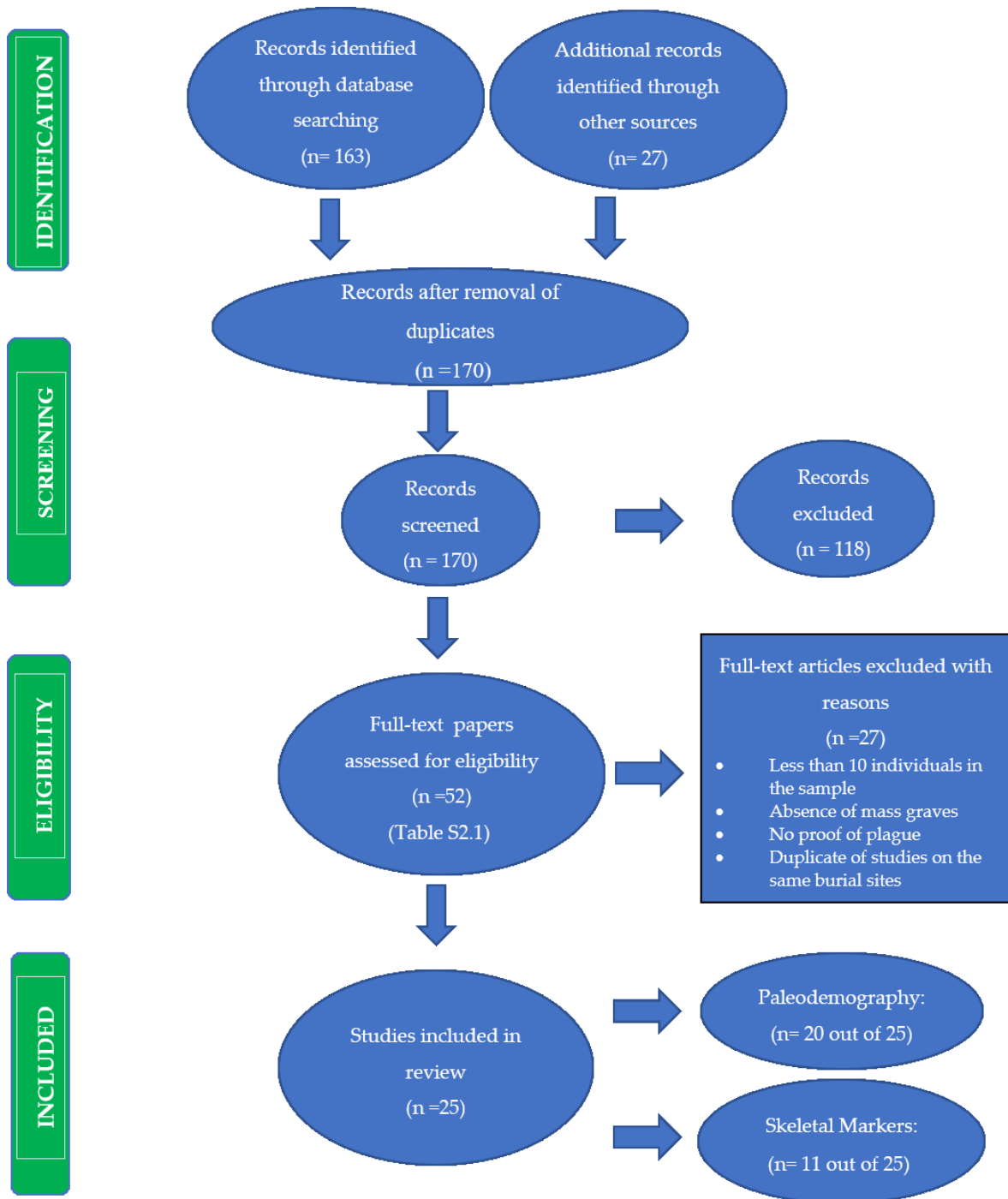
Skeletal Markers:
(n= 11 out of 25)

Figure 2.1: PRISMA Flowchart

2.4   RESULTS

170 publications were found in the bibliographic search. Of those, 118 studies were rejected because they did not meet the inclusion criteria described in the method section. Of the remaining 52 studies (Supplementary Table S2.1), only 25 (Table 2.1) met the inclusion criteria for further statistical analyses.

2.4.1   Paleodemography

Anthropological data of victims of the First Plague Pandemic are rare (Tab.2.1). Among the four sites previously investigated (Aschheim, Altenerding, Le Clos des Cordeliers, Vienne), neither the difference in the frequencies of adults and sub-adults ($p= 0.7545$) nor that between males and females ($p=0.1577$) was statistically significant. In the different archaeological contexts, the majority of the individuals were adults, only in the French site of Vienne the frequencies of adults and sub-adults were similar, although the sample is small (11 individuals). The individuals died at the age of 20-40 years and 15-19 years showed the highest incidence in all the samples of this period. Regarding sex, we notice that females are slightly more represented than males.

Concerning the Second Plague Pandemic, the anthropological samples of the Black Death (14th century) showed some anthropological differences to each other. The difference between sub-adults and adults is statistically significant among sites ($p= 0.0036$). We observed a slightly higher prevalence of adults in four sites from four nations (UK, F, IT, E), and a slightly higher incidence of sub-adults at Manching Pichl (D), Dreux (F), Montpellier (F) and Villanau (F). In the United Kingdom, Waldron et al. (1992; 2001) studied 600 plague victims and identified a much higher mortality of adults than subadults.

The most represented class of age in these samples from the 14th century was that of adults died at 20-40 or more. The only exception was the sample of London with higher mortality of sub-adults between 5 and 15 years of age.

Samples of the 16th and 17th centuries showed similar frequencies of adults and sub-adults, with no relevant differences ($p =0.2039$). Consistently with previous data for this period, the classes of age most represented were again those of children between 5 and 15 (prevalent in the French sites of Puy St. Pierre and Les Fedons, and in the Belgian site of Maria Troon's abbey in Dendermonde), and young adults of 20-35/40 years of age in the Italian site of Imola. At Alghero (Italy), the two age-at-death classes 3-11 and 12-20, were equally represented.

The most recent plague pits submitted to anthropological analyses were from sites of the 18th century in France and Denmark. The incidence of adults was higher (sometimes much higher - see the two sites of Marseilles) than that of sub-adults; the difference among sites of this period was statistically significant ($p =0.0356$). The classes of age most represented in Marseille were again adults of more than 20 years of age and sub-adults between 5 and 19 years, whereas in Martigues young adults (20-35) and children 0-5 were equally represented.

As for the Second Plague Pandemic in its entirety (14th-19th c.), the comparisons among sites showed significant differences in the frequency of adults and sub-adults ($p=$ 0.0000), with a tendency to a higher prevalence of adults in the 14th century and similar rates in the subsequent centuries.

A comparison between sexes of victims from the second pandemic showed no statistically significant difference ($p=$0.3895). Female and male frequencies in the Second Pandemic sites differed from site to site, although they were usually near 50%.

The comparison between adults and subadults across the two pandemics gave a statistically relevant p-value ($p=$0.0002). In both cases, adults are prevalent, although the difference between adults and subadults for the first pandemic was higher than for the second one (Fig. 2.2).

The comparison between frequencies of males and females from the First and Second Pandemic did not provide a significant difference ($p=$ 0.2662) (Fig. 2.2).

Table 2.1- Studies on skeletal remains of plague victims: sex and age at death

Geographic area	Site	Period	Sample n.	M n. (%)	F n. (%)	sex ND n. (%)	Sub-Adults n. (%)	Adults n. (%)	Age ND n. (%)	Age class most represented		Ref.
										Age class	n. (%)	
Southern Germany	Aschheim	5th-7th cent.	77	27 (35%)	40 (52%)	10 (13%)	26 (34%)	50 (65%)	1 (1%)	20-40 40-60	23 (30.3%) 21 (27.4%)	(Staskiewicz, 2007)
Southern Germany	Altenerding	5th-7th cent.	20	5 (25%)	6 (30%)	9 (45%)	9 (45%)	11 (55%)		20-40 0-6 7-12	9 (45%) 4 (20%) 4 (20%)	(Helmuth and Ankner, 1996)
Northern France	Le Clos des Cordeliers, Sens, Yonne	5th-6th cent.	73	14 (31.11%)	17 (37.8%)	14 (31.11%)	28 (38%)	45 (62%)				(Castex and Kacki, 2016)
Southern France	Place Camille Jouffray, Vienne, Isère	760-880	11	1 (9%)	3 (27%)	7 (64%)	6 (55%)	5 (45%)		15-19 40+	3 (27.3%) 3 (27.3%)	(Signoli et al., 2009)
Second Pandemic: 14th-18th cent.												
UK	Royal Mint, East Smithfield, London	1348-1349	600	210 (35%)	167 (28%)	223 (37%)	231 (38.5)	367 (61%)	2 (0.5%)	5-15 25-35	109 (18%) 99 (16.5%)	(Waldron, 2001)
UK	Hereford cathedral	14th cent.	185	40 (38%)	52 (49%)	14 (13%)	79 (43%)	106 (57%)		5-9 20-39	28 (15%) 25 (13.5%)	(Kacki, 2016)
Southern Germany	Manchin Pichl, Ingolstadt	1250-1500	21	13 (62%)	6 (29%)	2 (9%)	12 (57%)	9 (43%)		20-40 0-6	6 (28.6%) 5 (23.8%)	(Seifert, 2014)
Northern France	Sain-Pierre à Dreux (eure-et-Loire)	14th cent.	69	11 (34%)	15 (47%)	6 (19%)	37 (54%)	32 (46%)		20-50+ 1-4	32 (46.3%) 15 (21.7%)	(Kacki, 2016)
Northern France	Bondy	1297-1373	12	3 (43%)	1 (14%)	3 (43%)	5 (42%)	7 (58%)				(Le Forestier, 2012)
Southern France	Vilarnau, Roussilon	14th cent.	19	3 (37.5%)	5 (62.5%)		11 (58%)	8 (42%)				(Passarrius et al., 2008)
Southern France	Saint Come et Damien, Montpellier	14th cent.	13	1 (17%)	3 (50%)	2 (33%)	7 (54%)	6 (46%)				(Crubézy et al., 2006)
Northern Italy	Lazzaretto Vecchio, Venice	14th-17th cent.	331				92 (28%)	192 (58%)	47 (14%)			(Gambaro et al., 2001)
Spain	Basilica of Saint Just et Pastor, Barcelona	14th cent.	120	7 (10%)	6 (9%)	57 (81%)	50 (42%)	70 (58%)		5-14 20-50+	13 (11%) 13 (11%)	(Kacki and Castex, 2014)
Belgium	Maria Troon, Dendermonde	16th cent.	99	20 (45%)	18 (41%)	6 (14%)	55 (56%)	44 (44%)		5-9 10-14	22 (22.2%) 17 (17.2%)	(Kacki, 2016)
Southern France	Les Fedons, Lambesc	16th cent.	133	30 (50%)	26 (43%)	4 (7%)	73 (55%)	60 (45%)		5-9 20-30	24 (18%) 17 (12.8%)	(Kacki, 2016)
Southern Italy	Lo Quarter, Alghero	1582-1583	185	43 (46%)	51 (54%)		89 (48%)	96 (52%)		12-20 3-11	38 (20.5%) 36 (19.5%)	(Milanese, 2010)
Southern France	Puy St. Pierre, Lariey	1629-1630	34	8 (47%)	8 (47%)	1 (6%)	17 (50%)	17 (50%)		40-60 5-9	8 (23.5%) 7 (20.6%)	(Ardagna et al., 2012)
Northern Italy	Osservanza, Imola	1629-1630	92	33 (36%)	39 (42%)	20 (22%)	43 (47%)	49 (53%)		20-35 12-20	27 (29%) 20 (22%)	(Rinaldo et al., 2014)

| Region	Site	Date	N									Reference
Southern France	l'Observance Marseille	1720-1722	179	59 (46%)	58 (45%)	11 (9%)	51 (28.5)	128 (71.5%)		5-9 20+	20 (11.2%) 128 (71.5%)	(Bello et al., 2006)
Denmark	Copenhagen	1711-1712	24				8 (33%)	16 (67%)				(Fiscella et al., 2008)
Southern France	Le Delos, Martigues	1720-1722	39	9 (41%)	5 (23%)	8 (36%)	17 (44%)	22 (56%)		0-5 20-35	6 (15.4%) 6 (15.4%)	(Bianucci et al., 2008)
Southern France	Le Couvent des Capucins de Ferrieres, Martigues	1720-1722	208	56 (46%)	53 (43%)	14 (11%)	86 (41%)	122 (59%)				(Tzortzis and Signoli, 2009)

ND: undetermined
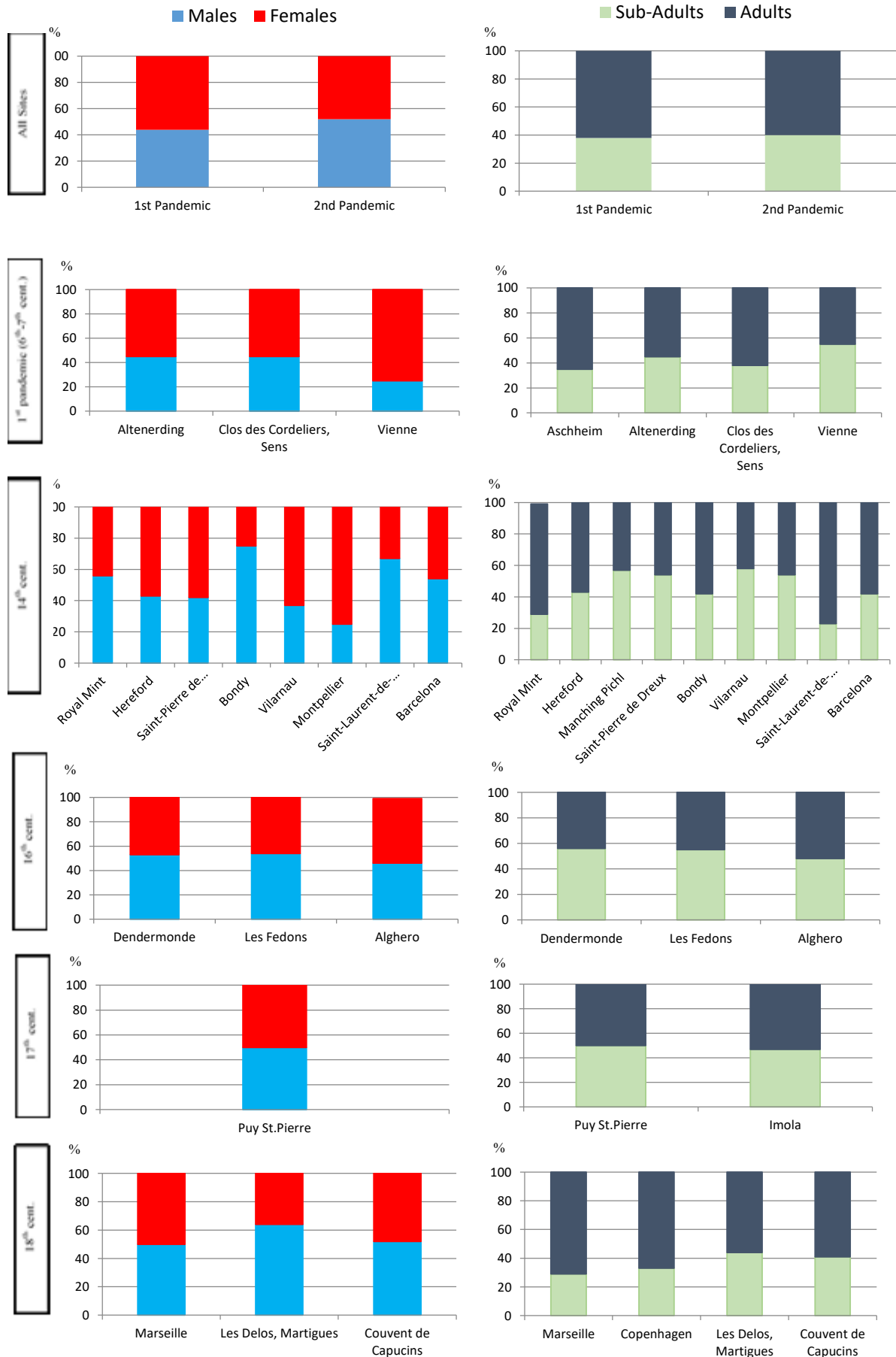
Figure 2.2: Frequencies % of sex and age at death of plague's victims. Absolute values can be found in Table 1.1

Historical sources indicate that plague arrived from the East in the Mediterranean area and then spread northwest across Europe. We attempted a comparison between the sites of the Mediterranean area and the northern sites in both pandemics, to see if there was a difference in the mortality for plague, possibly due to climatic and ecological differences. Concerning the First Plague Pandemic, there was no statistically significant difference (p=0.2041) between the southern sites and the northern ones, regarding the presence of adults and sub-adults in the samples.

Furthermore, we observed a slightly higher frequency of females both in the northern and southern region. Yet, the difference in the incidence of male and female individuals between the two geographic areas was not significant (p= 0.4460). It should be considered anyway that we had very few samples of the First Pandemic. Concerning the Second Pandemic, the difference in the frequency of adults and sub-adults (p= 0.9386), and males and females (p= 0.0866) between the samples from northern Europe and the Mediterranean area was not relevant as well, although, in the north European samples, the frequency of males was higher than that of females (Fig. 2.3).
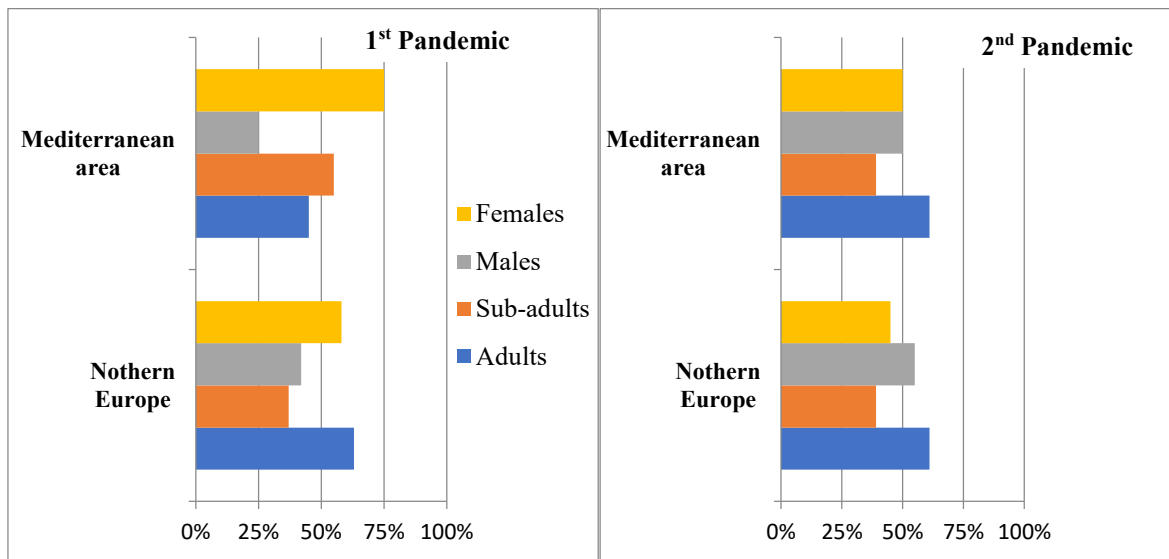

Figure 2.3: Frequencies in percentage of adults and subadults, males and females of the First and Second Pandemic, subdivided into two geographic areas.

2.4.2   Skeletal Biomarkers of Stress

Since plague does not leave visible signs on the skeletons, the traces left on the bones are due to other physiological or pathological stress conditions during life and can give us a view of the health status of the victims. Perhaps, for this reason, only very few researches have considered and described osseous lesions of plague victims. Some of the authors (Dutour et al., 1994; Waldron, 2001) gave only a general description of skeletal markers, the majority of which were linked to natural ageing processes in adults. Alternatively, anthropologists provided a general description of the health status of the victims in a narrative form. In Table 1.2, we reported about the skeletal markers of stress retrieved in some studies. The only data regarding the First Pandemic in Europe were from the site

of Aschheim (D), for which we had data on CO and LEH. CO and PH are usually associated with episodes of anemia, both genetic or acquired (Ortner, 2003; Walker et al., 2009), as we have discussed in the first chapter. The frequency of CO was very heterogeneous, with low percentages in the site of Ashheim and all the samples of the Second Pandemic (less than 30%), apart from the samples from Dreux and Maria Troon. In these last burial sites, CO was present almost in 50% of the skeletons. Concerning PH, it was present in less than 30% of the individuals in all the samples, apart from the Royal Mint's one (90 % of the individuals presented PH).

The analysis of teeth can also furnish interesting information: Enamel Hypoplasia is, for instance, an unspecific stress marker that denotes malnutrition during childhood. In the sample of Aschheim, LEH was less frequent than in the individuals from the following centuries, in which LEH was generally present in higher frequencies, particularly in the British samples. It is well known that before the Black Death, in 1315-17 a great famine occurred in all Northern Europe; thus, it is not surprising that plague victims from this area showed signs of malnutrition during infancy and childhood. There were also other skeletal lesions commonly related to the poor health status of the individuals, like Harris lines in subadults, and periosteal reactions, mainly in adults. Harris lines are the result of slowing or total arrest of the growth of a long bone at the cartilage plate; they appear radiographically as horizontal lines or bands of increased radiopacity, mostly in the metaphysis of the long bones. Like Enamel Hypoplasia, a high prevalence of Harris lines in a population is evidence for the exposure to an elevated level of stress during childhood. The incidence of Harris lines was higher than 50% in Maria Troon's sample and the sample from southern France (Marseilles), while among the victims of Copenhagen, the frequency was lower.

Non-specific periostitis occurs when the inner surface of the periosteum reacts to trauma or infections by forming new woven bone. It can be in an acute or a chronic form. In all the samples in which periostitis was analysed, the frequency of this skeletal marker was between 30% and 40%, with the exception of Puy St. Pierre (18%). Short stature in respect of the mean stature of the population is also considered as a skeletal marker of biological stress. Though, only the study carried out on the sample of the Royal Mint, London (Dewitte and Slavin, 2013), took this marker into account and annotated the frequency of individuals with a femoral length shorter than the average observed in the sample. Another study (Dewitte, 2012) of the Royal Mint, London, also considered periodontal disease in adult individuals. Periodontitis is a common chronic disease in living populations, and it is associated with an increased risk of mortality (Ajwani et al., 2003; Ogden, 2008; DeWitte and Bekvalac, 2010; Dewitte, 2012; Marklein et al., 2016).

In his Doctoral thesis, Dr Sacha Kacki (2016) also considered endocranial remodelling as a skeletal marker of the individual health status, following the suggestion of other scholars (Lewis, 2004). Endocranial remodelling is symptomatic of meningeal inflammation. The retrieved frequencies were less than 20% in all the samples considered.

Comparing the sites for skeletal markers of biological stress, we observed that, regarding the frequency of periostitis, there was no statistically significant difference across the samples ($p=$ 0.1966), whereas all the other skeletal markers frequencies showed high variability among the different samples (Fig. 2.4). The difference in frequency was, in fact, statistically significant for every skeletal marker: PH ($p < 0.0001$), for which the London sample differs significantly from the others, showing the highest frequency; CO ($p= 0.0004$); LEH ($p < 0.0001$); Harris lines ($p< 0.0001$) and endocranial remodeling ($p= 0.0528$).

Table 2.2- Studies on skeletal remains of plague victims: skeletal bio-markers

| Geographic area	Site	Period	Sample n.	Low Stature	Cribra Orbit.	Porotic Hyperost.	Hypopl.	Harris lines	Periostitis	Endocr. Remodelling	Osteoarthritis	Co-morbidities %	Ref.
Southern Germany	Aschheim	5th-7th cent.	77		12/77		7/77						(Staskiewicz, 2007)
UK	Royal Mint, East Smithfield, London	14th	491	A 5/44	A 17/93	A 85/94	A 74/98					Periodontal disease: 80/81	(Dewitte, 2012; Dewitte and Slavin, 2013)
UK	Royal Mint, East Smithfield, London	1348-1349	600								15-45+: M=(29.9%) F= (19.4%)		(Waldron, 1992)
UK	Hereford	14th	185		A 4/31 S 16/38	A 12/36 S 7/50	A 33/37 S 39/48		A 32/78 S 6/40	2/88			(Kacki, 2016)
Northern France	Sain-Pierre à Dreux (eure-et-Loire)	14th	69		A 5/16 S 10/16	A 2/40 S 6/40	A 9/16 S 9/15		A 8/16 S 4/19	A 1/13 S 5/30			(Kacki, 2016)
Spain	Basilica of Saint-Just et Pastor, Barcelona	14th	120		A 1/9 S 2/10	A 1/11 S 1/13	A 4/9 S 9/15					Caries: 2/9	(Kacki and Castex, 2014)
Belgium	Maria Troon, Dendermonde	16th	99		A 9/25 S 12/23	A 8/30 S 7/29	A 15/20 S 24/30	36/69	A 8/24 S 16/27	A 4/24 S 2/21			(Boucherie et al., 2016; Kacki, 2016)
Southern France	Les Fedons, Lambesc	16th	133		A 9/45 S 13/30		A 42/55 S 34/53			S 1/34			(Kacki, 2016)
Southern France	Puy St. Pierre, Lariey	1629-1630	34				A 1/17 S 2/17		A 2/17 S 4/17			TBC: 1/34	(Ardagna et al., 2012)
Northern Italy	Osservanza, Imola	1629-1630	92				51/92					Caries: 65% Calculus: 53% Abscess:9%	(Rinaldo et al., 2014)
Denmark	Copenhagen	1711-1712	24					3/24					(Fiscella et al., 2008)
Southern France	Delos, Martigues + L'observance, Marseille	1720-1722	113				72/113						(Chaumoitre et al., 2007)
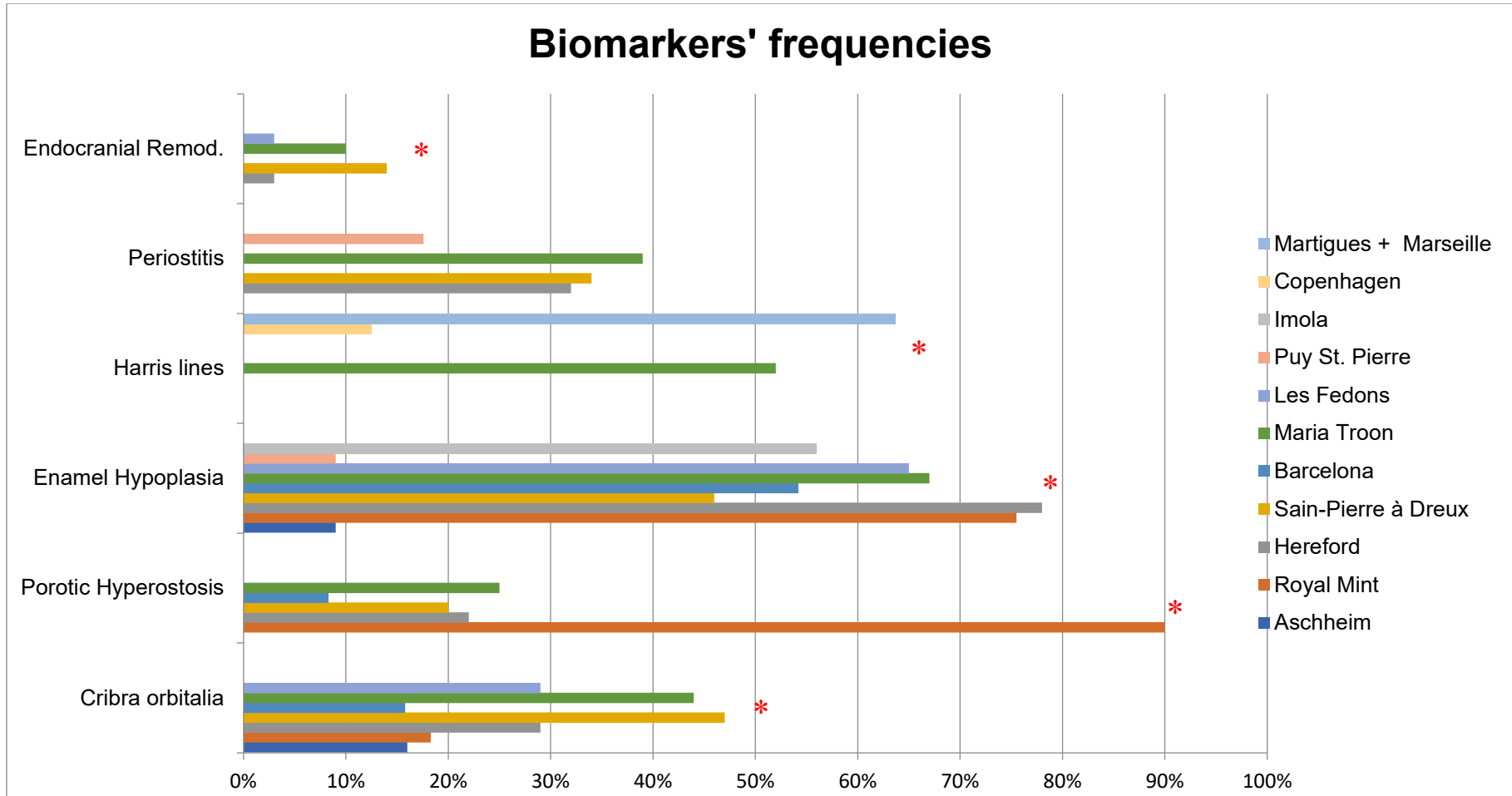
Figure 2.4: Frequencies in percentage of biological stress markers in plague victims.

2.4.3 Predictor models

The linear regression model was used on seven plague sites (Tab. 2.3), for which enough data were provided on the frequency of skeletal biomarkers of stress, mainly limited to LEH's, CO's and PH's frequency. We used Multiple Linear Regression analysis to test for the best predictor among the three indicators of health in adults, using the sex-ratio of each site as a continuous dependent variable. The different outcomes of the analyses are listed in Table 2.4.

All the independent variables showed a strong statistical correlation with the sex-ratio. Among these, Cribra Orbitalia showed a low association with the sex ratio ($\beta = 0.163$), while porotic hyperostosis showed the highest linear association in this model.

LEH exhibited values lower than those of hyperostosis; however, the association was reversed. The negative value ($\beta = -0.260$) indicates that sex-ratio increased in the population with a low level of hypoplasia. In other words, there were more males among plague victims with a lower stress level. The overall variance of the sex-ratio explained by the model was 83%, which means that the association with the skeletal markers was very high.

Although there was a significantly high level of correlation between all independent variables and the sex-ratio, LEH was the only variable that has been reported in the 7 studies under consideration; therefore, we decided to use it as a proxy for health status in the second model. Moreover, PH, that was present with a high frequency (especially at the sites from the UK) may have been attributed to a misinterpretation of the normal microporosity of the outer table of the cranial vault. For sure, there were different levels of severity both in PH and in CO (see chapter 3 of this work), but neither the severity nor the possible level of healing has been considered in the plague studies.

Boldsen (Boldsen, 2007) has observed that "individuals who experienced childhood stress, as evidenced by LEH, had an increased risk of death at all ages for both men and women". Thus, we tested whether in plague populations with a low state of health represented by high levels of LEH more males died from plague, employing a second model with the addition of another independent variable (latitude) and a categorical one (period) (Tab. 2.4). In this second model, the association between sex-ratio and LEH remained negative, indicating that, in plague victims' assembles with lower frailty, males were more represented, although the reduction in sex-ratio for each unit of increase in hypoplasia was low (0.0104). Latitude, which could be related to different environments in Europe, played a better role in this model, showing that more males died of plague in the North: the ratio between the sexes increases by a value of 0.0229 for each unit of latitude increase. However, the greatest effect was due to the change of period: comparing the 16th century with the 14th century, we found an increase in the sex-ratio, similar to the comparison between the 16th century and 17th century (sex-ratio parameter = 0.3022 and -0.5107, respectively). Time fluctuation was not strongly associated with the general health status of the victims, at least using LEH as a proxy. In other words, there was no general trend which could be evinced in respect to sex-ratio and other variables based on the results of our linear multiple regression models. Even if all the independent variables were

statistically associated with the sex-ratio, the variance explained by the models was low (13%). The difficulties in establishing a general trend of association between LEH and the sex-ratio in the data are evident in Fig. 2.5, where we plot the two variables from the different sites for graphical comparison. The multiple linear regression models used here proved to be accurate, no anomalous values were found (see the graphs of the normal probabilities of the residuals in Figure S2.1). The correlation between the variables was statistically significant, but they did not show any high linear association, even if classified in periods and considering their latitude. In other words, based on the data published so far, it is impossible to generally support the hypothesis that plague negatively selected males in poor health status (LEH took as a proxy) or killed more females in a healthier population as stated previously (DeWitte, 2010). Not even in England, where the phenomenon was observed by DeWitte (2010), is visible a coherent tendency. If for London, our analysis (see Fig. 2.5) confirmed the trend previously reported, the opposite trend is noticeable for Hereford, which most likely was also hit by the Black Death (Haensch et al., 2010).

Table 2.3- Frequency of biomarkers of stress in adult victims of plague from different archaeological sites, periods and latitudes.

Site	Period	Latitude	Sample (sexed adults)	Sex ratio	Freq. of Hypoplasia	Freq. of Cribra Orbitalia	Freq. of Hyperostosis
St. Pierre de Dreux	14th c.	48.737112	26	0.73	56.2	31.3	17.6
Barcelona	14th c.	41.386527	13	1.17	44.4	11.1	9.1
Royal Mint	1348-49	51.50267	377	1.26	75.5	18.3	90.0
Hereford	14th c.	52.055908	92	0.77	89.0	13.0	33.3
Les Fedons	16th c.	43.655419	56	1.15	76.4	20.0	
Maria Troon	16th c.	51.025078	38	1.11	75.0	36.0	24.1
Puy St. Pierre	1629-30	44.53332	16	1.00	5.9		

Table 2.4- Results of Multiple Linear Regression Model

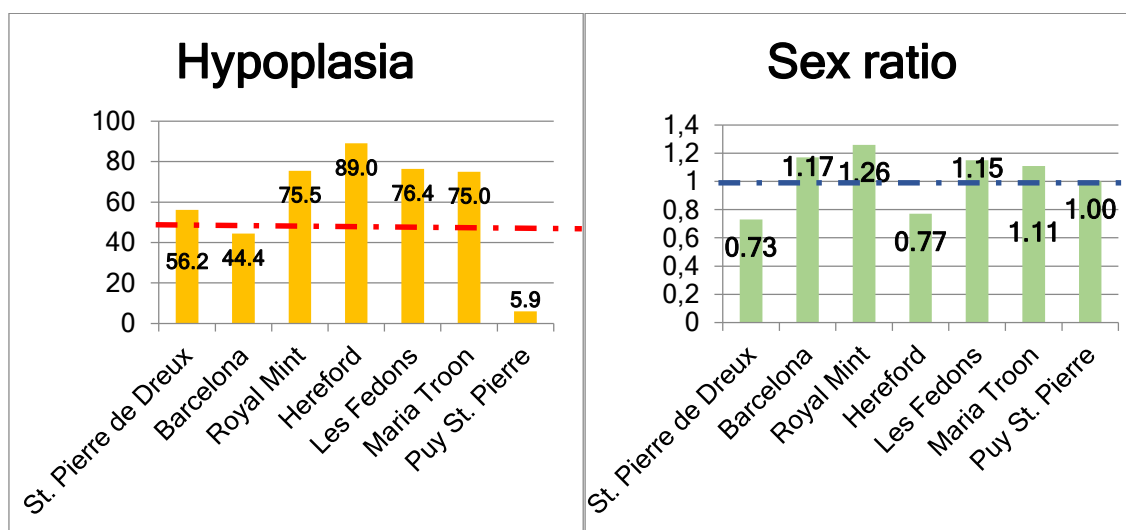| Predictor variable	Model 1			Model 2		
	β	t	*p* value	β	t	*p* value
Cribra orbitalia	0.163	8.178	<0.001			
Porotic Hyperostosis	0.895	48.728	<0.001			
Hypoplasia	-0.260	-13.472	<0.001	-0.741	-9.283	<0.001
Latitude				0.339	5.453	<0.001
Period						
16th c. (Period 1)				1.141	8.816	<0.001
1630 (Period 2)				-1.231	-9.209	<0.001
R²	0.831			0.135		
Adjusted *R²*	0.830			0.130		
***p* value**	<0.001			<0.001		

Fig. 2.5 Graphical comparison of LEH-frequencies and sex-ratio in the same populations of plague victims.

2.5 DISCUSSION AND CONCLUSION

From our research, it seems that plague mortality was not oriented in selecting a specific group; in fact, the distribution in age and sex of the victims was very heterogeneous. Adults were generally the most represented group, while sex-ratio was heterogeneous. The only real constant was the presence in almost all the sites of a peak of mortality around 20-35 years of age and a second peak of children between 5-10 years of age. Similarly, in epidemiological studies carried out on plague patients during the Third Pandemic in India, it was reported that the age range most exposed to risk was that between 20 and 35 years, and that in adult age plague seems to be more virulent (Nathan, 1898). A possible explanation for the observed phenomenon might be higher exposure levels, possibly due to work activities, and thus a higher mortality risk for this class of age. Unfortunately, it was not possible to investigate this hypothesis statistically due to the lack of detailed historical information.

Moreover, the methodological differences in age estimation and the age class groups identified at each site differed consistently. It is crucial that the methods used for the anthropological analysis and the age classes are the same, and thus comparable. Maybe using raw data, it will be possible to see if there was effectively a selection on the age of the victims. Moreover, having data on the original composition of the population would be a significant additional element.

Regarding sex, apart from a slightly more prominent presence of women among the victims of the First Pandemic, we couldn't find any statistically significant difference, as well as no univocity, with a sex constantly prevalent on the other. Even the predictive models couldn't identify a common pattern for plague mortality: using LEH as a proxy for frailty, we didn't saw any clear trend of sex-selectivity across different periods and geographic regions. Latitude seemed to play a significant role in the model, showing that more males died in northern Europe, but for the First Pandemic (Fig. 1.3) the opposite is true. Some demographic studies supported the hypothesis that male individuals were more liable to plague if they lived during times of high stress (Macintyre, 2002; Curtis and Roosen, 2017), others state that plague had operated a sex-selection against females (Perez Moreda, 1987; Ell, 1989; Signoli et al., 2002; Zapnik, 2007; Frandsen, 2010; Curtis and Roosen, 2017): from our dataset and statistical analysis we couldn't support any of the hypotheses. No trend of sex-bias is visible in the study of anthropological data from different sites and epochs.

We can, therefore, state that, from the anthropological data published until 2016, it is not possible to reveal a general trend in plague's mortality in regard to the sex of the victims and their health status, a piece of evidence which seems to corroborate the hypothesis that plague killed indiscriminately. However, it should be considered that the data have some bias: first of all, frailty was assessed in the model using LEH as a proxy, because it was the skeletal biomarker of stress investigated on more samples. It is, therefore, necessary to investigate more skeletal markers of stress on plague victims, to see if there was no pattern in *Y. pestis*'s selectivity. The most important biomarkers to detect may be those connected to anemia, given that iron is an essential player in the immunological reaction to infectious diseases.

Secondly, many plague sites are still to be found, and the lack of representative samples from different countries could be an important bias.

Thirdly, heterogeneity in sex and age distribution of the plague victims in the single sites can be a product of population composition or a reflex of cultural aspects in burying practices.

Further research and the standardisation of the anthropological analysis, in particular, concerning the investigation of skeletal stress biomarkers for the estimation of frailty, will be necessary to exhaustively answer our questions about plague mortality.

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2.7 SUPPLEMENTARY

Table S2.1: Studies on skeletal remains of plague victims

Author	Site	Country	Period		n° ind.	Basis of Plague determination			Anthropological studies				note
			Date	C^{14}		DNA	Immun.	Historical texts	sex	age	pathologies	Biological Stress markers	
Staskiewiz 2007	Aschheim	Germany	5th-7th cent.	530-630 (Seifert, 2014)	77	✓ (Feldman et al., 2016)			✓	✓	✓		
Seifert 2014 PhD thesis	Aschheim	Germany	5th-7th cent.	530-630	19	✓ (Feldman et al., 2016)			✓	✓			
Helmuth and Ankner, 1996	Altenerding	Germany	5th-7th cent.		20	✓ (Feldman et al., 2016)			✓	✓			
Castex, 2007	Le Clos des Cordeliers, Sens, Yonne	France	5th-6th cent.		68	✓ (Drancourt et al., 2004)	✓ (Malou et al., 2012)			✓			Graph with mortality rates
Castex, 2008	Le Clos des Cordeliers, Sens, Yonne	France	5th-6th cent.		73	✓ (Drancourt et al., 2004)	✓ (Malou et al., 2012)		✓	✓			Graph with mortality rates
Castex, Kacki, 2016	Le Clos des Cordeliers, Sens, Yonne	France	5th-6th cent.		73	✓ (Drancourt et al., 2004)	✓ (Malou et al., 2012)		✓	✓			
Castex, Kacki, 2016	Poitiers, Poitou-Charentes	France	5th-6th cent.		53								
Signoli et al. 2009	Place Camille Jouffray, Vienne (Isère)	France	760-880	610-1040	11	✓ (Drancourt et al., 2007)	✓ (Bianucci et al., 2008)		✓	✓			
Rubini 1991	Castro dei Volsci, Frosinone	Italy	6th cent.		148				✓	✓	✓	✓	
Rubini et al. 2016	Castro dei Volsci, Frosinone	Italy	6th cent.		179	✓			✓	✓			
Waldron 1992	Royal Mint, East Smithfield, London	UK	1348		600	✓ (Bos et al., 2011)			✓	✓	✓		

Author	Site	Country	Period		n° ind.	Burial Type*	Anthropological studies	note	Included				
			Date	C^{14}			DNA	Immun.	Historical texts	sex	age	pathologies	Biological Stress markers	
Waldron 2001	Royal Mint, East Smithfield, London	UK	1348		600		✓ (Bos et al., 2011)			✓	✓	✓		
Gowland and Chamberlain, 2005	Royal Mint, East Smithfield, London	UK	1348		600		✓ (Bos et al., 2011)				✓			Graph with percentage of age classes
DeWitte, 2006 PhD thesis	Royal Mint, East Smithfield, London	UK	14th cent.		491		✓ (Bos et al., 2011)			✓	✓	✓	✓	
DeWitte, 2010	Royal Mint, East Smithfield, London	UK	14th cent.		299		✓ (Bos et al., 2011)			✓		✓	✓	
DeWitte, 2010b	Royal Mint, East Smithfield, London	UK	14th cent.		337		✓ (Bos et al., 2011)				✓			Graph with percentage of age classes
De Witte 2012	Royal Mint, East Smithfield, London	UK	14th cent.		161		✓ (Bos et al., 2011)				✓	✓		analysis of periodontal diseases
DeWitte and Hughes-Morey, 2012	Royal Mint, East Smithfield, London	UK	14th cent.		127		✓ (Bos et al., 2011)			✓			✓	
De Witte, Slavin 2013	Royal Mint, East Smithfield, London	UK	14th cent.		491		✓ (Bos et al., 2011)						✓	
Rubini et al. 2016	Royal Mint, East Smithfield, London	UK	1348-1350		636		✓ (Bos et al., 2011)				✓			Graph with percentage of age classes
Grainger and Phillpotts, 2011	St Mary Grace Abbey, East Smithfield, London	UK	1350-1400		199		✓ (Bos et al., 2011; Feldman et al., 2016)			✓	✓	✓	✓	
Kacki 2016 PhD thesis	Hereford cathedral	UK	14th cent.		185		✓ (Haensch et al., 2010)	✓ (Haensch et al., 2010)		✓	✓	✓	✓	
Castex, Kacki, 2016	Hereford cathedral	UK	14th cent.		185		✓ (Haensch et al., 2010)	✓ (Haensch et al., 2010)		✓	✓			
Prechel 1996	Lubeck	Germany	14th cent.	1260-1390	671					✓	✓			

Author	Site	Country	Period		n° ind.	Basis of Plague determination			Anthropological studies				note
			Date	C^{14}		DNA	Immun.	Historical texts	sex	age	pathologies	Biological Stress marker	
Lütgert, 2000	Lubeck	Germany	14th cent.	1260-1390	816				✓	✓			not all are plague's victims
Wiechmann et al. 2010	Manchin Pichl, Ingolstadt	Germany	1250-1500		6	✓ (Wiechmann et al., 2010)			✓	✓			
Seifert 2014 PhD thesis	Manchin Pichl, Ingolstadt	Germany	1250-1500		21	✓ (Wiechmann et al., 2010)			✓	✓			
Castex 2007	Saint-Pierre à Dreux	France	14th cent.		2	✓ (Drancourt et al., 2004)				✓			Graph with mortality rates
Castex 2008	Saint-Pierre à Dreux	France	14th cent.		72	✓ (Drancourt et al., 2004)			✓	✓			Graph with mortality rates
Castex, Kacki, 2016	Saint-Pierre à Dreux	France	14th cent.		72	✓ (Drancourt et al., 2004)			✓	✓			
Kacki 2016 PhD thesis	Sain-Pierre à Dreux	France	14th cent.		69	✓ (Drancourt et al., 2004)			✓	✓	✓	✓	
Passarius et al. 2008	Vilarnau	France	14th cent.		19	✓			✓	✓			
Le Forestier 2012	Bondy	France		1297-1373	12	✓ (Tran et al., 2011a)	✓ (Malou et al., 2012)		✓				
Kacki et al., 2011	Saint-Laurent-de-la-Cabrerisse	France	14th cent.	1269-1409	9	✓ (Haensch et al., 2010)	✓		✓	✓			
Crubezy et al. 2006	Saint Come et Damien, Montpellier	France	14th cent.		13	✓ (Raoult et al., 2000)			✓	✓			
Kacki, Castex 2004	Basilica of Saint Just et Pastor, Barcelona	Spain	14th cent.		120	✓ (Spyrou et al., 2016)			✓	✓	✓	✓	
Castex,Kacki, 2016	Basilica of Saint Just et Pastor, Barcelona	Spain	14th cent.		120	✓ (Spyrou et al., 2016)			✓	✓			

Author	Site	Country	Period		n° ind.	Basis of Plague determination			Anthropological studies				note
			Date	C^{14}		DNA	Immun.	Historical texts	sex	age	pathologies	Biological Stress marker	
Gambaro et al. 2001	Lazzaretto Vecchio, Venice	Italy	14th-17th cent.		331	✓ (Tran et al., 2011b)	✓ (Malou et al., 2012)	✓		✓			
Signoli et al., 2008	Lazzaretto Vecchio, Venice	Italy	1478-1486		184	✓ (Tran et al., 2011b)	✓ (Malou et al., 2012)	✓		✓			
Bizot 2005	Les Fedons, Lambesc	France	16th cent.		133	✓ (Drancourt et al., 1998)	✓ (Bianucci et al., 2008)	✓	✓	✓			
Castex 2007	Les Fedons, Lambesc	France	16th cent.		133	✓ (Drancourt et al., 1998)	✓ (Bianucci et al., 2008)	✓ (Bizot, 2005)		✓			Graph with mortality rates
Castex 2008	Les Fedons, Lambesc	France	16th cent.		133	✓ (Drancourt et al., 1998)	✓ (Bianucci et al., 2008)	✓ (Bizot, 2005)	✓	✓			Graph with mortality rates
Bianucci et al. 2008	Les Fedons, Lambesc	France	1590		133	✓ (Drancourt et al., 1998)	✓	✓ (Bizot, 2005)	✓				
Kacki 2016 PhD thesis	Les Fedons, Lambesc	France	16th cent.		133	✓ (Drancourt et al., 1998)	✓ (Bianucci et al., 2008)	✓ (Bizot, 2005)	✓	✓	✓	✓	
Castex, Kacki, 2016	Les Fedons, Lambesc	France	16th cent.		133	✓ (Drancourt et al., 1998)	✓ (Bianucci et al., 2008)	✓ (Bizot, 2005)	✓	✓			
Bianucci et al., 2009	Sainte-Croix Abbey, Poitiers	France	After 1524		6		✓		✓	✓			
Boucherie et al. 2016	Maria Troon, Dendermonde	Belgium	1579-1584	End 16th-early 17thcent.	99			✓ (Goudie Falkenbach E; Ryssaert C, Brion M, Castex D, Rouzic M, Colombo A, 2012)	✓	✓		✓	
Kacki 2016 PhD thesis	Maria Troon, Dendermonde	Belgium	16th cent.		99			✓ (Goudie Falkenbach et al., 2012)	✓	✓	✓	✓	

Author	Site	Country	Period		n° ind.	Basis of Plague determination			Anthropological studies				note
			Date	C¹⁴		DNA	Immun.	Historical texts	sex	age	pathologies	Biological Stress marker	
Castex, Kacki, 2016	Maria Troon, Dendermonde	Belgium	16th cent.		99			✓ (Goudie Falkenbach et al., 2012)	✓	✓			
Milanese, 2010	Lo Quarter, Alghero	Italy	1582-1583		185		✓ (Bianucci et al., 2012)		✓	✓			
Bianucci et al. 2012	Lo Quarter, Alghero	Italy	1582-1583		10		✓		✓	✓			
Seifert 2014 PhD thesis	Brandenburg	Germany	1618–1648		9	✓			✓	✓			
Signoli et al. 2007	Puy St. Pierre, Lariey	France	17th cent.		34		✓ (Bianucci et al., 2008)		✓	✓			
Bianucci et al. 2008	Puy St. Pierre, Lariey	France	1628-1632		34		✓		✓	✓			
Ardagna et al. 2012	Puy St. Pierre, Lariey	France	1629-1630		34		✓ (Bianucci et al., 2008)		✓	✓	✓	✓	
Bianucci et al. 2008	La Butte aux Herbes, Draguignan	France	1649-1650		8	✓	✓		✓	✓			
Bianucci et al. 2009	La Chaize-le-Vicomte	France	1600-1700		6		✓		✓	✓			
Hadjouis et al., 2006	Saint-Maurice, Charenton le Pout	France	17th cent.		3	✓				✓	✓	✓	
Castex 2007	St. Benedict, Prague	Czech Republic	17th cent.		120					✓			Graph with mortality rates
Castex et al., 2012	St. Benedict, Prague	Czech Republic	17th cent.		95				✓	✓			Graph with mortality rates
Rinaldo et al. 2014	Osservanza, Imola	Italy	1629-1630		92		✓ (Rubini et al., 2016)	✓ (Cervellati, 1986)	✓	✓	✓	✓	Only dental pathologies
Rubini et al. 2016	Osservanza, Imola	Italy	1629-1630		114		✓ (Rubini et al., 2016)	✓ (Cervellati, 1986)		✓			
Caruso et al., 2013	Viale Sabotino, Milan	Italy	17th cent.		240				✓	✓	✓	✓	

Author	Site	Country	Period		n° ind.	Basis of Plague determination			Anthropological studies				note
			Date	C14		DNA	Immun.	Historical texts	sex	age	pathologies	Biological Stress marker	
Signoli and Dutour, 1998	Rue Leca, l'Observance, Marseille	France	1720-1722		303	✓ (Bos et al., 2016b)	✓ (Bianucci et al., 2008)	✓ (Dutour et al., 1994)		✓			
Bello et al. 2006	l'Observance Marseille	France	1722		179	✓ (Bos et al., 2016b)	✓ (Bianucci et al., 2008)	✓ (Dutour et al., 1994)	✓	✓			
Bianucci et al. 2008	l'Observance Marseille	France	1722		172	✓ (Bos et al., 2016b)	✓	✓ (Dutour et al., 1994)					
Fiscella et al. 2008	Copenhagen	Denmark	1711-1712		24			✓		✓		✓	
Dutour et al. 1994	Rue Leca, Marseille	France	1720-1722		22	✓ (Bos et al., 2016b)	✓ (Bianucci et al., 2008)	✓	✓	✓			
Signoli and Dutour, 1997	Rue Leca, l'Observance, Marseille	France	1720-1722		22	✓ (Bos et al., 2016b)	✓ (Bianucci et al., 2008)	✓ (Dutour et al., 1994)	✓	✓			
Signoli et al., 2002	l'Observance Marseille	France	1720			✓ (Bos et al., 2016b)	✓ (Bianucci et al., 2008)	✓ (Dutour et al., 1994)		✓			Graph with percentage of age classes
Signoli, 2008	l'Observance Marseille	France	1720		216	✓ (Bos et al., 2016b)	✓ (Bianucci et al., 2008)	✓ (Dutour et al., 1994)		✓			Graph with percentage of age classes
Chaumoitre et al. 2007	Delos, Martigues + L'observance, Marseille	France	1720-1722		113	✓ (Bos et al., 2016b)	✓ (Bianucci et al., 2008)	✓ (Dutour et al., 1994; Signoli et al., 1995)		✓		✓	
Signoli et al., 1995	Le Delos, Martigues	France	1720-1722		39		✓ (Bianucci et al., 2008)	✓ (Signoli et al., 1995)	✓	✓			
Bianucci et al. 2008	Le Delos, Martigues	France	1720-1722		39		✓ (Bianucci et al., 2008)	✓ (Signoli et al., 1995)	✓	✓			
Bianucci et al. 2008	Le Couvent des Capucins de Ferrieres, Martigues	France	1720-1722		210	✓ (Drancourt et al., 2007)	✓	✓ (Tzortzis and Signoli, 2009)					
Tzortzis, Signoli 2009	Le Couvent des Capucins de Ferrieres, Martigues	France	1720-1722		208	✓ (Drancourt et al., 2007)	✓ (Bianucci et al., 2008)	✓	✓	✓			
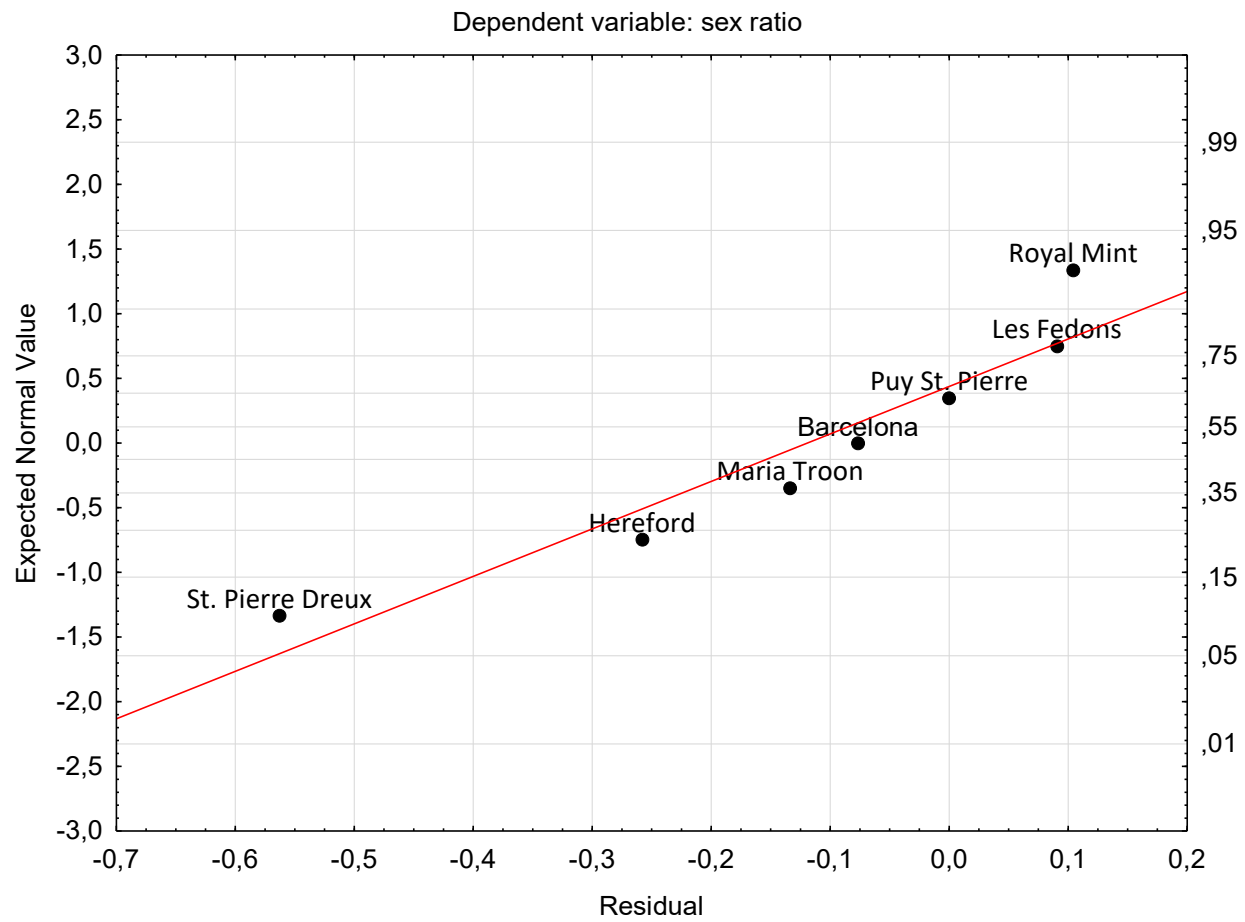
Figure S2.1. Normal Probability Plot of Raw Residuals

CRIBRA ORBITALIA AND POROTIC HYPEROSTOSIS: METHODS OF EVALUATION

Porous lesions of the skull (Porotic Hyperostosis, PH) and of the orbits (Cribra Orbitalia, CO) are generally recognised as an indicator of anemia, either genetic or acquired, but their etiology is still debated. Although they are among the lesions more retrieved in skeletal remains of individuals from the past, there are still no internationally accepted guidelines for their scoring. Moreover, not only the presence but also both the degree of healing and severity should be recorded when analysing these lesions.

Here we propose two new evaluation forms, one for CO and one for PH, that consider all the aspects that may be informative for the analysis of porotic lesions. Besides, we propose an original quantitative method for the analysis of the frequency of the pores. We have analysed the inter and intra-observer error of both qualitative and quantitative analysis of the lesions and demonstrated a good level of agreement.

These new guidelines could be essential in the future analysis of anemia in the past and to discern the exact etiology of the porous lesions.

3.1   INTRODUCTION

Anemia is one of the most common blood diseases, and today it affects 24.8% of the global population (de Benoist, Buno; McLean, Erin; Egil, Ines; Cogswel, 2008). Usually, it is due to iron deficiency, which represents "the most frequent nutritional deficiency in the world" (Poskitt, 2003). Anemia is also one of the most common chronical disease identified in the skeletal remains of past individuals (Poskitt, 2003). Frequently, human skeletal remains show small holes in the outer compact bone of the skull and the surface of the orbits, as well as on the proximal epiphysis of the humerus and femur (Ortner, 2003; Poskitt, 2003). These small holes, called Cribra, were first attributed to Porotic Hyperostosis. The name Porotic Hyperostosis was first used by Angel (1966) to refer to an overgrowth of the spongy marrow of the skull and porous lesions on the vault. This condition was soon related to anemic conditions. Anemia causes disruption of the blood cell production (hematopoiesis) in the hematopoietic centres, mainly in the marrow of the trabecular bone, resulting in overproduction of the blood cells or an increasing of their size (hyperplasia or hypertrophy) and subsequent expansion of the diploe of the cranium. This process results in an irregular remodelling of the outer cranial table, which is reabsorbed with time, becoming thinner and with visible porous lesions, developing, in the most severe cases, in the exposition of the spongy bone of the diploë (Martin and Goodman, 2002; Rivera and Mirazón Lahr, 2017). The exact etiology

or etiologies of these lesions is still debated, as we discussed in the first chapter, but generally they are considered a manifestation of chronic anemia (Martin and Goodman, 2002; Rivera and Mirazón Lahr, 2017) either genetic or acquired, due to iron deficiency (Wapler et al., 2004; Oxenham and Cavill, 2010), vitamin B deficiency (Walker et al., 2009), or to chronic disorders (Rivera & Mirazón Lahr, 2017). Still, porous lesions on the skull or orbit surface can also be induced by other pathological processes, such as those associated with chronic scalp infections and scurvy (Ortner, 2003; Walker et al., 2009). In-deep analysis of the other part of the skeleton or the use of radiological and CT scan analyses can help in distinguish between anemia and other etiologies.

Recently, researchers have highlighted the independent appearance in some cases, of porotic lesions either on the cranial vault or on the orbital roof, concluding that the two forms of Cribra seem to have distinct etiologies (Wapler et al., 2004; Walker et al., 2009; Rothschild, 2012; Rivera and Mirazón Lahr, 2017). This distinction has led to discriminate between 'Porotic Hyperostosis' (PH), term, which is used for the porous lesions of the skull, and 'Cribra Orbitalia' (CO), which indicates the lesions of the orbital bones.

While Cribra are extensively used in anthropological research as an indicator to assess hygienic conditions, health and nutritional status of past populations (Lallo et al., 1977; Mensforth et al., 1978; Facchini et al., 2004; Masson et al., 2015), there is not yet an international standard in assessing PH or CO. Both pathological conditions can be expressed on the skeleton at different degrees of severity. Stuart - Macadam (1985) identifies four degrees of severity, from the least serious (Grade 1) which is represented by scattered fine foramina to the most severe (Grade 4) that presents outgrowth in trabecular structure from the normal contour of the outer bone table; Salvadei et al. (2001) distinguish only three categories; in the Data Collection Codebook (Steckel et al., 2006) four degrees of severity, from 0 to 3, are proposed, while Hengen (1971) describes even 12 categories.

Some scholars also recognise different degrees of lesion's healing or bone remodelling, that can be described as "smooth lamellar texture with new bone filling of the peripheral pores" (Mensforth et al., 1978; Mittler and Van Gerven, 1994). Salvadei et al. (2001) describe four categories of healing for Cribra, ranging from active without any sign of bone remodelling (Grade 1), to fully healed lesions (Grade 4).

All the methods proposed for assessing the degree of Cribra are qualitative, based on descriptions and sometimes on black and white pictures of the different degrees provided by the authors. Thus, the assessment is subjective and depends on the observer experience. A study carried out by Jacobi and Danforth (2002) reports about inter-observer scoring patterns in PH and CO, demonstrating a significant variation among 22 scorers' observations, therefore revealing that the scoring methods available represent an issue. There is a need for a more objective and quantifiable approach to describe Cribra.

We propose a new evaluation form for the assessment of PH and CO that takes in consideration qualitative aspects, degree of severity (Stuart-Macadam, 1985), healing status (Salvadei et al., 2001), but also position of the lesions and dimension of the area affected on the cranial bones and orbital

roofs. The method proposed does not require the completeness of the skull and can also be used on single bones. Moreover, we offer a new quantitative evaluation method that enables to quantify the frequency of pits or holes in 1 cm^2.

We evaluated the reliability and reproducibility of the new recording sheets through statistical analyses.

3.2   THE NEW EVALUATION FORM

Considering the probable different etiology of the two pathological conditions, we developed two scoring sheets, one for PH and one for CO (fig 3.1 and 3.2).

For the analysis of PH, we took into consideration only the individuals with at least one cranial bone preserved on which at least 50% of the external surface was observable. Cribra were recorded on frontal, occipital and parietal bones, and to locate the lesions, every bone considered has been divided into four virtual quarters (Supplementary Figure S3.1). For the entire skull and subsequently for each cranial bone, PH has been evaluated indicating its presence/absence or not observability (P/A/NO), its degree of severity, its degree of healing, the location of the lesions within the quarters, the size of the area with lesions (less or more than 50% of the total area of the cranial bone) and the frequency of the pits/1 cm^2.

As regard to CO, only the skulls with at least one orbital roof preserved (50% observable surface at least) has been taken into consideration. For each individual and each orbital roof, the presence/absence of CO has been recorded, as well as the degree of severity, the degree of healing and the size of the area of the lesion measured directly with a non-stretchable metric tape.

For the assessment of the severity of both PH and CO, we decided to use the scale proposed by Stuart-Macadam (1985), that divided the degree of severity (Fig. 3.3) as:

0: absence of lesions;

1: presence of small, scattered holes;

2: presence of small and large scattered holes;

3: presence of holes that join within the trabecular structure;

4: marked development of the trabecular bone, which protrudes toward the outside surface.

For the degree of healing (Fig. 3.4), a distinction between active and non-active Cribra was made following the definition of Mittler and Van Gerven (1994) and using the classification proposed by Salvadei et al. (2001):

1: Lesions with no healing activity (active);

2: Lesions with a healed area of less than 50%;

3: Lesions with a healed area greater than 50%;

4: Fully healed lesions (non-active).

When different degrees of severity of the lesions coexisted in the cranial bones (for PH) and the right and left orbits (for CO), the general degree assessed for the individual is the higher degree observed. Concerning the degree of healing, the general score is assessed by evaluating the amount of cranial vault or orbital roofs in which healed or healing lesions are present: score 1, if no healing is present in the cranial bones or the orbital roofs, score 2, if healing is started but only for a total area is less than 50%, score 3, if the healing/healed area is more than 50% and finally score 4, if all lesions are completely healed.

In addition to the evaluation of severity and degree of healing, we developed a new technique to evaluate the frequency of the pitting lesions for every bone in a fixed area of 1 cm^2. For this purpose, the observer selected the area of each bone with more pits and took a zenith picture of the selected area after applying a squared photo scale. The scales may be easily reproduced using a flexible material (e.g. paper) and were created to be adapted to the rounded surface of the orbital roof, (Fig.3.5), or of the cranial bones (Fig.3.6). The number of pits for each cranial bone and orbital roof were then counted manually with the aid of the image-processing program ImageJ (https://imagej.net/Welcome).

RECORDING FORM for CRIBRA ORBITALIA

Observer _____   Date_____

Skeleton code_____   Stratigraphic Unit (US)_____

Site_____   Collection_____

GENERAL INFORMATION	
Ancestry: _____	Sex: _____
Age: _____	Stature: _____

INVENTORY (ORBITAL ROOFS) Codes: P – present / A – absent / NO – not observable

RIGHT ORBITAL ROOF _____   LEFT ORBITAL ROOF: ____

ASSESSMENT OF CRIBRA ORBITALIA (PER INDIVIDUAL)

Presence/absence of CO   _____ (Codes: P – present / A – absent)

General degree of severity (0-4)   _____

General degree of healing (1-4)   _____

ASSESSMENT OF CRIBRA ORBITALIA (FOR EACH ORBITAL ROOF)					
RIGHT ORBITAL ROOF			**LEFT ORBITAL ROOF**		
Degree of severity (0-4)			Degree of severity (0-4)		
Degree of healing (1-4)			Degree of healing (1-4)		
Frequency of pits in 1 cm^2	$n=$		Frequency of pits in 1 cm^2	$n=$	
Size of area with CO	Length _____mm	Width _____mm	Size of area with CO	Length _____mm	Width _____mm

Figure 3.1: Recording form for Cribra Orbitalia (Rinaldo et al., 2019).

RECORDING FORM for POROTIC HYPEROSTOSIS

Observer _____   Date_____

Skeleton code_____   Stratigraphic Unit (US)_____

Site_____   Collection_____

GENERAL INFORMATION

Ancestry: _____   Sex: _____

Age: _____   Stature: _____

INVENTORY (CRANIAL BONES) Codes: P – present / A – absent / NO – not observable

FRONTAL: _____   RIGHT PARIETAL: ____

OCCIPITAL: _____   LEFT PARIETAL: ____

ASSESSMENT OF POROTIC HYPEROSTOSIS (PER INDIVIDUAL)

Presence/absence of PH _____ (Codes: P – present / A – absent)

General degree of severity (0-4) _____

General degree of healing (1-4) _____

ASSESSMENT OF POROTIC HYPEROSTOSIS (FOR EACH CRANIAL BONE)

RIGHT PARIETAL			LEFT PARIETAL		
Presence of PH within the quarters (P/A/NO)	2	1	Presence of PH within the quarters (P/A/NO)	1	2
	4	3		3	4
Degree of severity (0-4)			Degree of severity (0-4)		
Degree of healing (1-4)			Degree of healing (1-4)		
Frequency of pits in 1 cm^2	*n=*		Frequency of pits in 1 cm^2	*n=*	
Size of the area affected by PH	<50%	>50%	Size of the area affected by PH	< 50%	>50%
FRONTAL			**OCCIPITAL**		
Presence of PH within the quarters (P/A/NO)	1	2	Presence of PH within the quarters (P/A/NO)	1	2
	3	4		3	4
Degree of severity (0-4)			Degree of severity (0-4)		
Degree of healing (1-4)			Degree of healing (1-4)		
Frequency of pits in 1 cm^2	*n=*		Frequency of pits in 1 cm^2	*n=*	
Size of the area affected by PH	<50%	>50%	Size of the area affected by PH	< 50%	>50%

Figure 3.2: Recording form for Porotic Hyperostosis (Rinaldo et al., 2019).

POROTIC HYPEROSTOSIS
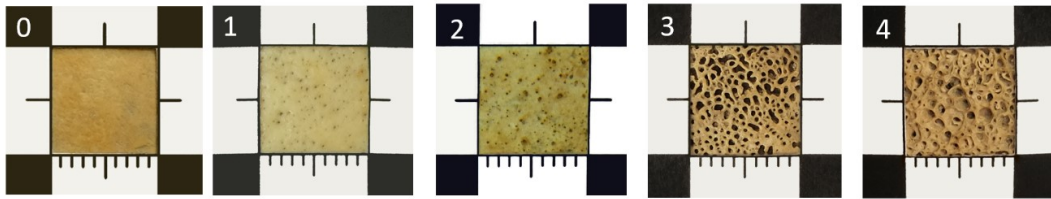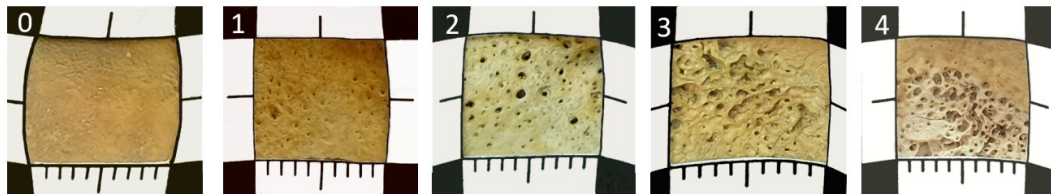
CRIBRA ORBITALIA

Figure 3.3: Degrees of severity from 0 absent to degree 4: for PH and CO (Rinaldo et al., 2019).

POROTIC HYPEROSTOSIS
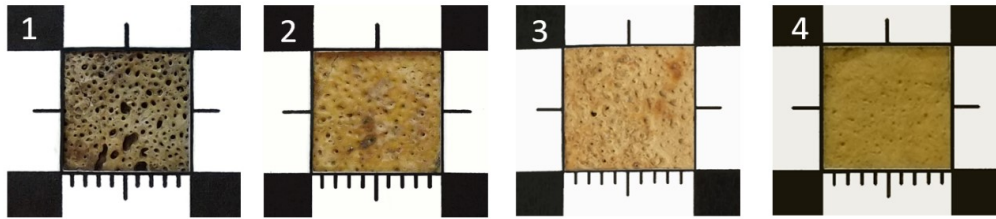
CRIBRA ORBITALIA
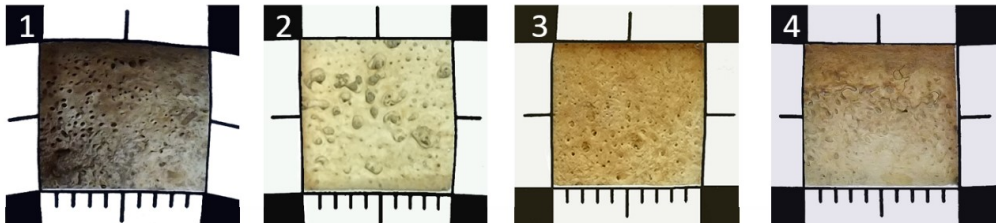
Figure 3.4: Degrees of healing from 1 active to degree 4 healed: for PH and CO (Rinaldo et al., 2019).

Figure 3.5: One cm^2 scale for the orbital roofs (Rinaldo et al., 2019).
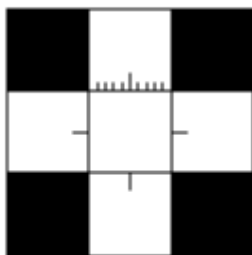
Figure 3.6: One cm^2 scale for the cranial vault (Rinaldo et al., 2019).

3.3 MATERIAL AND METHODS

3.3.1 Material

For the development of the new methodology, we examined skeletal remains of different archaeological sites and periods, covering a large variability of cases. All the skeletal series came from excavations at Imola (North-East Italy): the mass grave of the Imola's Lazzaretto "L'Osservanza", with victims of the plague epidemic of 1630 and 1632; single burials from "L'Osservanza" dated to the Late antiquity; single burials from the site of "Via Emilia" from the 7th-9th c. (Lombard Period), and from the mass graves of "Via Maghinardo" a site of the 14th century. Complete or partially complete skeletons were selected for this study for a total of 189 bones, of which 135 bones of the cranial vault and 54 orbital roofs. For the methodological purpose of this study, we decided to consider both sexes and all the classes of age at death.

3.3.2 Statistical Analysis

We tested the intra-observer and inter-observer variability for every variable considered, both qualitative, (degrees of severity and healing, position and size of the area with Cribra), and quantitative (number of pits in 1 cm^2). For each of the selected individuals, both scoring sheets (for PH and CO) were independently completed by two trained observers. Intra-observer reliability was tested between two different observations of the same operator (A1 and A2), while the inter-observer reliability was tested by comparing the results of the two different observers (A1 and B). For the qualitative variables weighted (degree of severity and degree of healing), and unweighted (presence/absence of the lesions) Cohen's Kappa was used to test the internal consistency between the two observations.

Furthermore, for the evaluation of the bias for the frequency of the lesions between the observations (quantitative variables), and the degree of concordance between the results, Bland Altman plot and Intraclass Correlation Coefficient (ICC) were calculated. In these analyses, we compared all the frequencies counted by the observers for each cranial bone (n=135) and orbital roof (n=54) for the evaluation of the presence/absence of the lesions. In the analysis of the degree of healing and severity, we excluded the bones in which both the operators observed no lesion because the high number of scores equal to zero could inflate the agreement among observers. This exclusion results in 70 cranial bones and 34 orbital roofs considered.

We performed the statistical analysis for validation of reliability and reproducibility considering CO and PH as a whole category, in general terms indicated as 'lesions'. This compromise should not affect the results of the tests since we are here estimating inter- and intra-observer differences in the evaluation of very similar lesions, even though they could have had distinct aetiologies. The scoring criteria we used are, in fact, identical for CO and HP in the assessment of the degree of severity and degree of healing, demonstrating that the localisation of the lesion cannot affect the evaluation of the operator.

All statistical tests were performed using MedCalc Statistical Software version 14.8.1 (MedCalc Software bvba, Ostend, Belgium).

3.4   RESULTS

The results of the Cohen's Kappa intra-observer agreement showed an almost perfect agreement for presence/absence, as well as degrees of severity and healing, according to the Landis and Koch classification (Landis and Koch, 1977) (Table 3.1). The inter-observer agreement resulted almost perfect in regard of presence/absence of the lesions, and "substantial" when the degree of severity and healing were tested, the lowest value of agreement (modest concordance) being for the assessment of the degrees of healing (Table 3.1).

The test-retest reliability for the frequency of the porous lesions was confirmed between the two replications of the analysis by the same operator (A1 – A2), which showed an ICC of 0.98 (95% CI 0.9293 – 0.9671) (Table 3.2), with an average difference of less than three pits (2.3 pits and a 95% confidence interval of -22.0 and +26.6) between the two different counts (Fig. 3.7). The ICC calculated for the observations of two different operators (A1 – B) showed a value of 0.91 (95% CI 0.8045 – 0.9577) (Table 3.4), with a mean difference between the two observations of -5.0 pits and a 95% confidence interval of -27.7 and +40.4. In this case, also, only one difference lied slightly underneath the lower limit of agreement (Fig. 3.8), showing exceptional reliability (Koo and Li, 2016).

If we consider the counts of the pits considering orbital roofs and cranial bones separately, the ICC values remained excellent, but for the inter-operator agreement on the orbital roofs' lesions, the ICC was 0.89 (95% CI 0.6798 – 0.9532), indicating a "good" agreement (Table 3.2). The average difference between the counts was -6.9 (95% CI -27.3 to +13.5). The Bland Altman Plot showing the count of the pits in orbital roofs and cranial bones separately are reported in the Supplementary (Fig. S3.2-S3.5)

Table 3.1 Intra and inter-observer reliability for presence/absence, degree of severity and degree of healing of Cribra,

			Intra-observer error		Inter-observer error	
Error test	N	Feature score categories	Kappa	Landis and Koch kappa "strength of agreement"	Kappa	Landis and Koch kappa "strength of agreement"
All cranial bones and orbital roofs						
Presence/absence of lesions	189	2	1.000	Almost perfect	1.000	Almost perfect
Degree of severity	104	4	0.776	Substantial	0.670	Substantial
Degree of healing	104	4	0.815	Almost perfect	0.726	Substantial
Orbital roofs						
Presence/absence of lesions	54	2	1.000	Almost perfect	1.000	Almost perfect
Degree of severity	34	4	0.603	Moderate	0.586	Moderate
Degree of healing	34	4	0.857	Almost perfect	0.645	Substantial
Cranial bones						
Presence/absence of lesions	135	2	1.000	Almost perfect	1.000	Almost perfect
Degree of severity	70	4	0.861	Almost perfect	0.703	Substantial
Degree of healing	70	4	0.619	Substantial	0.603	Moderate

Table 3.2 Intra-class Correlation Coefficient (ICC) values resulting from the test of repeatability and reproducibility of the pits' frequency and their related 95% CI

Parameters	Intra-observer variability	Inter-observer variability
	A1 – A2	A1 - B
All cranial bones and orbital roofs (n=104)		
ICC values	0.9517	0.9516
95% CI	0.9293 to 0.9671	0.9124 to 0.9711
Orbital roofs (n=34)		
ICC values	0.9488	0.8887
95% CI	0.9006 to 0.9740	0.6798 to 0.9532
Cranial bones (n=70)		
ICC values	0.9386	0.9496
95% CI	0.8995 to 0.9622	0.9139 to 0.9698
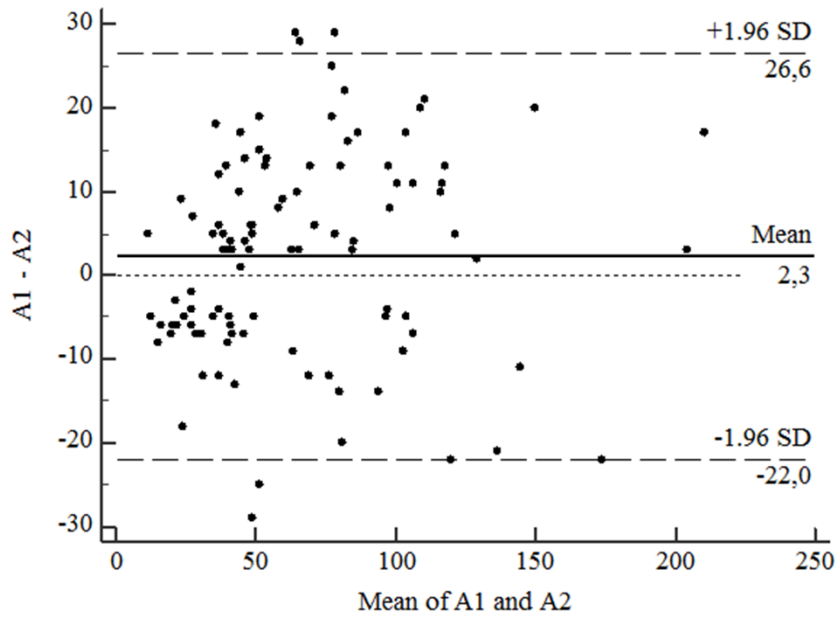
Figure 3.7: Bland Altman plot evaluating the intra-observer variation between the count of the frequency of the lesions. X-axis: the average of the two measures; Y-axis: the difference between the two measures (Rinaldo et al., 2019).
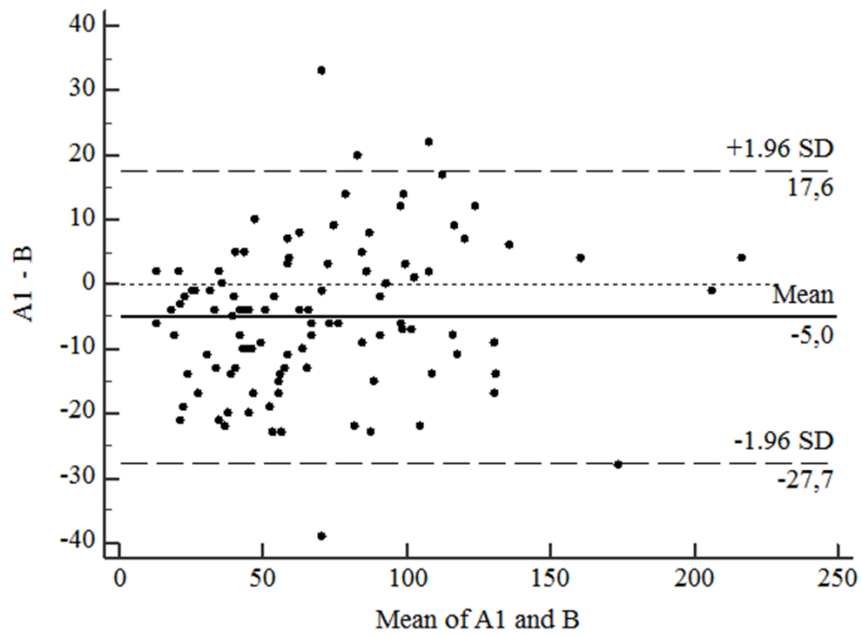
Figure 3.8: Bland Altman plot evaluating the interobserver variation between the count of the frequency of the lesions. X-axis: the average of the two measures; Y-axis: the difference between the two observers (Rinaldo et al., 2019).

3.5   DISCUSSION AND CONCLUSIONS

Analysing PH and CO in past populations is essential to understand their health and nutritional status. While Cribra are often detected in individuals from the past and used to assess their living conditions, there is no standard guideline used internationally. Moreover, the evaluation of the severity and healing of the lesions is often overlooked.

We propose an evaluation form that considers all the evaluable aspects for the analysis of PH and CO. Degrees of severity and healing are essential: the presence or absence of the lesions alone is not enough to determine a condition of frailty in the individuals of the past. Different degrees of severity may be related to a difference in the progression or intensity of the anemic condition or even indicate different etiologies. Healed lesions indicate an illness which has been overcome, thus are not indicative of frailty conditions at death. Conversely, they may indicate a better health status of the individual, who was able to overcome the illness. Moreover, the description of severity and healing of PH and CO could be fundamental in future studies to determine the exact etiology of both.

Moreover, the new colour pictures proposed for all the degrees of CO and PH are references of considerable help, as the good level of inter, and intra-observer concordance demonstrate, although they are based on subjective observations. Intra and inter-observer evaluations showed a good agreement for the qualitative analysis of the lesions, both presence/absence and degrees of severity and healing.

The addition of the location of the lesions and size of the affected area in the evaluation form represents another crucial element in the study of these lesions. As demonstrated by Brickley (2018), the location of the lesions might be an important observation, due to the different distribution of the three types of marrow (red, yellow and mixed marrow) in the different regions of the skull. Therefore, the location of the lesions could lead to discern the etiology of the lesions. More data are needed to test this theory.

We have also proposed a new quantitative method to analyse the porous lesions of the skull: as expected, it appears to be more reliable than the qualitative methods, as the outcomes of the Bland Altman and ICC showed. The importance of the pores frequency in CO and PH has already been hypothesised by Nathan and Haas (1966), by Webb (1982) and by Jacobi and Danforth (2002). The number of pores/unit area, in fact, should be directly related to the diameters of the pores and therefore to the severity of the pathology's expression. Indeed, the researches need to be extended in this direction.

If routinely used in many anthropological labs, we are confident that our evaluation sheet will be useful to detect anemia in past individuals, and that it could even become determinant in understanding the nature of the different expressions of Cribra.

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3.7 SUPPLEMENTARY
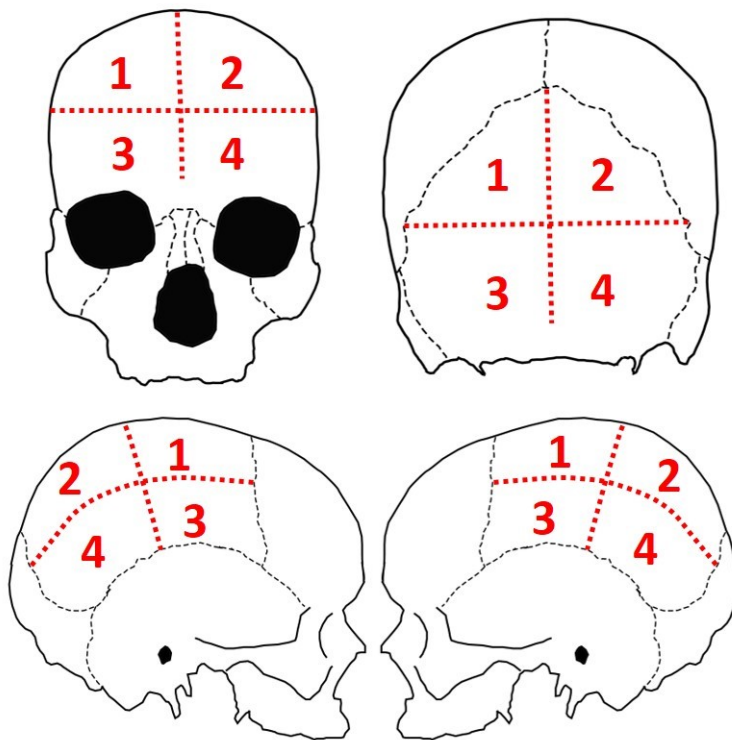
Figure S3.1: Partition of each cranial bone considered into four virtual quadrants for the evaluation (Rinaldo et al., 2019).
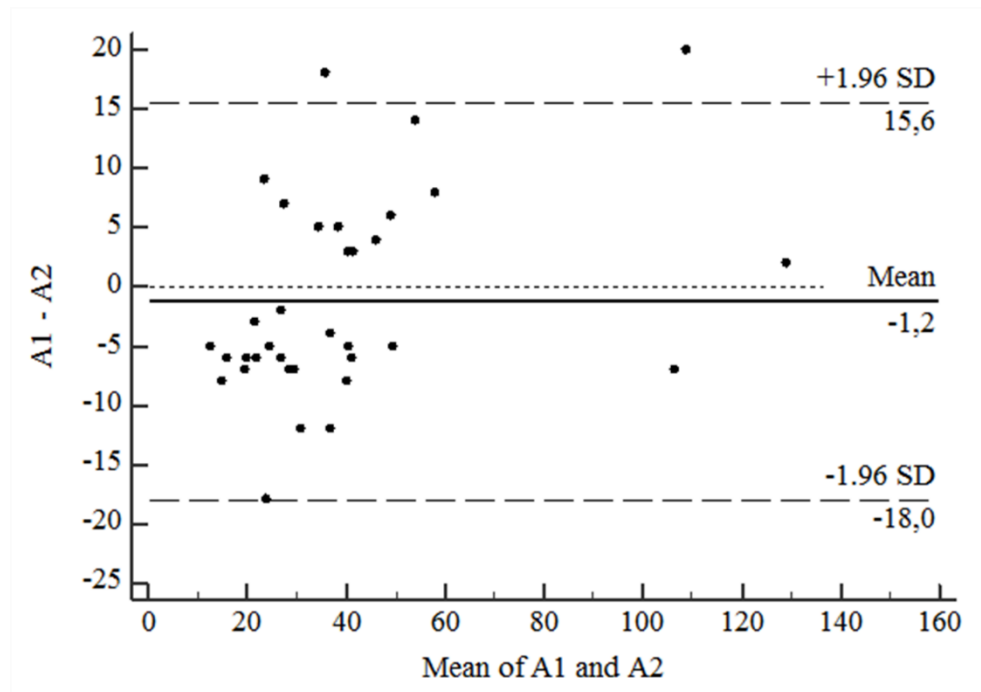
Figure S3.2: Bland Altman plot evaluating the intraobserver variation for the count of the frequency of the lesions in the orbital roofs. X-axis: the average of the two measures; Y-axis: the difference between the two measures (Rinaldo et al., 2019).
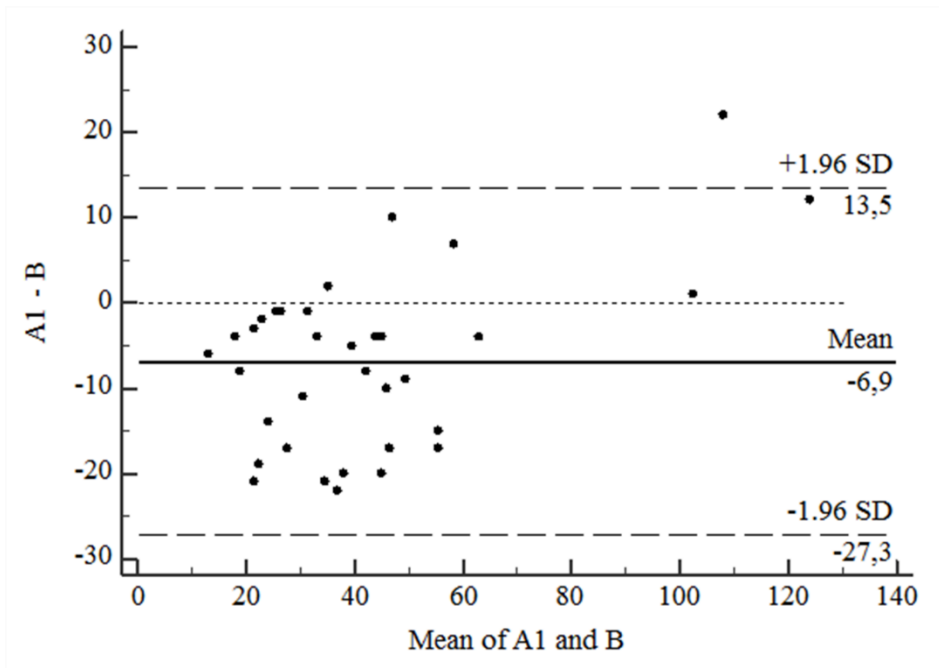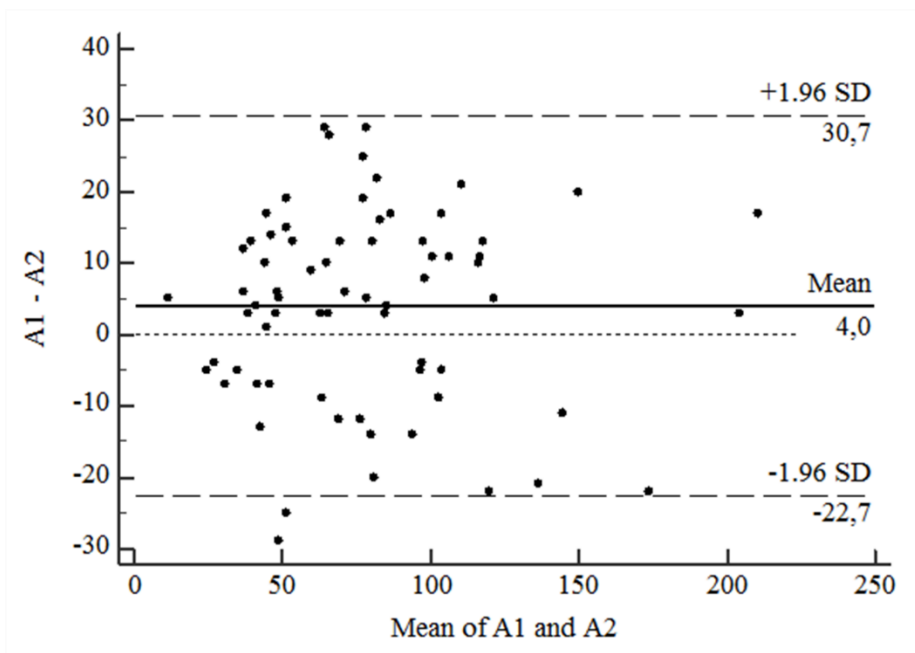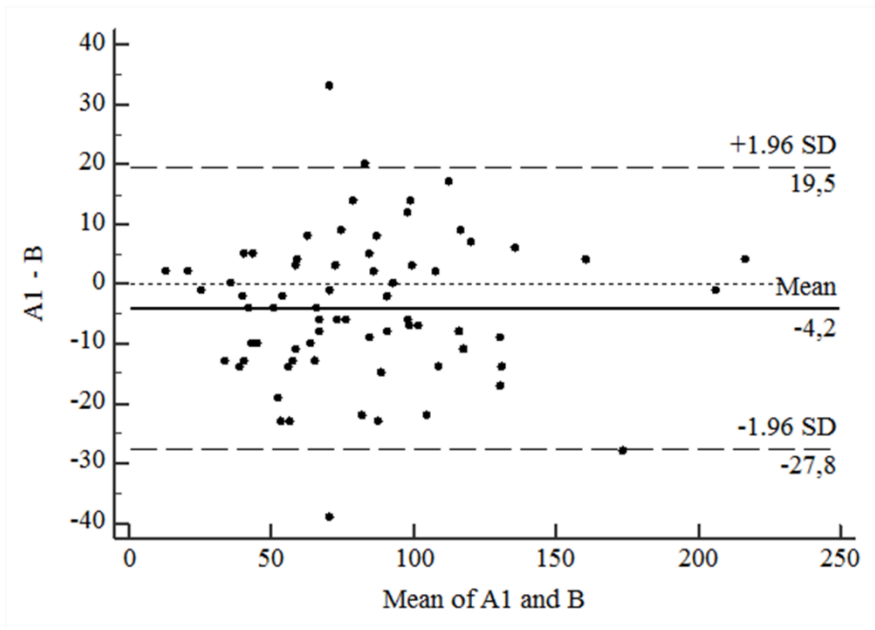
Figure S3.3: Bland Altman plot evaluating the interobserver variation between the count of the frequency of the lesions in the orbital roofs. X-axis: the average of the two measures; Y-axis: the difference between the two measures (Rinaldo et al., 2019).

Figure S3.4: Bland Altman plot evaluating the intraobserver variation for the count of the frequency of the lesions in cranial bones. X-axis: the average of the two measures; Y-axis: the difference between the two measures (Rinaldo et al., 2019).

Figure S3.5: Bland Altman plot evaluating the interobserver variation for the count of the frequency of the lesions in cranial bones. X-axis: the average of the two measures; Y-axis: the difference between the two measures (Rinaldo et al., 2019).

A NEW INDEX OF FRAILTY

Health in past populations is traditionally assessed by observing the presence of different biomarkers of biological stress. Some attempts have been previously undertaken to propose an index that brings together all biomarkers in order to describe the frailty of single individuals and of populations. However, these indexes have some fundamental limitations: on one side, they cannot be used on incomplete skeletons. On the other side, they do not take into account the degree of severity and healing of the lesions and do not give a weight to the different biomarkers.

Here we propose a new index for skeletal remains to evaluate frailty in individuals of the past. Through statistical analyses of a large amount of raw data from the Museum of London (MoL Wellcome Osteological Research Database), we came to attribute a different weight to each biomarker. Doing so, we could provide a new index of frailty, which weight different biomarkers of biological stress, can be used on incomplete skeletal remains and takes into account the severity and healing of some biomarkers.

4.1   INTRODUCTION

The concept of frailty has been highly debated in recent years, in both medical and bioarchaeological studies (Vaupel et al., 1979; Kiple, 2004; DeWitte, 2010; Marklein et al., 2016; Scott and Hoppa, 2018; Wallace et al., 2019).

Among scholars, frailty has been defined as "vulnerability to diverse outcomes and proximity to death" (Wallace et al., 2019, p 179); "set of susceptibility and risk factors that alters their chances of death at different ages" (Vaupel, 1988, p 277); "an individual's relative risk of death compared to other members of the population" (DeWitte, 2010); "proportion of accumulated defect […] reflecting severity of illness and proximity to death" (Mitnitski et al., 2001, p 324).

Although many definitions of frailty exist, all correlate it to physiological stress and the risk of death. Physiological stress, mainly if it occurs during childhood, may generate a weakened response to future illness, thus increase the risk of death (Goodman and Armelagos, 1989; Armelagos et al., 2009). Therefore, frailty, that should not be confused with bone fragility, can be defined as the load of physiological stress that an individual sustained in life and that makes him more susceptible to disease and death, without causing them directly.

As previously written, the evaluation of frailty is fundamental in medical studies because it is one of the primary causes of premature mortality, especially in older people (Dent et al., 2016). In recent years, its importance in bioarchaeological studies has also been stressed because frailty can give crucial indications on the health status of ancient populations (e.g. Goodman et al., 1980; Goodman and Martin, 2002; Steckel, 2005; DeWitte, 2010, 2017).

Frailty can be assessed in bioarchaeology trough the study of skeletal biomarkers of stress. Biomarkers are the signs left on the skeleton by physiological stress during the life of the individual; they are the manifestation of the allostatic load of the individual or " the price the body pays over long periods for adapting to challenges " (McEwen, 2001, p 44)[1].

Different physiological stressors like malnutrition, infections or other factors, which may produce disruptions in the growth, can generate lesions on the skeleton.

Usually, in bioarchaeological studies, these biomarkers are analysed independently (e.g. Kyle et al., 2018; Novak et al., 2018; Lowman et al., 2019), and used as proxies. By doing so, the comprehensive understanding of the global health status of the individuals may be lost. Few previous attempts exist to create an index of frailty which is the result of the evaluation of several stress biomarkers jointly. One of them provided the Health Index of Steckel and Rose (Steckel and Rose, 2002), which considers seven skeletal biomarkers; the second attempt produced the Skeletal Frailty Index (SFI) of Marklein and colleagues (Marklein et al., 2016; Marklein and Crews, 2017) that uses 13 biomarkers. Despite the advantages in comparison to the use of single biomarkers, both indexes have some limitations: firstly, they both consider all the skeletal markers to have the same weight, which means that each of them contributes equally to the individual's frailty. Moreover, only one of the proposed indexes considers that some lesions can be in an active or healed status at the time of the death of the individual (Marklein et al., 2016), while none consider that biomarkers can have different degrees of expression or severity. Finally, both the indexes can only be used on very well preserved and almost complete skeletons; therefore, they cannot be used on the majority of ancient and poor preserved skeletal remains.

Here we propose a new index of frailty, the Biological Index of Frailty (BFI), that aims to overcome all the limitations of the former indexes.

4.2 BIOMARKERS OF PHYSIOLOGICAL STRESS

All the biomarkers chosen for the new Biological Index of Frailty indicate physiological stress occurred during life; all of them are connected to a higher risk of death but are not the direct cause of death.

4.2.1 Low stature

Stature is defined by the interaction between genetic and environmental factors (Johnston, 2001; Wasterlain et al., 2018) and can be influenced by different stressors, like nutrition, socioeconomic status, physical activity, climate and others. The two acknowledged principal stressors, which may have negatively impacted the statures in the past, are diseases and malnutrition (King, S. E., & Ulijaszek, 1999; Lewis, 2006; Wasterlain et al., 2018).

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[1] The concept of allostatic load was coniated by McEwen after Sterling and Eyre (Sterling P. and Eyer J., 1988), who introduced the concept of 'Allostasis', or the ability of the human body to "achieve stability trough change"(Ice and James, 2012).

When subjected to stress, the growth velocity may decrease until not stressful conditions are re-established. The more intense the stress is, the more it affects the rate of growth (Bogin, 1999; Wasterlain et al., 2018). A poor nutritional status leads in general to a decline of the immune response, while the infections itself causes disruption of the immune response by inducing, among other consequences, malabsorption and protein catabolism (King, S. E., & Ulijaszek, 1999; Wasterlain et al., 2018).

Individuals with a compromised immune system due to episodes of stress (malnutrition and infection) during childhood and adolescence may consequently have a slower growth pace and thus be shorter in stature once they become adults. Also, the weak immune response of these individuals leads to a higher risk of mortality (Larsen, 1997; DeWitte and Wood, 2008; DeWitte and Hughes-Morey, 2012).

Many reliable methods exist in literature to estimate adult stature in skeletonised individuals through the measurement of long bones (Pearson, 1899; Trotter and Gleser, 1952; Gualdi-Russo et al., 2018). Once stature has been estimated, the first quartile (below the 25th percentile) of the population's height, considering males and females separately, correspond to short stature. At least one complete long bone should be present and measurable. This biological marker can be considered in the index of frailty only in adult individuals whose sex is assessed.

4.2.2   Low body mass

In modern populations, especially in developing and developed countries, overweight and obesity are one of the main threats to health (World Health Organization., 2000), but in underdeveloped countries and in past populations underweight and undernutrition can be considered major factors contributing in increasing the risk of death. Low weight and malnutrition are associated with higher mortality, especially in older individuals (Fried et al., 1998).

To assess body mass from skeletal remains there are different methods (Lacoste Jeanson et al., 2017): using the femoral head diameter (Ruff et al., 1991, 2012; McHenry, 1992; Grine et al., 1995); through the measurement of the bi-iliac breadth (Ruff, 1994; Ruff et al., 2005); and with the measurement of the thickness of the femoral diaphysis cortical bone (Elliott et al., 2016).

The femoral head diameter is demonstrately the most correlated measure to the weight at the moment of death (Ruff et al., 1997), and also the easiest to measure. Yet, the equations based on this measure are not always accurate, and more population-specific equations are needed. For this reason, we use the direct measure of the femoral head diameter as a proxy of undernutrition in our index, without the indirect calculation of the body mass.

The individuals whose measurements fall in the first quartile of the population, after having separated males and females, are considered as having a low body mass. All the others will be regarded as not having a low body mass. At least one complete femoral head should be present. In our index, body mass can be estimated only in adult individuals whose sex is assessed.

4.2.3   Linear Enamel Hypoplasia

The mineralisation of the dental enamel (amelogenesis) starts during gestation in deciduous teeth and soon after birth in permanent dentition. Amelogenesis is very susceptible to both environmental and physiological strains, and they may cause its temporary cessation or disruption (Goodman et al., 1980; Kinaston et al., 2016; Masterson et al., 2017). The defects resulting from amelogenesis disruption are still visible in adults because teeth do not remodel.

Linear Enamel Hypoplasia (LEH) is the most common enamel defect; it manifests as grooves on the crown surface of the teeth (Fig. 4.1A); each groove is an interruption of the mineralisation process. It's possible to estimate the age of the individual at the formation of each groove, trough the measurement of the distance between the line and the cementum-enamel conjunction and applying equations (Goodman et al., 1980) or evaluating the portion of the teeth with the defect (Reid and Dean, 2000, 2006).

The most common environmental stressors in early life are malnutrition and infection. Different studies have noticed a lower life expectancy for the individuals who present LEH, supporting the hypothesis that physiological stress early in life brings consequences in adult life and subsequent more significant risk of premature death (Steckel and Rose, 2002; Boldsen, 2007; Miszkiewicz, 2015).

LEH can manifest in single or multiple lines. Considering that one line corresponds to a single event of stress and multiple lines to chronic stress (Goodman et al., 1980; Miszkiewicz, 2015), LEH is considered present if at least one tooth present multiple lines of enamel hypoplasia. To determine if hypoplasia is not present, all teeth should be without defects or only showing a single line of hypoplasia in one tooth. At least one canine must be present and observable.

4.2.4   Cribra Orbitalia and Porotic Hyperostosis

As explained in the previous chapter, although both CO and PH are considered a manifestation of anemia, they should be considered separately. Active lesions indicate the presence of the stress at the moment of the death, while healed lesions suggest that the stressful situation has been overcome. Some scholars argue that healed lesions are signs of strength because the individual has overcome the stressful factor (illness, malnutrition, etc.) (Wood et al., 1992). Still, even though the stress has been overcome, especially if it happened during childhood, it can increase the risk of dying in comparison to individuals of the same age cohort without signs of stress (Steckel, 2005).

As uncertainty remains in the interpretation of these lesions, in our analysis, we consider both active lesions and healing/healed lesions of both PH and CO, and this in four separated evaluation forms (see chapter 3). Moreover, it has been noted that degree 1 of severity of both PH and CO (fine scattered holes on the cranial vault or orbital roof surface), is very often present in skeletal remains, and it can be easily confused with normal microporosity of the cranial vault (Roberts and Manchester, 2005), or be due to a scalp infection.

Accordingly, grade 1 of severity for both pathologies has been analysed separately from the other higher degrees of severity. For a detailed description of these biomarkers and their degrees of severity and healing, see chapter 3.

PH and CO are present if the lesions are observable in at least, respectively, one cranial bone and one orbital roof. Both healing and severity degree should be reported. The pathologies are absent if no lesions are present, with at least half cranial vault and one orbital roof observable.

4.2.5   Rickets/osteomalacia

Vitam D is a pro-hormone that maintain the calcium homeostasis; it is essential to the mineralisation process both during bone growth and in adult life for the bone remodelling (Ortner, 2003; Meyer, 2016). Vitamin D deficiency causes rickets in children and adolescents and osteomalacia in adults. Both pathologies manifest as defects in the mineralisation process of the bone and bring to the softening and deformation of some bones, especially the legs' and pelvic's bone, which are more affected by gravitational forces (Brickley et al., 2005; Mays and Brickley, 2018). Other signs of vitamin D deficiency are, in case of osteomalacia, pseudofractures, or incomplete fracture, with a poorly mineralised bone callus, usually on ribs or scapula (Mays and Brickley, 2018).
Vitamin D is crucial to human life; its deficiency increases susceptibility to other diseases and also the risk of mortality (Mays et al., 2006; Mays and Brickley, 2018; Ngari et al., 2018).

If any signs of vitamin D deficiency is recognised in the skeleton (i.e. mainly deformation of tibia and femur), then the individuals can be diagnosed with rickets or osteomalacia. Since the change is generally both evident and bilateral, we consider it absent if at least one femur or tibia is observable and without deformations.

4.2.6   Periodontal Disease

Inflammation of the gums, or gingivitis, if left untreated, can worsen and develop into periodontal disease, resulting in reabsorption of the alveolar bone and eventually tooth loss (Regezi et al., 2000; Ortner, 2003) (Fig. 4.1B).
In modern populations, a correlation has been noted between periodontitis and a higher risk of mortality: it is often associated with cardiovascular disease in older individuals and in general with systemic poor health (Hillson, 1996; Garcia et al., 2001; Ajwani et al., 2003). A correlation between periodontal disease and a higher risk of mortality has been demonstrated also in past populations (DeWitte and Bekvalac, 2010; Marklein et al., 2016).
To diagnose periodontal disease, some researchers use to measure the distance between the cementum-enamel junction and the alveolar crest (Brothwell, 1981). Nonetheless, the retraction of the alveolar margin and root exposal can be a natural retraction due to age and is a function of the compensatory eruption. For this reason, the aspect of the alveolar bone should also be taken into consideration. Ogden (Ogden, 2008) proposed a scoring system that takes in account both the root

exposition and the aspect of the alveolar bone: when periodontitis is absent the alveolar margin meets the tooth at a knife-edged acute angle (degree 1). Mild periodontitis corresponds to an alveolar bone with a blunt flat-topped and slightly raised rim (degree 2), while moderate periodontitis produces a rounded and porous alveolar margin, with 2-4 mm between the tooth and the alveolus (degree 3). When the alveolar margin become ragged and porous with an irregular trough of more than 5 mm between the tooth and the alveolus, the periodontitis is severe (degree 4).

Periodontal disease should be considered present if at least one tooth manifest degree 2 to 4 of the Ogden's periodontitis score. The first and the second molars, and in general posterior teeth are the most affected by periodontal disease (Hillson, 1996; Ogden, 2008). Therefore, if periodontitis is not present and at least one posterior tooth and a half jaw is observable, we can consider the dental pathology as absent.

4.2.7 Non-specific Periostitis

The periosteum, a tissue membrane that surrounds most bones, houses the osteoblasts and therefore, is involved in bone growth and repairs. The periosteum is often the first tissue to respond to any insult that affects the bone, like trauma, infections or tumours (Dwek, 2010).

Periosteal new bone formation can be aggressive, with rapid deposition of new woven and disorganised bone: this occurs immediately after an insult and is also called active periostitis (Fig. 4.1C). New bone formation can also be a slow process, in case of less intense insults or from the remodelling and replacement of the woven bone (inactive periosteal reaction). This condition is characterised by an organised layering of new bone, with a plaque-like surface texture of the bone similar to that of a healthy bone (Rana et al., 2009; Weston, 2012) (Fig. 4.1D). A mixture of these two types of new bone formation can be found during the substitution of the woven bone.

Periostitis can be found associated with specific infections like tuberculosis or leprosy, metabolic disease like scurvy, or with trauma, but it can also result from Non-specific infections (Ortner, 2003). Non-specific periostitis is often used in bioarchaeology to evaluate the health status of past populations (Goodman and Martin, 2002; Steckel and Rose, 2002; DeWitte, 2014; Marklein et al., 2016). Differentiating between active and healing/healed new bone formation is important. In fact, it has been proven that the risk of death is different between the two forms, with a higher risk for active lesions (DeWitte, 2014).

If new bone formation is present and is not due to trauma or a specific disease, periostitis is scored as present, and we can differentiate between active or mixed/healed new bone formation. Since tibia is considered the bone most affected by this pathology and the manifestation is often bilateral (DeWitte, 2014; Marklein et al., 2016), if no signs of Non-specific periostitis are observed and if at least one tibia is present, then the biomarker is scored as absent.

4.2.8 Joint disease

Joints are essential for the flexibility and the movement of the skeleton. Joints can be damaged by many types of injuries or diseases, the most common worldwide being Osteoarthritis (OA), that affects mostly middle-aged and elderly individuals (Cleveland and Callahan, 2017; Büchele et al., 2018). OA and all other joints diseases, in general, reduce the mobility of the joint creating considerable musculoskeletal stress. Especially in older individuals, joints dysfunctions are associated with other pathologies (e.g. cardiovascular and gastrointestinal disorders) and with a higher risk of mortality (Hochberg, 2008; Cleveland and Callahan, 2017).

In OA and all other degenerative joint diseases, we can observe two different mechanisms: either we have erosion and loss of bone density due to inflammation, or a hypertrophic reaction, with osteophytosis and bone proliferation. Sometimes, both processes are present at the same time (Rogers and Waldron, 1995). If the deterioration of the joint progresses, eburnation of the joint surface can happen.

To diagnose the presence of OA, it is necessary the presence of eburnation, or at least two other signals of the three possible ones, osteophythosis, change in the joint's contour and pitting of the surface, as suggested by Waldron (Rogers and Waldron, 1995) (Fig. 4.1G).

Other joint diseases, like dysplasia, rotator cuff disorder and other erosive arthropathies, should be diagnosed using specific guidelines (Rogers and Waldron, 1995; Ortner, 2003).

If OA or other joint diseases are found in one of the 16 major joints (shoulder, elbow, wrist, hand, hip, knee, ankle and foot of both sides), joints disease is considered as present.

Absence of joint disease is confirmed if no joints are affected and at least 2/3 of the major joints (10/16) are observable.

4.2.9 Vertebral disease

The spine is composed of vertebrae and is divided into three parts: cervical, thoracic and lumbar spine. The backbone supports the musculoskeletal system and protects the spinal cord and its nerves. Between the vertebrae, there is a fibro-cartilagineous disc that has the function to absorb all the shocks that the spine undergoes during body activity (Rogers and Waldron, 1995).

Vertebrae may be affected by osteoarthritis (Fig. 4.1E), but they may also be suffering from intervertebral disc diseases, and other specific pathologies, like Diffuse Idiopathic Skeletal Hyperostosis (Fig. 4.1H) and spondylitis. Intervertebral disc disease (IVD) reflects the degeneration of the cartilaginous disk between the vertebrae; it manifests itself in the form of pitting and bone growth on the disc surface (Rogers and Waldron, 1995; Ortner, 2003; Boncal, 2014).

Degenerative disorders affecting the spine reduce mobility and cause significant pain. They create a higher risk of death, especially if the lumbar or cervical vertebrae are affected (Neva et al., 2001; Kauppila, 2009). Some characteristics of vertebral degeneration are more painful than others; the

most painful are the cracks in the intervertebral disc or herniations, and the structural changes to the endplates of the vertebral body (Adams and Dolan, 2012).

Vertebral disease is scored as present if OA of the spine, IVD, or another specific degenerative disorder of the spine is visible. If the spine is free of disease and at least 2/3 of the vertebrae (i.e. 16 out of 24) are observable, the disease is scored as absent.

4.2.10 Trauma

Bone, like other connective tissue in the body, can repair itself (Ortner, 2003). In archaeological remains, traces of injury healed during the life-time can be often easily recognised by the bone callus formed in case of a fracture, while perimortem traumas are more challenging to identify (Ortner, 2003). Traumas are used in bioarchaeology to define the social contest of the individuals, because they represent external influences on the body, and are influenced by culture and activity during life (Ortner, 2003; Agnew et al., 2015).

Physical traumas, like the psychological ones, causes stress in the individual (Marklein et al., 2016); injuries to the head and the spine are the ones most connected to a higher risk of death (Puisto et al., 2008, 2011; Boldsen et al., 2015). Traumas can also be a consequence of osteoporosis; in particular, vertebral fractures (Fig. 4.1F), Colles' fractures and fractures of the femoral head are often due to osteoporosis (Curate et al., 2016).

Perimortem traumas are not considered in our index because they are timely too close to the death of the individuals and do not represent their health status during life. Trauma is considered present if any signs of a bone callus or other signs of healed injuries, and also osteoporotic vertebral fracture (Curate et al., 2016), are visible in the skeleton. If no signs of trauma are present and at least 2/3 of the skeleton is observable, trauma is considered absent.

4.2.11 Osteoporosis

Osteoporosis is a skeletal metabolic disorder characterised by the reduction of bone mineral density and the deterioration of the bone microstructure that leads to an increased risk of fracture (NIH Consensus Development Panel on Osteoporosis Prevention, Diagnosis, and Therapy, Diagnosis and Therapy, 2001; Agarwal et al., 2004; Rinaldo et al., 2018).

Bone loss commonly occurs during ageing in both women and men, but the process is accelerated in post-menopausal women. Therefore, osteoporosis is often present in older individuals, especially in older women (primary osteoporosis). Osteoporosis, and in general poor bone mineral density, can be caused by other factors, such as poor bone growth in childhood, some pathologies and nutrition deficiencies. In this case, it is known as secondary osteoporosis (NIH Consensus Development Panel on Osteoporosis Prevention, Diagnosis, and Therapy, Diagnosis and Therapy, 2001).

In bioarchaeology, osteoporosis is macroscopically diagnosed through the presence or absence of osteoporotic fractures (Curate et al., 2016; Marklein et al., 2016), like fractures of the vertebral

bodies, hip and wrist. However, we already consider those fractures as traumas in our index. Yet, fractures are not the only risk in osteoporosis: osteopenia, or low bone mineral density itself, has been associated with increased Non-traumatic mortality and is possible to observe osteopenia without fractures (Browner et al., 1991; Rinaldo et al., 2018).

To diagnose osteopenia and osteoporosis, different methods have been used in the last years (Agarwal and Grynpas, 1996; Beauchesne and Agarwal, 2017; Rinaldo et al., 2018): the most used are dual X-ray absorptiometry (DXA), that calculates the amount of hydroxyapatite in the bone (Golob and Laya, 2015; Beauchesne and Agarwal, 2017), radiogrammetry, that measures the amount of cortical bone (Ives and Brickley, 2004; Beauchesne and Agarwal, 2017), and quantitative ultrasonometry (QUS), that assesses bone mineral status trough ultrasound signals (Rinaldo et al., 2018).

Osteoporosis is diagnosed if the individual falls in the range of osteoporotic risk defined by each methodology, otherwise, it is considered as absent.

Figure 4.1: Biomarkers of biological stress: A= Linear Enamel Hypoplasia; B= Periodontitis; C= active periostitis; D= healed periostitis; E= Osteorthtritis of a vertebral facet; F= osteoporotic fracture, crush of a vertebral body; G= osteoarthritis of the sternal epiphysis of the clavicle; H= example of vertebral disease, vertebral fusion, probable DISH.

4.3 MATERIAL AND METHODS

4.3.1 Material

For the creation of this index, we have chosen to analyse data from Monastic and Non-Monastic English cemeteries (12th–16th centuries CE) from the open-access MoL Wellcome Osteological Research Database, WORD (http://archive.museumoflondon.org.uk/Centre-for-Human-Bioarchaeology/Database/).

The same skeletal data have been previously investigated by DeWitte and colleagues and by Marklein and colleagues (the proposers of the Skeletal Frailty Index), to compare frailty and health status of Monastic and Non-Monastic individuals in Medieval times (Dewitte et al., 2013; Marklein et al., 2016; Marklein and Crews, 2017). The choice to use the same database allows the comparison of the results of our study with those obtained previously with the other indices. The Monastic cemeteries considered are St. Merton Priory (1117-1538 CE) and Bermondsey Abbey (1066-1540 CE), while the Non-Monastic are Guildhall Yard (1140-1350 CE), Spital Square (1200-1500 CE), St. Benet Sherehog (1250-1666 CE), and St. Mary Graces (1350-1538 CE).

4.3.2 Methods

4.3.2.1 Data collection

The data on biomarkers of stress, sex and age of the individuals have been obtained and published by researchers at the MoL's CHB, using the Human Osteology Method Statement (Power, 2012). Unfortunately, not all the biomarkers we have proposed before have been scored, e.g. periodontal disease was scored using the measurement of alveolar retraction without the observation of alveolar morphology (Ogden, 2008). Moreover, there are very few cases of PH recorded, possibly because a different method was used for its evaluation, nor we have data about the healing degrees of CO (Power, 2012).

Only individuals over 12 years of age, adolescents and adults according to the Buikstra and Ubelaker classification (Buikstra and Ubelaker, 1994), have been chosen for our analysis. Children skeletons are fragile and often under-represented or are partial in skeletal assemblies (Mays et al., 2017); therefore, we have excluded them from the whole analysis. Moreover, children have more difficulty coping with infections, especially during infancy when the immune system has not had time to form; therefore they present an increased risk of dying at an early age without reaching adulthood (Simon et al., 2015). Marklein and colleagues selected only adult individuals whit sex determination but considering that in medieval England adolescents were already part of the social community (Lewis, 2016), we believe that it would be interesting to consider also them for the estimation of the health status of the populations. Sex estimation was carried out only on adults through a macroscopic assessment on skull and pelvis (Power, 2012).

Data for each cemetery were recorded in separate delimited files in the WORD database, so it was first necessary to merge them into a single excel database, that included all individuals over the age of 12 years along with all information and measurements required to determine the presence or absence of the biomarkers of interest.

Scoring of the biomarkers was made in accordance with the following guidelines:

- Low bone robusticity: femoral vertical head diameter was annotated for each individual. If the femoral head diameter was in the first quartile of the adult males or females measurements, then low robusticity was considered as present. If the femoral head diameter was not in the first quartile of the adult males and females measurements, the individual was scored as having average robusticity. At least one femoral head should be observable and measured for the scoring.

- Short stature: For each individual, the stature was estimated using Pearson equations (Pearson, 1899) on long bones whose growth was complete. The equations were applied to the left side if available, otherwise to the right side. The final stature of the individual was the average of the heights estimated from each long bone. The individual was considered short if his height was in the first quartile of the adult males or females statures (according to his/her sex; otherwise, short stature was absent. At least one long bone should be measured to estimate the stature.

- Linear Enamel Hypoplasia: If at least one tooth with visible grooves due to hypoplasia was observed, hypoplasia was considered as present; otherwise, it was absent, if at least one canine was observable.

- Periodontal disease: Periodontitis was scored in the Word database through the measurement of the alveolar retraction (Brothwell, 1981), not considering the morphology of the alveolar bone. If there was a retraction of at least 2-3 mm, periodontitis was present; otherwise, it was absent. At least one observable posterior tooth and a half jawbone were necessary.

- Periostitis: it was considered as present if at least on one bone new bone formation was visible. We recorded if the new bone formation was active, remodelled or mixed. If no bone showed new bone formation and at least one tibial shaft was observable, periostitis was considered as absent.

- Rickets/Osteomalacia: if any sign of deformation of the legs or other signs of vitamin D deficiency were observed, rickets were scored as present. If no sign of vitamin D deficiency was visible, and at least one leg's long bone was observable, it was considered as absent.

- Cribra Orbitalia: it was present if porous lesions were observed on the orbital roofs. Degree of severity and healing were scored (Rinaldo et al. 2019). If no porous lesions were visible on the orbital roofs and at least one orbital roof was observable, it was considered as absent.

- Porotic Hyperostosis: It was excluded from our analysis because there was only one case.

- Joint Disease: it was considered present if Osteoarthritis or other diseases were present on at least one joint. If there was no joint disease and at least ten joints are observable, it was considered as absent.

- Vertebral disease: it was considered as present if there was IVD or Osteoarthritis on at least one vertebra. Otherwise, it was deemed to be absent, if at least 16 vertebras were observable.

- Trauma: it was present if there was at least one healed trauma. If no trauma was visible, and at least 2/3 of the skeleton was observable, it was considered absent.

- Osteoporosis: it was not considered in our analysis, because osteoporosis was determined in the WORD database through the study of osteoporotic fractures, that should instead be considered as trauma.

The scoring sheet for the Biological Index of Frailty can be found in the Supplementary material (Fig. S4.1).

4.3.2.2 *Statistical analysis*

To assign an importance/weight to each biomarker, we performed a multivariate logistic regression model (Logit) that evaluated the association between each stress biomarker considered (predictor variables), and the risk of dying prematurely (dependent variable). First of all, using the same database, we created a life table to establish life expectancy at birth (Brothwell, 1981; Ubelaker, 1989; Mallegni and Lippi, 2009). The mean life expectancy of the six necropoles was 34 years of age (Supplementary Table 1). This information was used to distinguish premature deaths.

In our database, each biomarker was included as a categorical variable as follow:

1=present; 0=absent

Regarding periostitis, it was also considered the degree of healing, and for CO the degree of severity:

- Periostitis/osteomyelitis degrees: 1=active, 2=mixed, 3=remodelled;
- CO degrees of severity: 1= degree 1, 2=degree 2, 3= degree 3, 4= degree 4

We tested the odd ratio of each biomarker for the possibility of dying younger than 35 years of age. For CO, we differentiated between degree 1 and the other degrees of severity, for the reasons exposed before.

The results were reported as odds ratio (ORs) with 95% confidence interval (CI). Individuals with missing data were excluded from the analysis. According to the results of the analysis, we assigned a weight to each biomarker.

Afterword, we applied the new Biological Index of Frailty on the Monastic and Non-Monastic skeletal series from the Museum of London. On the data collected, we performed *t*-test, analysis of variance (ANOVA), and analysis of covariance (ANCOVA) adjusted for age, to compare the health status of the two groups (Monastic and Non-Monastic), and to make comparisons between sexes.

Gross frequencies of the biomarkers in the two main groups (Monastic and Non-Monastic) were compared with the Chi-Squared Test.

The *p*-values <0.05 were considered statistically significant. All statistical analyses were conducted using STATISTICA (version 11, StatSoft, Tulsa, OK).

4.4   RESULTS

4.4.1   The Biological Index of Frailty (BIF)

In Table 4.1, are reported the results of the Logit model: none of the biomarkers alone is significantly relevant in causing the premature death of the individual, and some of them resulted negatively correlated with a short life expectancy (odds < 1). This outcome does not disagree with what was said before: in fact, each of the biomarkers has the potential of increasing the risk of dying but is not the direct cause of death. We were interested in the contribution that each indicator has in creating frailty, thus higher probability of dying prematurely, as expressed by a bigger odds ratio. Accordingly with the results of the multivariate logistic regression model (Logit), we attributed the biomarkers with an odds ratio < 1, a weight of 1; those markers with a ratio between 1 and 1,9, a weight of 2, and finally those with a ratio equal to or greater than 2, a weight of 3, since they probably are the best indicators for frailty, independently from the age of the individuals (Tab.4.2).

Unfortunately, there were not enough cases of PH in our database; therefore, we could not assess the weight of it. We decided to assign to PH the same values of CO, because, even if their etiology is not the same, both are consequences of a deficiency, probably of iron (see chapter 3 for more information of CO and PH).

Considering the process of healing of the lesions, we only had information for periostitis, for which, as shown in Table 4.1, the odds ratio was higher for the active lesions than for the healing /healed ones, as expected. We decided, therefore, to apply the same concept to CO and PH, assigning to healing/healed lesions half the value of the active ones (Tab. 4.2).

Regarding Osteoporosis, it was not possible to evaluate the effect in our sample because, in the WORD database, this biomarker is assessed using the presence of osteoporotic fractures, which we considered trauma. Therefore, we assigned to osteoporosis the same value (1), like periodontitis, joint and vertebral diseases. All these indicators are, in fact, skeletal markers that manifest more commonly in older age.

The value of frailty is the weighted mean of the scores:

$$BIOLOGICAL\ INDEX\ OF\ FRAILTY = \frac{\Sigma(Weight*Score)}{\Sigma(Weight)}*100$$

Using a weighted mean, we do not underestimate the skeletons in which only a few characters are assessable, but we make a proportion between the number of characters observed and their weight. Of course, at least three biomarkers should be observable.

Table 4.1 Logit estimates of the correlation between stress biomarkers with relative odds of premature death, 95% confidence intervals are reported

Biomarkers	Odds Ratio	Lower CL 95,0%	Upper CL 95,0%	p
Short Stature	0.7034	0.1892	2.6148	0.5994
Low Body Mass	**2.4831**	0.6654	9.2667	0.1759
Linear Enamel Hypoplasia	**3.3431**	0.4943	22.6110	0.2159
active Periostitis	**1.4290**	0.3165	6.4526	0.6425
healing Periostitis	0.1766	0.0144	2.1617	0.1748
healed Periostitis	0.5735	0.1186	2.7734	0.4893
Periodontal Disease	0.0817	0.0102	0.6563	0.0185
CO Severity 1	0.0000	0.0000		0.9972
CO Severity 2-4	**3.6470**	0.3218	41.3306	0.2962
Rickets/Osteomalacia	0.0000	0.0000		0.9992
Joint disease	0.1513	0.0215	1.0656	0.0579
Vertebral disease	0.2804	0.0895	0.8781	0.0290
Trauma	**1.4555**	0.329917	6.4214	0.6201

Table 4.2 Form for the evaluation of the Biological Index of Frailty

Skeletal biomarker	Weight		Score	W * S
Short Stature	1			
Low Body Mass	3			
Linear Enamel Hypoplasia	3			
Rickets/Osteomalacia	1			
Periodontal Disease	1			
Cribra Orbitalia (severity 1)	1 active	0.5 healing/healed		
Cribra Orbitalia (severity 2-4)	3 active/absent	1.5 healing/healed		
Porotic Hyperostosis (severity 1)	1 active	0.5 healing/healed		
Porotic Hyperostosis (severity 2-4)	3 active/absent	1.5 healing/healed		
Periostitis	2 active/absent	1 healing/healed		
Joint diseases	1			
Vertebral disease	1			
Trauma	2			
Osteoporosis	1			
BIOLOGICAL INDEX of FRAILTY (at least 3 biomarkers)	**Max .22**			

4.4.2 Application of the new index to the Monastic vs the Non-Monastic datasets

We have applied the new index on the data from the Monastic and the Non-Monastic skeletal samples. The index was used on all individuals over 12 years of age at death and on those, whose skeletons showed at least three observable biomarkers of stress. Doing so, we obtained a total of 1009 individuals, 692 Monastic and 317 Non-Monastic ones.

As we see in Table 4.3, the Monastic population had a significantly higher frailty index, males had higher frailty than females, when considering both groups together, and, again considering the two groups together, we observed a statistically significant difference among different classes of age. Mean BIF values for the two groups, divided for each class of age and both sexes, are represented in Figure 4.2.
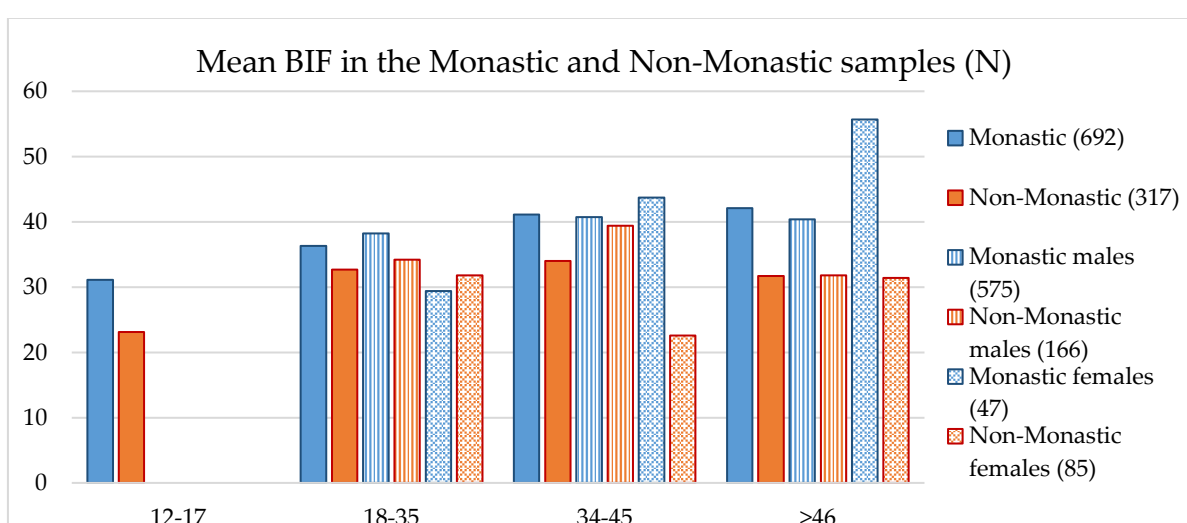
Figure 4.2: Mean Biological Frailty Index values (y-axis) for each class of age and sex. For individuals under the age of 18, we do not considered sex determination.

Table 4.3: Mean Biological Frailty Index in the sample and comparison (*t*-test and ANOVA) between groups (lifestyle; sex; age).

	Mean *BIF* (St. d.)	*p*-Value
Lifestyle (N)		**< 0.0001**
Monastic (692)	40.28 (22.57)	
Non-Monastic (317)	31.58 (22.99)	
Sex (N)		**0.0083**
Male (741)	39.56 (22.60)	
Female (132)	33.94 (21.98)	
Age (N)		**0.0004**
12-17 (43)	26.24 (25.40)	
18-35 (264)	34.90 (21.87)	
36-45 (387)	39.38 (21.89)	
>46 (166)	38.84 (21.20)	

Considering the significant difference between age at death classes, we tested the difference between the two groups, representative of different lifestyles, for each age classes (Tab. 4.4). After pinpointing that difference existed for the two older classes of age, we performed an ANCOVA adjusted for age (Tab. 4.5). Again, the general difference between the Monastic and the Non-Monastic groups was significant. However, when we considered males and females separately, we saw that Monastic men were Non-significantly different from their Non-Monastic counterpart, even though they showed a higher mean BIF. Females, meanwhile, showed a significant difference: Monastic women had higher frailty than the Non-Monastic ones. It should be considered anyway that the female sample was smaller than the male one (sex was estimated only for 8% of the Monastic individuals, see Tab. S4.2).

Table 4.4: T-test results between Monastic and Non-Monastic groups for each age class

	Monastic	Non-Monastic	
	Mean *BIF* (St.d)	Mean *BIF* (St.d)	*p*-Value
12-17 (n=43)	31.08 (24.01)	23.08 (26.25)	0.3181
18-35 (n=264)	36.33 (21.40)	32.74 (22.49)	0.1921
36-45 (n=387)	41.05 (21.42)	34.04 (22.66)	**0.0072**
>46 (n=166)	42.07 (20.14)	31.73 (21.94)	**0.0033**

Table 4.5 ANCOVA between Monastic and Non-Monastic individuals

Monastic vs Non-Monastic	Monastic (n= 692)	Non-monastic (n=317)		
	Mean *BIF* (St.d)	Mean *BIF* (St.d)	F	*p*-Value
Tot (n=1009)	40.28 (22.57)	31.58 (22.99)	**17.610**	**< 0.0001**
Males (n=741)	40.71 (22.44)	35.58 (22.78)	2.691	0.1014
Females (n=132)	43.23 (20.86)	28.80 (20.99)	**11.655**	**0.0009**

When we considered the frequencies of the biomarkers individually (Fig. 4.3), we saw that all the biomarkers connected to malnutrition (Short Stature, Low Body Mass, Linear Enamel Hypoplasia, Cribra Orbitalia and Rickets) were more present in Non-Monastic individuals, but the difference was statistically significant only for Linear Enamel Hypoplasia (*p*=0.0004). Conversely, we noticed a significantly higher presence of aspecific infections (*p*=0.0001), joint and vertebral disease (respectively *p*= 0.0093 and *p*<0.0001) in Monastic individuals. When we considered men and women separately, we observed that the difference was still significative in men regarding LEH (*p*=0.0011), periostitis (*p*=0.0236) and vertebral disease (*p*=0.0092) (Fig. 4.4). In women, anyway, only the difference in the frequency of vertebral disease was still statistically significant (*p*=0.0073) (Fig. 4.5).
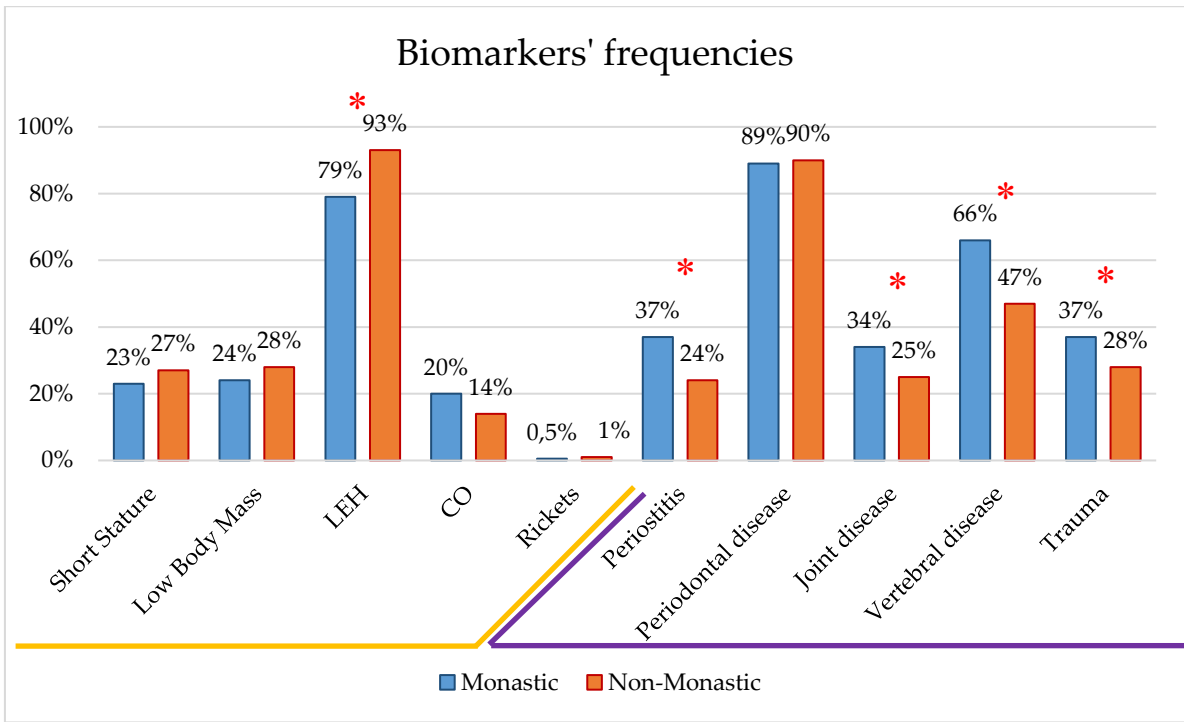
Figure 4.3: Biomarkers' frequencies in the Monastic and Non-Monastic groups. Yellow box= malnutrition biomarkers; purple box= aspecific infections and activity/older age biomarkers.
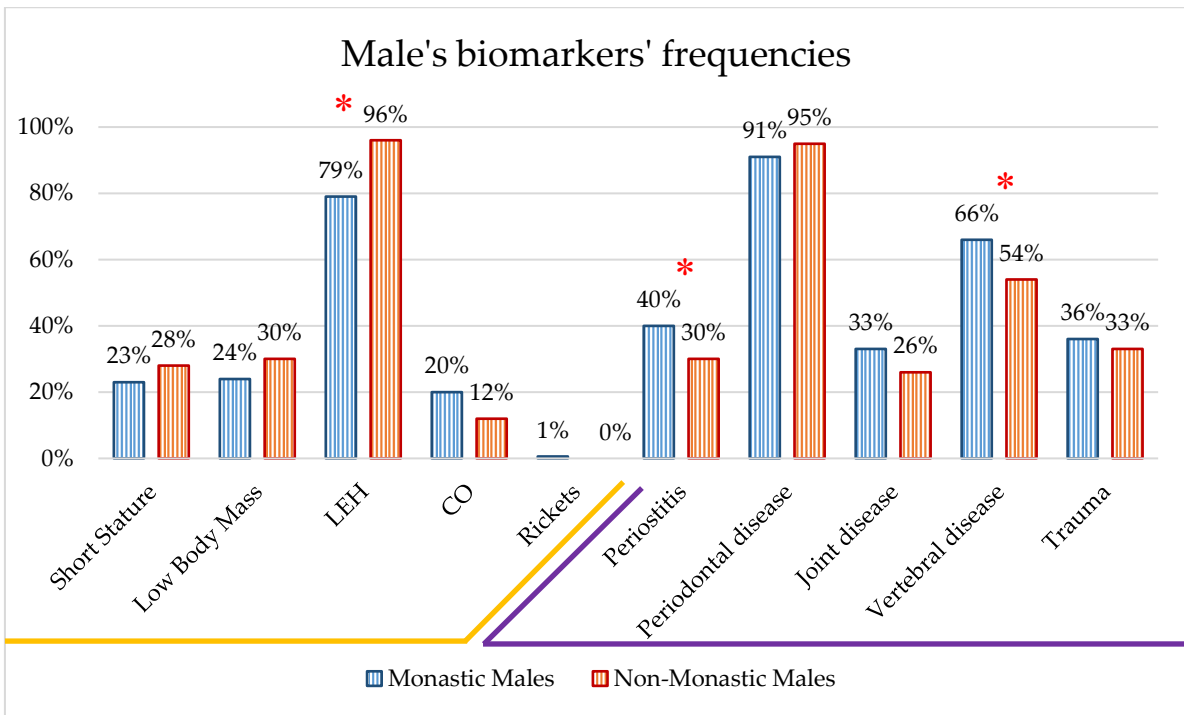
Figure 4.4: Biomarkers' frequencies in the Males, Monastic and Non-Monastic. Yellow box= malnutrition biomarkers; purple box= aspecific infections and activity/older age biomarkers.
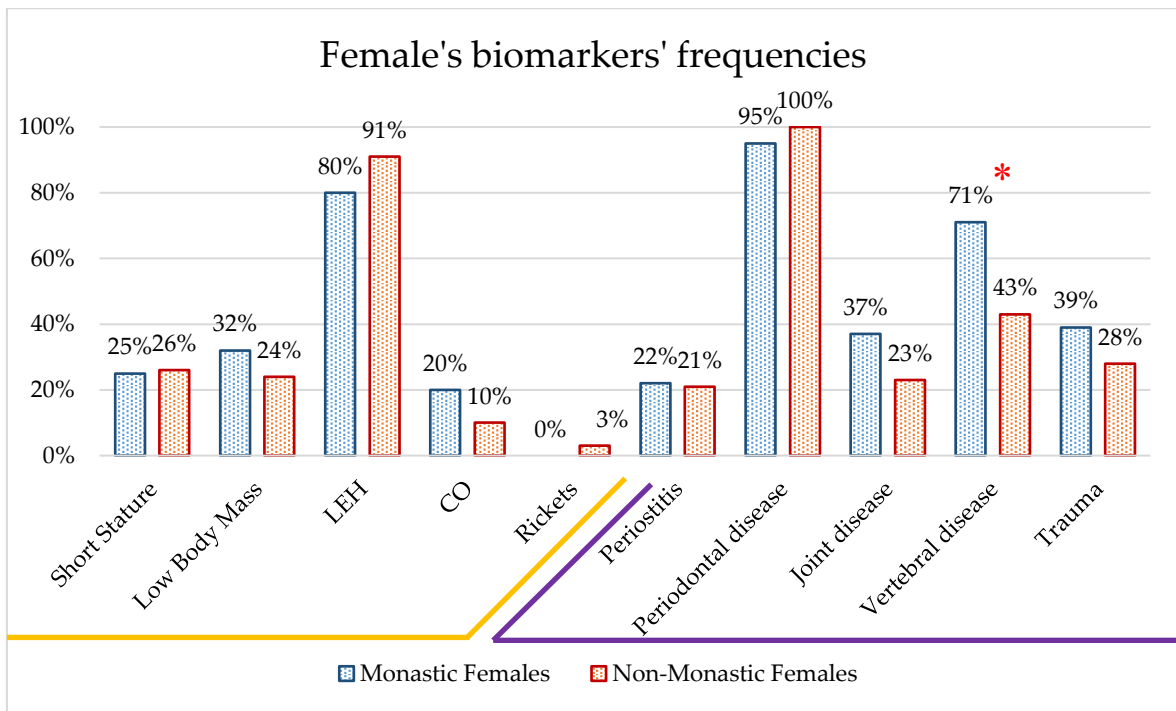
Figure 4.5: Biomarkers' frequencies in the Females, Monastic and Non-Monastic. Yellow box= malnutrition biomarkers; purple box= aspecific infections and activity/older age biomarkers.

4.5   DISCUSSION

In this chapter, we proposed a new index to estimate frailty in ancient human skeletal remains, the Biological Index of Frailty (BIF). The evaluation of frailty is becoming increasingly important in bioarchaeological studies because it can be used to assess the general health status of a population or the predisposition or not of the individuals to diseases. The need to have a new index that encompasses all indicators of health fragility has been underlined several times in the literature, as evidenced by the attempt to create specific indexes.

Frailty indices for the living population have been proposed before (e.g. Abete et al., 2017; Burn et al., 2018; Sacco et al., 2018; Wallace et al., 2019b), however, so far and at our knowledge, only two indices have been proposed for skeletal remains (Steckel and Rose, 2002; Marklein et al., 2016; Marklein and Crews, 2017). Although they were innovative and had the merit of considering all stress biomarkers together for the first time, the two existing indices (Health index and SFI) had some non-indifferent limitations. In this study, we have tried to overcome these limits.

The results demonstrated how the new Index of Frailty could be successfully used on archaeological skeletal remains. BIF is fundamentally different from the previously existing indexes of frailty: the first innovative feature of BIF is that it gives a different weight to each skeletal biomarker of stress, based on their importance in increasing the risk of premature death for the individuals. The Logit Model has allowed us to assign each biomarker a weight that reflects its potential in increasing the risk of death of the individual who exhibits it.

Secondly, for the first time, both the severity and the healing status of the lesions are considered in the index. On the database of the Museum of London, healing was considered only for aspecific periostitis, and we noticed that active lesions had a higher odd ratio than healed ones. Even if it was not possible to test if the same relationship existed for the porous lesions of the cranial vault (PH) and orbital roofs (CO), we consider it as a feasible inference, that should be verified in the future.

Thirdly, and most importantly, the new index can also be used on incomplete skeletal remains, assuming that at least three skeletal biomarkers are observable, making it possible to investigate frailty even in ancient and poor preserved skeletal series. This is possible because we implemented a weighted mean, which allows to not underestimate the skeletons in which only a few characters are assessable. Indeed, the more biomarkers can be observed, the more precise the analysis is, but the weighted mean of at least three biomarkers ensure a reliable estimation.

The application of the new index on Monastic and Non-Monastic medieval populations from the Museum of London revealed the suitability and applicability of BIF. The same general differences previously observed by Marklein and colleagues (Marklein et al., 2016) were detected in our analysis as well: higher frailty in the Monastic group than in the Non-Monastic one (Fig. 4.6).
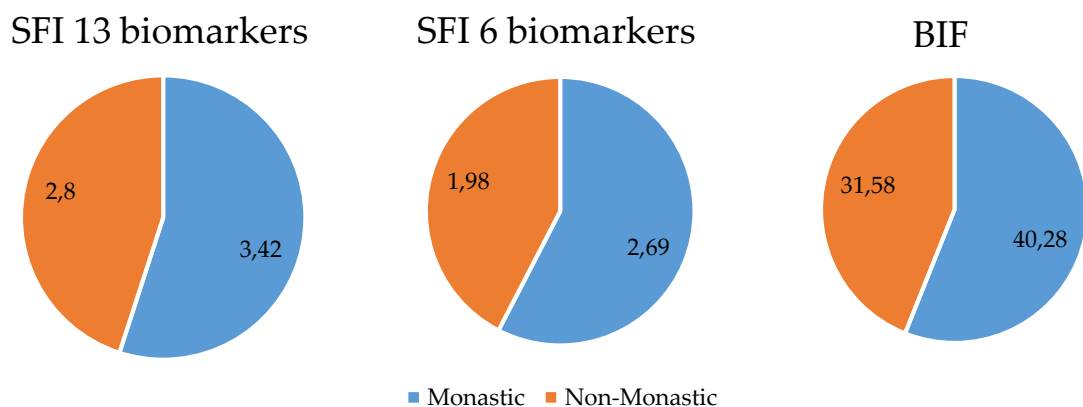
Figure 4.6: mean BIF and SFI values for the Monastic and Non-Monastic groups.

Contrarily to the SFI of Marklein and colleagues, the BIF could be applied to more individuals, which is a great innovation, if we consider that even Marklein and colleagues stressed out the need to have a higher number of individuals. This was the reason why they have proposed later an SFI with a reduced number of biomarkers (Marklein and Crews, 2017). Nevertheless, with BIF, it was possible to analyse 1009 individuals, compared to the 134 initially investigated by Marklein in 2016 (Marklein et al., 2016) and the 517 examined, always by Marklein, in 2017 (Marklein and Crews, 2017) with the modified SFI composed by 6 biomarkers.

Using age as the covariate in the ANCOVA, we noticed how only for women the difference in frailty between the two groups still existed. This phenomenon might be due to the lower number of females

recovered in the two monastic cemeteries (only 8% of the Monastic sample was composed of women) compared to males. Nevertheless, women were not few (Table S4.2; Fig. 4.2), and the difference between Monastic and Non-Monastic women was statistically significant. It is also interesting to notice how, comparing the frequencies of the biomarkers individually, there was not a statistically significant difference between Monastic and Non-Monastic women if not for vertebral diseases (Fig. 4.5).

It was previously observed by Dewitte (Dewitte et al., 2013) that Monastic individuals from medieval times lived longer than Non-Monastic people. As we can see in the Supplementary Table S4.2, there were many elders in the Monastic population respect to the Non-Monastic one. The monastic population was composed of clerical men and women, yet, also noble individuals were sometimes buried with the clericals (Dewitte et al., 2013). Moreover, during the Middle Ages, those who joined the clergy were mostly people from noble families, usually second children who could not inherit the assets of their family. Therefore, the longer lifespan of the Monastic population was probably due to better living conditions and diet, but they were subjected to higher morbidity, as the higher frailty score, both the SFI (Marklein et al., 2016) and the BIF, testified.

Observing the gross frequencies of the biomarkers, we noticed that all malnutrition-related stress markers were highly present in the Non-Monastic population, denoting its worst living condition. Monastic population, however, had a statistically higher frequency of periostitis, probably due to the role of infirmary that some monastery assumed during medieval times (Dewitte et al., 2013), allegedly increasing the possibility to contract infections. Moreover, joint and vertebral disease, as well as trauma, were more frequent in the Monastic sample: both activity and old age can cause joints problems, and, indirectly, fractures, trough bone loss. Supposedly, it was the longer lifespan the cause of the phenomenon in this case.

Still, observing frailty levels, we noticed that, while frailty increase with age, i.e. with the increasing of the allostatic load on each individual, in the last classes of age, particularly the last one (>46), the level of frailty seemed to slow its increase or even to slightly decrease (Fig. 4.7). Apparently, only individuals who do not overcome a determined frailty can survive the average life expectancy. Of course, more research should be done before establishing a cut-off, as other variables (historical time, geographic region, and others) could affect the data, as the difference between Monastic and Non-Monastic mean BIF reveals.
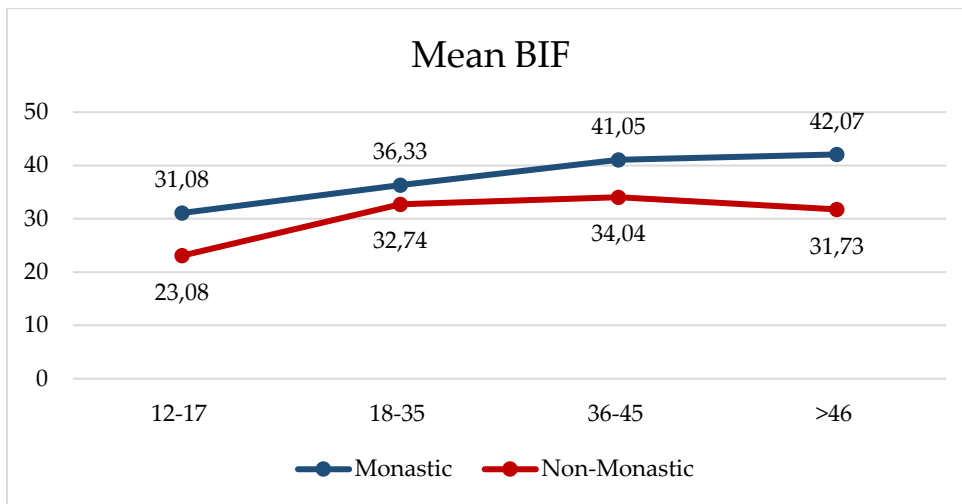
Figure 4.7: mean BIF for each class of age in the monastic and Non-Monastic group.

As we underlined, both biomarkers' frequencies and frailty index are necessary to correctly describe and analyse the health status of past populations: frailty, evaluated trough the BIF, give information on the cumulative physiological stress load and general health conditions of an individual as well as of a population, and can be used to compare two or more populations. Gross frequencies of the biomarkers and a proper analysis of the historical sources may shed light on which stress has led to frailty.

There are also limitations in our study, namely that we could not assess the importance of PH, the healing status of the porotic lesions and the weight of osteoporosis evaluated with imaging techniques. It will, therefore, be necessary to subsequently integrate the study using another database that also considers those variables to confirm the validity of this method.

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4.7   SUPPLEMENTARY

Table S4.1: Life table of the London Cemeteries

x	dx	d'x	Ix	I'x	qx	px	Lx	Tx	ex
0/11 mesi	9	0,860421	1046	100	0,860421	99,13958	99,56979	3386,902	33,86902
1/5	40	3,824092	1037	99,13958	3,857281	96,14272	486,1377	2900,765	29,2594
6/11	58	5,544933	997	95,31549	5,817452	94,18255	555,2581	2345,507	24,60782
12/17	58	5,544933	939	89,77055	6,176784	93,82322	521,9885	1823,518	20,3131
18/25	91	8,699809	881	84,22562	10,32917	89,67083	639,0057	1184,512	14,06356
26/35	207	19,78967	790	75,52581	26,20253	73,79747	656,3098	528,2027	6,993671
36/45	409	39,10134	583	55,73614	70,15437	29,84563	361,8547	166,348	2,984563
>46	174	16,6348	174	16,6348	100	0	83,174	83,174	5

Table S4.2:Sample of Monastic and Non-Monastic individuals used for statistical analysis, divided by sex and class of age (ND=not-determined).

	Monastic			Non-Monastic		
	Males	**Females**	**Sex ND**	**Males**	**Females**	**Sex ND**
12-17	-	-	17	-	-	26
18-35	145	8	6	61	39	5
36-45	253	30	12	64	23	5
>46	101	8	6	31	20	1
Age ND	76	1	29	10	3	29
Tot	**575**	**47**	**70**	**166**	**85**	**66**

Figure S4.1: Biological Index of Frailty Scoring Form

BIOLOGICAL INDEX OF FRAILTY

Site		Individual Code	
US / N. tomb		Operator	

GENERAL INFORMATION

Ancestry: _____   Sex: _____

Age: _____   Stature: _____

(da: Perizonius, 1982)
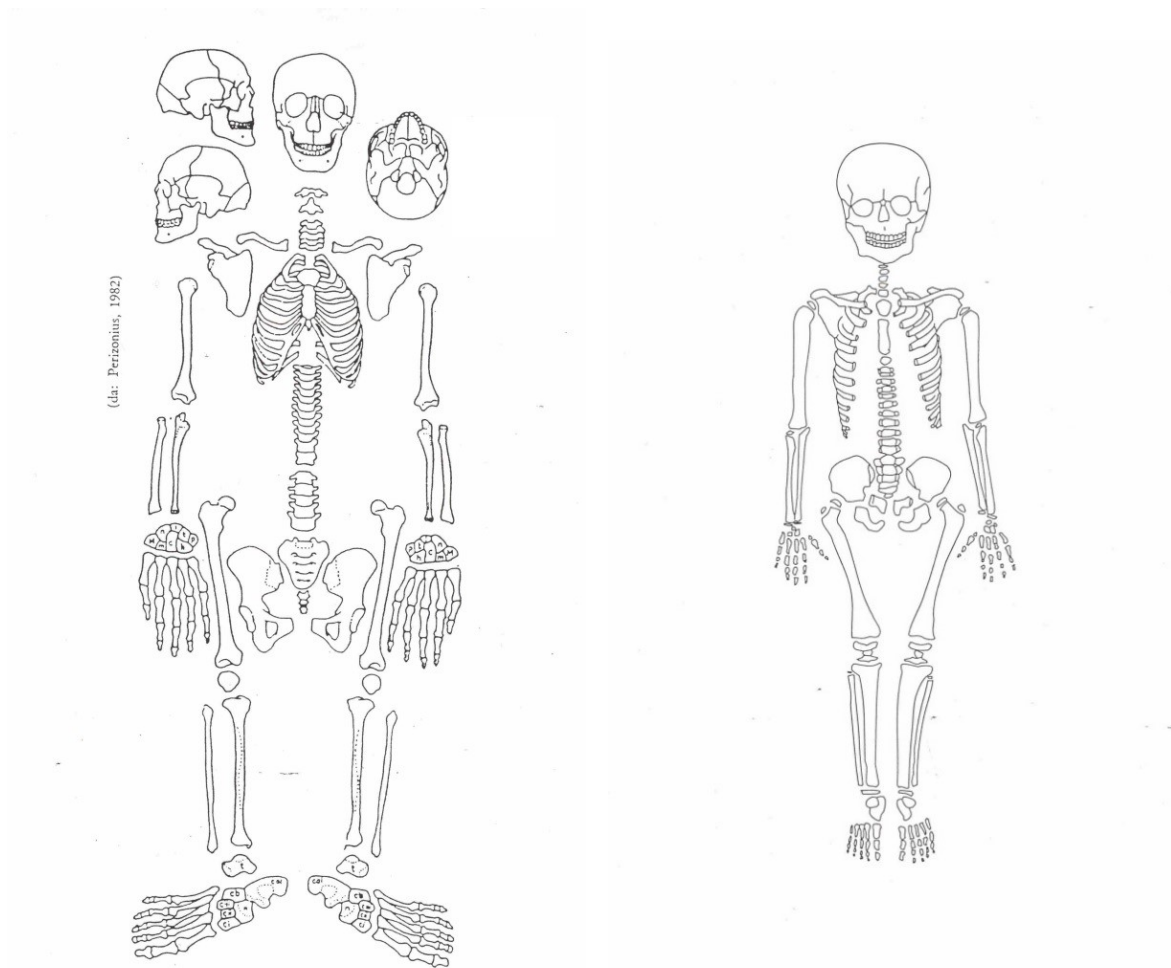
STATURE

PRESENCE/ABSENCE SKELETAL PART

LOWER LIMB	
FEMUR R: _____	FEMUR L: _____
TIBIA R: _____	TIBIA L: _____
FIBULA R: _____	FIBULA L: _____

UPPER LIMB	
HUMERUS R: _____	HUMERUS L: _____
RADIO R: _____	RADIO L: _____
ULNA R: _____	ULNA L: _____

MEASUREMENT

LONG BONES' MAXIMAL LENGHT	R	L
Humerus	mm	mm
Radio	mm	mm
Ulna	mm	mm
Femur (maximum length)	mm	mm
Femur (physiological length)	mm	mm
Maximal femur head diameter	mm	mm
Tibia	mm	mm
Fibula	mm	mm

ESTIMATION OF STATURE

(adults only)

METHODS			
Use left limbs, if present, otherwise the right ones.			
Trotter e Gleser (1952, 1958)	±		cm
	±		cm
	±		cm
	±		
	±		
Manouvrier (1892, 1893)	±		
	±		
Gualdi, Bramanti, Rinaldo (2018)			

STATURE
Mean stature (Trotter e Gleser) =
Mean stature (Manouvrier) =
Mean stature (Gualdi et al.) =
Mean stature (other methods)
Mean stature (other methods)

LINEAR ENAMEL HYPOPLASIA

(permanent teeth)

Presence/absence:_____ (Codes: P – present / A – absent / NO – not observable)

MAXILLA

	P/A	HYPOPLASIA	N° DEFECTS	POSITION
Dx M3				
M2				
M1				
P2				
P1				
C				
I2				
I1				
Sx I1				
I2				
C				
P1				
P2				
M1				
M2				
M3				

MANDIBLE

	P/A	HYPOPLASIA	N° DEFECTS	POSITION
Dx M3				
M2				
M1				
P2				
P1				
C				
I2				
I1				
Sx I1				
I2				
C				
P1				
P2				
M1				
M2				
M3				

Note:

LINEAR ENAMEL HYPOPLASIA

(deciduous teeth)

Presence/absence:_____ (Codes: P – present / A – absent / NO – not observable)

MAXILLA

	P/A	HYPOPLASIA	N° DEFECTS	POSITION
Dx				
m2				
m1				
c				
i2				
i1				
Sx				
i1				
i2				
c				
m1				
m2				

MANDIBLE

	P/A	HYPOPLASIA	N° DEFECTS	POSITION
Dx				
m2				
m1				
c				
i2				
i1				
Sx				
i1				
i2				
c				
m1				
m2				

Note

PERIODONTITIS

Presence/absence:_____ (Codes: P – present / A – absent / NO – not observable)

MAXILLA

	P/A	PD LEVEL (1-4)	MISURA R. M. ALV.
Dx M3			
M2			
M1			
P2			
P1			
C			
I2			
I1			
Sx I1			
I2			
C			
P1			
P2			
M1			
M2			
M3			

MANDIBLE

	P/A	PD LEVEL (1-4)	MISURA R. M. ALV.
Dx M3			
M2			
M1			
P2			
P1			
C			
I2			
I1			
Sx I1			
I2			
C			
P1			
P2			
M1			
M2			
M3			

PD levels: 1-healthy, 2-light periodontitis; 3-moderate periodontitis; 4-severe periodontitis (Ogden 2008)

Note:

CRIBRA ORBITALIA

<table>
<tr><td colspan="2">INVENTORY (ORBITAL ROOFS) Codes: P – present / A – absent / NO – not observable
RIGHT ORBITAL ROOF _____   LEFT ORBITAL ROOF: ____</td></tr>
</table>

ASSESSMENT OF CRIBRA ORBITALIA (PER INDIVIDUAL)

Presence/absence of CO _____ (Codes: P – present / A – absent)

General degree of severity (0-4) _____

General degree of healing (1-4) _____

ASSESSMENT OF CRIBRA ORBITALIA (FOR EACH ORBITAL ROOF)

RIGHT ORBITAL ROOF			LEFT ORBITAL ROOF		
Degree of severity (0-4)			Degree of severity (0-4)		
Degree of healing (1-4)			Degree of healing (1-4)		
Frequency of pits in 1 cm^2	$n=$		Frequency of pits in 1 cm^2	$n=$	
Size of area with CO	Length _____mm	Width ____mm	Size of area with CO	Length _____mm	Width ____mm

From (Rinaldo et al., 2019)

Note:

POROTIC HYPEROSTOSIS

INVENTORY (CRANIAL BONES) Codes: P – present / A – absent / NO – not observable	
FRONTAL: _____	RIGHT PARIETAL: ____
OCCIPITAL: _____	LEFT PARIETAL: ____

ASSESSMENT OF POROTIC HYPEROSTOSIS (PER INDIVIDUAL)				
Presence/absence of PH _____ (Codes: P – present / A – absent// NO – not observable)				
General degree of severity (0-4) _____				
General degree of healing (1-4) _____				
ASSESSMENT OF POROTIC HYPEROSTOSIS (FOR EACH CRANIAL BONE)				
RIGHT PARIETAL			**LEFT PARIETAL**	
Presence of PH within the quarters (P/A/NO)	2	1	Presence of PH within the quarters (P/A/NO)	1	2
	4	3		3	4
Degree of severity (0-4)			Degree of severity (0-4)		
Degree of healing (1-4)			Degree of healing (1-4)		
Frequency of pits in 1 cm^2	$n=$		Frequency of pits in 1 cm^2	$n=$	
Size of the area with PH	<50%	>50%	Size of the area with PH	< 50%	>50%
FRONTAL			**OCCIPITAL**	
Presence of PH within the quarters (P/A/NO)	1	2	Presence of PH within the quarters (P/A/NO)	1	2
	3	4		3	4
Degree of severity (0-4)			Degree of severity (0-4)		
Degree of healing (1-4)			Degree of healing (1-4)		
Frequency of pits in 1 cm^2	$n=$		Frequency of pits in 1 cm^2	$n=$	
Size of the area with PH	<50%	>50%	Size of the area with PH	< 50%	>50%

From (Rinaldo et al., 2019)

Note:

PERIOSTITIS

Presence/absence:_____ (Codes: P – present / A – absent / NO – not observable)

PERIOSTITIS TIBIA

	P/A lesions	Type of lesions ACTIVE/ REMODELLED/ MIXED	Note
TIBIA R			
TIBIA L			

PERIOSTITIS

Bone	Type of lesions ACTIVE/ REMODELLED/ MIXED	Note

Note:

JOINT DISEASE

Presence/absence: _____ (Codes: P – present / A – absent / NO – not observable)

UPPER JOINTS

		Right		Left	
		P/A/NO	Description	P/A/NO	Description
Shoulder	scapula				
	clavicle				
	Humerus (head)				
Elbow	Humerus (distal end.)				
	Ulna (proximal end)				
	Radio (proximal end)				
Wrist	Ulna (distal end.)				
	Scaphoid				
	Radio (distal end.)				
	Semilunar				
Hand	Carpals				
	Metacarpals				
	Hand Phalanges				

JOINT DISEASE

LOWER JOINTS

		Right		Left	
		P/A/NO	Description	P/A/NO	Description
Hip	Coxal				
	Femur (head)				
Knee	Femur (distal end.)				
	Patella				
	Tibia (proximal end)				
Ankle	Tibia (distal end.)				
	Fibula (distal end.)				
	Astragalus (trochlea)				
Foot	Talus				
	Calcaneus				
	Tarsus				
	Metatarsus				
	Foot Phalanges				

TRAUMA

Presence/absence fractures: _____ (Codes: P – present / A – absent / NO – not observable)

OSTEOPOROTIC FRACTURES

vertebra	P/A vertebra	Deformation degree	Fracture shape
T4			
T5			
T6			
T7			
T8			
T9			
T10			
T11			

vertebra	P/A vertebra	Deformation degree	Fracture shape
T12			
L1			
L2			
L3			
L4			

OTHER TRAUMA

BONE	Healing (P/A)	Description

VERTEBRAL PATHOLOGIES

Presence/absence:_____ (Codes: P – present / A – absent / NO – not observable)

VERTEBRA	P/A/NO pathologies	DESCRIPTION
C1		
C2		
C3		
C4		
C5		
C6		
C7		
T1		
T2		
T3		
T4		
T5		
T6		
T7		
T8		
T9		
T10		

VERTEBRAL PATHOLOGIES

VERTEBRA	P/A/NO pathologies	DESCRIPTION
T11		
T12		
L1		
L2		
L3		
L4		
L5		
S1		
S2		

RICKETS/OSTEOMALACIA

Presence/absence:_____ (Codes: P – present / A – absent / NO – not observable)

Description:

OSTEOPOROSIS

Presence/absence :_____ (Codes: P – present / A – absent / NO – not observable)

Methodology	Treshold for osteoporosis	Value

FRAILTY IN PLAGUE AND NON-PLAGUE VICTIMS FROM ROMAGNA, ITALY (17TH CENTURY)

Does plague kill indiscriminately? This is one of the questions about plague that historians, bioarchaeologists, biomolecular scientists have often tried to answer in the last years. Plague is one of the infectious diseases that affected the human population with catastrophic results throughout almost its entire history and is nowadays still present in some parts of the world. Here, we attempt to address the question of selective plague mortality through the analysis of frailty's conditions in two 17th century skeletal series: one of plague's victims and one from a conventional (attritional) cemetery of the same geographic area. Since in an attritional cemetery we can find the majority of people coming from the frailer cohorts, we assumed that similar scores of frailty in the two populations could be indicative of selective mortality due to plague. Alternatively, lower scores of frailty in the individuals died by plague could be interpreted assuming that plague also affected healthy individuals indiscriminately.

5.1 INTRODUCTION

Different theories regarding plague mortality have been proposed, and we have reported and discussed them in the first chapter of this thesis. Some authors have tried to test whether plague killed indiscriminately or selected its victims between the frailest, analysing the skeletal remains recovered in plague mass graves. In particular DeWitte and Wood (2008) argued that plague selected its victims within the frailer cohort, while Kacki (Kacki, 2016), in his doctoral thesis, in which he compared French plague victims and non-plague victims, claimed that within plague victims we could find healthier individuals than in an attritional cemetery. None of them, however, analysed the frailty of the individuals using an index but compared every single biomarker independently. Here, we aim to test the theories about plague selectivity on the weakest applying the index of frailty, that we described in the previous chapter, to two skeletal samples, one of plague victims and one from an attritional cemetery. In particular, we designed our research to check whether a difference exists in the health status of plague victims in comparison to that of non-plague's victims. If plague admittedly affected the frailer individuals, as other infectious diseases tend to do (Thorburn, 2009; Drew et al., 2017), frailty scores of its victims should be similar to those of the non-plague's victims, who died in consequence of their poor health status. But, if plague affected all individuals, even those in good health, there should be a higher number of individuals in good health status (lower frailty scores) among the plague victims.

Moreover, analysing porous lesions of the cranial vault and orbital roofs, which are generally acknowledged signal of an anemic status (see chapter 3), we can investigate if iron played a significant role in plague mortality.

To carry out this investigation, which would have allowed us to address several issues that were raised during this PhD-work, we chose two samples from two distinct archaeological excavations from the same geographic area and the same historical period. Environmental conditions should have influenced the frailty of all the individuals in the same way: similar food availability, living conditions, exposition to infections as well as historical events they were subjected to, should have similarly modulated the health status of all the individuals living in the same region.

Thus, choosing two sites from the same geographic and historic environment, we aimed to ensure that the plague was the main discriminating variable. The plague site is represented by the necropolis of L'Osservanza Lazaret of Imola, in use during the epidemic of 1630-32, whereas the conventional necropolis is represented by the graveyard of the San Biagio Church in Ravenna (17th century CE); both cemeteries are located in the region Emilia-Romagna (North-Eastern Italy) (Fig. 5.1).

Figure 5.1: Romagna (Emilia Romagna, Italy), geographic area of the two samples analysed.

5.2 MATERIALS AND METHODS

5.2.1 Materials

We analysed a sample of plague victims buried during the epidemic of 1630-32 at the Imola's Lazzaretto and a sample of non-plague victims of the 17th century CE from the cemetery of the church San Biagio in Ravenna. As the aim was to investigate health and frailty in the two populations, we

chose to apply the frailty index only on individuals older than or equal to 12 years of age at death, for the same reasons we detailed in the previous chapter.

We analysed 133 skeletal remains from 4 mass graves (tomb 3,6,7 and 8) excavated in 2007 in the complex of L'Osservanza at Imola (BO); and 58 skeletons from the lower level of the cemetery of San Biagio in Ravenna.

All the skeletons were stored at the Laboratory of Archaeo-Anthropology and Forensic Anthropology of the University of Ferrara.

5.2.1.1   Imola (1630-32)

Between 1629 and 1632, Northern Italy was hit by an epidemic of plague that killed more than 25% of the population (Hays, 2005). The plague was introduced in Italy by troops of soldiers crossing the Alps during the Thirty Years War, and reaching first the city of Milan in 1629. The Italian writer Alessandro Manzoni portrayed this event in his famous novel "*I Promessi Sposi*" ("The Betrothed"). Soon, the plague spread in all northern and central Italy till Tuscany, encountering a population already weakened by wars, an epidemic of typhus in 1628-29 and famine (Del Panta and Livi Bacci, 1977), while it reached southern Italy during the second wave in 1656 (Capasso et al., 2011). The plague reached the city of Imola in 1630, after several attempts to stop the spread of the contagion in its course (Cervellati, 1986). Historical sources clearly explain the symptoms of the individuals affected and describe buboes among other characteristic signals (Cervellati, 1986); therefore we can assume with confidence that *Yersinia pestis* was the causative agent of the pestilence. The convent of L'Osservanza was situated south of the medieval city walls and was used as a "Lazzaretto", a place for the care of infected people from both the city and the countryside, as well as a burial place for the dead (Cervellati, 1986). Only part of all the mass graves reported in the historical sources (Cervellati, 1986) was excavated by the Superintendence for Archaeological Heritage of Emilia-Romagna in 2007, consisting of 4 mass graves (Fig. 5.2) and a total of 133 individuals.

5.2.1.2   Ravenna (17th century)

Ravenna, located in the same geographic area of Imola (Fig. 5.1), was not spared by the plague. Like many other cities of the Emilia Romagna, it was hit by the disease during the years of 1630-32. Like their neighbours, the inhabitants of Ravenna suffered wars, famines and diseases during the 17th century (Manzoni, 1989). In the village of San Biagio, at the edge of Ravenna, there was a small church erected at the beginning of the 17th century (Caravita, 2008). The cemetery of the church was used from 1602 to 1817, as attested in the Parish register of the Archdiocese of San Biagio, but it was not used for plague victims. Archaeological excavation started in 2013, recovered two batches of burials dating to 1600 until the start of 1800 (Fig. 5.3). The lower burials date back, most likely, to the 17th century, and are the ones used in our analysis.

Figure 5.2: Tomb 8 from L'Osservanza, Imola. (Rinaldo et al., 2014).

Figure 5.3: Excavation of the cemetery of San Biagio in Ravenna. Courtesy of the "Soprintendenza dei Beni Culturali e del Paesaggio" of Emilia Romagna.

5.2.2 Methods

5.2.2.1 *Age and sex estimation*

The skeletal remains were carefully cleaned and restored; age at death and sex estimation were assessed for each individual. Sex and age at death estimation for the Imola sample was carried out by Dr Natascia Rinaldo (Guellil et al.- in preparation; Rinaldo et al., 2014; Rubini et al., 2016).

To assess sex in adult individuals and adolescents older than 15 years of age, morphological methods based on sexual dimorphism of the skull and pelvis (Acsadi, G.; Nemeskeri, 1974) were employed, if applicable; otherwise, and only for the adults, metric methods related to the post-cranial skeleton were used (Scheuer and Elkington, 1993; Bass, 1995; France, 1998; Gualdi-Russo, 2007; Manolis et al., 2009). To estimate the age-at-death in subadults, we employed several different methods: we evaluated the degree of development and eruption of teeth (Ubelaker, 1989), and the degree of fusion of the ossification centres (İşcan and Kennedy, 1989; Belcastro et al., 2008; Ríos and Cardoso, 2009; Cardoso and Severino, 2010), as well as the evaluation of the length of the long bones (Ubelaker, 1989; Scheuer and Black, 2004). For the determination of the age at death in adults, different methodologies were applied as well: we observed the modifications due to aging of the morphology of both the pubic symphysis (Todd, 1920; Suchey et al., 1986), and the auricular surface of the ileum (Buikstra and Ubelaker, 1994); the degree of obliteration of the ectocranial sutures (Meindl and Lovejoy, 1985), and the degree of dental wear (Brothwell, 1981; Lovejoy, 1985). Individuals were then grouped into age classes according to the division proposed by Buikstra and Ubelaker (1994).

5.2.2.2 *Biological Index of Frailty (BIF)*

The same index and biomarkers analysis we described in chapter 4 was applied to the two samples (plague victims and non-plague victims). Following the guidelines outlined in the previous chapter, we collected data on 11 biomarkers (osteoporosis was not evaluable). The weight attributed to each biomarker is described in Table 4.2

Each biomarker was evaluated as a categorical variable as follow:

- Short stature (at least one long bone needs to be present to estimate the stature): 1=present, the stature of the individual falls in the first quartile of the population's stature distribution; 0=absent, the stature of the individual falls outside the first quartile of the population's stature distribution.

- Low Body Mass (at least one femoral head should be measurable): 1=present, the femoral vertical head diameter of the individual falls in the first quartile of the population's distribution; 0=absent, the femoral vertical head diameter of the individual is not in the first quartile of the population's distribution.

- Linear Enamel Hypoplasia (LEH) (at least one canine is observable): 1=present, at least one tooth presents multiple lines of hypoplasia; 0= no tooth exhibit multiple linear hypoplasia.

- Cribra Orbitalia (CO) (at least one orbit should be observable): 1 = present, at least one orbital roof showed porotic lesions, the weight of the biomarker changes if CO's severity is

1 or 2-4, and if the lesion is active or healing/healed (Table 2-chapter 4); 0= orbits have no porous lesions.

- Porotic Hyperostosis (PH) (at least half cranial vault should be observable): 1 = present, the cranial vault showed porotic lesions, the weight of the biomarker changes if PH's severity is 1 or 2-4, and if the lesion is active or healing/healed (Table 2-chapter 4); 0= cranial vault has no porous lesions.

- Rickets/osteomalacia (at least one leg bone should be observable): 1 = present, signs of deformation of legs' bones or other signs of vitamin D deficiency on the skeletal remains; 0= no signs of vitamin D deficiency are detectable.

- Periodontal disease (at least one molar tooth and half jaw should be observable): 1 = present, at least one tooth manifests degree 2 to 4 of Ogden's periodontitis score; 0= absent, no teeth and alveolar bone showed signs of periodontal disease.

- Periostitis/osteomyelitis (at least one tibia should be observable): 1= present, the individual showed the presence of aspecific new bone formation; the weight of the biomarker changes if the periostitis is active or mixed/remodelled (Table 2-chapter 4); 0= absent, no new bone formation was present.

- Joint diseases (at least 2/3 (10/16) of the major joints should be observable): 1= present, at least one joint presents sign of osteoarthritis (OA) or other joint diseases; 0= no signs of joint disease.

- Vertebral diseases (at least 2/3 (16 out of 24 vertebrae) of the column should be observable): 1= present, at least one vertebra presents signs of Intervertebral disk disease (IVD), osteoarthritis (OA) or other vertebral diseases; 0= no signs of vertebral disease.

- Trauma (at least 2/3 of the whole skeleton should be observable): 1= present, at least one healed trauma or osteoporotic fracture is present; 0= no trauma is present.

At least three of these biomarkers should be observable on the skeleton to apply the BIF.

5.2.2.3   *Statistical analysis*

Differences between BIF values of plague victims and non-plague victims and between the two sexes were assessed using a *t*-test, the test U of Mann-Whitney, and the analysis of covariance (ANCOVA) adjusted for age. We then performed Chi-squared tests on the frequencies obtained for each biomarker to identify statistically significant differences between the two groups (plague and non-plague victims), and between sexes. The *p*-values <0.05 were considered statistically significant. All statistical analyses were conducted using STATISTICA (version 11, StatSoft, Tulsa, OK), and MedCalc Statistical Software version 14.8.1 (MedCalc Software bvba, Ostend, Belgium).

5.3 RESULTS

The index of frailty was applied to all individuals over 12 years of age at death, and on whose skeleton there were at least three biomarkers of stress observable. Under these stringent conditions, it was anyway possible to estimate the frailty index BIF in 64 individuals from Imola and 52 individuals from Ravenna.

As reported in Table 5.1, we found no significant difference between plague victims and non-plague victims, regarding mean frailty values. Nonetheless, we noticed that the mean BIF value was higher in the sample of Ravenna (46.10 Ravenna vs 40.08 Imola). When we compared men and women separately using an ANCOVA adjusted for age, we observed a small difference between males of the two groups, with plague victims showing a slightly higher mean frailty than non-plague victims. Quite different was the situation of females, who displayed a slightly higher dissimilarity, even if not statistically significant, with lower frailty values in the Imola's sample (Tab. 5.1), indicative of general better health status in female plague victims. Yet, the difference in frailty between males and females within each population did not give significant results (Imola $p=$ 0.5465; Ravenna $p=$0.4390). We compared the differences of frailty in each class of age with the U Mann Whitney test, and while there are some noticeable differences, none is statistically significant (Tab. 5.1). We noted a high value of mean BIF in the adolescents of Ravenna, with a high standard deviation, that is due to the low number of individuals (2) in this class. We also noted that, although not statistically significant, the mean BIF value was always higher in the non-plague victims, with the only exception of the group 18-34 years.

Table 5.1: Mean Biological Frailty Index in the sample and comparison (ANCOVA e U Mann-Whitney) between groups (Plague victims and Non-plague victims). In bold are indicated the higher values of the two groups, plague and non-plague victims.

	Plague Victims (Imola)		Non-Plague victims (Ravenna)		
	N	Mean *BIF* (s.d.)	N	Mean *BIF* (s.d.)	*p*
Total	64	40.08 (23.59)	52	**46.10 (21.11)**	0.1474*
Males	25	**44.27 (21.58)**	27	42.52 (18.62)	0.9622*
Females	26	40.48 (27.16)	22	**48.40 (20.83)**	0.1620*
12-17	13	31.23 (18.19)	2	**58.82 (58.23)**	0.4969+
18-34	32	**46.67 (24.60)**	10	41.10 (18.08)	0.4785+
35-50	14	35.91 (23.89)	22	**48.12 (20.12)**	0.2175+
50+	5	32.58 (22.13)	14	**46.29 (17.43)**	0.3085+

*Comparisons performed using ANCOVA adjusted for age
+ Comparisons performed using U Mann Whitney statistical test

Regarding the gross frequencies of each biomarker, we saw (Fig. 5.4) few significant differences between the two groups: all the biomarkers related to an older age (Periodontitis, Joint diseases, Vertebral diseases) displayed higher frequencies in the group from Ravenna, as well as Periostitis. The only statistically significant differences, was for Vertebral diseases (p=0.0127) and Periostitis (p= 0.0254), while for Joint diseases, the difference was very close to the significance threshold (p= 0.0518). If we consider only the males (Fig. 5.5), there was no significant difference between Imola and Ravenna, while for females (Fig. 5.6), the frequencies of Vertebral diseases in the non-plague sample were significantly higher than in the plague sample (p= 0.0030).
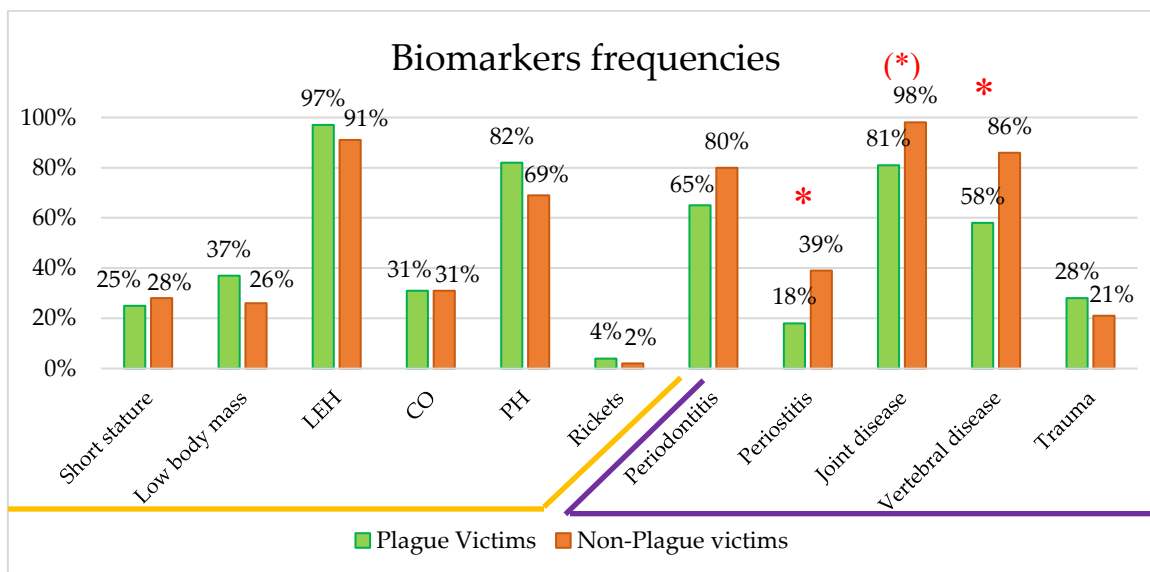
Figure 5.4: Biomarkers' frequencies in Plague's and Non-Plague's victims.
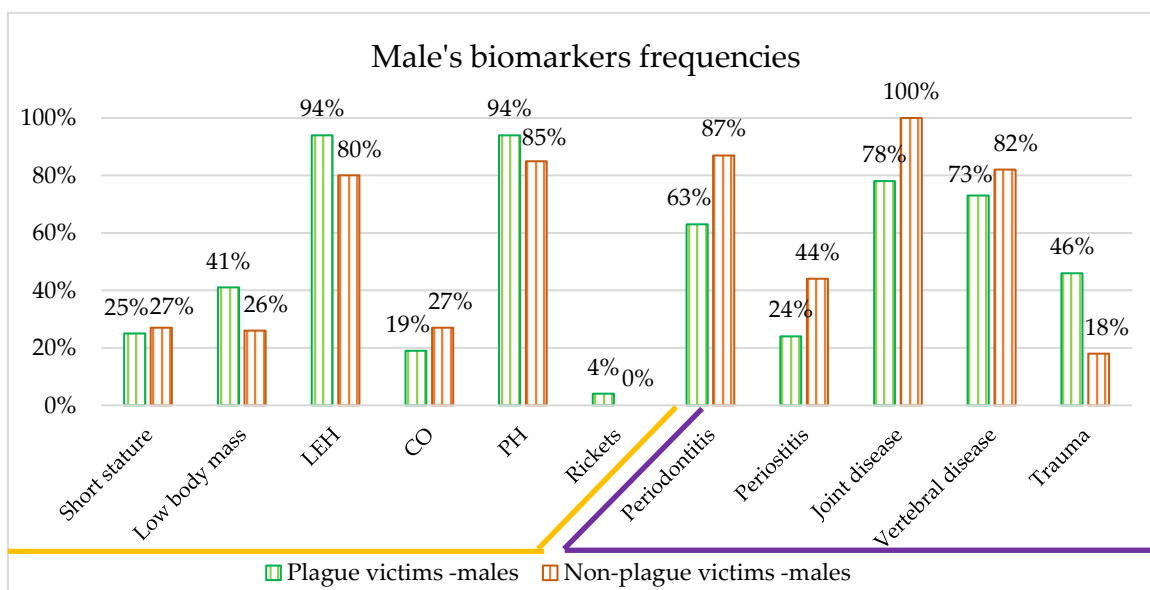
Figure 5.5: Biomarkers' frequencies in the Male Plague's victims and Non-Plague's victims.
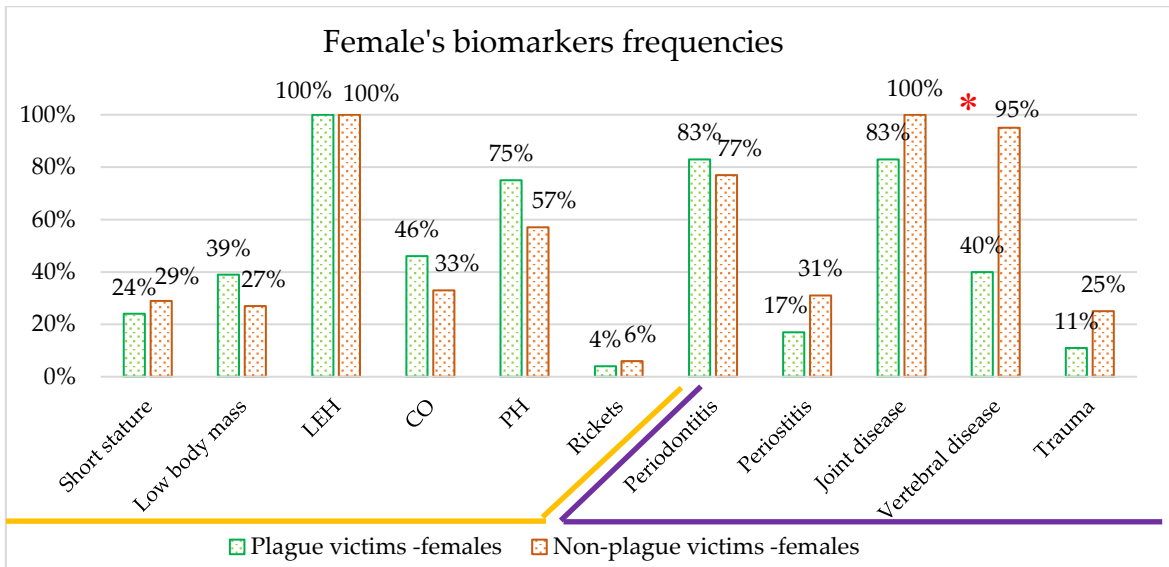
Figure 5.6: Biomarkers' frequencies in the Female Plague's victims and Non-Plague's victims.

We also considered if there was any dissimilarity in the presence of active and healed Porotic Hyperostosis (PH) and Cribra Orbitalia (CO), since a healed status (in the logic of the osteologic paradox (Wood et al., 1992; Dewitte et al., 2015)), would be indicative of a higher robustness of the individuals. We saw that no statistically significant difference existed for PH and CO in both groups (Fig. 5.4). Males and females of both groups did not show any distinct behaviour, in this respect as well (Fig. 5.5, 5.6). The few differences between the groups regarding the degrees of severity and healing were not statistically significant (Fig. 5.7, 5.8, 5.9, 5.10). No difference was found between men and women within each population, as well. Relative frequencies (in percentage) were calculated on the total number of individuals that manifested porous lesions.
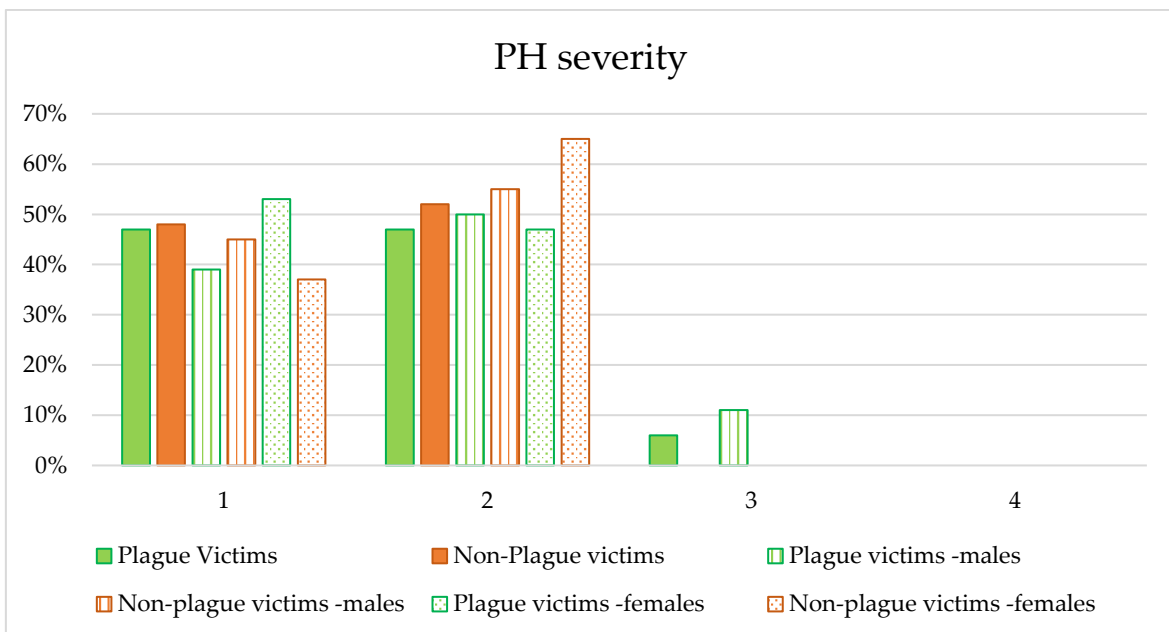
Figure 5.7: Relative frequencies in percentage for each degree of severity of active PH in Plague's Victims and Non-Plague's Victims and both sexes (on those individuals on which sex was estimated).
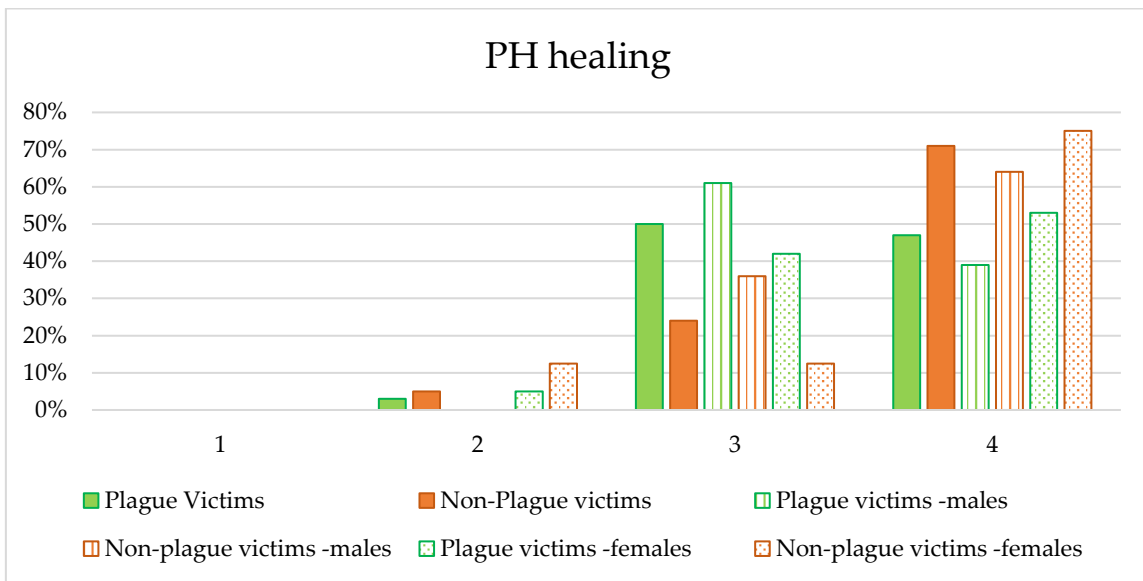
Figure 5.8: Relative frequencies in percentage for each degree of healing of PH in Plague's Victims and Non-Plague's Victims and both sexes (on those individuals on which sex was estimated).
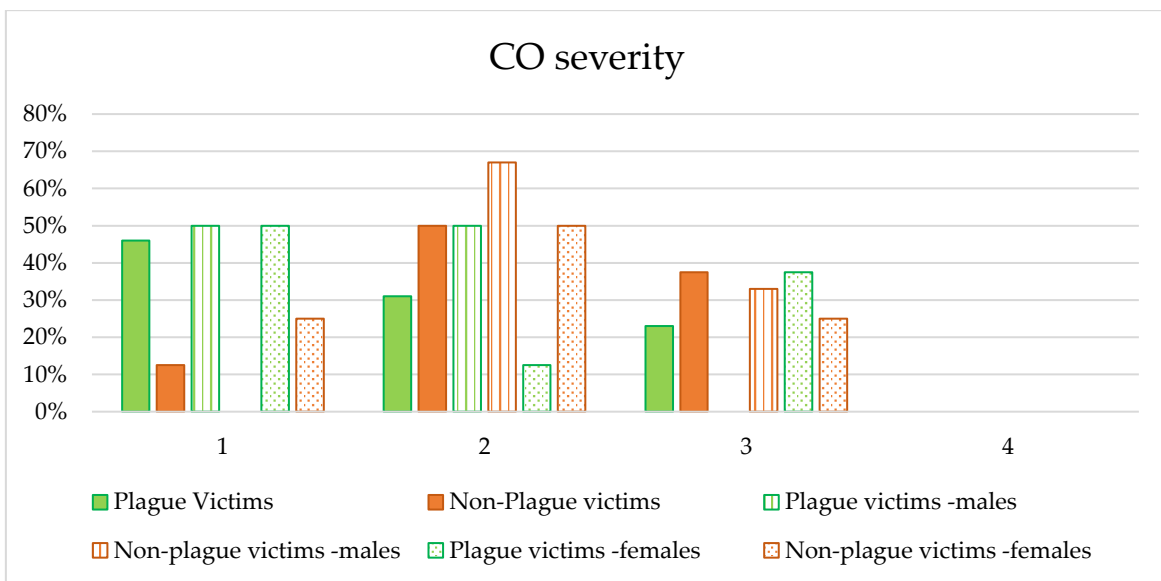
Figure 5.9: Relative frequencies in percentage for each degree of severity of CO in plague victims and non-plague victims and both sexes (on those individuals on which sex was estimated).
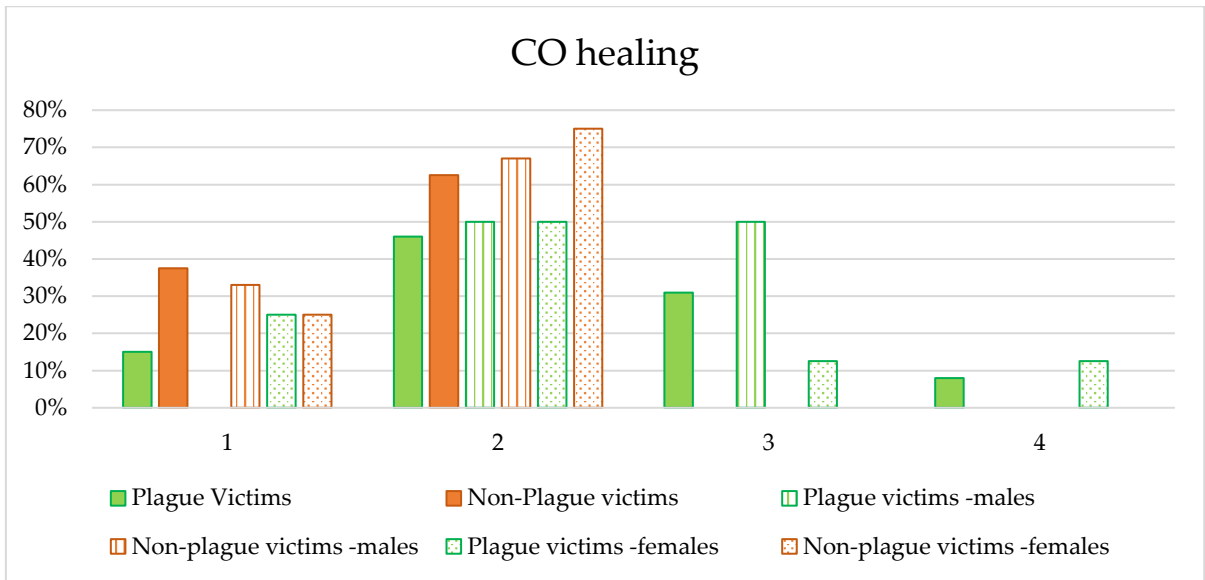
Figure 5.10 Relative frequencies in percentage for each degree of healing of CO in Plague's Victims and Non-Plague's Victims and both sexes (on those individuals on which sex was estimated).

Concerning CO, the degree of severity appeared to be irrelevant to distinguish the two populations. Nevertheless, we observed that the individuals showing a healed CO (degree 3-4) were all plague victims (both males and females), a situation that suggests better health conditions for this group (Fig. 5.10).

5.4   DISCUSSION

In this chapter, we discuss if plague selected its victims based on their health status, as some scholar suggested (DeWitte and Wood, 2008; DeWitte and Hughes-Morey, 2012; DeWitte, 2018), or not. As we have discussed in the first chapter, this question was highly debated in the last years, since plague played an important role in the history of the humankind, with high death-tolls and psychosocial, political and historical consequences. Moreover, plague still exists in some parts of the world and represents a consistent threat in times of globalisation.

In our review of all anthropological data on plague victims (which is depicted in the second chapter of this thesis), plague seemed not to select a determinate group of weak people. Using LEH as a proxy for frailty, the statistical model did not disclose, any selectivity of plague (chapter 2). Nonetheless, the investigation methods used by the researchers in the different studies, which we considered for our review, were consistently dissimilar, and none had made use of an index of frailty before.

Here, we proposed the first study of plague victims employing a newly developed index of frailty. The skeletal sample of the Imola's lazaretto was compared with a group from a regular cemetery from the same geographic region and the same historical period. Belonging to the same geographical area and historical timeframe means that the environmental, dietary and climatic conditions, as well as the social and historical circumstances, that can influence the frailty, are similar between the groups, making plague the main discriminant variable.

From the inferential statistic outcomes obtained, we could not appreciate any significant difference between the two groups, a condition which might suggest that plague selected its victims from the frailer group, those which have a health status comparable to that of a normal population of people who reached its biological limit of life. Nonetheless, by observing the data obtained for the mean BIF of the populations, we noticed that the people buried in the lazaretto of Imola were less frail, especially the women. Except for the class of age 18-35, all other age classes also appeared to be less frail in plague than non-plague individuals.

Indeed, in our previous study (Bramanti et al. 2018), we had seen that the mortality rates in different plague-cemeteries were similar. We can see in Figure 5.11 that the mortality rates of Imola were very similar to those of the other plague sites we compared in chapter 2: a smaller peak of mortality during late childhood and a second more prominent peak for the young adults (18-34). The mortality rates are very different from those of the regular cemetery of Ravenna, where mortality increases with age, particularly after 35 years of age, and the difference between the two groups is statistically significant ($p < 0.0001$).

If we compare the mean BIF values for each class of age (Fig. 5.12), we notice that the main BIF in the adolescents of Ravenna was very high (it should be considered anyway the low numerosity), while the 13 subadults killed by plague show lower frailty values. The same situation occurs in middle adults (35-50 years old) and old adults (>50 years old), but not in young adults (18-34 years

old), for whom the values of frailty are higher in the plague victims. Therefore, while it's true that the difference in frailty is not statistically significant, there are some differences that should be considered. If we separate male and female individuals (Fig. 5.13, 5.14), the difference in frailty is diminished between the male fractions of the two groups but is more prominent between the female representatives.

The results suggest that plague selected the frailer individuals of both sexes, but also some healthy individuals, considering the lower frailty scores in the sample of Imola. Moreover, it seems that it selected more healthy women than healthy men.
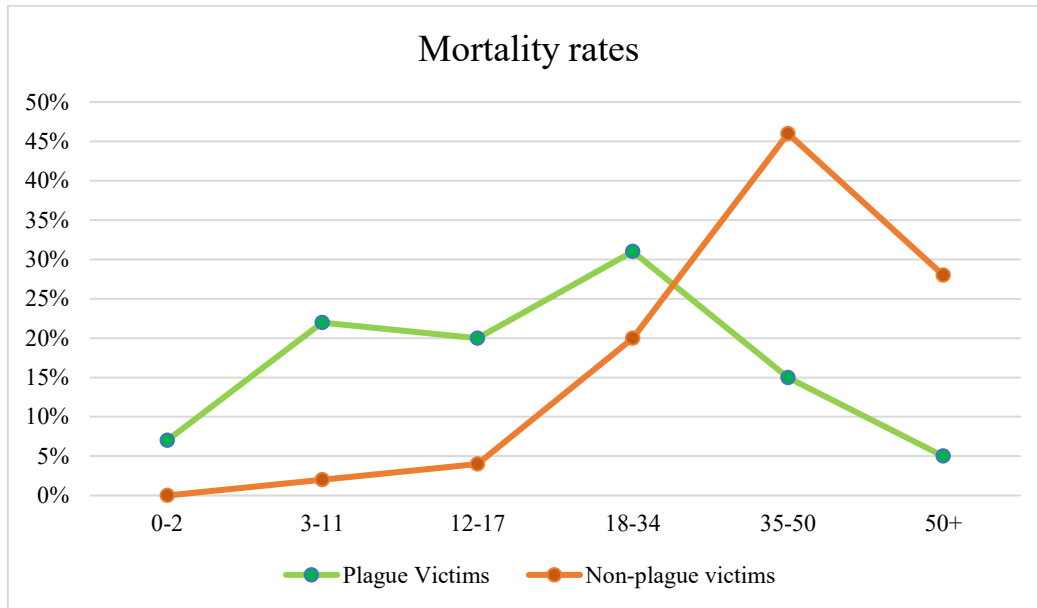
Figure 5.11: Mortality rates of the two samples (Plague Victims and Non-Plague Victims) calculated as percentual amount of victims for each age class, in the two populations.
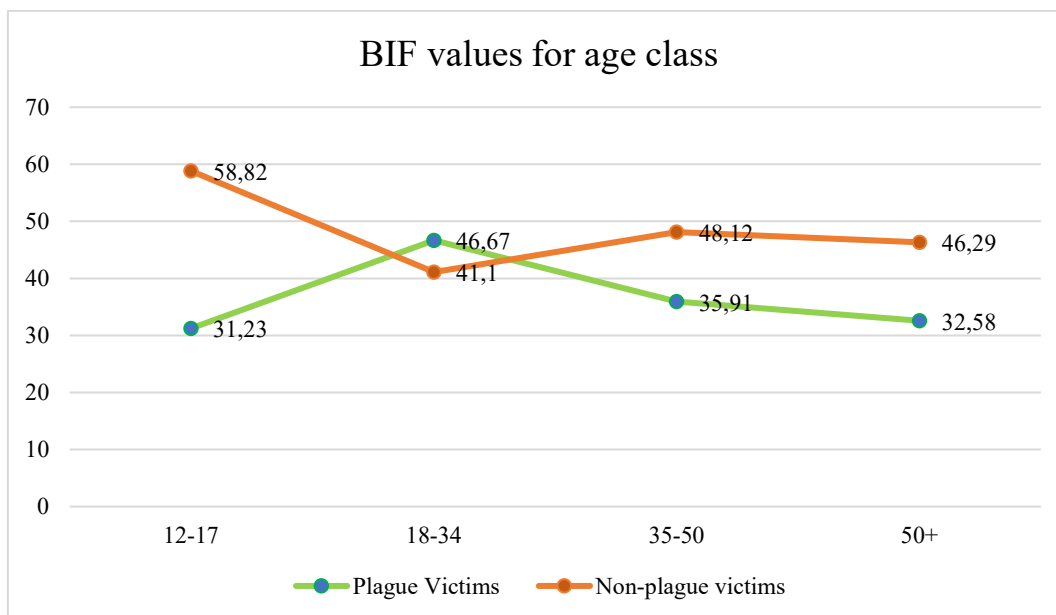
Figure 5.12: Mean BIF values for each class of age in the two groups, Plague Victims and Non-Plague Victims.
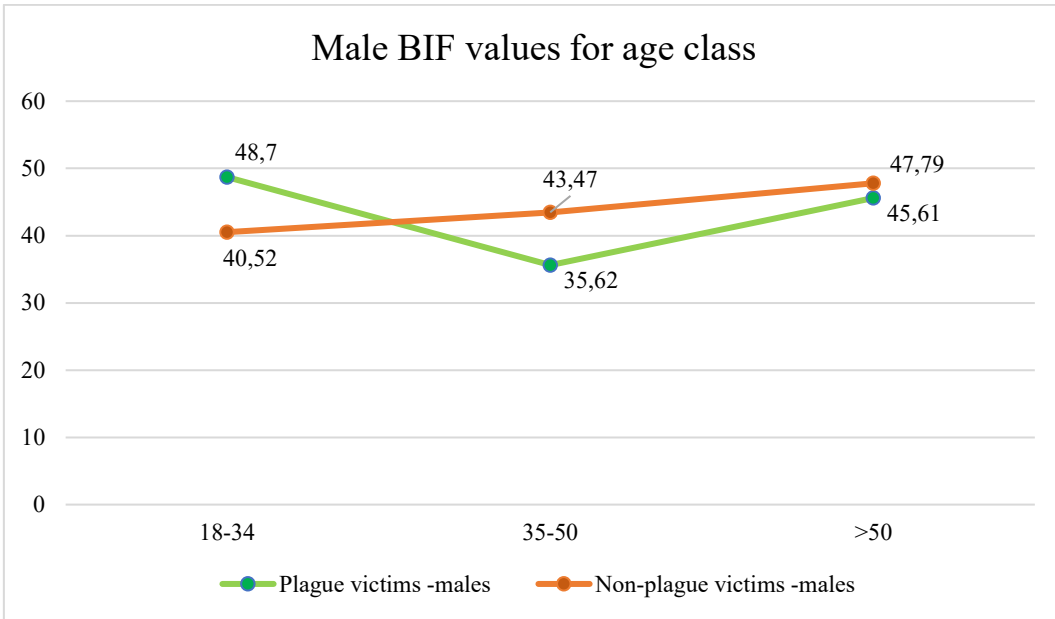
Figure 5.13: Mean BIF values for each class of age in the males of the two groups, Plague Victims and Non-Plague Victims (only individuals whose sex was estimated).
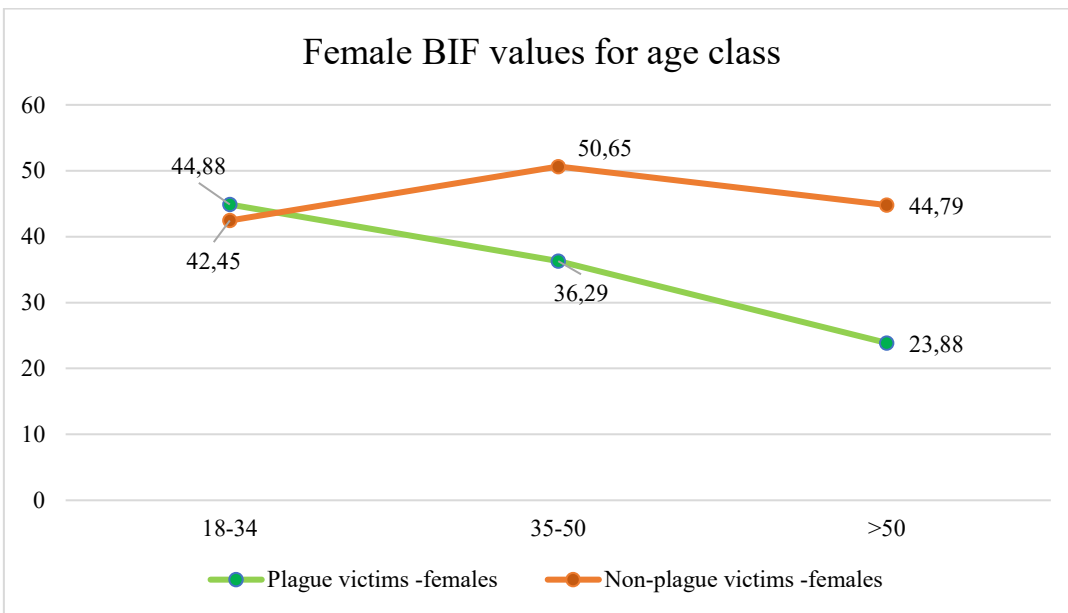
Figure 5.14: Mean BIF values for each class of age in the females of the two groups, Plague Victims and Non-Plague Victims (only individuals whose sex was estimated).

Regarding the comparison of the gross frequencies of each biomarker, it was not surprising for us to observe few significant results between the two groups, with higher frequencies of vertebral and joint diseases, as well as of periostitis, in the individuals from Ravenna (Fig. 5.4). The higher frequencies of those biomarkers in Ravenna were mostly associated with the older age found in this attritional cemetery in comparison to the plague pits of Imola (respectively, 14 vs 5 individuals were >50 years old). When we compared male and female individuals separately, anyway, we saw that the differences decreased, especially between males (Fig. 5.5, 5.6).

Concerning PH and CO, we found again no statistically significant differences between the two groups, both for presence and absence, but also the severity and healing of the lesions (Fig. 5.7, 5.8, 5.9, 5.10). This condition seems to indicate that probably there was not a difference in iron levels, between the plague's victims and the individuals of the cemetery of Ravenna. Nonetheless, we noticed that the majority of healed individuals, at least regarding CO, are among plague victims, indicating that these individuals were more robust than the ones of the Ravenna's population since they had circumvented a pathological status due to anemia of whatever nature.

As already discussed in chapter 1 and 3, the etiology of PH and CO is still debated, and while it seems to be connected to a deficiency of iron (due to genetic, infective or nutritional causes), we yet don't know the timing of the onset of the lesions after the state of anemia has begun. Moreover, as we explained in the first chapter, different types of anemia can trigger (or be induced by) different alterations of the iron homeostasis (Pak et al., 2006; Camaschella and Silvestri, 2011). Some of them (anemia due to chronic diseases) may have increased the possibility of dying from plague. Therefore we cannot be sure that iron levels were similar between the two groups without further investigation. There are other methods to detect levels of iron in the skeletons, and they are described in the first chapter. One possibility is to analyse the aDNA of the individuals to search for mutations in genes responsive of the regulation of iron in the body (iron deficiency or excess). aDNA was extracted in the frame of the project MedPlag (for methods see e.g. (Namouchi et al., 2018)), from the teeth of the plague victims of Imola to investigate the presence of *Y. pestis* DNA (Guellil et al.- in preparation). Using shotgun technology, aDNA was sequenced with an Illumina Hiseq 2500 (125bp PE) system at the Norwegian Sequencing Centre and produced raw sequences of each organism represented (host, pathogen, soil bacteria and other microorganisms). We cleaned, merged and mapped human sequences to a reference human genome (Gr38) to investigate 87 SNPs in 34 genes connected to iron regulation (see chapter 1) and immunity deficiencies. Unfortunately, the human endogenous aDNA content was poor, and the genes covered fractions did not reach 30% in almost any sample (Supplementary Table S5.1). Because of the poor condition of the aDNA, probably due to diagenetic factors (Guellil et al.- in preparation), it was not possible to determine any polymorphism in the human aDNA of Imola. More analyses should be done, to better understand the genetic regulation of the iron content in plague victims and its role in determining mortality.

Back to the results of our comparison with the BIF rate, S. DeWitte (DeWitte, 2010), analysing the victims of the London plague, found similar differences to ours in the health status of male and female plague victims:

> "the lower excess mortality associated with stress markers among women compared to men as indicated by the estimated values of the parameter representing the effect of the sex covariate might indicate that the Black Death killed more otherwise healthy women than healthy men." (DeWitte, 2010, p 14).

However, in her study, the individuals from London were not compared with non-plague victims from the same geographical area and historical time. Therefore, it is possible that her results reflect the normal differences in the population of medieval London. Moreover, without considering the healing and severity of PH and CO and assigning a different weight to the biomarkers (as we instead did with the BIF), we cannot be sure that the results obtained in the cemetery of London reflect the real frailty of the individuals. Nonetheless, if we compare the relative frequencies of some stress biomarkers of Imola with those of the plague victims of London (Waldron, 1992; DeWitte and Hughes-Morey, 2012; Dewitte and Slavin, 2013), we notice that there are some other interesting likeliness, particularly for CO and PH frequencies, if we do not consider the healing and severity of the lesions.

On the other hand, there is a statistically significant difference between the two plague pits regarding the LEH, which is higher in the individuals from Imola, while in the London sample the presence of periodontitis was significantly higher. Contrarily, regarding Joint disease the difference is noticeable: in the sample from London the rate is lower than in Imola, even if we could not test this difference statistically (Tab. 5.2). These dissimilarities may be due to the different historical periods and geographic areas.

Table 5.2: Difference in biomarkers frequency between Imola's (1630-32) and London's (1348-49) plague's victims.

	Imola (1630-32)	London (1348-49)	p
Short stature	25%	11%	0.0973
LEH	97%	75%	0.0014
CO	31%	18%	0.1087
PH	82%	90%	0.1585
Periodontitis	65%	99%	< 0.0001
Joint disease	81%	~25%	-

To further investigate the consistency of CO and PH frequencies, we compared our results for Imola with other data collected in the review of anthropological studies on plague victims (see chapter 2), regarding these two biomarkers (Fig. 5.15). We noticed that all the other sites, apart from Imola and London, have lower rates of PH in their adults. One explanation could be the difference in the methodology used to assess the porous lesions of the skull. Moreover in the sample from London and Imola the adolescents were included, whereas they were not in the other studies. Therefore, the comparison may be biased. As we underlined in the second chapter, only with a standardisation of

the methodology used in assessing sex and age at death, a comparison between more sites can be possible.
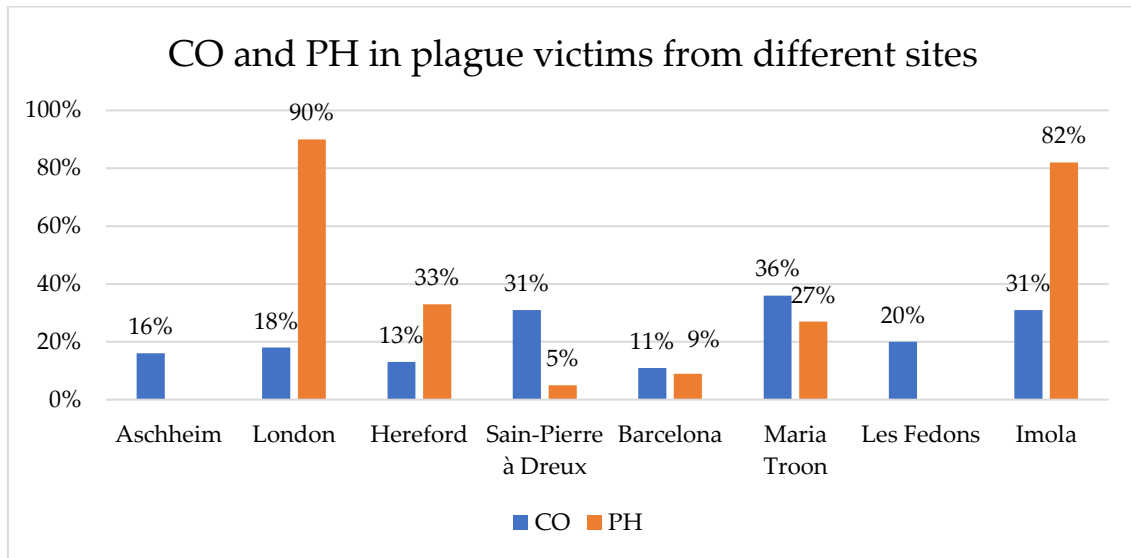
Figure 5.15: CO and PH frequencies in adults plague victims from different sites and epochs.

5.5 CONCLUSION

The analysis of frailty from individuals died from plague in 1630-32 at Imola, and that of the individuals died from other causes in an attritional cemetery at Ravenna from the same century, indicates that there is no statistically significant difference in frailty between the two groups. Yet, we observed differences in frailty and mortality rates among the different classes of age and between the sexes: plague victims had lower frailty (in particulars women), and died more during childhood and between 18 and 35 years of age, while individuals from Ravenna had a higher frailty score and mainly died at an older age. This observation leads us to propose that plague, at least during the epidemic of 1630-2 in Northern Italy, killed not only the frailer individuals but also some of the healthier ones. Moreover, plague seems to have killed more healthy (less frail) women than healthy men. The lack of significance in frailty scores in comparison with a regular cemetery might indicate that plague did not spare healthy individuals.

It would be useful to investigate other plague contest using the Biological Index of Frailty (BIF), to increase the sample size and thus determine if the trend that we have seen here is a rule or just a local effect.

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5.7 SUPPLEMENTARY

Table S5.1: Covered reads fractions of the genes of interest in the four samples from Imola. In red are the fraction less than 20%covered, in yellow the ones covered between 20% and 29%.

chr	start	end	strand	Gene Name	IMO2a				IMO3a				IMO7a				IMO16B			
					Reads Map.	Bases Cov	Feature Length	Frac Cov	Reads Map.	Bases Cov	Feature Length	Frac Cov	Reads Map.	Bases Cov	Feature Length	Frac Cov	Reads Map.	Bases Cov	Feature Length	Frac Cov
1	155305052	155532324	-	ASH1L	701	42413	227273	19,0%	26	1214	227273	1,0%	39	2545	227273	1,0%	1	42	227273	0,0%
1	145413191	145417545	-	HJV	12	777	4355	18,0%	1	46	4355	1,0%	1	75	4355	2,0%	0	0	4355	0,0%
2	190425316	190448478	-	SLC40A1	76	4516	23163	19,0%	2	96	23163	0,0%	3	185	23163	1,0%	0	0	23163	0,0%
2	102979093	103015230	+	IL18R1	132	7826	36138	22,0%	0	0	36138	0,0%	6	368	36138	1,0%	0	0	36138	0,0%
2	103035254	103069025	+	IL18RAP	111	7320	33772	22,0%	6	255	33772	1,0%	5	213	33772	1,0%	0	0	33772	0,0%
3	148880197	148939832	-	CP	184	10498	59636	18,0%	4	213	59636	0,0%	12	788	59636	1,0%	0	0	59636	0,0%
3	46395235	46402413	+	CCR2	32	1913	7179	27,0%	0	0	7179	0,0%	1	96	7179	1,0%	0	0	7179	0,0%
3	46411633	46417697	+	CCR5	17	1134	6065	19,0%	1	70	6065	1,0%	2	191	6065	3,0%	0	0	6065	0,0%
3	133464977	133497850	+	TF	105	6240	32874	19,0%	3	149	32874	0,0%	5	308	32874	1,0%	0	0	32874	0,0%
3	159706623	159713806	-	IL12A	20	1663	7184	23,0%	0	0	7184	0,0%	2	147	7184	2,0%	0	0	7184	0,0%
3	50643885	50649262	-	CISH	18	993	5378	18,0%	1	73	5378	1,0%	1	47	5378	1,0%	0	0	5378	0,0%
5	150226085	150228231	+	IRGM	12	601	2147	28,0%	0	0	2147	0,0%	1	27	2147	1,0%	0	0	2147	0,0%
6	26087422	26096438	+	HFE	35	1868	9017	21,0%	2	86	9017	1,0%	2	91	9017	1,0%	0	0	9017	0,0%
6	32605169	32612152	+	HLA-DQA1	15	1125	6984	16,0%	0	0	6984	0,0%	1	54	6984	1,0%	0	0	6984	0,0%
6	32627241	32634466	-	HLA-DQB1	9	477	7226	7,0%	1	47	7226	1,0%	2	44	7226	1,0%	0	0	7226	0,0%
7	117120017	117308719	+	CFTR	635	40693	188703	22,0%	11	527	188703	0,0%	28	1766	188703	1,0%	0	0	188703	0,0%
7	99102273	99131445	+	ZKSCAN5	92	6237	29173	21,0%	8	393	29173	1,0%	13	847	29173	3,0%	0	0	29173	0,0%
7	100218039	100240332	-	TFR2	70	4402	22294	20,0%	2	86	22294	0,0%	3	148	22294	1,0%	1	25	22294	0,0%

7	77423045	77427747	-	TMEM60	27	1572	4703	33,0%	0	0	4703	0,0%	0	0	4703	0,0%	0	0	4703	0,0%
9	120466453	120479769	+	TLR4	55	3376	13317	25,0%	2	64	13317	0,0%	2	125	13317	1,0%	0	0	13317	0,0%
11	10874251	10879620	-	ZBED5	25	1479	5370	28,0%	0	0	5370	0,0%	0	0	5370	0,0%	0	0	5370	0,0%
11	66886740	67025553	+	KDM2A	453	26427	138814	19,0%	12	654	138814	0,0%	20	1417	138814	1,0%	0	0	138814	0,0%
11	5246696	5248301	-	HBB	6	218	1606	14,0%	0	0	1606	0,0%	0	0	1606	0,0%	0	0	1606	0,0%
12	111843720	111889427	+	SH2B3	132	7698	45708	17,0%	9	476	45708	1,0%	12	833	45708	2,0%	1	44	45708	0,0%
16	4560676	4588816	-	CDIP1	100	5459	28141	19,0%	3	190	28141	1,0%	7	446	28141	2,0%	0	0	28141	0,0%
16	50727507	50766990	+	NOD2	146	8996	39484	23,0%	4	193	39484	0,0%	5	283	39484	1,0%	1	49	39484	0,0%
17	26083792	26127555	-	NOS2	132	8182	43764	19,0%	9	362	43764	1,0%	9	501	43764	1,0%	0	0	43764	0,0%
17	71228372	71245098	+	C17orf80	61	3643	16727	22,0%	0	0	16727	0,0%	3	212	16727	1,0%	0	0	16727	0,0%
17	32582296	32584222	+	CCL2	6	464	1927	24,0%	1	39	1927	2,0%	1	77	1927	4,0%	0	0	1927	0,0%
19	17186591	17324104	+	MYO9B	411	24077	137514	18,0%	18	839	137514	1,0%	35	2149	137514	2,0%	17	159	137514	0,0%
19	18170371	18197806	-	IL12RB1	87	5624	27436	20,0%	6	330	27436	1,0%	5	267	27436	1,0%	0	0	27436	0,0%
19	35773410	35776046	+	HAMP	10	612	2637	23,0%	0	0	2637	0,0%	2	86	2637	3,0%	0	0	2637	0,0%
19	49467659	49470136	+	FTL	14	701	2478	28,0%	0	0	2478	0,0%	1	70	2478	3,0%	0	0	2478	0,0%
21	34602231	34636831	+	IFNAR2	114	6705	34601	19,0%	5	261	34601	1,0%	4	231	34601	1,0%	0	0	34601	0,0%
22	37461476	37499693	-	TMPRSS6	98	6112	38218	16,0%	2	109	38218	0,0%	7	510	38218	1,0%	1	31	38218	0,0%
23	55035488	55057497	-	ALAS2	74	4226	22010	19,0%	0	0	22010	0,0%	6	411	22010	2,0%	0	0	22010	0,0%

6

SEX-RELATED PLAGUE SUSCEPTIBILITY AND MORTALITY

The research exposed in the previous chapters dealt with skeletons of plague victims and therefore focused solely on the concomitant causes of plague mortality. While skeletons can provide much information on the life and death of the individuals from the past, other information is missing. Regarding plague, we cannot know of which form of plague (bubonic, pulmonary or septicemic) the individuals have died and, of course, we do not have information on those that managed to recover from the infection.

During the 19th and 20th centuries, different detailed reports have been compiled by medical staff and researchers, regarding the infection, death by plague or recovery of those that manifested symptoms of plague infection. We have reviewed many of these records and collected data on singular individuals from all around the world between 1813 and 1945.

With the analysis of these data, we aimed to investigate the differences in morbidity and mortality between sexes and between different ages, in particular, trying to find an explanation for the phenomenon that we have observed in our previous work, i.e. the higher mortality rate for individuals between 25 and 35 years of age.

6.1 INTRODUCTION

In this work, we have attempted to address the question of whether susceptibility to contracting the plague and mortality are influenced by the sex and the age of its victims. From our analysis of the anthropological data from plague mass graves, we could not find a general trend of selectivity for mortality, with the exception of the higher mortality of children aged 5-10 years and that, even higher, of young adults around 20-35 years of age. In the last chapter, we saw that plague seems to select not only frailer individuals as other infectious diseases tend to do (Thorburn, 2009; Li et al., 2011; Drew et al., 2017), but also less frail ones. Among the plague victims of Imola (1630-32), in fact, the mean value of the biological index of frailty (BIF) was lower than that of the attritional cemetery of the same geographical area and same historical time (Ravenna, San Biagio, 17th c.), even if the difference was not statistically significant. The differences in frailty observed between males and females and age classes were not statistically significant in the two samples analysed (Imola and Ravenna), but significant was the difference in the mortality rate between the age at death classes ($p < 0.0001$). How plague distinguished between sexes and different ages is still unclear. To limit the research to

the skeleton of plague victims from the past may preclude the access to other information, for example, the infection rate, which might be crucial to answering our questions. In this chapter, we broaden our research, investigating data from historical sources, thus adding to our previous work the missing variable, the information about contagion and recovery of individuals affected by plague.

In the Middle Ages, records of deaths during the epidemics were often reported in parish documents, but during the 19th and 20th centuries, and in particular, after the discovery of the bacterium by Alexandre Yersin in 1894, much more considerable attention was given to the study of plague. There are many records, reports and descriptions of epidemic events and singular cases in the pre-antibiotic era (i.e. before the 1950s), often reporting name and surname of the infected, their sex and age, the kind of symptoms, the timing of their admission to hospitals or lazarettos, and the timing of their death or recovery.

We have collected many of these reports from different parts of the world, and from 1813 to 1945.

In the literature, this is not the first time that data have been used to analyse mortality or susceptibility patterns. For example, Cohn and Alfani (Cohn and Alfani, 2007) studied some parish records of Nonantola and the "*Books of the Dead*" from Milan that reported the deaths during the 1630 epidemic of plague, to analyse how the contagion spread through households. In Rubini et al. (Rubini et al., 2016), on the other hand, the mortality data of Madagascar's plague epidemics registers were compared with the mortality data of skeletonised victims of historical pandemics to analyse the mortality peaks by age group. This is the first time, however, that clinical data from many different countries of the world have been collected and analysed together.

6.2 DATA COLLECTION

Starting from a previous publication of the MedPlag group (Bramanti et al., 2019), we systematically searched for online publications providing detailed information on plague outbreaks (due to *Y. pestis*), with data on infected individuals and their fate (death or recovery). We started our research on online search engines, such as Google Scholar, PubMed and others, using several keywords: "plague", "third pandemic of plague in Europe", "plague in", "medical reports of plague", "clinical data of plague victims", "cases of plague" in combination with "recovery", "healed", "sex", "age", "death". We extended the research to publications in languages other than English (Italian, French and Spanish). From the high number of texts retrieved, we selected 34 papers, which provided data on 1034 individuals from 17 different countries around the world. These individuals, according to the sources, showed signs of plague infection and were mostly admitted to hospital facilities. All the cases belonged to the end of the Second Plague Pandemic and the Third, encompassing the period from 1720 to 1945 (Supplementary Table 1).

For the statistical analyses, we considered only bubonic plague cases, excluding primary pulmonary or septicemic plague, whose mortality is close to 100%. A further criterion for inclusion was the presence of information regarding sex, age and fate (death or recovery from the plague). After applying all criteria, the number of cases was reduced to 404, which could be classified into four age classes, according to Buikstra and Ubelaker (1994), by merging the classes of young and mature adults: children (0-11.9 years), adolescents (12-19.9 years), adults (20-49.9 years) and the elderly (over 50 years). Data are reported in Tab. 1. We merged the adults in a unique class after observing that their age was mostly approximated.

6.3   STATISTICAL ANALYSIS

We performed different comparisons between sexes and among the different age groups through the Chi-squared test, to highlight differences regarding contagion, mortality and healing.

A multivariate logistic regression model (logit model) was carried out to test the association between biological characteristics (sex and age), as categorical variables, and the variable 'death by plague or recovery'. As reference values, we used the female category and the last age group (the elderly). Individuals with missing data were excluded from the analyses.

Other predictive variables were later added to the model (adjusted model) as alleged confounding variables: latitude, longitude and Plague Pandemic. Latitude and longitude were included as continuous variables, while for the variable Plague Pandemic, we attributed to the Second Plague Pandemic all episodes (outbreaks or single cases) until 1894, and to the Third Plague Pandemic those from 1894 onwards.

The results of the multivariate logistic regression model were reported as odds ratios (ORs) with 95% confidence interval (CI). In our outcomes, an odds ratio higher than 1 indicates an increased probability of dying by plague in comparison to the reference, while values less than 1 mean reduced odds of dying and increased probability of recovering.

Values of $p < 0.05$ were considered statistically significant. All statistical analyses were conducted using STATISTICS (version 11, StatSoft, Tulsa, OK).

6.4   RESULTS

The complete database encompasses 1034 cases (610 males and 410 females), recorded in 17 countries and five continents. Of these, one individual was affected and died by septicemic plague, 347 out of 353 individuals died from pneumonic plague (only six individuals survived), while 571 were cases of bubonic plague (Supplementary Table S6.1). The probability of dying in cases of septicemic or pulmonary plague is close to 100%, therefore probably independent by sex and age.

For this reason, we have considered only individuals affected by bubonic plague for further statistical analyses on sex- or age-related susceptibility and mortality.

From this reduced dataset consisting of 571 patients affected by bubonic plague, we have further excluded individuals whose sex, age, and whether they have died or healed after infection was not recorded. We ended with 404 individuals (Tab. 6.1). In Figures 6.1 we report a graphic representation of the number of infected patients stratified by age and sex.

Most of the infected individuals were in the classes of adolescents and adults, for both sexes (Fig. 6.1). Males were the most infected in all age groups. It seems, therefore, that males were more exposed to the infection, especially after childhood. However, when we considered plague mortality, we saw that the rates of mortality were very similar between males and female (Fig. 6.2). The frequency of the individuals who died in respect to those who recovered, anyway, was different between males and females: for almost all age groups, except for individuals older than 50 years, the incidence of female deaths was higher than that of males (Fig. 6.3). In other words, despite being the most affected by the plague, males showed a higher percentage of recovered cases than females. Female mortality due to bubonic plague represented 62.6% of all female cases, while male mortality represented 52.2% of all male cases: the difference is statistically significant (p=0.0300). When we considered each age class separately, we noticed that this difference was still substantial during adolescence (p=0.0141), while it was no longer significant in the mature class (p=0.3053), despite the fact that women represented the higher percentage, nor is it significant in the children's class (p=0.6748), or in the elder class (p=0.7866).

From these observations, we deduce that, although women were less infected, they died relatively more than men, particularly in the adolescent and fertile age. Pre-pubertal individuals, on the other hand, showed similar mortality rates for males and females, while among the elderlies, we had a reversal, with a relatively higher percentage of deaths for men.

To determine whether factors other than sex could be implicated in this phenomenon, we used logistical regression models, in which we tested what was the probability of dying from plague once infected, adding several possible confounding variables. The results of the logistic regression model with only the sex and age variables of individuals are reported in Table 6.2. We saw that being a man was a protective factor in case of plague mortality compared to being a woman (odd ratio less than 1), while adolescents were less likely to die than the elderly. Mature individuals and children were also less likely to die than the elderlies, but the results were not statistically significant.

When we added to the variables the geographical position (latitude and longitude) and which Plague Pandemic (Second or Third) the cases belonged to, we still observed that men compared to women and adolescents compared to the elderlies were less likely to die after being infected by plague (Table 6.2; Fig. 6.4). It seems, therefore, that there was no difference in the lethality of the plague between

the end of the Second and the Third Pandemics, and also that the geographical position did not influence the data significantly.
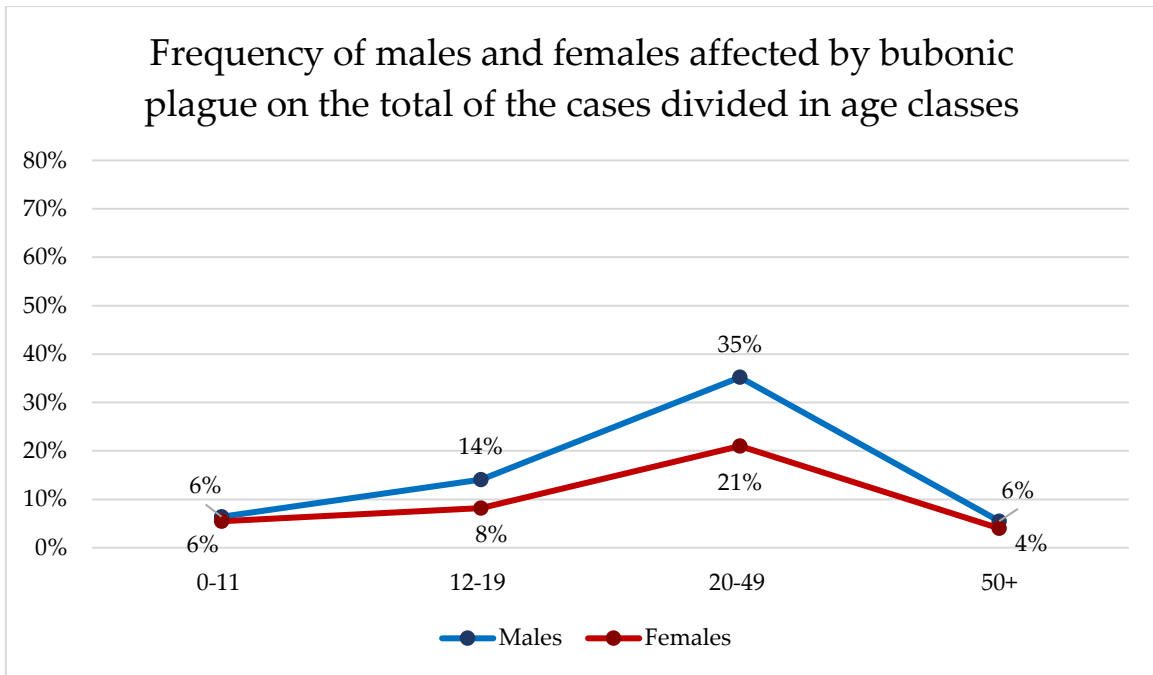
Figure 6.1 Frequency of males and females affected by bubonic plague on the total of the cases (404) divided into age classes.
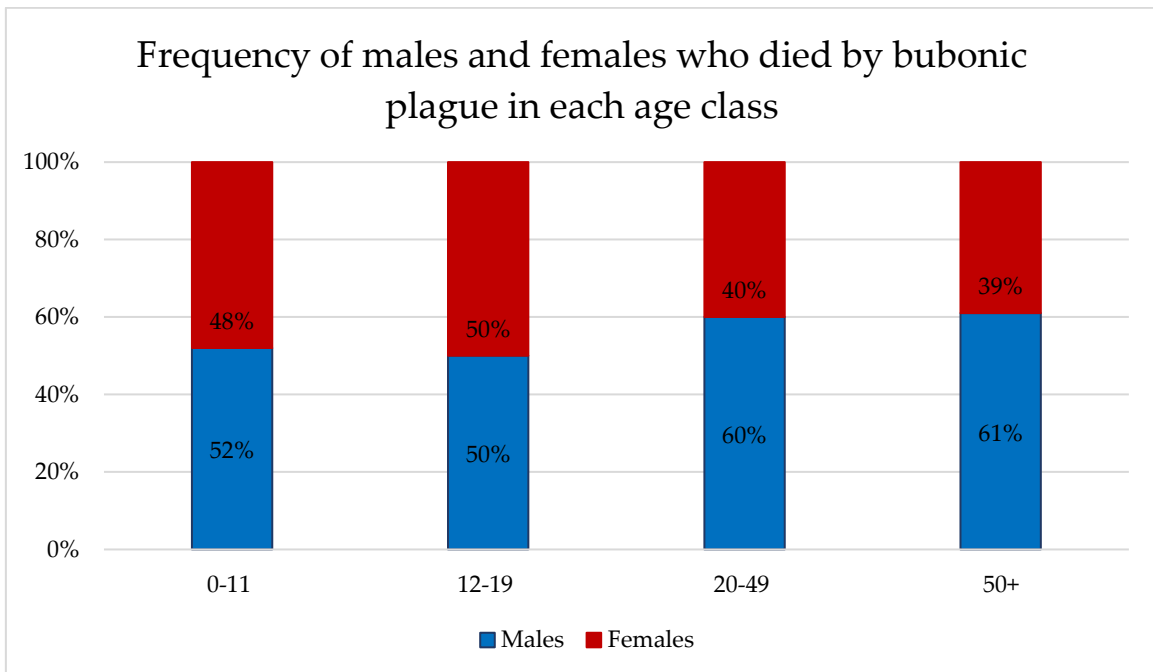
Figure 6.2: Frequencies of males and females affected by bubonic plague on the total of each age class
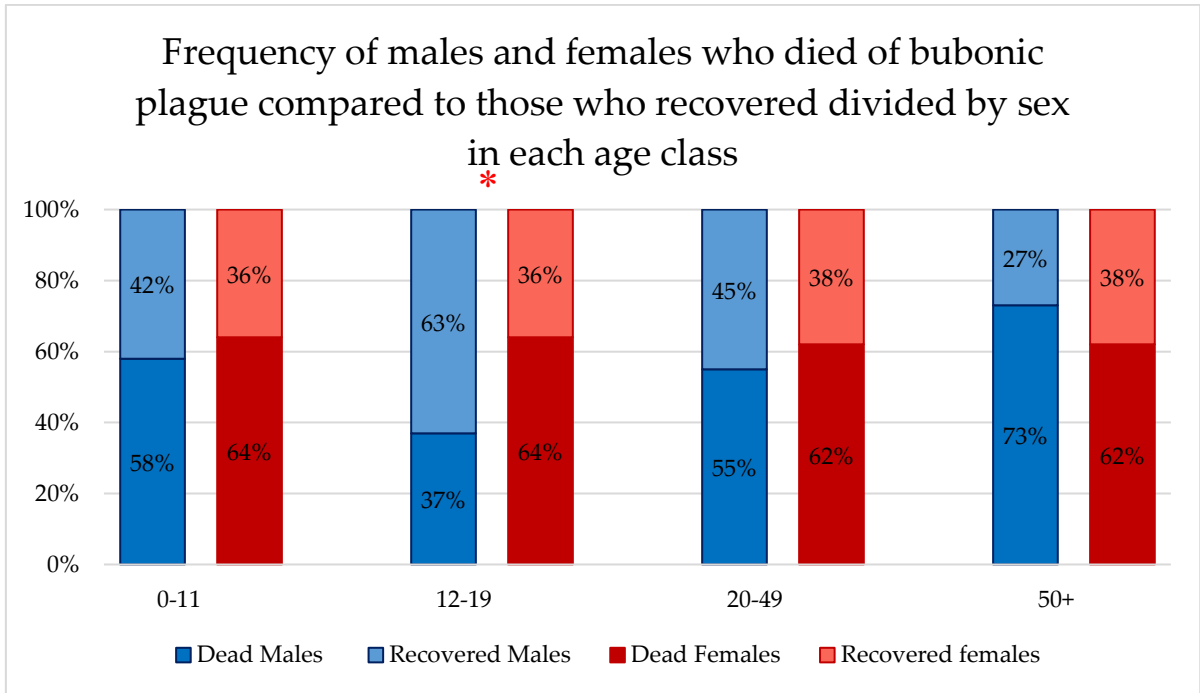
Figure 6.3: Frequencies of males and females who died of bubonic plague compared to those who recovered divided by sex in each age class.

Table 6.1: Individual affected from Bubonic plague divided by geographic area, period, sex, age and their fate, died (D) or recovered (R)

Geographic area	Site	Period	N° infected	Sex M n°		Sex F n°		Age M n°				Age F n°			
				D	R	D	R	0-11	12-19	20-49	50+	0-11	12-19	20-49	50+
France	Marseille	1720	5	1	0	4	0	0	0	3	0	0	0	0	1
Malta	La Valletta	1813 1819	66	25	21	10	10	1	3	18	0	4	1	6	2
Greece	Malecchia, Potami	1816	4	2	0	2	0	0	1	0	0	1	0	1	0
Greece	Corfù	1816	13	7	0	5	1	1	0	0	0	0	1	0	0
Russia	Michailovka, Lower Volga -	1879	1	0	1	0	0	0	0	1	0	0	0	0	0
India	Bombay	1897	215	66	51	67	31	17	28	58	14	12	20	57	9
Portugal	Oporto	1899	12	2	6	1	3	0	2	5	1	0	0	3	1
Annam	Natrang	1899	3	0	2	0	1	0	0	1	0	0	1	0	0
UK	Glasgow	1900	11	6	0	5	0	1	0	3	1	2	1	2	1
UK	Cardiff	1900	1	1	0	0	0	0	0	1	0	0	0	0	0
California	San Francisco	1900	21	16	0	5	0	0	13	3	1	2	2	0

Country	City	Year													
France	Frioul	1901	32	9	23	0	0	0	3	6	1	0	0	0	0
South Africa	Cape Town	1901	3	1	0	2	0	0	0	0	0	0	0	0	0
UK	Liverpool	1901	2	1	0	1	0	0	0	0	0	0	0	0	0
Australia	Fremantle	1903	8	4	2	0	2	0	2	3	1	0	1	1	0
Australia	Brisbane	1904	28	8	11	3	6	0	8	10	0	0	2	6	0
Australia	Rockhampton	1906	8	4	3	0	1	0	4	3	0	0	0	1	0
UK	East Suffolk	1909-1910	8	1	3	3	1	1	1	1	1	0	1	0	0
Italy	Catania	1914	9	3	5	1	0	1	0	6	1	0	0	1	0
Spain	Barcelona	1931	26	3	13	5	5	1	3	12	0	3	2	2	3
Italy	Taranto	1945	17	10	5	0	2	0	1	2	0	0	1	0	0

Table 6.2: Logit estimates of correlation of individual sex and age with relative odds of dying by plague (crude model and adjusted model). 95% confidence intervals in brackets. Significant associations are highlighted in bold.

	Crude OR (95% CI)	*p*	Adjusted OR (95% CI)	*p*
Sex				
Males	**0.659 (0.452; 0.866)**	**0.0480**	**0.607 (0.394; 0.819)**	**0.0210**
Females	1 (reference)		1 (reference)	
Age classes				
Children	0.842 (0.353; 1.331)	0.5721	0.927 (0.427; 1.428)	0.4577
Adolescents	**0.462 (0.073; 0.851)**	**0.0207**	**0.496 (0.089, 0.902)**	**0.0353**
Adults	0.735 (0.421; 1.051)	0.9718	0.754 (0.429; 1.078)	0.9140
Old adults	1 (reference)		1 (reference)	
Latitude			0.998 (0.988; 1.009)	0.7761
Longitude			1.000 (1.000; 1.000)	0.3431
Plague pandemic				
2° plague pandemic			0.600 (0.256; 0.943)	0.1446
3° plague pandemic			1 (reference)	
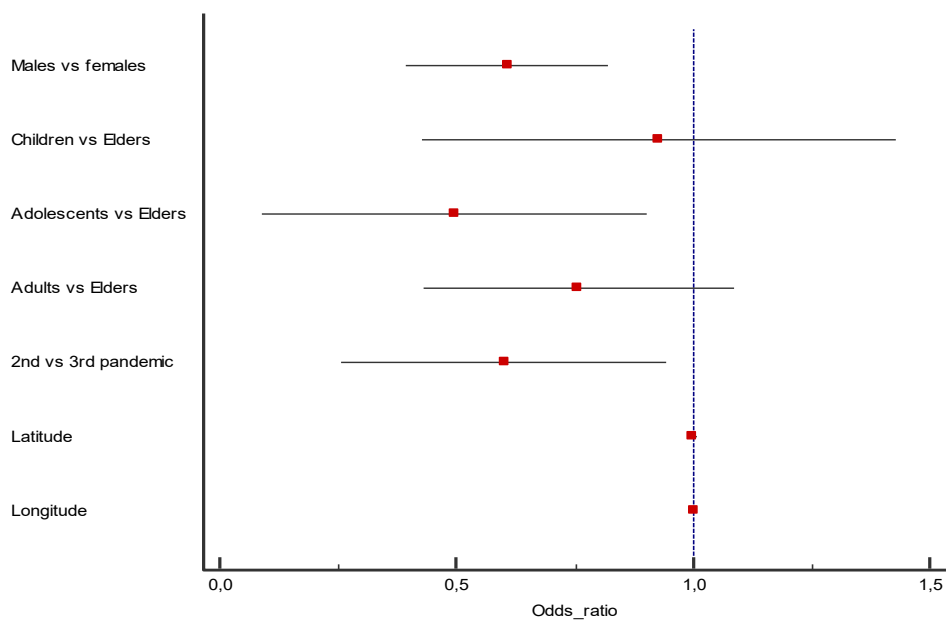
Figure 6.4: Forest plot displaying the odds ratio of being dead by plague relative to have recovered from plague.

6.5   DISCUSSION AND CONCLUSION

This study aimed to better understand the causes underneath the differences in mortality rates between sex and age classes we noticed in the previous studies.

The results we obtained from the data collected seem to demonstrate that males were more susceptible to bubonic plague infection than females (Fig. 6.1). Although the absolute frequencies of the deaths from bubonic plague displayed similar frequencies of man and women in all age groups (Fig. 6.2), we saw that infected females died from bubonic plague in a higher percentage than males, especially in the age from puberty until maturity (Fig. 6.3).

The mortality rates of our sample divided by sex (Fig. 6.5) differed slightly from those of the past epidemics and those from Madagascar during the 2014–2015 outbreak (Rubini et al., 2016), where the highest death rates were registered in children between 5–9 and 20–29 years of age. In our sample, we again observed a major peak in correspondence to young and mature adults, but we noticed that children and adolescence rates of mortality were similar. Not having differentiated between young children (0-4 years old) and prepubertal children could explain the missing second peak we usually found in the mortality rate of plague.
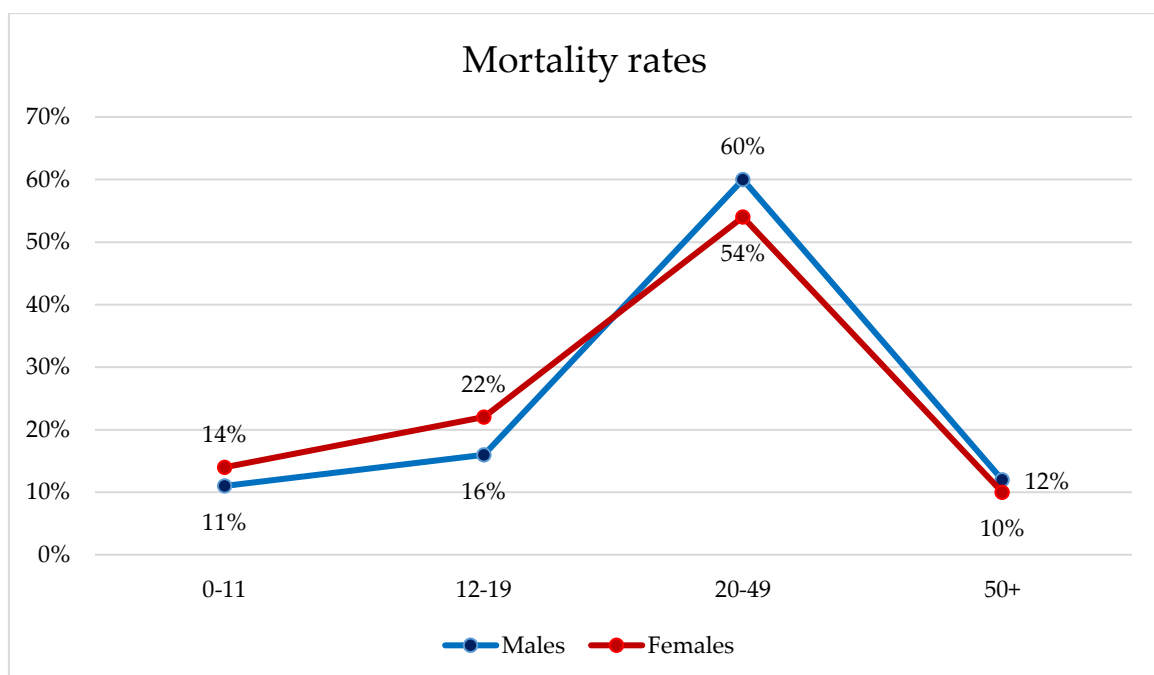
Figure 6.5: Mortality rates of our sample for each sex and age class.

In literature, we could not find a precise trend on plague mortality concerning sex. Both anthropological papers (see chapter 2) and paleodemographic articles have showed an heterogeneous picture: some reported a majority of females (e.g.(Guellil et al.- in preparation; Cervellati, 1986; Signoli et al., 2002), others of males (e.g. (Hollingsworth and Hollingsworth, 1971), others again did

not notice a difference in mortality rates between the sexes (e.g. (Whittles and Didelot, 2016; Alfani and Murphy, 2017). Yet, in recent outbreaks in Madagascar, different studies noted a prevalence of male cases of plague, in almost all age groups (Chanteau et al., 2000; Migliani et al., 2006). One study (Migliani et al., 2006) used logistic regression analysis to deduce the risk factors of plague death. Neither sex nor adolescence was however associated with an increased risk of plague death. However, it should be noted that the patients considered in this study were partly treated with antibiotic therapies and that, although bubonic plague was the most common (92.6%), other forms of plague were not excluded from the analysis. Therefore, a comparison with our study can only concern the prevalence of male cases.

Other scholars (Pollitzer, 1954), have previously suggested that differences in mortality rates between men and women during plague epidemics were only a consequence of different exposure to the bacteria. Men spend more time outside, and this can enhance the chance of infection. Yet, women are notoriously the ones who care for sick people both in the family and at a social level (Who, 2007). This condition increases the possibility of being infected by plague as well, as some researchers have proposed (Dean et al., 2019).

Thus, gender differences in exposition to the infection by plague may hardly be the only explanation for the pattern we have found. As we observed in the first chapter, women are more resistant to infectious diseases than men because they have a more effective immune response (Klein, 2012; Schurz et al., 2019), and mortality rates for many infectious diseases are notoriously in favour of women (Who, 2007). Immunity is strengthened by genes on the X chromosome (Schurz et al., 2019) and by estrogen (Walker, 2011), but a third component may be in play, the physiological dissimilarity in iron content of men and women.

It is confirmed that testosterone downregulates hepcidin (Luque-Ramírez et al., 2011; Ikeda et al., 2012; Sangkhae and Nemeth, 2017), the key regulatory protein of the iron homeostasis, thus increasing extracellular iron and diminishing the intracellular storage iron (see chapter 1 for more information on hepcidin). In adolescents and adult men, therefore, there is more extracellular iron, and this can be the biological cause for their significantly higher susceptibility to plague infection (Fig.6). At the same time, less iron is stored in their macrophages; thus, when *Y. pestis* is inoculated at the flea's bite, the bacteria multiply more slowly within the macrophages, and the number of pathogens that reaches the lymph nodes should be less. At this point other immune defences intervene, and the individual can recover from the infection with a higher probability (Fig. 6).

In women, the role played by the estrogen on the iron homeostasis is still not clear: some experiments *in vitro* and cell cultures showed that estrogen downregulates hepcidin as testosterone does (Hou et al., 2012; Yang et al., 2012; Lehtihet et al., 2016) – possibly an evolutionary response to balance iron deficiencies due to menstrual blood loss. Other experiments on rats anyway, demonstrated that estrogen upregulates hepcidin expression (Ikeda et al., 2012). Recent studies moreover have found

that some steroid molecules, like some female hormones (progesterone and mifepristone), upregulate hepcidin both *in vitro* and *in vivo* (i.e. in women) (Li et al., 2016).

If indeed estrogen or some other female hormones would upregulate hepcidin, we could have found an explanation for the phenomenon we saw in our dataset (i.e. more susceptibility for men but a significantly higher mortality risk for women once infected). In case of upregulation of hepcidin, less extracellular iron would be on disposal for *Y. pestis* to multiply once inoculated, thus first immunity (mainly neutrophils), that is very strong in females, could efficiently block the infection. Yet, when *Y. pestis* is encompassed by the macrophages, which contain much storage iron due to the upregulation of hepcidin, and they start circulating in the lymphatic system, *Y. pestis* would multiply in high number and could, therefore, easily overcome the immunity system of the host. Girls during adolescence and fertile women would, therefore, be less susceptible to infection but have less chance of recovery (Fig. 6).

This hypothesis would explain the outcomes of our research, in term of dissimilarities observed both between sexes (males more prone to infection, females to death) and age classes (both sexes are more affected during the puberty and the reproductive age). Yet, the role estrogen or other female hormones play on hepcidin and the effect of differentiated iron contents on *Y. pestis* infection should be further investigated through additional experimental tests in appropriate facilities.
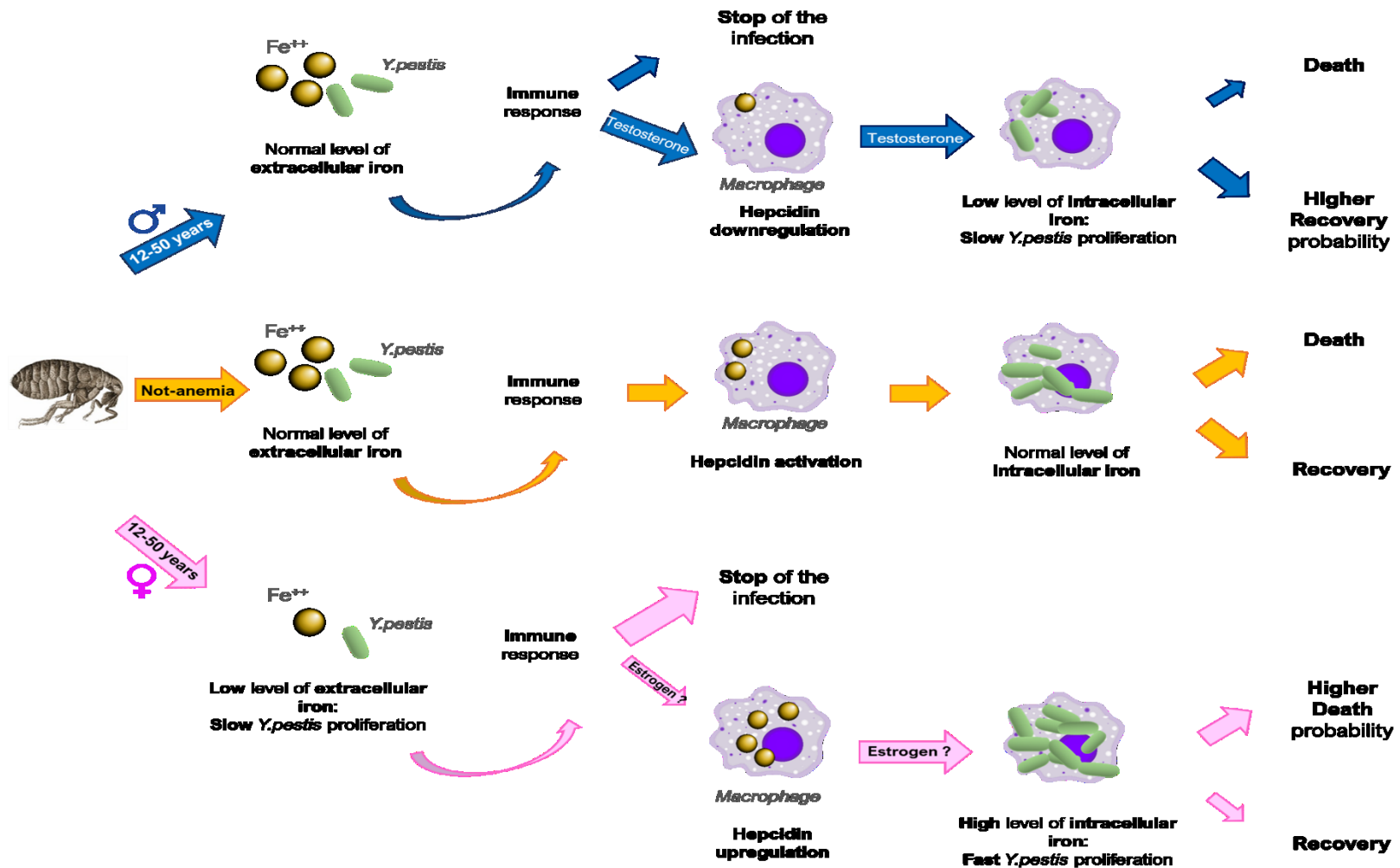
Figure 6.6: Model proposed for *Y. pestis* infection in a general non-anemic human host (yellow arrows), a woman (pink arrows) and a man (blue arrows) aged between 12 and 50 years.

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6.7 SUPPLEMENTARY

Table S6.1: Plague cases between 1720 to 1945 reported in 34 publications.

Geographic area	Site	Period	Plague	N° infected	References
France	Marseille	1720	Bubonic	11	(Bertrand and Plumptre, 1805)
Malta	La Valletta; Maltese Bombard "Sta.Trinità" ; Maltese Brig "Costanza"	1813 1814 1819	Bubonic; not-defined	115	(Stafford, 1816; Faulkner, 1820; Burrell, 1854; De Piro, 1879)
Gozo	Gozo	1813	not-defined	15	(Burrell, 1854)
Greece	Malecchia, Potami; Corfù; Cephalonia	1816	Bubonic	22	(Tully, 1821)
Russia	Lower Volga; Vetlianka;	1878 1879	Pneumonic; Bubonic	324	(Eyre and Spottswoode, 1881; Clemow, 1899)
India	Bombay	1897	Bubonic	265	(Gatacre, 1898)
Austria	Vienna	1897	Pneumonic	3	(Anon, 1898)
Tagikistan	Anzob; Marzitch	1898	Not-defined	3	(Clemow, 1899)
Portugal	Oporto	1899	Bubonic	13	(Da Cunha Coelho, 1900)
Annam	Natrang	1899	Bubonic	3	(Da Cunha Coelho, 1900)
UK	Glasgow; Cardiff; Liverpool	1900 1901	Bubonic; Septicemic	60	(Anon, 1900; Walford, 1900; Boyle and Hope, 1901)
California	San Francisco	1900	Bubonic	21	(Kellogg, 1900; Montgomery, 1900)
France	Frioul	1900 1901	Bubonic	33	(Pellissier, 1902)
Australia	Fremantle; Brisbane; Rockhampton	1900-1907	Bubonic	57	(Watson, 1903; Burnett, 1907)
South Africa	Cape Town	1901	Bubonic	3	(Mitchell, 1983)
UK	East Suffolk	1909-1918	Bubonic; Pneumonic	22	(Van Zwanenberg, 1970)
Italy	Catania	1914	Bubonic	16	(Privitera, 1917)
Spain	Barcelona	1931	Bubonic	26	(Cartana Castella and Collado, 1934)
Malta	La Valletta	1936	Bubonic	3	(Burrell, 1854)
Italy	Taranto	1945	Bubonic	17	(Leone, 2000)

CONCLUSIONS

Plague is an infectious disease that has shaped the history of humankind and is still today a threat to health in many parts of the world. There are still unresolved questions, about how the plague affects its victims, and if there are differences between subjects in susceptibility and mortality to it, both in past pandemics and in the present time. This doctoral thesis endeavours to answer these questions. As we described in the first chapter, different factors can influence the susceptibility and mortality of an infectious disease: sex and age of the victims, their status of frailty and pre-existing health conditions. In particular, iron levels of the body affect susceptibility to different infections, and a moderate iron deficiency anemia may be a protective factor to many infectious diseases (Fig. 1.2).

We started with a review of all published data we could find on anthropological studies on plague victims. We collected data from the First (6th -7th c.) and Second (14th -18th c.) Pandemic of plague regarding sex, age and some biomarkers of biological stress to evaluate their health status. From our analysis we could not find a general pattern of mortality, if not regarding the age of the victims: most of those who died from plague were children between 5 and 9 years and young adults between 20-35 years of age. Both prepubertal children and young adults in their reproductive years should have a stronger immune system, compared to younger children and the elderlies. It seemed, therefore, that plague did not select its victims based on their frailty, and instead affected also healthy individuals. Nonetheless, the methodology used by the various authors to evaluate sex, age and to assess heath conditions was not the same for all studies; therefore, much information was lost, and comparison between sites was not always possible or resulted in some bias. Indeed, there is a need to standardise the methodology between the authors, in particular regarding the assessment of frailty and health. For this reason, we decided to first create some guidelines for the analysis of porotic lesions of the skull (Porotic Hyperostosis, PH) and the orbits (Cribra Orbitalia, CO). These lesions are generally considered a symptom of anemia, either genetic or acquired, although there are still doubts about their exact etiology. The new evaluation forms consider all the aspect necessary to evaluate PH and CO, most importantly, their degrees of severity and healing.

Then, we created a new index for the evaluation of frailty in human skeletal remains. Frailty is the biological stress load that an individual accumulate during life; frailty affects the immune system, making the individuals more susceptible to diseases. In past populations, frailty is estimated through the analysis of skeletal biomarkers of physiological stress, but few attempts have been made in creating an index that comprised all the biomarkers. The new index (Biological Index of Frailty) we have proposed takes into consideration both the healing and severity of some lesions (mainly PH and CO) and, for the first time, each biomarker of stress was attributed a different weight. Moreover, the index can be used even on partial skeletal remains, with excellent results.

Using the new index of frailty, we analysed two samples: one of plague victims from the plague epidemic of 1630-32 at Imola (Italy) and one of non-plague victims, from an attritional cemetery of Ravenna (Italy), dated to the 17th century. The aim was to investigate whether any difference in frailty was present, and therefore deduce if plague selected its victims from the frailer cohort as other researchers have proposed before. We could not find any significant difference in frailty scores of the two groups, but we noticed that female plague victims had a lower frailty score than non-plague victim women, while men were more similar in frailty to the other group. In other words, it seems that healthy men died less from plague than healthy women. The evaluation of anemia trough the study of PH and CO did not give any significant result: more specific analysis would be necessary to detect the iron content of the individuals at the moment of their death.

Finally, in the last chapter, the paleoepidemiological analysis of data collected from medical reports on plague individuals who were affected by plague and died or recovered from the infection, permitted us to gain information not evincible from skeletons. The data collected came from reports on plague patients between 1813 and 1945 and from all over the world. We analysed only individuals who were affected by bubonic plague, as the pulmonary and septicemic forms of the plague have higher levels of virulence, and very few individuals managed to recover from it without antibiotic treatments. We noticed that males were the most susceptible in contracting plague, independently from their age. At the same time, deceases frequencies of males and females were very similar, i.e. men recovered in higher frequencies than women. We observed this phenomenon in particular for adolescents, whose differences were statistically significant, and for adult, whereas for children and elderlies the rates of deads and recovered were similar between the sexes. We have proposed (Fig. 6.6) a possible explanation for this phenomenon: hormone levels increase during adolescence, and sex hormones are present during all adulthood till they decrease slowly in old age in men and drop drastically after the menopause in women. Testosterone is known to downregulate hepcidin, the iron homeostasis regulatory hormone, thus reducing iron storing in macrophages and helping individuals (in good health) to overcome the infection. The role of estrogen on hepcidin is still debated, but if it acted as an up-regulator of hepcidin, this effect, coupled with the low circulating iron levels of fertile women, could lead women to a less susceptibility to first infection. On the other side, their higher iron content in macrophages, may forster a higher tendency to death. Of course, only an experimental analysis can verify this hypothesis or find an alternative explanation.

Nonetheless, the evidence of less frail women and more frail men we found in the skeletonised victims of plague of Imola, can also find an explanation if our hypothesis is correct: men were more susceptible, but have higher chance to recover if their frailty and health did not impact on the immune system, while frailer men succumbed to the bacterium. Women, even less frail ones, had more possibility to die once infected.

We cannot, anyway, explain the high frequency of prepubertal children in the skeletal assemblies of plague victims: iron could be again the answer, but only with further analyses, this circumstance may be proved.

Therefore, did plague selected its victims? It seems that the answer is not a single one.

While it seems to select frailer men, it does not seem to do the same with women. Only additional experimental analyses will be able to clarify the reason for this mechanism. By analysing other skeletal series of plague and non-plague victims of different periods with the Biological Index of Frailty and with the new guidelines for the analysis of PH and CO, it will be possible to evaluate whether the phenomenon we found in Imola, and which the paleopidemiological data seem to confirm, was not limited to single historical periods or geographical places. Besides, by analysing the iron content with other methodologies (for instance quantifying ferritine in skeletons with proteomics), it will be possible to directly verify whether iron has played any role in modulating the mortality from plague.

To establish the iron content in skeletons anyway, is not the only possibility to further explore this issue, also the study of the adaptive immune response through the analysis of regulatory genes in the aDNA of past individuals might help in discovering other mechanisms that could have influenced plague mortality. Laayouni et al. (Laayouni et al., 2014) already noticed a selection of some Toll-like receptor due to an adaptive evolutionary response to plague. Are there some genes that make people more susceptible to plague? This could be a new fascinating field of research that could integrate the information we have collected.

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