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Studying the plague: retrieving information from the past

Ph.D. Thesis

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Abstract

Recently, due to COVID-19 pandemic, epidemic preparedness programs have received particular attention. Unfortunately, the importance of preparedness and rapid response to epidemic events reached politicians and the public only when it was too late to prevent or limit this infectious disease. This pandemic has rapidly exposed the enormous vulnerabilities of modern human societies; globalization, fast transport, climate change, high population density, and ecological transitions are all aspects of modern societies that can favour and influence the emergence of new and old human pathogens. Although it is currently impossible to predict the emergence of a new pathogen before it actually emerges, analysing past microorganisms and epidemics can allow us to learn from past successes and mistakes, so that present and future surveillance and monitoring programs can be improved.

With these premises, this thesis project incorporates biological and historical investigations to examine which information related to microorganisms and epidemics from the past can be derived from remains of organic material and historical records.

In the first part of the project, human teeth, recovered from an archaeological site dated to the period of the Milan plague of 1629-1631, were processed to investigate the presence of traces of *Yersinia pestis*, the causative agent of plague. Both DNA and proteins associated with the pathogen of interest have been searched. Preliminary results for these analyses were inconclusive, but the metagenomic analysis is still in progress and the results will be available in the next few months.

During the second part of the project, historical and epidemiological investigations were carried out on textual sources associated with past plague epidemics. The first study (Manuscript n. 1) involved the development of a new informatic tool aimed at extracting useful information from a huge amount of textual data. This tool was applied to derive information regarding the 1720–1722 plague of Marseille and the 1629–1631 plague in northern Italy. The analysis of text related to these two epidemic episodes revealed that plague-related words were associated with the words “merchandise”, “movable”, “tatters”, “bed” and “clothes”, while no association was found with rats. These results support the hypothesis of a role of human ectoparasites during the second plague

pandemic. Moreover, the results suggested a potential future application of this tool for the prediction of pathogen, responsible for a described disease, in ancient texts.

The second study (Manuscript n.2) concerned the analysis of the progress of the plague epidemic that hit the city of Milan during the years 1629-1631. The registers of the deaths of the city of Milan for the year 1630 were digitized and subsequently used for the spatio-temporal analysis of the epidemic and historical events related to it; in particular, the effect that a religious mass gather had on the spread of the epidemic in the city was analysed.

The last part of the thesis project was focused on the research and study of the bibliography concerning the paleomicrobiological studies performed on ancient human microbiota (Manuscript n.3).

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1. Introduction

1.1 The study of ancient microorganisms and epidemics

"*Over the past fifty years, more than three hundred infectious diseases have either newly emerged or reemerged, appearing in territories where they've never been seen before. Ninety percent of epidemiologists expect that one of them will cause a deadly pandemic sometime in the next two generations. It could be Ebola, avian flu, a drug-resistant superbug, or something completely new. While we can't know which pathogen will cause the next pandemic, by unravelling the story of how pathogens have caused pandemics in the past, we can make predictions about the future*."

From the synopsis of the book Pandemic: Tracking Contagions, from Cholera to Ebola and Beyond by Sonia Shah (Shah, 2016).

The SARS-CoV-2 pandemic was a big shocking event for humanity. When this new disease began to spread in December 2019 in the city of Whuan, China (Riou & Althaus, 2020), all the governments in the world were caught unprepared (Frutos et al., 2021), even the ones that were considered most prepared, like North America and Europe (Oppenheim et al., 2019). Within months, this new disease spread to all regions of the world, causing, according to some, an unprecedented pandemic event (Nkengasong, 2021).

Is this true? Have humanity never had to face similar events in the past? And even better, could have we done something to make us better prepared?

Echoing the words of Sonia Shah, a science journalist who in 2016 published her book about pandemics (Shah, 2016), the vast majority of epidemiologists expected a deadly pandemic in the near future. She even reported precise hot spots for the potential emergence of new deadly diseases, among which China's wet markets were found. The most interesting thing is that, she was not the only one, nor the first to evidence the great risk that wet markets pose as potential disease sources (Karesh et al., 2005; Wan, 2012; Woo et al., 2006). The evidence for this epidemiological interest into some specific hot spots, such as the wet markets, can be found in the biology, ecology, and history of human epidemics. The ecosystems and the living beings that compose it are all linked in a network of relationships that can influence the survival dynamics of each individual. This

concept is at the base of the scientific approach called “One health”, a “collaborative, multisectoral, and transdisciplinary approach with the goal of achieving optimal health outcomes recognizing the interconnection between people, animals, plants, and their shared environment” (https://www.cdc.gov/onehealth/; Figure 1). One thing that is not clearly stated into this definition is that past epidemics and pathogens can be retrospectively studied. History can help us in this. Studying what happened in the past, how our ancestors reacted and behaved during, before and after an epidemic, can help us understand the best way to fight the emergence of new epidemic events. Therefore, it can help us avoid making the same mistakes and avoid being caught unprepared for the next epidemic/pandemic.

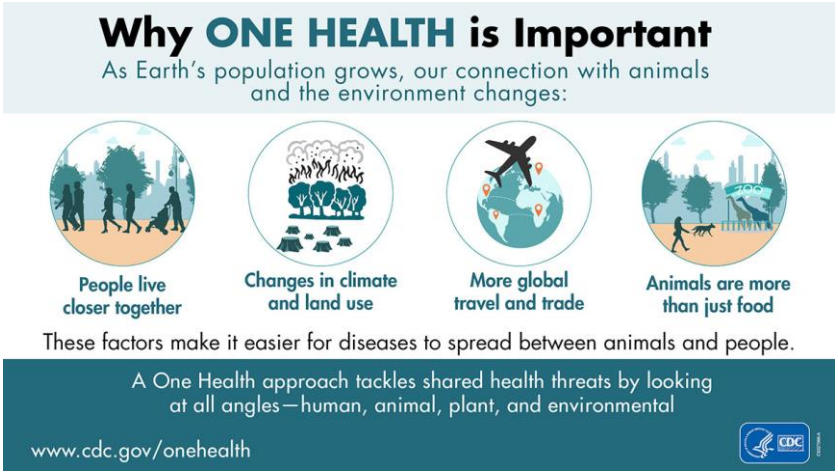

Figure 1. One Health. Main concepts of One Health approach and why it is important (CDC).

Studying ancient microorganisms and, more specifically, pathogens can be a valuable tool in answering many historical and biological questions. Analysing and studying problems in the past can allow us to see patterns in present day problems. For instance, studying the environmental and social conditions, present before the emergence of an epidemic in the past, might be useful to individuate critical points on which we can act upon.

Some ecological and health-related questions which can be analysed through an historical perspective, are here summarized: what could have caused the high mortality of past diseases; how pathogens spread across continents; how vectors and host reservoirs contributed to their virulence and transmission of infectious diseases; from where pathogens come from and how do they adapt to humans and other species; what was the diversity of pathogens in the past. The answers to these questions can give us information about the present state of pathogens and, maybe, also about the future. Understanding how pathogens evolved and infect humans, so that we can intervene

preventively to counter the adaptation or the diffusion of future pathogens (mutations, speciation events, adaptation to vectors, and acquisition of antibiotic resistance and virulence genes). Moreover, this can also help to understand the effect on human/host evolution.

1.2 How to study ancient microorganism and epidemics

There are two main approaches that can be applied to investigate ancient pathogens:

- The historical approach, based on the study of written record or other documented communication (Cunha & Cunha, 2008);
- Paleomicrobiology, based on the study of molecules in ancient remains (Raoult & Drancourt, 2008).

In general, historical data are useful to know where and what to investigate, while paleomicrobiology can provide scientific proof of the presence of a causative agent during a past period (Raoult & Drancourt, 2008). However, sometimes it is not possible to use paleomicrobiological techniques due to the absence of well preserved and suitable samples. In these cases, historical analysis remains the most helpful tool for the study of ancient epidemics (Cunha & Cunha, 2008).

1.2.1 Historical approach

> "*… but I shall talk about its course, and explain the symptoms, by which it could be recognised in the future, having knowledge of it beforehand. For I myself was ill and saw others suffer from it.*"
>
> *Thucydides on The Plague of Athens (430–426 B.C.)*

Thucydides gave a perfect example on the importance of historical records. Writing about the characteristics of an epidemic event and the associated disease can be useful for the people that

will come and that might have to deal with the same or a similar event. Therefore, many physicians took the time to record what they saw during epidemics or other relevant events.

Historical records, not only give us information about past diseases, but also indicates the existence of a disease/epidemic event in the past. Without historical records, we would have probably forgot all the tremendous events that afflicted our species. As stated by Cusani (Ripamonti, 1841, p. 10), even the terrible and now famous epidemic of 1630 in Milan would probably have gone forgotten if it had not been for the work of Alessandro Manzoni "I promessi sposi", a masterpiece of Italian literature set in northern Italy during the early XVII century (Manzoni, 1827).

Plague is probably the most known disease from the past. Its fame is due to both the great mortality and devastation brought by the disease (Ditchburn & Hodgkins, 2019)and also to the great number of books written during and after on this subject (Cohn, 2010). In fact, considering only the XVI century, more than 300 books were published in Italy alone (out of a total of 1500 published books) (Cohn, 2010, p. 22). In addition, during the second pandemic of plague, several cities started to keep detailed daily or weekly records of deaths. An example is the very detailed registers of the dead (*Mortuorum libri*) for the city of Milan, that in an almost continuous series for the period 1451-1801 (with some gaps) recorded all the deaths that occurred in the city, with particular regard to possible suspicions of plague (*Atti di Governo, bb. 118-119*). Similar records are available for other cities, like Venice (Lazzari et al., 2020) or, outside Italy, in London where weekly bulletins of the dead in the city were published (London bills of mortality) (Bellhouse, 1998; Boyce, 2020; Heitman, 2020). Therefore, it appears evident that the multitude of historical information preserved in books, city registers, but also tax records (Alfani & Bonetti, 2019), church registries (Alfani & Bonetti, 2019; Weisdorf, 2016) or coroners' reports (Butler, 2014) can provide invaluable information about the epidemiology and characteristics of some diseases of the past (Cunha & Cunha, 2008).

The historical approach can help us to identify the aetiology (*i.e.* the cause) of an epidemic episode base on the author description of the symptoms and the context of the disease (Cunha & Cunha, 2008). It can also be used to obtain important information about the characteristics of a pathogen in ancient times and the dynamics of epidemics caused by them. For example, the transmission and vectors involved in a past epidemic can be studied by analysing the seasonality of epidemics (Alfani & Cohn Jr, 2007), the temporal and spatial spread of the disease (Alfani & Bonetti, 2019; Christakos

et al., 2007; Dean et al., 2018), or even by the precautions taken by contemporary people (Barbieri et al., 2022). Moreover, mortality rates, signs and symptoms differences between past and modern epidemics can provide information about the virulence of a pathogen (Carmichael, 2008; Cohn, 2010).

In the absence of scientific proof and despite some major problems that afflict the field (Box 1), the historical method remains the backbone of the analytical approach to the study of ancient epidemics.

Box 1. Problems of the historical approach:

- *Epidemics not recorded or information lost (Martin & Martin-Granel, 2006);*
- *Difficulties of description and interpretation (Major, 1978);*
- *Translator interpretation of the languages in which they were first described (Cunha & Cunha, 2008);*
- *The observers or recorders of the descriptions of ancient epidemics varied in their observational and descriptive capabilities as well as in their knowledge of medical terms used at the time (Page, 1953; Parry, 1969).*

1.2.2 Paleomicrobiology

Ancient molecules, such as ancient DNA (aDNA), from both pathogenic and commensal microorganisms, can provide information about the health status of ancient individuals as well as past dietary habits and the ecology of diseases (Orlando et al., 2021).

Until three decades ago, the only way to study ancient pathogens, and the epidemics caused by them, was through the methods described before, i.e., the study of historical records, or, for some specific cases, the use of palaeopathology (Box 2). Both of these methods offer fundamental information about the past and the pathogens that may have caused an epidemic event. Nevertheless, the only way to confirm a suspected diagnosis on ancient times is through paleomicrobiology (Drancourt & Raoult, 2005).

> ***Box 2. Paleopathology.***
>
> *A third approach used to study past diseases is paleopathology.*
>
> *Paleopathology is the study and application of methods and techniques for investigating diseases, nutritional deprivation, and mechanical stress from skeletal and soft tissue in human remains (Smith, 2013). Despite this field is very useful to derive information about some infectious diseases, such as tuberculosis (Nerlich & Lösch, 2009) and syphilis (C. Meyer et al., 2002), it cannot be used if the pathogen left no traces on the remains (e.g. Y. pestis, P. falciparum, S. enterica, …).*

Paleomicrobiology is the study of microorganisms associated with ancient material (Drancourt & Raoult, 2005). The pioneering work of Drancourt et al. 1998, paved the way for this field, by identifying for the first time traces of *Yersinia pestis* DNA in the teeth extracted from skeletons of French graves of persons thought to have died of plague between the 16th and 18th century (Drancourt et al., 1998). This work proved, not without an initial opposition by a portion of the scientific community (Drancourt & Raoult, 2014), that the molecules associated with the microorganisms persist in archaeological records. Anyway, this field is also afflicted by some major problems that greatly reduce the efficacy of paleomicrobiological analysis. Some of these major problems are:

- Rarity of well-preserved and suitable samples for analysis;
- Exogenous modern contaminations;

- Ancient molecules are often highly degraded and usually found in low concentrations (Dabney, Meyer, et al., 2013);
- Differential colonisation from infection (the mere recovery of an organism from an ancient preserved specimen does not necessarily imply a role for this organism as the cause of the death (Cunha & Cunha, 2008);
- Difficulties related to the absence of reference genomes or information for an organism for sequence/genome comparison.

Today, there are entire scientific research groups dedicated to this field and the study subject is not limited to the pathogen *Y. pestis*. Many other pathogenic microorganisms have been isolated from ancient samples, among which we can find:

- bacteria, such as *Mycobacterium tuberculosis* (Bos et al., 2014)*, Mycobacterium leprae* (Schuenemann et al., 2018)*, Treponema pallidum* (Schuenemann et al., 2018)*, Yersinia pestis* (Bos et al., 2011)*, Helicobacter pylori* (Maixner et al., 2016)*, Salmonella enterica* (Vågene et al., 2018)*, Vibrio cholerae* (Devault et al., 2014);
- viruses, such as HBV (Mühlemann et al., 2018), HIV (Worobey et al., 2016), influenza virus (Taubenberger et al., 2005), VARV (Duggan et al., 2016);
- eukaryotes, such as *Plasmodium falciparum* (Marciniak et al., 2016)*, Plasmodium vivax* (Gelabert et al., 2016)*, Phytophthora infestans* (Yoshida et al., 2013)*, Taenia solium* (Søe et al., 2018)*.*

The primary sources for paleomicrobiological studies on humans are different types of remains, e.g. bones, teeth, mummified tissues, hair, paleofeces (coprolites), dental calculus (tartar), and the analysed molecules are not limited to nucleic acids, but also include ancient proteins (Barbieri et al., 2017; Warinner, Rodrigues, et al., 2014) and lipids (Lee et al., 2012). Among the different samples that can be used, dental pulp is considered the sample of choice for blood-borne diseases (Mai et al., 2020). Dental pulp is a connective tissue, situated inside the teeth, that contains an abundant vascular system and nerves. Blood-circulating microorganisms could reach and colonize the vessels in the dental pulp (La et al., 2008). Moreover, after the death of the individual, this tissue remains

enclosed in the dental cavity, which is well protected from external contamination and degradation by enamel and dentin (Mai et al., 2020).

Without doubts, DNA is the most studied ancient molecule and it is also the one that can provide the majority of information. There are multiple methods and techniques that are used for the detection and isolation of pathogen DNA from ancient human specimens (Figure 2; (Spyrou, Bos, et al., 2019).

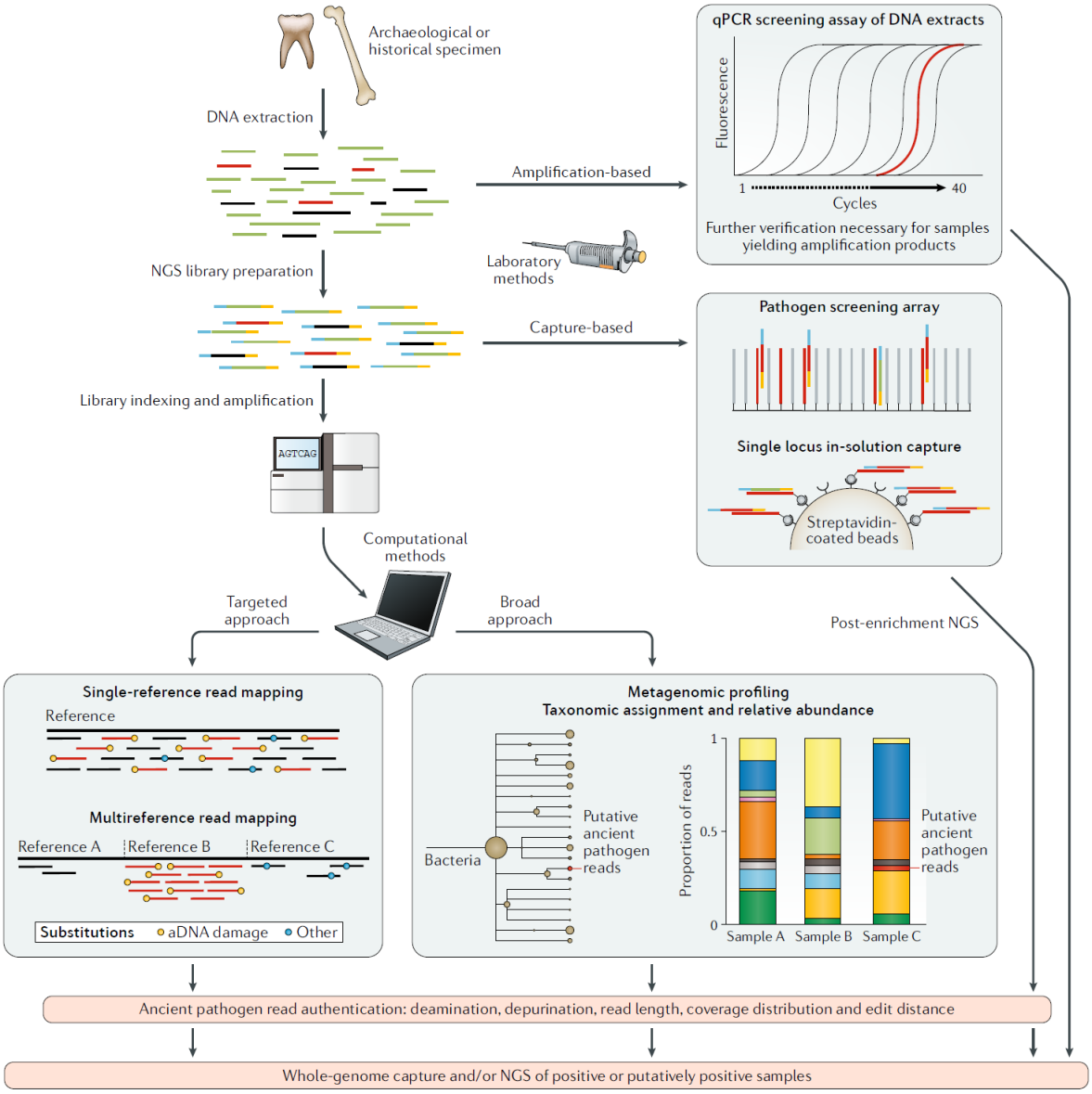

Figure 2. Workflow for the paleomicrobiological analysis of ancient DNA (Spyrou, Bos, et al., 2019).

In any case, every analysis begins with the extraction of the DNA from the specimens (Dabney, Knapp, et al., 2013). The extracted DNA can then be directly screened for the presence of genic markers of specific organisms of interests through PCR analysis (Drancourt et al., 1998; Schuenemann et al., 2011) or it can be used for the preparation of Next Generation Sequencing (NGS) libraries and subsequent bioinformatic analysis (Meyer & Kircher, 2010; Spyrou, Keller, et al., 2019). In general, the "simple" detection of pathogens through amplification-based techniques it is fast and cost-effective but should be coupled with NGS analysis for further confirmation (Spyrou, Bos, et al., 2019). In cases in which a causative agent is suspected, the analysis can be improved by selectively sequencing DNA fragments of interest (e.g. through in solution capture) or by mapping the reads generated by NGS against reference genome/s (Bos et al., 2014). In the last 10 years, more than 80 genomes of *Y. pestis* spanning the last 6,000 years have been reconstructed (Barbieri et al., 2020). The analysis of these ancient genomes allowed the identification of a temporal sequence of genetic changes that lead to increased virulence and the emergence of the bubonic plague (Spyrou, Keller, et al., 2019).

NGS libraries can also be analysed through metagenomic profiling methods (Vågene et al., 2018). This method is useful when there is no historical information about the possible causative agent responsible for the death of individuals from an archaeological site, or even for microbiome analysis (Groussin et al., 2017; Warinner et al., 2017, p. 201; Weyrich et al., 2017). The reconstruction of the microbial communities associated with ancient humans, or other hominids, is used to analyse and describe, not only the relation with pathogenic microorganisms, but also the changes in human lifestyle (e.g. in diet, medical innovations, hygiene, and antibiotics) that are characterized by changes in the microbiome composition (Nodari et al., 2021).

Sometimes, fragmentation and decomposition are so extensive that the analysis of aDNA is not possible, while proteins are naturally more resistant and can be detected even after millions of years (Barbieri et al., 2017; Demarchi et al., 2016). Paleoproteomics, the field that studies ancient proteins, has revealed important information about the dietary habits of past human populations (Warinner, Hendy, et al., 2014), pathogens (Barbieri et al., 2017; Warinner, Rodrigues, et al., 2014), host immune systems (Warinner, Rodrigues, et al., 2014), and the microbial population from the digestive tract microbiome of ancient individuals (Jersie-Christensen et al., 2018).

1.3 History of Plague

The term “plague” comes from the Latin “pestis” which literally means "destruction, ruin, epidemic" (https://www.treccani.it/enciclopedia/peste_%28Universo-del-Corpo%29/). In the past, the term was associated with epidemics that spread quickly causing high number of deaths and/or cause terror (Alfani & Murphy, 2017). This pose a serious problem for historical studies on past epidemics since historical sources, especially those preceding early modern times, often refer to generic “plague epidemics” even when the diseases in play were different (Alfani & Murphy, 2017). Biologically, the term plague is the infectious disease caused by the bacterium *Y. pestis*, but it is not rare to find past plague epidemics that had no actual relation with *Y. pestis*, *e.g.* the plague of Athens (Papagrigorakis et al., 2006).

Plague has very close roots with the history of man. Molecular studies identified the presence of *Y. pestis* in ancient remains dated back till 3000 BCE (Rascovan et al., 2019). Moreover, written records mentioning plague epidemics from ancient Egypt (1350 BCE) have been also recovered (Panagiotakopulu, 2004). There is also a mention of a plague epidemic in the biblical book 1 Samuel (approximately 1000 BCE), in which the Philistines territories were hit by a deadly disease that was associated with rodents and caused tumours in its victims (Freemon, 2005; Griffin, 2000).

Three pandemics from the last 2000 years have been attributed (and paleomicrobiologically confirmed) to *Y. pestis*.

1.3.1 First pandemic

The first plague pandemic started in the city of Pelusium (Egypt) in 541 CE (Little & Walsh, 2007). From there, the disease quickly reached Constantinople and all the areas of the Mediterranean region. Eventually, the pandemic reached all the "known world" (Europe, North Africa, Arabia, central and southern Asia) (Figure 3)(Perry & Fetherston, 1997).

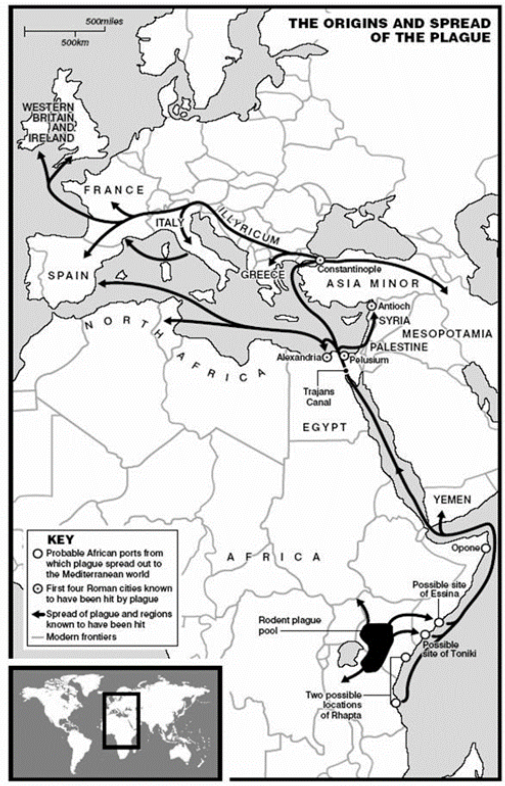

Figure 3. Possible origin and diffusion of the first plague pandemic (Keys, 1999).

The first pandemic is also known as Justinian Plague, from the name of the Emperor of the Eastern Roman Empire at the time: Justinian I, which ruled on the Empire from 527 to 565 (Barbieri et al., 2020). This first epidemic episode (541 – 544 CE) was followed by 14 to 21 additional plague waves until half VIII century (Mordechai et al., 2019). This cyclical reoccurrence of plague epidemics is a typical characteristic of plague pandemics that was also reported for the second and the third pandemic. The exact source of the first pandemic is still not known. According to contemporaneous chronicles, the plague originated in Eastern Africa, in the modern Ethiopia (Bramanti et al., 2016; Dols, 1974), but also an Asiatic origin has been proposed by historians, in particular the regions of India (Glatter & Finkelman, 2021) or the Great Steppe region (Rosen, 2007).

After 200 years of devastation and an estimated population loss of 50 to 60% (Perry & Fetherston, 1997), plague disappeared (Keller et al., 2019).

1.3.2 Second pandemic

Around the year 1000 A.D. there were probably no more than 30-35 million people in all of Europe (including Russia and the Balkans). From the 10th century until the beginning of the 14th century the European population grew slowly but steadily. In the years 1330-1340 the total population of Europe must have been at least 80 million (Cipolla, 1976; Perry & Fetherston, 1997). Although wars,

famines and epidemics were not unknown before the 14th century, low population densities limited the devastating effects of epidemics. However, with the growth of the population and the increase of density in the cities, the nature of the problem changed. In fact, at the beginning of the 14th century, several areas of Europe were overpopulated (e.g. San Gimignano in 1332 reached a population greater than that of the same city in 1951) (Cipolla, 1976). This important increase in population was not followed by improvement in medicine and hygiene practices. This imbalance between population growth on the one hand, and a lack of medical and health development on the other, reached a critical point in the early 14th century with garbage, human and animal waste piled up on the streets; overabundant rats, fleas and lice; unsafe water wells; non-existent health facilities; soap was rarely used, and personal hygiene was a little-known practice. All these conditions set a fertile soil for the spread of epidemics.

The origin of the second pandemic is attributed to central Asia at the end of the 1330s, probably in present-day Kazakhstan, Russia, or China (Namouchi et al., 2018; Spyrou, Keller, et al., 2019). According to historical records, plague may have travelled by the Mongolian Army to the West. During the besiege of the Genoese settlement of Kaffa (1346), on the Crimean Peninsula, the Mongols allegedly catapulted the bodies of their comrades who died of the disease beyond the walls of city, in the first recorded episode of bacteriological warfare (Inglesby et al., 2000). The disease was further transported via Genoese ships fleeing from Kaffa reaching Italy (Bramanti et al., 2016). From there plague spread all over, covering all Europe in few years, in the epidemic event which later became known as the Black Death.

In just seven years (Benedictow & Benedictow, 2004), the Black Death killed an estimated 17 million to 28 million Europeans (1/3 of the European population at the time) (Perry & Fetherston, 1997). Moreover, the Black Death marks the beginning of the second pandemic, with a succession of cyclical plague epidemics throughout Europe during the following 400 years. Epidemic cycles afflicted all the main (and also most of the minor) European cities of the time, with recurring plague epidemics (Figure 4) (Perry & Fetherston, 1997).

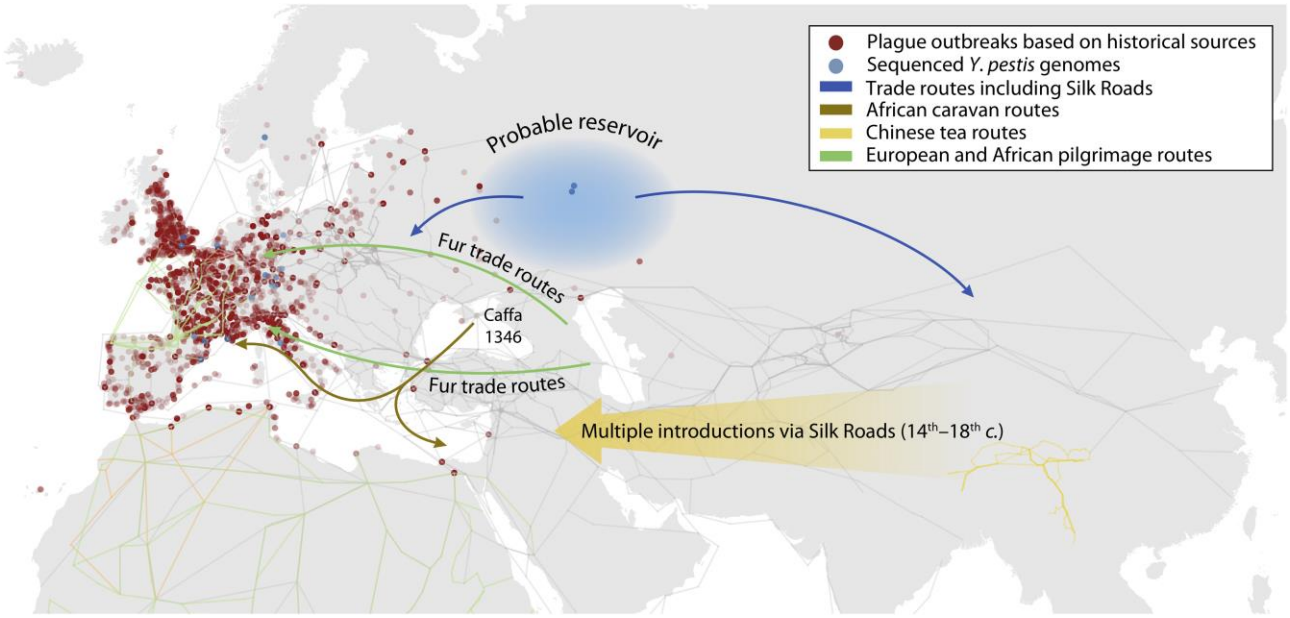

Figure 4. Map of the second plague pandemic, including major movement and trade routes (Barbieri et al., 2020).

It is difficult to define the end date for the second pandemic (Barbieri et al., 2020). The last official epidemic occurred in Moscow in 1771 (Cohn, 2008), although other outbreaks were also recorded in nineteenth century in the Mediterranean region (Cohn, 2008; Valensi, 1969).

As for the first pandemic, the reasons for the decline of the second pandemic have not yet been identified. The various hypotheses involve changes in climate, abundant rodent populations (from the invasion of the grey mouse and the disappearance of the black mouse), public health problems (alleged improvements in construction, to better ways to bury corpses) and changes in the etiological agent of the disease *Y. pestis* (Cipolla, 1976; Perry & Fetherston, 1997), but all have turned out to be inconclusive.

1.3.3 Third pandemic and modern cases

The third pandemic is likely to have originated from the Chinese province of Yunnan where plague cases were recorded since mid XVIII century (Bramanti et al., 2016; Xu et al., 2011). In the 1850s and 1860s it became endemic in southwestern China (Perry & Fetherston, 1997). From there, plague spread to the East reaching Hong Kong and Canton in 1894 and Bombay in 1898 (Perry & Fetherston, 1997; Yersin, 1894). Between 1899 and 1900 steamships and trains rapidly disseminated the disease

to all the continents (excluding Antarctica) (Perry & Fetherston, 1997). It was during the 1894 epidemic in Hong Kong that a Swiss-French doctor isolated for the first time the causative agent of the plague: what is now called *Yersinia pestis* (Yersin, 1894). At the same time, he individuated into rats a clear carrier of the disease, especially because of the fact that human epidemic usually broke out following a large die-off of rodents (Yersin, 1894)(https://www.epicentro.iss.it/peste). Three years later, during the Indian epidemic of 1897, Paul-Louis Simond discovered the role of the flea in the transmission of the plague, in particular the rat flea of the species *Xenopsylla cheopis* (Simond, 1898).

Between 1894 and 1903, the plague reached 77 ports on five continents (Riedel, 2005)(Figure 5). In the following years, a series of small epidemics occurred around the world. India and China were the regions most affected by the third plague pandemic. In 1903, in India alone, the plague killed one million people a year, and in total an estimated 12.5 million Indians died of the plague between 1898 and 1918 (Perry & Fetherston, 1997).

The death rate and the spread of sporadic plague outbreaks have dropped significantly over the past hundred years compared to previous pandemics, largely due to the advent of effective public health measures. In particular since about 1950, antibiotics, on the one hand, and the application of powerful insecticides such as DDT, on the other, have made the plague a curable and controllable disease (Pollitzer, 1954). According to the WHO, the pandemic was considered active until 1959, when cases around the world dropped to fewer than 200 per year. Despite this, plague has not completely disappeared and endemic areas are still present today in Asia, Africa and America, where wild rodent populations act as reservoir for the disease (Figure 5)(Barbieri et al., 2020).

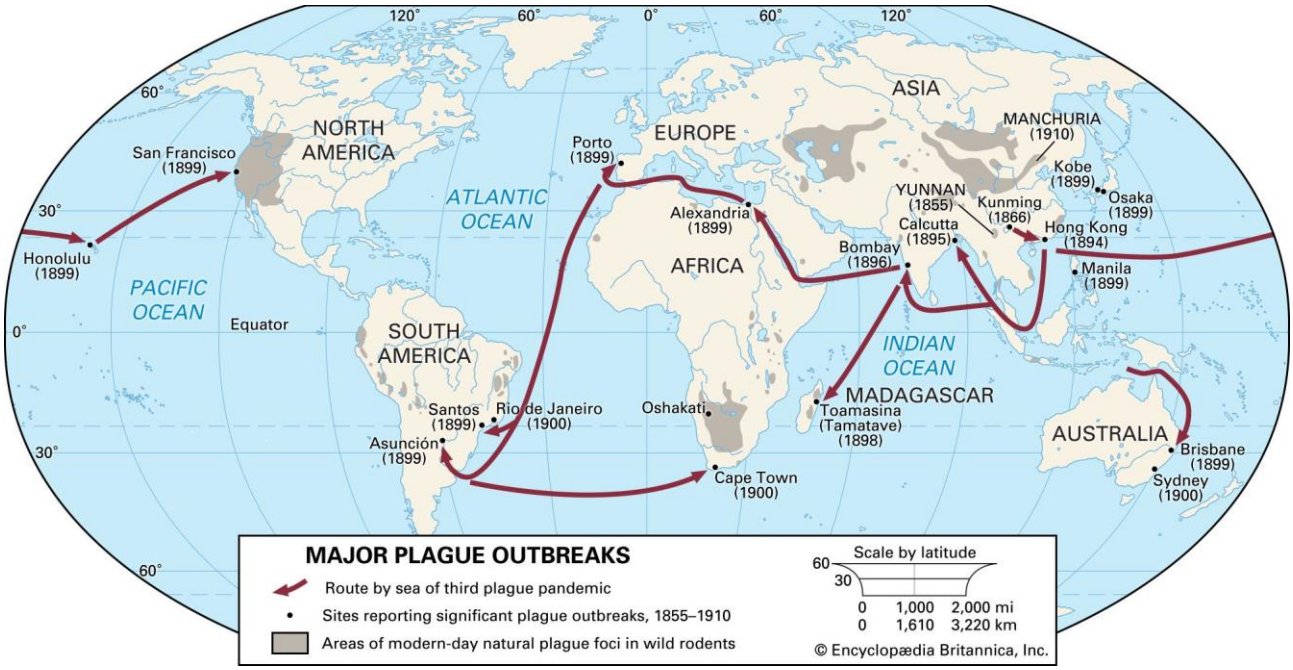

Figure 5. Map of the third plague pandemic and modern-day natural plague foci in wild rodents (https://www.britannica.com/science/plague/History).

As for the previous pandemics, plague during modern times has the ability to re-emerge and cause another outbreak in areas that have long remained unaffected (ECDC, 2021). While no case has been reported in Oceania or Europe since the 1945, nearly 50,000 human cases of plague were reported to the WHO from Africa, Asia and America between 1990 and 2020 (https://www.pasteur.fr/en/medical-center/disease-sheets/plague). In 1994, an epidemic of pneumonic plague erupted in India, a country where the disease was thought to be eradicated 30 years earlier (Campbell & Hughes, 1995). The plague has also reappeared in the regions of Mozambique, Perú, Jordan, Algeria, Congo, Libya, Kyrgyzstan and Russia after 30 or even 80 years without epidemics. More recently, a pneumonic plague outbreak hit Madagascar in 2017 causing 2417 cases and 209 deaths, in a region where no cases had been reported since 1950 (Rakotosamimanana et al., 2021).

1.3.4 The Great Plague of Milan (1629-1631) and the condition the favoured the epidemic.

In 1629 northern Italy was afflicted by a tremendous epidemic of plague that killed hundreds of thousands of people. The precise number of dead people is not clear. Only in the city of Milan this

number range from 80 thousands to 160 thousands (Ripamonti, 1841, p. 262; Tadino, 1648). What is certain is the fact that this epidemic left northern Italy, and Milan, severely damaged in both human loses and economical ones. Recent studies have in fact proposed that the epidemics of plague in the XVII century in Italy were the main reason for the economic decline of Italy in that period respect to northern Europe (Alfani, 2020).

Northern Italy in the XVII century was a very divided land (Figure 6), with multiple territories under direct or indirect control of other greater European nations. During the first half of that century northern Italy was the battlefield for wars between Spain, the Empire and France. In particular, two wars destabilized this region of Italy: the Valtellina War and the succession war of Mantua and Monferrato. Both these two wars were integral part of the Thirty Years' War, one of the most destructive wars in European history.

Figure 6. Map of northern Italy in 1559 (https://www.doppiozero.com/dossier/disunita-italiana/atlante-storico-dellitalia).

Valtellina was a fertile valley with a strategic location under the Alps. It connected the Duchy of Milan to the Empire and was also important in trade. The Valtellina war started in 1620 due to tensions between Catholics and Protestants in the region. The three leagues, supported by France, Savoy and Venice, had every interest in maintaining dominance over these territories, but under the pretext of religious tensions, Spain and the Empire used the opportunity to occupy the territory. Peace was achieved in 1626 with the maintenance of the Valtellina under the control of the three leagues (Priorato, 1672, p. 165). Actually, Spain remained in the occupied territories, prolonging the conflict for several years. The tensions between the major European forces over northern Italy continued

one year later. The succession war of Mantua and Monferrato started in 1627, with the death of Vincenzo II Gonzaga, duke of Mantua and Monferrato. Who had the right to succeed Vincenzo II were Carlo duke of Nevers and Don Ferrante Duke of Guastalla (Priorato, 1672, p. 268). The strategic position of these territories would have allowed a position of political control over all of northern Italy to those who had managed to win control. For this reason, from a simple legal dispute, the succession of the Duchy of Mantua turned into a war, dragging Europe's major powers to battle in northern Italy. Louis XIII King of France sided in favour of the Count of Nevers together with Venice. Spain, the Empire and the Savoys lined up in favour of the Duke of Guastalla. This situation led to the arrival of thousands of soldiers both from the North, the Alemans, and from the West, the French.

Starting from 1627, northern Italy was also afflicted by a tremendous famine, brought by unusual climatic conditions, which led to poor harvests, and by the high request of food to maintain all the army in the territory. The famine led to an increase in the price of raw materials and bread to figures too high for the populace. In addition to this, the stories of the time tell us of bread "stretched" with waste material, not edible and even harmful to health. These conditions led to mass revolts, the most famous of which took place in Milan during the day of St. Martin in 1628.

The wars, the famine and all that these two wounds bring with them, created the ideal conditions for the rapid and inexorable spread of the disease.

Apparently, there were no plague outbreaks since the outbreaks of Turin in 1600 and Trieste in 1601 in northern Italy (Corradi, 1870). As reported by many authors of the time, plague was brought into northern Italy by Aleman soldiers (Tadino, 1648). Both French and Aleman soldiers marched towards Mantua in the above mentioned “War of Mantua and Monferrato”. Aleman soldiers came from the North. They started from the city of Lindau. They passed through Grigioni Castles in Switzerland, and entered in Valtellina (Tadino, 1648). It was a known thing at the time that the German soldiers were afflicted by a contagious disease. Moreover, they were also aware of the chaos and destruction that that would have brought to every village and city they’d have passed. Direct stories speak of how the passage and raids of German soldiers were followed by the spread of the infection. For this reason, the city of Milan tried in every way to prevent the spread of infection in the city and the

inner lands. Confident and capable people were assigned to go and examine the villages around the city from which the soldiers passed (Tadino, 1648).

Despite the efforts of the Milanese council and physicians, in October, plague officially entered the city of Milan. The first case of plague was Pietro Antonio Lovato, a soldier stationed in Lecco who entered the eastern gate village (Borgo di Porta Orientale) of Milan on 22 October 1629 along with various vestiges of Aleman soldiers. He was taken straight to the major hospital, with signs of tumour on his arm, bubo under his armpit, and high fever. It died after four days (Tadino, 1648). Anyway, despite few localized cases of people that mostly had contact with the first case, the epidemic seemed to disappear during the winter and then re-emerged in March 1630 (Ripamonti, 1841; Tadino, 1648). Some contemporary chroniclers highlighted how the cases peeked immediately after a specific religious event: The San Carlo procession held the 11th June 1630 (Ripamonti, 1841; Tadino, 1648). According to the contemporary chroniclers, the death cases rose after the procession from about 100 to about 1700 per day (della Somaglia, 1653, p. 484; Ripamonti, 1841, p. 53). The epidemic continued until the end of the year, with some cases registered also during 1631.

1.4 The etiological agent of plague: *Yersinia pestis*

Yersinia pestis is an aerobic, gram-negative, coccobacillus, nonmotile, and nonsporulated that grows within a temperature range of 4 to 40°C (with an optimum between 28 and 30°C) at pH condition ranging from 7.2 to 7.6 (Barbieri et al., 2020; Perry & Fetherston, 1997). Due to its inability to produce some basic metabolic requirements (e.g. L-isoleucine, L-valine, L-methionine, L-phenylalanine, glycine, biotin, thiamine, pantothenate, and glutamic acid), *Y. pestis* has to rely on host organisms, making it an obligate parasite (Brubaker, 1972, 1991). It has been reported that more than 200 species of mammals can become infected with *Y. pestis* (Oyston & Williamson, 2011). Among these, the most important hosts for this microorganism are rodents (Oyston & Williamson, 2011).

The survival of *Y. pestis* is maintained by the cyclical transmission between rodents and fleas, which acquire the pathogen during the blood meal on an infected mammal and then transmit it to another

mammal during the following blood meals. Occasionally, humans can become infected, but they play no role in the long-term survival of the pathogen (Perry & Fetherston, 1997). Plague is normally well tolerated in rodent population (Gage et al., 1994) but sometimes it can cause the death of the rodent host. Due to this, fleas left without hosts can more easily pass to humans. For this reason, a typical indication for the beginning of a plague epidemic, during the third pandemic, was the death of large number of rats (Dennis, 1994; Oyston & Williamson, 2011). The schematic summary of the complex life cycle of *Y. pestis* is represented in Figure 7.

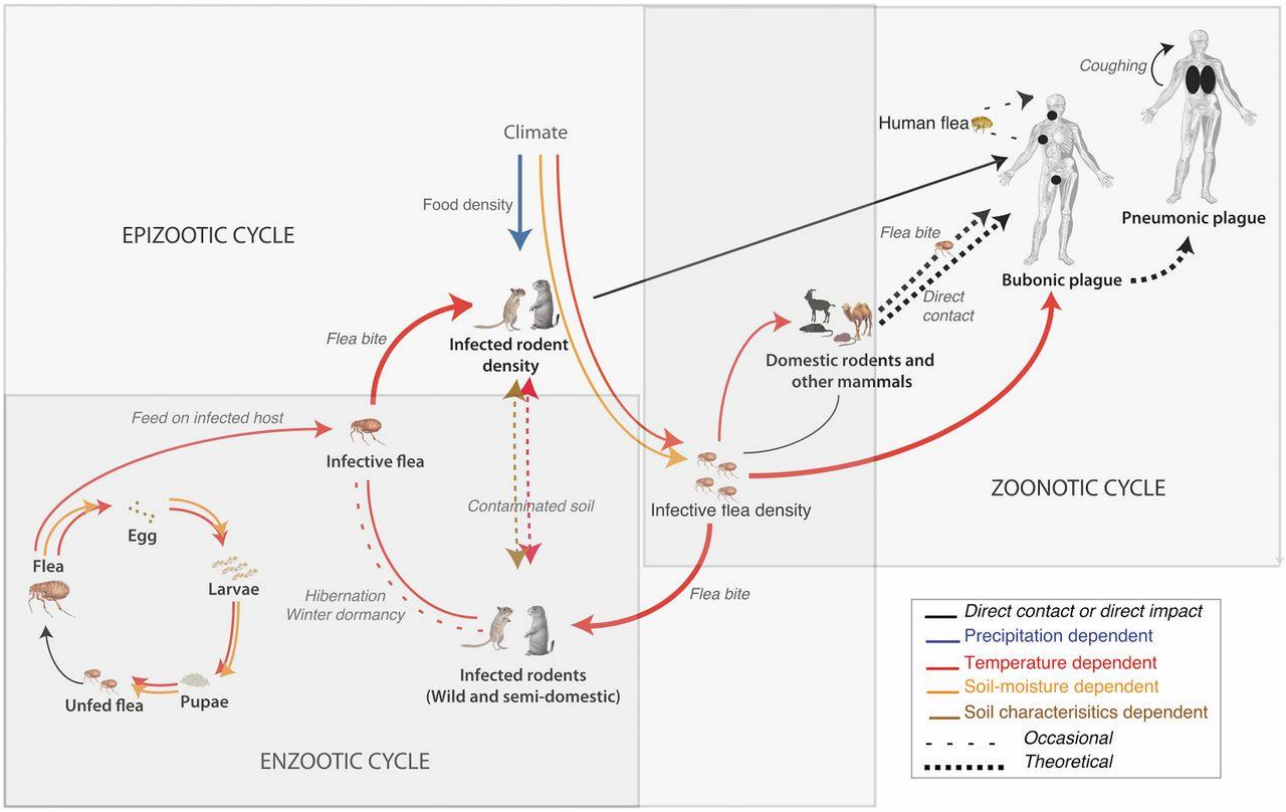

Figure 7. Schematic life cycle of Yersinia pestis (Ben Ari et al., 2011).

Paleogenomic analysis on ancient *Yersinia* strains performed in the last decade, uncovered many information about the evolutionary history of this pathogen (Demeure et al., 2019). At the genomic level, *Y. pestis* is highly similar to the enteric pathogen *Y. pseudotuberculosis,* a most closely related species. The speciation of *Y. pestis* from *Y. pseudotuberculosis* can be dated back to 5700 - 6000 years ago with the acquisition of two plasmids and the inactivation of a gene associated with virulence (Rasmussen et al., 2015). In fact, the genetic material of *Y. pestis* is split between 1 single

chromosome and three plasmids (Perry & Fetherston, 1997): pYV/pCD1, which is also present in other *Yersinia* species (e.g. Y. pseudotuberculosis), pFra/pMT1 e pPla/pPCP1, which are unique among *Yersinia* genus (Perry & Fetherston, 1997). These two plasmids contain the genes that are commonly used as *Y. pestis* markers in both modern plague detection and paleomicrobiology analysis on ancient samples: *pla* and *caf1* (Haensch et al., 2010; Riehm et al., 2011). Another important step in the evolution of *Y. pestis* was the adaptation to the rat flea. A series of genes mutations between 3800 and 5000 years ago have led to the appearance of the biofilm-forming ability that allow *Y. pestis* to colonized the anterior part of fleas' digestive tract, dramatically increasing its transmissibility (Hinnebusch et al., 2016; Sun et al., 2014).

1.5 Plague vectors and transmissions

Despite the majority of reported cases of plague are transmitted through rat fleas (which cause bubonic plague), *Y. pestis* can be transmitted by multiple ways resulting in the development of different clinical forms in humans, as shown in Figure 8. Other routes include transmission trough: respiratory droplets (which cause pneumonic plague); consumption of uncooked contaminated meat (which causes gastrointestinal plague); contact with infected, dead or alive, animals (causing conjunctivitis, skin plague or pneumonic plague) (Yang, 2018). In addition, some historians and scientists

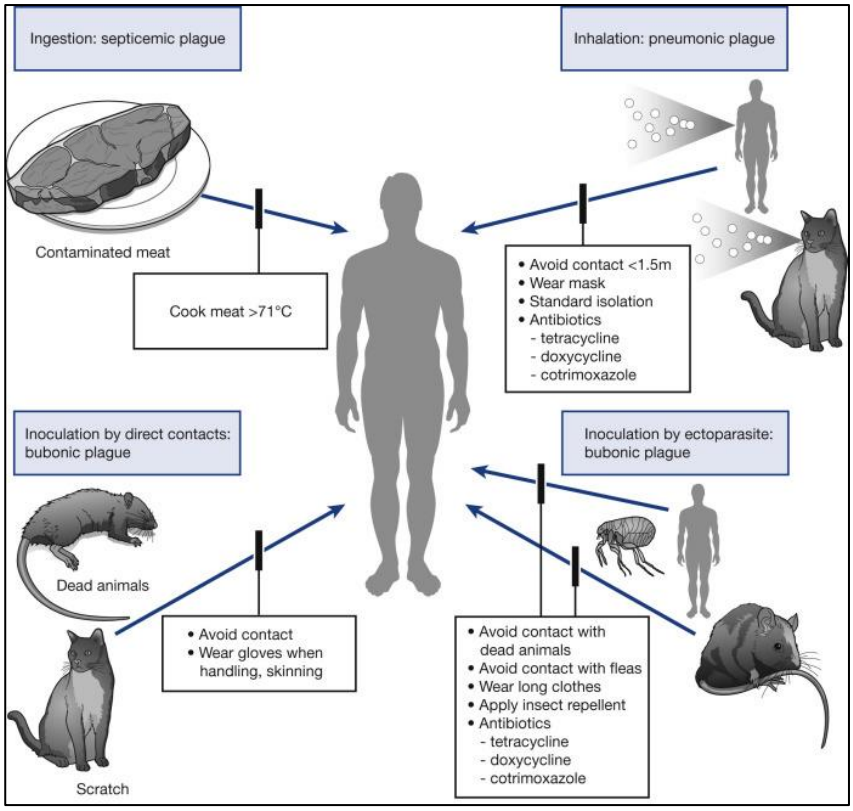

Figure 8. Sources and prevention of plague (Jones et al., 2019).

suggested a possible involvement of human ectoparasites, like body lice or species-specific human fleas, as vectors of *Y. pestis* (Blanc & Baltazard, 1942; Delanoë, 1932; Houhamdi et al., 2006; Lethem, 1923). The ability of human fleas and lice to carry and transmit *Y. pestis* from one mammal to another has been observed in laboratory experiments (Ayyadurai et al., 2010; Houhamdi et al., 2006; Zhao & Yin, 2016) and in the field studies have shown the presence of infected human ectoparasites in areas with plague cases (Blanc & Baltazard, 1942; Drali et al., 2015; Piarroux et al., 2013; Ratovonjato et al., 2014). Moreover, this hypothesis is supported by epidemiological (Dean et al., 2018, 2019), historical (Davis, 1986) and archaeological studies (Hufthammer & Walløe, 2013) that depict a crucial role of human ectoparasites in the second plague pandemic.

1.6 Clinical forms of plague in humans

The plague occurs mainly in three different forms, which at times can also be present together (https://www.epicentro.iss.it/peste/; https://www.cdc.gov/plague/symptoms/index.html) (Inglesby et al., 2000; Prentice & Rahalison, 2007; Salam et al., 2020; Yang, 2018):

- **Bubonic plague**: it is the most common form of plague and occurs following the bite of infected ectoparasites or by direct contact between infected material and skin lesions of a person. Typical manifestation of this form is the development of buboes, inflamed swellings of the lymph glands, followed by fever, headache, chills, and weakness. The incubation period for this form of plague is between 2-8 days. If the patient is not appropriately treated, the infection can spread to other parts of the body, leading to other forms of plague;
- **Pneumonic plague**: occurs when the bacterium infects the lungs. This form of the disease can be transmitted from person to person through the air or aerosols of infected people (primary pneumonic plague) and therefore constitutes one of the most dangerous forms due to the epidemic potential that characterizes it. The pulmonary form can also result from the degeneration of the other forms if they are not treated promptly (secondary pneumonic plague). The incubation for primary pneumonic plague is between 1 to 3 days;

- **Septicemic plague**: derives from the multiplication of *Y. pestis* in the blood, and can be a consequence of complications of the two previous forms. It can be contracted from bites of infected fleas or from handling an infected animal, and is not transmitted from person to person. It causes fever, chills, abdominal pain, shock and prostration, bleeding of the skin and other organs, but does not manifest with buboes. The incubation period for this form is poorly defined but likely occurs within days of exposure.

If plague signs are not recognized and treated in time, it can easily be fatal for the infected individual. Mortality rates of untreated cases varies among the different clinical forms of plague: bubonic plague has a mortality between 40 and 60 %, while both pneumonic and septicemic plague have a mortality of 100% (Perry & Fetherston, 1997). Nowadays, the majority of human cases can be treated successfully with effective antibiotics that are commonly used against Enterobacteriaceae, such as streptomycin, gentamicin, levofloxacin, ciprofloxacin, doxycycline, moxifloxacin, and chloramphenicol (Yang, 2018)(https://www.cdc.gov/plague/healthcare/clinicians.html).

1.7 Aim of the project

With the premises discussed up to now, this thesis project incorporates biological and historical studies to examine how remains of organic material and historical records can provide information about the dynamics of past epidemics. Due to the highly interdisciplinarity nature of the study, the project was generated by the collaboration of researchers specialized on different aspects of health-related fields:

- Prof. Claudio Bandi, Prof. Sara Epis, and Dr. Francesco Comandatore from the laboratory of Molecular and Evolutionary Parasitology (University of Milan), experts in parasitology, microbiology, and bioinformatics;
- Prof. Cristina Cattaneo from the LABANOF group (University of Milan), expert in anthropology;
- Prof. Massimo Galli (University of Milan), expert in medicine and historical epidemiology;
- Prof. Didier Raoult, Prof. Michel Drancourt, and Dr. Rémi Barbieri from the IHU Méditerranée infection (from the AIX University of Marseille), experts in infectious diseases and paleomicrobiology.

The first part of the project was focused on the application of paleomicrobiological analysis on teeth recovered in an archaeological site situated in Milan and dated back to the period of the Great Plague of Milan 1629-1631 (Caruso et al., 2013). The teeth were provided by the LABANOF group. All the paleomicrobiological analyses were performed at the IHU Méditerranée infection in Marseille, where I spent 15 months working under the supervision of Prof. Michel Drancourt and Prof. Didier Raoult. Prof. Drancourt and Prof. Raoult are considered pioneers in this field since they were the first to recover and identify traces of *Yersinia pestis* from a past epidemic (Drancourt et al., 1998). During this period, the samples were analysed for the presence of ancient DNA or protein molecules associated with a *Y. pestis* infection.

In the second part of the project, the attention was focused on historical and epidemiological analysis.

The first paper I presented, titled “Differential word expression analyses highlight plague dynamics during the second pandemic.”, is a study on the application of a mostly unbiased automatic tool to

analyse the words present in plague related texts respect to a group of control text (not related to plague). This tool was tested to determine its capability to extract useful information from a huge quantity of textual data related to past epidemics.

The second paper titled “The plague of 1630 in Milan: had the procession with the body of San Carlo a role in the spread of the epidemic?” is a study focused on the spatio-temporal distribution of plague-related deaths and the role of a religious mass gathering on the dynamics of the epidemic that hit Milan in the year 1630. The starting materials for this study are the registers of the deaths of the city Milan. The digitalization and the analysis of the data contained in the registers were done in collaboration with Prof. Massimo Galli and the palaeographer Dr. Luca Fois.

In the third part of this project, I contributed to the collection and simplification of data of historical origin. In particular, the studies performed on the ancient human microbiota, an extremely important field that is still in its infancy as regards the quantity of material analysed, were reviewed. The third paper, entitled “Paleomicrobiology of the human digestive tract: A review”, is a review in which the bases for the analyses on the human microbiota of the past have been summarized, retracing the main discoveries of the field with a focus on the analysed samples and the techniques used.

1.8 Paleomicrobiological analysis for the detection of traces of *Yersinia pestis* in the dental pulp of individuals recovered from a 1630 mass grave in Milan

As stated above, a portion of the project described in this thesis was focused on the analysis and the search of *Yersinia pestis* traces in ancient teeth retrieved in an archaeological site situated in the city of Milan and dated back to the period of the plague epidemic in northern Italy (1629-1631). Recently, ancient genomes of *Y. pestis,* from two sites located in the Alps and dated back to the period around the epidemic, were reconstructed (Guellil et al., 2020; Seguin-Orlando et al., 2021). Nevertheless, no published analysis has detected DNA traces of the pathogen responsible for the epidemic in the city of Milan.

A total of 59 teeth, provided by the LABANOF group, were collected from the mass grave in Viale Sabotino (Milan, Italy) (Caruso et al., 2013). Associated ceramics date the mass grave to the period around the 1630 plague. This site was discovered in 2006 during roadworks just outside the "Spanish "walls that delimitated the city of Milan in the XVII century.

All the paleomicrobiological analyses on the teeth were performed at the IHU Méditerranée infection in Marseille. Each sample were treated following the best practices used to prevent contamination, among which: the use of dedicated laboratory, reagents, and equipment; no use of positive controls; inclusion of several negative controls handled strictly in parallel with investigated samples (Poinar & Cooper, 2000; Drancourt et al., 2005).

Dental pulp from each tooth was extracted following the protocol of Drancourt et al. (1998) (Figure 9). Then, the extracted dental pulp was analysed for the presence of ancient DNA or protein molecules associated with a *Y. pestis* infection.

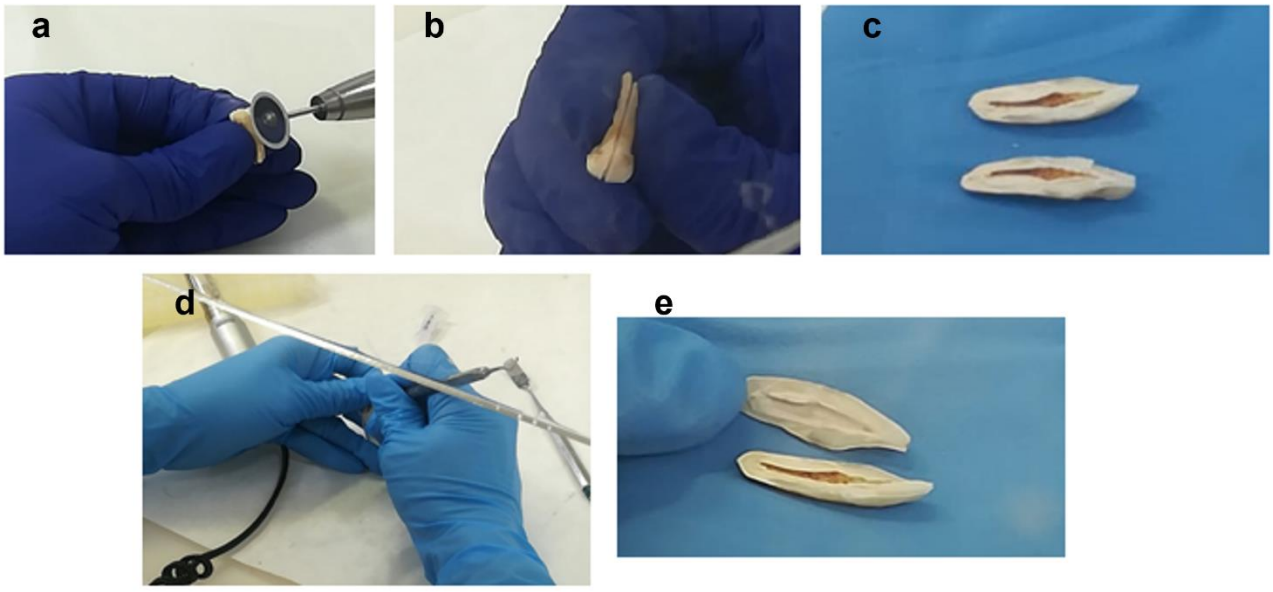

Figure 9. Dental pulp extraction. The tooth is cut on the longitudinal axis using a rotating diamond blade (a and b); dental pulp (c) is then scratched from the dental cavity using a dental excavator or a scalpel (d) till all the pulp is removed (e).

Protein extraction for paleoproteomic analysis was obtained through the use of a series of washes and centrifugations with extraction buffers containing guanidine hydrochloride, monosodium phosphate (NaH2PO4), disodium phosphate (Na2HPO4), and ethylenediaminetetraacetic acid (EDTA), as described in Shaw et al. (2019). Purified proteins were then digested with trypsin and then analysed through Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometric (LC/ESI-MS/MS) as described in Barbieri et al. (2017).

Unfortunately, the only good quality peptides detected through the paleoproteomic analysis were of human origin, mainly collagen alpha and albumin.

DNA extraction was performed following the protocol of Spyrou et al. (2019). Briefly, dental pulp was incubated in a lysis buffer consisting of EDTA and proteinase K for 18h at 37 °C on a rotating wheel, after which a binding buffer consisting of guanidine hydrochloride, isopropanol, Tween-20, and sodium acetate (Dabney et al., 2013) was added to each sample. DNA was then purified using the MinElute purification kit (Qiagen). DNA was finally eluted using ultrapure water. Then, a qPCR screening for the detection of *Y. pestis* target genes, *pla* and *caf1,* was performed using the primers and thermal profile described by Schuenemann et al. (2011).

No samples gave positive result for any of the two genes. To exclude the possibility that the negative results were due to errors in the procedure, the same protocol for DNA extraction and qPCR detection of the two aforementioned genes were also tested on other ancient samples, using the same reagents, and performed by the same operator. Since the detection of both two genes were possible in some of the other samples tested, we can reasonably exclude that the negativity was due to errors in the procedure. Another possible explanation for the negative results is that the samples were too much degraded for the detection of these specific two genes.

As the next step for the investigation, a shotgun metagenomic analysis was planned to detect every possible trace of *Y. pestis* or any other pathogen responsible for the deaths of these individuals. Unfortunately, the pandemic emergency has heavily influenced the progress of this part of the project for which the results will be produced in the next few months. The plan is to perform a metagenomic analysis through Illumina platform on NGS libraries prepared following Meyer & Kircher (2010), and Kircher et al., (2012).

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Sitography

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2. Selected Papers

2.1 Manuscript n°1

Differential word expression analyses highlight plague dynamics during the second pandemic.

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Title: Differential word expression analyses highlight plague dynamics during the second pandemic.

Short Title: Informatics analyses of plague-related texts

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Abstract

Research on the second plague pandemic that swept over Europe from the 14th to 19th centuries mainly relies on the exegesis of contemporary texts and is prone to interpretive bias. By leveraging certain bioinformatic tools routinely used in biology, we developed a quantitative lexicography of 32 texts describing two major plague outbreaks, using contemporary plague-unrelated texts as negative controls. Nested, network and category analyses of a 207-word pan-lexicome, comprising overrepresented terms in plague-related texts, indicated that “buboes” and “carbuncles” are words that were significantly associated with the plague and signalled an ectoparasite-borne plague. Moreover, plague-related words were associated with the terms “merchandise”, “movable”, “tatters”, “bed” and “clothes”. Analysing ancient texts using the method reported in this paper can certify plague-related historical records and indicate the particularities of each plague outbreak, which can inform on the potential sources for the causative *Yersinia pestis*.

Introduction

Plague, a deadly zoonosis caused by the bacterium *Yersinia pestis* [1,2], has been incontrovertibly identified via paleomicrobiological research on numerous historically described burial sites in Europe, ending decades-long controversies regarding the aetiology of the so-called “Black Death” (1346-1353) and the related episodes that formed the second plague pandemic, which lasted from forming the 1346 until the 19th century [3–9]. The ancient texts related to these plague episodes reported massive mortality rates [10–13], with an estimated 30 million deaths attributed solely to the “Black Death” [6,13]. Similar figures have not been observed during the plague outbreaks, from 1894 to the current third plague pandemic [14,15], leaving the forces that shaped the plague dynamics during the second plague pandemic somewhat speculative [15–18].

Readings of second plague pandemic descriptions have been made amidst the anachronistic background of cumulative observations from the third pandemic, which may not be applicable to the second pandemic [19,20]. Accordingly, an extensive analysis of the differences between the second and third plague pandemic historical descriptions demonstrated that the two pandemics exhibited such different epidemiological and clinical descriptions that they may even have to be considered two different diseases [21]. For example, the sources of plague during the second pandemic could be related to clothes and goods, with no reference to epizootic episodes among rats [21]. Additionally, the incidence of infection was higher for individuals living in the same household, suggesting that the plague was able to spread from humans to humans [22–24].

Therefore, the sources of and routes for the spread of *Y. pestis* in medieval European populations remain speculative, deriving as they do from the mathematical modelling of ancient plague episodes [15], which obviously cannot be verified.

In the next perspective of contributing to that matters, we here designed a novel quantitative method for the automatic analysis of ancient plague texts, derived from a method routinely used in biology to measure the relative expression of genes in a set of biological samples, including negative control samples [25]. This model uses ancient plague-related text sets and negative control texts to measure the differential expression of plague-related words, creating a core-lexicome (core signature) for the ancient plague and an accessory-lexicome that is specific to each ancient plague

outbreak, thereby opening an avenue for studying the pan-lexicome of the plague. The method described here was developed based on positive control texts describing the paleomicrobiologically confirmed Great Plague that affected the city of Marseille and Provence at large in 1720-1722 [4,8,26,27] as well as the plague episode that ravaged Northern Italy in 1629-1631 [3,27–29].

Results

Building the Marseille word database

A word repertoire was drawn from 16 historical French texts describing the microbiologically documented 1720-1722 plague of Marseille, France [4,8,26] and from 11 contemporary French texts that are unrelated to the plague (Data files S1 and S2), following a verification that the maximum Jaccard index between any two texts was 0.08 using the "Jaccard" package in Rstudio (Fig. S1A). This ensured that the 27 texts were original enough (i.e., without significant plagiarism) to be used as independent samples. This repertoire comprised a total of 2,049,566 words directly imported (without any modification) from Google Books into Rstudio; 473,681 words (23.1%) were poorly digitized due to damage resulting from preservation conditions and therefore could not be included in further analysis. Finally, 1,575,885 words (76.9%), representative of 10,315 unique words, were cleaned by removing noncharacter letters and spaces, replacing ancient characters with homologous characters in the current alphabet (i.e., the "long S" was replaced with "s"), filtering (with a French glossary of 336,631 words, conjugated verbs and proper nouns) and deleting words with fewer than five occurrences in all 27 texts (the complete script is available as Supplementary Text 1). Altogether, the final 1,575,885-word database comprised 904,005 words from the 16 plague-related texts and 671,880 words from the 11 negative control texts.

Analysing the Marseille word database

The word database we created was used to compare the relative expression of words in the 16 plague-related texts and the 11 negative control texts (Fig. 2A). This comparison was performed by leveraging informatics methods and employing cut-off values commonly used in the field of

transcriptomics (for the analysis of RNA sequences, i.e., the significant letter series using the universal biological code, in biological samples) (Fig. 1). Accordingly, using the DESeq2 package originally developed to identify dysregulated genes in transcriptome analyses, we generated a list of 70 words (log2fold change >0 and P adjusted<=0.05) (0.0007%), which were more overrepresented in plague-related texts than in control texts, as presented in WordCloud (Fig. 2; Table S1; Data file S3). two words were represented twice, i.e., “contagious” and “infected”. Our results confirmed the significance of two plague signs, namely, “bubo” (P adjust= 5e-07) and “carbuncle” (P adjust= 0.003). Notably, these two signs were continuously described, first by Procopius in antiquity [30], then by Guy de Chauliac in medieval times [31] and by Yersin and Simond at the beginning of the third and current plague pandemic [32,33]. The significant detection of these two words, constituting internal positive controls, validated our method and allowed us to consider other enriched words significantly associated with the plague. Notably, the words “rats” and “fleas” appeared only 11 and 5 times in plague-related texts versus 5 and 3 times in negative control texts, respectively; the differences were not significant. Further careful examination revealed that the word “rat” was misleading in 5 cases, resulting from the abbreviation of the word “magistrate”. Ultimately, there were 6 true occurrences in one text reporting dead rats in the streets and rats fleeing from houses where plague was declared, but the author describes plague in general, and in any case, he does not mention rats during the plague of Marseille [34]. Likewise, the word “flea” was mainly used to describe spots on the dura, observed during autopsy, that looked “like flea bites” and only once to describe spots on the belly of a plague patient as “like flea bites”. Only in one case did “flea” unambiguously refer to the ectoparasite but this was in an out-of-plague context.

Categorizing the Marseille word database

By an analogy with bioinformatics, where clusters of orthologous genes (COGs) are defined as functional categories [35], the 70 plague words were classified into 19 categories of words, with each category comprising words related to the same function in the plague-related texts (Data file S4). The category of “Other” was the most abundant, comprising 15/70 (21%) unclassified words, followed by the categories of “Consequence” (10/70 words, 14%), “Public Response” (8/70 words, 11%) and “Nature of Plague” (7/70 words, 10%). Some words, i.e., “police”, “physician”, “hospital”

and “infirmary”, were classified into two different categories: “Prevention” and “Public Response”. Indeed, it appeared that these professions or places already existed before the plague surged due to the catastrophic situation, as demonstrated by the construction of additional infirmaries and hospitals and the increase in the numbers of doctors and police personnel. To address the assignment of some words to several categories, we specifically consolidated the category “Sources” by analysing every sentence in which each word of this category was used to determine the number of its occurrence as a bona fide plague source. This analysis indicated that the word “movable” was used in 194/207 (94%) occurrences as a plague source; “tatters” was used in 196/225 (87%) occurrences as a plague source; “clothes” was used in 128/153 (83%) occurrences as a plague source; and “merchandise” was used in 561/810 (69%) occurrences as a plague source (Table 1).

Marseille word database: Nested and network analyses

A nested analysis was used to contextualize the 70 words overrepresented in the plague-related texts by extracting 25 of these overrepresented words downward and 25 upward and then testing the significance of each. A WordCloud for each overrepresented word was generated using a P adj < 0.001 (Data file S5; Fig. S3). As an illustration of this nested analysis representation, the word “merchandise” (Fig. 3) in the category of “Source” was significantly associated with “Levant”, “smugglers”, “June”, “fur”, “ship”, “clear”, “unseating”, “exposed”, “purged”, “tatters”, “transport”, “porter” and “Jarre” (the name of a quarantine island in the Marseille gulf) (P adj < 0.001) (Fig. 3). Likewise, in the “Symptoms” category, the word “carbuncles'' (the gangrenous lesions consecutive to the blisters that correspond to a microbe’s gateway through the skin) [33] had 625 occurrences and was significantly associated with “groin”, “parotid”, “arms'', “leg” and “thigh”; all words indicative of the topography of the lesions (P adj < 0.001). The word “bubo” (the lymph node proximal to the inoculation site (blister or carbuncle)) [33,36] had 948 occurrences and was significantly associated with “groin”, “armpit”, “arm”, “throat”, “parotid”, “ear”, “maxillary”, “jugular” and “thigh” (P adj<0.001) (Fig. 3). All words designating localizations were, unsurprisingly, similar to those associated with “carbuncles”, with 95% similarity. Concerning plague sources, the words “tatters”, “movable” (P adj<0.001), “cloth” and “air” (P adj<0.005) were found to be linked

with “infected” and multiple other plague-related words (Fig. 4). The word “tatters” was associated with infection (e.g., “kill”, “corrupt”, “communicated”, “filth”, “contaminates”, “miasms”, “infect”, “infection” P adj<0.001), disinfection-related words (e.g., “purge”, “burn”, “aromatic”, “confiscation”, “punished” P adj<0.001), and other interesting words related to the dissemination of the plague and its origin (e.g., “smugglers”, “Turkish”, “Tripoli” P adj<0.001). The word “movable” was linked to words from the same lexical field as “tatters” (e.g., “tatters”, “cloth”, “clothes” P adj<<0.001). It was also associated with infection (e.g., “dangerous”, “infection” P adj<0.001) and disinfection-related words (e.g., “burn”, “purge”, “destroyed”, “disinfection”, “perfume”, “purify”, “confiscation” P adj<0.001). All these associations suggest that contemporaries associated the terms tatters, clothes, and movable with plague sources that communicated the plague across the city. Likewise, the word “merchandise” was linked to multiple disinfection-related words (e.g., “purge”, “quarantine” P adj<0.001). The word “air” was dichotomously associated with contagion (e.g., “bad”, “corrupt” P adj<0.001, “infected” and “poisonous” P adj<0.005) and with purification (e.g., “purify”, “pure” P adj<0.001). In Fig. 4, it can be clearly seen that the word “infected” is linked with the words “tatter”, “cloth”, “movable”, “air” and “merchandise” (P adj<0.01). Some other networks for potential sources of plague and their relation with other words are reported in Figs. S5 and S6.

Documenting the 1629-31 plague in Northern Italy

We then processed 16 historical texts describing the 1629-1631 paleomicrobiologically confirmed plague outbreak in Northern Italy (Data file S6) [3,28]. Twenty Italian texts from the same historical period were used as negative control texts (Data file S7) (Jaccard index between two texts < 0.03) (Fig. S1B). The repertoire of words included 1,574,910, with a final word database of 1,063,483 total words, comprising 451,856 words from the plague-related texts and 611,627 words from the negative control texts. Using the DESeq2 package, we obtained a list of 147 overrepresented words (0.03%) (Adjusted P value <= 0.05 and log2fold change>0) (Fig. 5) (Table S2; Fig. S2B; Data file S8). Seven words were represented twice, i.e., “contagion”, “could”, “did”, “infected”, “our” and “stuff”, while “were” was represented three times. These 147 words were then classified into the same categories used for the Marseille text analysis (Data file S4), and the words in the “Source” category were consolidated as explained above (Table 1). In particular, the Italian words “roba” and “robba”,

which have been translated into the English word “stuff” and are used in a general way to indicate multiple terms such as movable, merchandise, food and especially clothes (Supplementary Text 2) appeared more than 400 times in plague-related texts, and in 90% of the cases, the term was used by the author to indicate a plague source. Interestingly, the Italian words for “rat” and “mouse” were present only four times in the plague-related texts; twice in a figurative sense, once as a general premonition of plague (“If the underground animals, which are worms, snakes, toads, and mice, flee their dens to come upon the earth, unable to live amidst the extreme putrefaction of their mother, then, being on the ground, they die; they are the cause of infection”) [37] and once in a statement that rats, together with dogs, cats, hens and doves, should be killed as a precaution to protect houses from the disease [37].

A nested analysis of Italian overrepresented words indicated that words such as “clothes”, “beds”, “air” and “rooms” were directly linked to infection, disinfection and danger-related words, indicating a clear association of them with potential plague sources (Data file S9; Fig. S4). In particular, the word “bed” was linked to infection (“infected”, “contagion”, “pestiferous” P adj <0.01), disinfection (“purge”, “perfume”, “air”, “boil” P adj<0.01), and “caution” (P adj <0.01). Similarly, the word “cloth” was found to be associated with plague-related words, such as “infected” (P adj<0.01), “caution” (P adj<0.001), and “poison” (P adj <0.01). The word “poison” was also associated with “clothes” (P adj<0.01). Moreover, the word “air” was linked to “infected” (P adj<0.001), “bed” (P adj<0.001), and “pestiferous” (P adj<0.01).

Plague pan-, core- and accessory- lexicomes

To define the plague pan-, core- and accessory- lexicomes (respectively, the complete collection of words associated with the plague, the plague words shared between the French and Italian lexicomes, and the plague words specific to either episode), all the French and Italian words were translated into English using the translation function in Microsoft Word. Misleading translations of four words (three from the French texts and 1 from the Italian texts) resulted from either a truncated word (e.g., "conta" for “contamination” or “contagion”) or from an isolated letter (e.g., "o"), whereby a DESeq2 analysis incorporated a total of 68 unique words for Marseille and more than

twice that number (139 unique words) for Northern Italy. This difference in the richness of the repertoires of the two datasets and the stronger association between words obtained from the nested analysis of the French texts (Datasets S5 and S9) could not be related to the profession of the authors (i.e., physician and non-physician) (Dataset S1 and S6) (Fig. S7) but may possibly result from political and linguistic fragmentation of 17th century Italy, as opposed to the 18th century centralized France. Additionally, the Italian texts describe an epidemic covering multiple cities in multiple small countries in Northern Italy, while the French texts cover a much more geographically limited region. The plague pan-lexicome was composed of 10,743 words and included a core-lexicome of 2,645 words: 4,063 words were specific to the Marseille's plague and 4,035 to the Italian plague. Regarding overrepresented unique words, there were 207 in the pan-lexicome (Fig. 6); the core-lexicome had 17 unique words. A total of 51 words specific to Marseille and 122 specific to Northern Italy constituted the two accessory-lexicomes. The words that were significantly enriched in the Marseille plague texts had a more significant probability of being enriched in the texts related to the 1629-1631 plague in Northern Italy than in the control texts (Fisher's exact test, performed with two conditions, P val<0.001***; P val<0.00001****). The French and Italian datasets were then graphically merged to create networks of common and unique words. The words of interest present in both datasets were compared and placed together to form a network that evidenced nested words that were unique or common to both datasets (P adj < 0.01). The network representing the significant symptoms of plague (bubo and carbuncle) showed an overlap between the Marseille and Northern Italy datasets corresponding to the most common locations for buboes and carbuncles ("groin", "armpit", "throat", "parotid", "leg", and "arm") (Fig. 7). Interestingly, three words, "air", "bed" and "cloth", from the category of "Sources", overlapped in the network for "infected" (Fig. 4). No other potential plague sources were observed to be common between the Marseille and Northern Italy datasets.

Discussion

The updated lexicographic analyses of ancient texts describing deadly outbreaks efficiently and effectively identified them as plague-describing texts and provided valuable information regarding the plague dynamics during the second pandemic.

We developed and used a method derived from automatic bioinformatics to obtain quantitative information from ancient texts. Our data, coupled with existing paleomicrobiological analyses [3,4,8,26,28], confirm that these ancient texts describe a plague caused by *Y. pestis*, as revealed by the significant presence of the words “buboes” and “carbuncles”, which were the two main symptoms of the plague during the second pandemic. Furthermore, our analysis confirmed some extant research results, such as the role of clothes and textiles in the spread of plague [21,38]. One contribution of our analysis is that it expands the scope of possibilities concerning the sources and routes of contamination during two plague outbreaks. Contemporaries did not identify animals as sources of plague. The historical texts did not significantly mention animals as a source of plague, which disrupts the traditional depiction of plague as a primary zoonosis during the second pandemic [16]. In particular, our analysis failed to obtain “rats” (or “mice”) and “fleas” from ancient texts, an observation already articulated by Cohn [39]. These terms did not reach significance and were not enriched in plague-related texts, revising our understanding of the role played by rats and fleas in the transmission of the plague during the second pandemic. Controversially, the absence of rats from medieval texts could be attributed to two factors: a disinterest of the authors in pests and their linguistic inability to identify rats or mice in Latin [40]. Such linguistic issues seem to have been resolved in the modern era with the use of French and Italian; both “rats” and “mice” were used as distinct words in more recent texts [41–43], and one entire treatise in French was dedicated to the role played by rats throughout human history [44]. Although a study has shown that rats were probably involved in a human plague epidemic in Gdansk (Poland) during the 15-16th centuries [45], the impact of rats on the spread of massive plague outbreaks was probably trivial. There is an absence of rats in ancient plague texts and a scarcity of archaeozoological evidence, as reflected in extant mathematical models [15,16,24,39,46]. Concerning alternate sources of zoonotic plague, the word “dog” was enriched in Marseille plague texts but never reached significance in the nested analysis. A careful reading of its 282 occurrences indicated that in 87 instances, dogs spread plague in Marseille by carrying infected tatters, clothes, dressings and bandages of plague victims, yet dogs were relatively resistant to plague, as demonstrated by contemporary experimental observations [47,48] and modern studies [49,50]. Other products derived from animal sources, such as meat or feathers, were also mentioned as possible plague sources, but a careful reading of texts disqualified the word “meat”, which was used as a remedy forming part of the broth to be given to plague

victims. Likewise, feathers were actually used to form pillows or mattresses, and as our results have shown, bedding elements were viewed with suspicion and considered potential sources of the disease.

In the texts here investigated, word usage was in the frame of the miasma and contagionist theories in fashion in the 17th-18th centuries Europe. Words such as "air", “levean”, “pestilential”, “venom”, “pestilent”, “pestilence”, “poison” belonged to the miasma theory repertoire after its conceptualization in Hippocratic texts; inferring that plague was caused by corrupted vapors filling the air, called “miasmas” [51]; whereas words such as “contagious”, “contagion”, “tatters”, “movable”, “stuff” belonged to the contagionist theory repertoire developed in the 16th century after scientist such as Girolamo Fracastoro; inferring that diseases were transmitted by direct contacts, clothing or air [52]. Data gathered in the present analysis confirms that any text here investigated could mix elements from both theories, (i.e., that plague arises from miasma and was spread by contagion [53]). Contemporaries traced plague outbreaks in Marseille and Northern Italy to imported “stuff”, a general word encompassing the significantly enriched words “movable”, “tatters”, “clothes”, and “merchandise”. During the 17-18th centuries in Europe, the plague and any other infectious disease was thought to be transmitted by textiles, according to contagionist theory [54–56]. Specific references were made to fur imported via the maritime route into Marseille, with a possibly unanticipated role played by smuggling and clothes brought by the French and German imperial soldiers into Northern Italy during the war of Mantua [28,57–59]. All such clothing was in direct contact with plague-stricken people, and contemporaries insisted on the dangers that these clothes represented for spreading plague. In addition to discussing the Marseille and Northern Italy outbreaks, numerous historical texts reported interhuman transmissions of plague through the transportation of movables, merchandise or clothes belonging to plague-stricken persons [57,60,61]. Accordingly, the people of those times identified infective potentiality in textiles and other merchandise that came from infected places or with which plague victims had been in contact, as reported by witnesses of the 1575-76 plague in Northern Italy [21]. Indeed, the trafficking of the clothes of deceased plague victims was a major problem in epidemic periods, given that in preindustrial Europe, wages were so low that buying clothes was a luxury that ordinary people could only afford a few times in their lives [62]. There are multiple direct accounts of episodes in which

buying, selling and stealing clothes was reported as having triggered plague in homes, small villages or even cities [57,62,63]. In Milan and Marseille, the purging and sequestering of all the clothes and movable goods of plague victims were two primary prophylactic measures.

Regarding the symptoms, in texts from both Marseille and Northern Italy, the words “carbuncle” and “bubo” were significantly enriched. Interestingly, the word “carbuncle”, referring to the skin ulcer that follows the introduction of *Y. pestis* through the skin [33], seems to be very representative of the second plague pandemic, whereas it is rarely used during the third pandemic and no longer reported in modern cases of plague. Notably, “buboes” and “carbuncles” were localized all over the body, including the face and the neck (specifically the ear, jugular, parotid gland, and maxillary regions); localizations that do not support a role for fleas because rodent fleas are known to mainly bite the lower part of the body (legs and thighs) [46]. Rather, the use of these terms is compatible with a role for ectoparasites human ectoparasites, including body lice and human fleas, as vectors of *Y. pestis*, which could explain the rapid spread of the epidemics [64–67], which is supported by epidemiological [15,68], historical [69] and archaeological evidence [16].

The text mining method that structures our analysis was based on the model of the bioinformatics method of quantitative analysis of differential gene expression to limit bias and give a differential expression of words with the unprecedented introduction of negative controls texts. Nevertheless, this method may not be exempt from biases, rendering data interpretation biases that it is necessary to be conscious of and that constitute major sources for future improvements. The quality of ancient text digitization can be extremely variable, with word loss ranging from 4.5% to 46.3% after word filtration (Supplementary Tables S1 and S2), potentially eliding valuable details. The choice of controls was also decisive because it makes it possible to orient the results on several levels. In this work, we stopped at the first level; that is, we used control texts (including medical texts) to neutralize the common words that were not specific to plague at that time. A second level of analysis could have been done, for example, by using control texts referring to other epidemic diseases to determine whether words such as 'bed', ‘clothes', “tatters”, “merchandise” or 'air' were plague specific or were associated with any infectious diseases according to miasmatic theory. However, we did not find any digitalized documents contemporary to the two plague outbreaks analysed here. Finally, translating the words from their mother language into English detracts from their linguistic

richness, often simplifying a relevant concept; for example, our analysis yielded 9.983 different words in French versus 6.707 in English and 9.909 in Italian versus 6.834 in English.

In summary, the method proposed here offers a new way to automatically analyse historical epidemics and historical events in general. In this era of digitalization, where historical data are becoming increasingly accessible each day, we propose a quantitative method that is able to rapidly analyse textual data by extracting not only a specific lexicome associated with a group of texts but also the relations and cooccurrence of words in these texts. This method is able to highlight interesting and potentially crucial pieces of information that can be used in parallel to close analyses of texts to help a reader interpret the dynamics of historical events.

Materials and Methods

Ancient texts

The texts were retrieved from Google Books, Archive.org and Buisante.parisdescartes using the following keyword combinations: plague & Marseille and plague & Italy, with specific period ranges, i.e., plague & Marseille & 1720-1820 or plague & Italy & 1629-1680. A total of 32 historical texts describing the 1720-1722 Great Plague of Marseille (16 complete books) and the 1629-1631 plague in Northern Italy, also known as the Manzoni plague, after the famous novel written by Alessandro Manzoni in 1827 [70] (13 complete books and 3 book chapters), were retrieved (Data files S1 and S6). Additionally, we retrieved 11 contemporary negative control texts for Marseille and 20 contemporary negative control texts for Northern Italy, all written during the 17th-18th centuries and describing surgery, plants, universal history, industry and architecture; these texts were scanned within the framework of the “Google Books” program, undertaken by Google and the Archives program (Data files S2 and S7). The Google Books format was used after we comparatively tested this format with the OCR software Wondershare PDFelement 6 Pro™ scanned format on the Plague_FR_02 from the Marseille Corpus text. After filtration, Google Books version yielded 51,984 words versus 51,418 words in the Wondershare PDFelement 6 pro™. Careful examination of both versions indicated that most of "words" scanned by Wondershare PDFelement 6 Pro™ were isolated

letters present in our dictionary as determinant possessive or verbs. As for an example, Google books yielded one word "Madame" translated by Wondershare PDFelement 6 Pro™ into six “words” "M", "A", "D", "A", "M", "E". This example suggested that using Wondershare PDFelement 6 Pro™ would imply an extensive control of texts before Deseq2 analysis, at the exact opposite of the method we aimed to develop. This observation was in agreement with Google Books was acknowledged to have one of the most efficient text digitization systems [71,72].

French texts’ disposition

To filter the raw text words (see the historical text mining analysis section), we used a list comprising 336,631 French words, conjugated verbs and proper nouns, compiled from the Français-Gutenberg Dictionary. The symbol “ſ” (“long S”, which was used in most languages in Europe until the industrial revolution) was automatically substituted with an “s” only if a modern version of the relevant term with an s existed on the list of French words. To standardize the words, plural forms ending with a “s” or “x” were converted to the singular form only if the singular word was present on the list of French words.

Italian texts’ disposition

Italian texts (plague-related texts and controls) presented ambiguities that could lead to errors in an analysis. Indeed, most Italian books present, on the upper part of each page, the name of the book or chapter. Another problem of Italian books is the repetition of the last word of each page at the beginning of the next page. To avoid problems due to the overrepresentation of some words, these two types of repetition were manually corrected by removing the name of the book/chapter from each page, together with the last word of each page. As reported above, the symbol “ſ” (“long S”) was automatically substituted with an “s”. However, we observed that OCR programs tend to recognize the “ſ” symbol as an “f” and not as an “s”. To avoid the loss of the Italian word “peste” (“plague”) due to an OCR mistranscription, the word “pefte” (no meaning) was changed to “peste” in all the texts. Accordingly, the only words identified were those present on the list of Italian words (including singular and plural forms, conjugated verbs, proper names, and surnames, for a total of

931,657 words). Of these words, 427 (32%) were discarded. Moreover, one-letter words were removed, and singular and plural forms of the words were unified, as much as possible, into one word by following Italian grammatical rules for plural formation (Supplementary Text 3). Plague-related and control text word counts were compared using the DESeq2 R package with default parameters (version 1.22.2).

Historical text mining analyses

After the raw texts were imported into R (see Supplementary data) in .txt format, all the words were separated and converted to lowercase; accents, empty cells and nonletter characters such as "? ", "! ", ",", "[.]", "-", "_", "%", "$", "€", "#", "\\", "+", "*", ":", ";", ">", "<", "§", "&", "•", "«", "»", "[{}]", "'", "\", and "‘" were removed. The number of occurrences was computed for each word in the 63 texts. Regarding the plague-related texts, 3,624,476 words were identified, with 2,049,566 words from the French texts and 1,574,910 words from the Italian texts. Given that we were not able to systematically repair ancient mistranscribed words, only words matching those in an exhaustive list of French words (including singular and plural forms and all conjugated verbs, for a total of 336,631 words) and an exhaustive list of French cities, towns and places or Italian words were considered. Of approximately 3.5 million words, 2,721,742 were conserved (75%), resulting in 25,659 and 44,475 unique words for the French and Italian texts, respectively. Then, we applied a cut-off value to remove all unique words occurring fewer than 5 times in the French and Italian texts before the DESeq2 analysis, resulting in 10,315 and 10,645 unique words for the French and Italian texts, respectively. Subsequently, the plague-related and control text word counts were compared using the DESeq2 R package with default parameters (version 1.22.2). This approach identified 70 French and 147 Italian terms that were overrepresented in plague-related texts compared to the control texts (P adjust < 0.05 and log2fold change >0) and 213 French and 313 Italian words that appeared at least 4 times more in plague-related texts than in control texts (Log2fold change >2) (Data files S3 and S8).

Nested analysis

In a second step, we used a “nested” analysis to identify the words that lay in close proximity to overrepresented words in the plague-related texts. We extracted words located +/-25 words apart from significant terms and compared them to the remaining part of the texts using DESeq2. Significant words (adjusted P value ≤ 0.001 and log2Fold change > 0) were plotted using the “WordCloud” R package (version 2.6) (Data files S5 and S9).

Word translation

All French and Italian words were translated into English using the translation function in Microsoft Word. Each overrepresented (only the words with P < 0.05 or log2fold change > 0) and nested (words associated with overrepresented words) word was controlled by the operators for mistranslation and then corrected. Words with no English translation were maintained as they were; this was the case for one French word, “corbeau” (a person responsible for burying plague victims), and 4 Italian words, namely, “monatti” (people responsible for burying plague victims), “espurgatori” (people responsible for stuff-purging), “untori” (people accused of intentionally spreading the disease using “special” ointments), “caldiera” (a tool used to untie the cocoons of silkworms), with no direct English translation. Words exhibiting an “out of context” translation were also manually corrected, such as “carbone”, “tacchi”, and “unti” in Italian and “preservatif” in French texts.

Network Representation using Gephi

A graphical representation of the nested analysis results was performed using Gephi 0.9.2 [73]. This software was used exclusively for representation purposes of the data and statistics obtained by the nested analysis. The results of this analysis were converted into nodes and edges to generate the network. At each node, a word was obtained from the nested analysis, and the edges between two words represented the association between them. Only words with strong statistical significance (P adj < 0.01) were represented. The spatial position of the words in the network has no statistical meaning. To maximize the efficiency of the comparison between the French and Italian datasets for the construction of the comparison networks, the words nested with each word of interest and the

words the word of interest was nested with were used. Therefore, an edge was created each time a word of interest was associated with another word with a significance determined by a P adj < 0.01.

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Author contributions: R.B. and R.N. performed data processing and analysis, interpreted the data, drafted the manuscript, and prepared figures. M.S. and S.E. interpreted the data and drafted the manuscript. D.R. and M.D. designed the study, interpreted the data, and drafted the manuscript. All authors reviewed the manuscript.

Competing interests: The authors declare no competing interests.

Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials.

Figures and Tables

Overrepresented or nested words identified as plague sources during the Great Plague of Marseille, 1720-1722			
Word	**Occurrence**	**Number of times the term is used as a plague source**	**Percentage**
Merchandise	810	561	69%
Tatters	225	196	87%
Movable	207	194	94%
Clothes	153	128	83%
Air	233	86	37%
Meat	133	8	6%
Dog	282	87	31%
Overrepresented or nested words identified as plague sources during the plague epidemic of 1629-1631 in Northern Italy			
Word	**Occurrence**	**Number of times the term is used as a plague source**	**Percentage**
Stuff	466	419	90%
Feather	29	26	90%
Clothes	89	55	62%
Bed Sheet	25	15	60%
Air	180	98	54%
Bed	146	58	40%

Table 1. Overrepresented or nested words identified as plague sources in the analysed records. Table representing the number of occurrences of each suspected plague source in plague-related texts from Marseille and Northern Italy, featuring the percentage that each word is used by ancient authors as a source of plague. Words for the "bona fide" analysis were selected from and depended on the results of the nested analysis for their association with plague-related words.

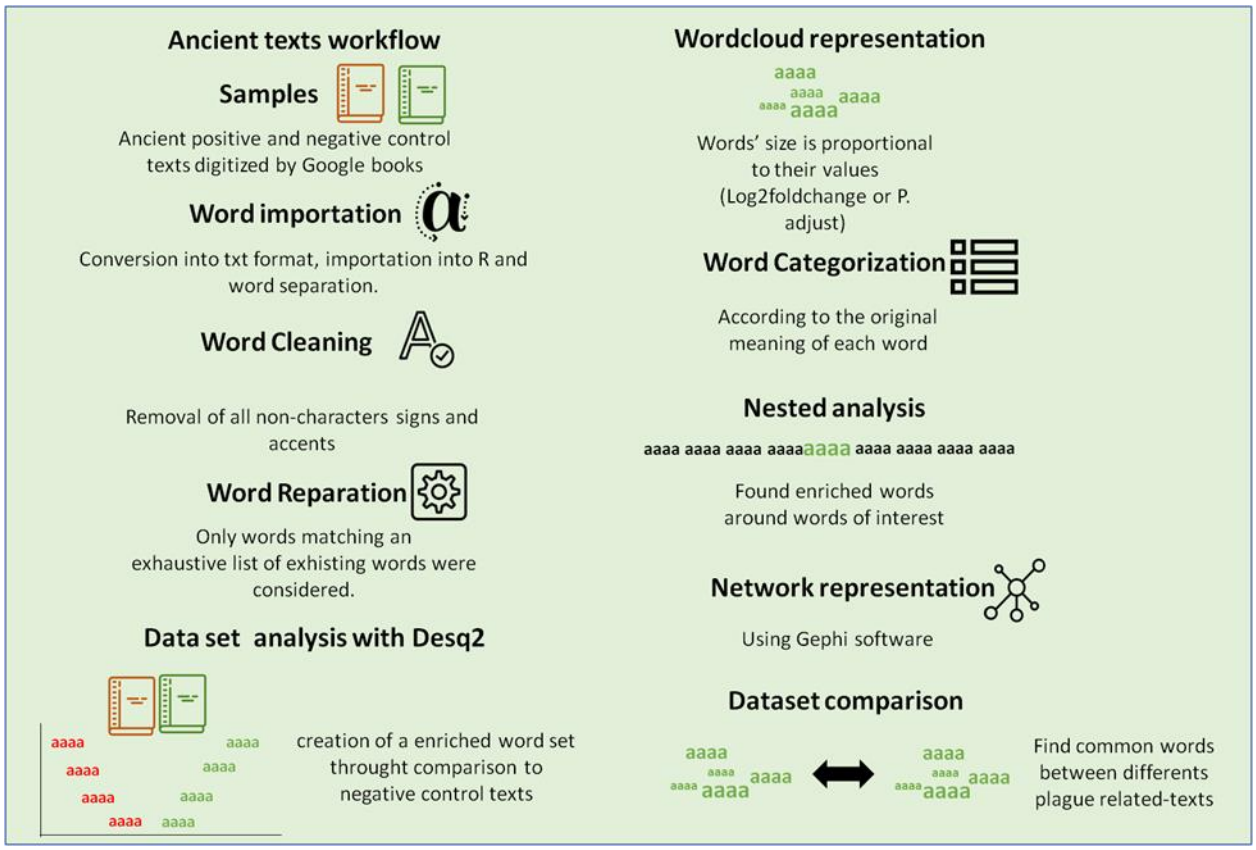

Fig. 1. Workflow summarizing the main steps of the method developed in this study.

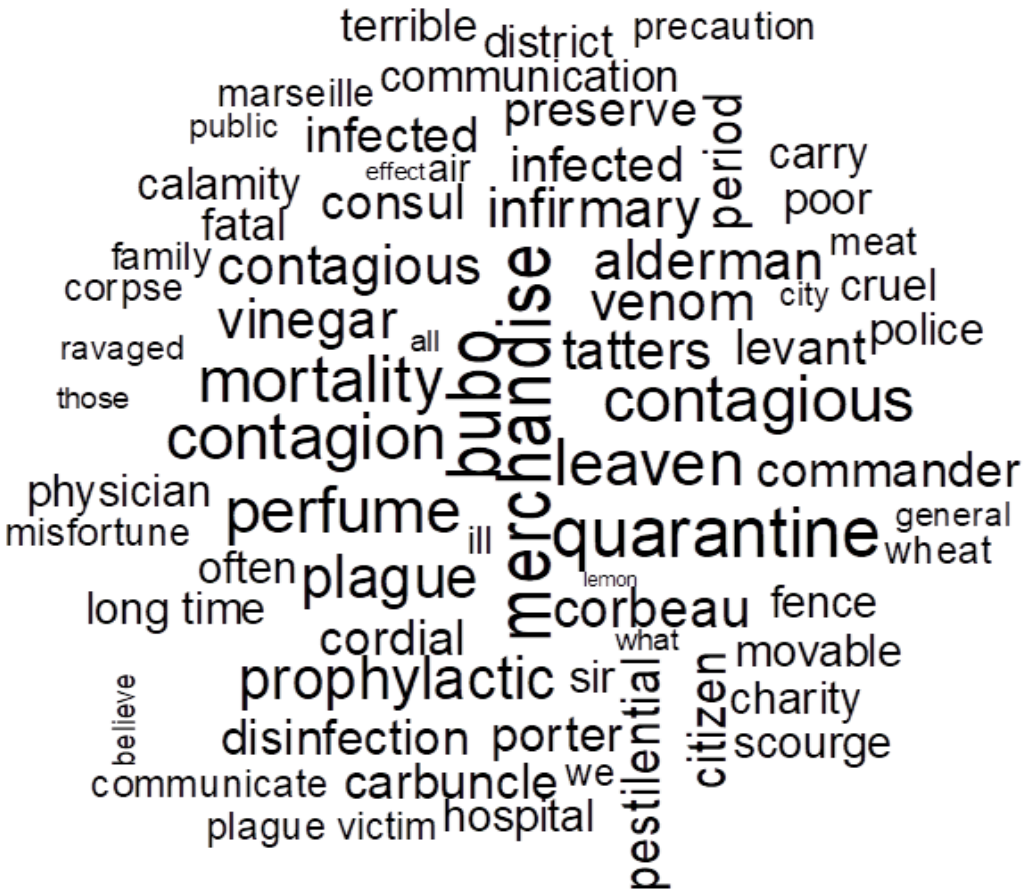

Fig. 2. WordCloud displaying the words that were overrepresented in the Marseille plague-related texts (P adj <=0.05 and log2fold change >0). The size of the words represents their adjusted P enrichment in plague-related texts compared to negative control texts.

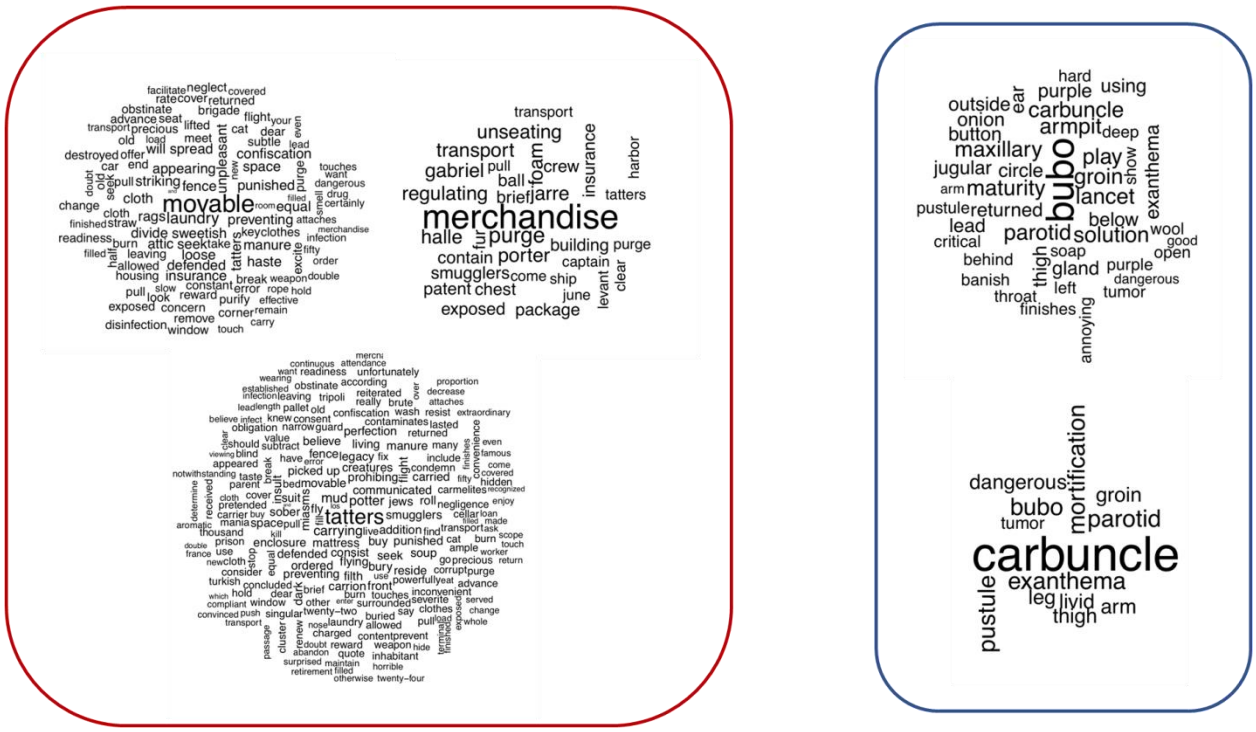

Fig. 3. WordCloud representations of the nested analysis, displaying the overrepresented words (P adj < 0.001) attached to the plague sources (movable, tatters and merchandise) (red panel, left) and plague symptoms (buboes and carbuncles) (blue panel, right) during the 1720-1722 plague of Marseille.

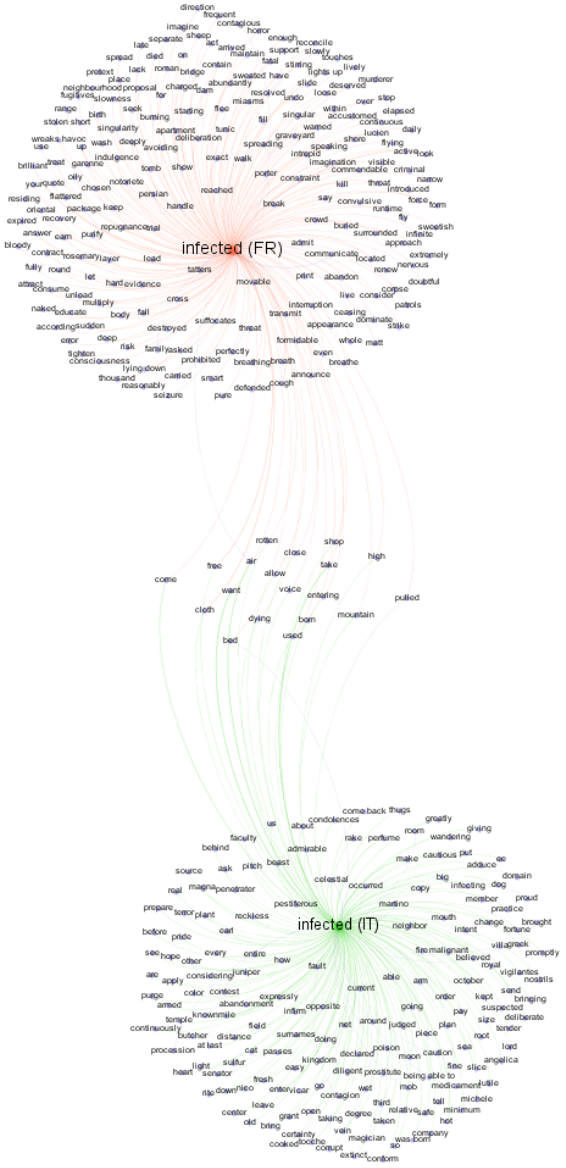

Fig. 4. Network representation of the relations between words, generated using Gephi software and based on the adjusted P values from the nested analysis (P adj<0.01) of the word “infected” (in green for Marseille and in orange for Northern Italy). Word label dimensions are proportional to the number of links with other words.

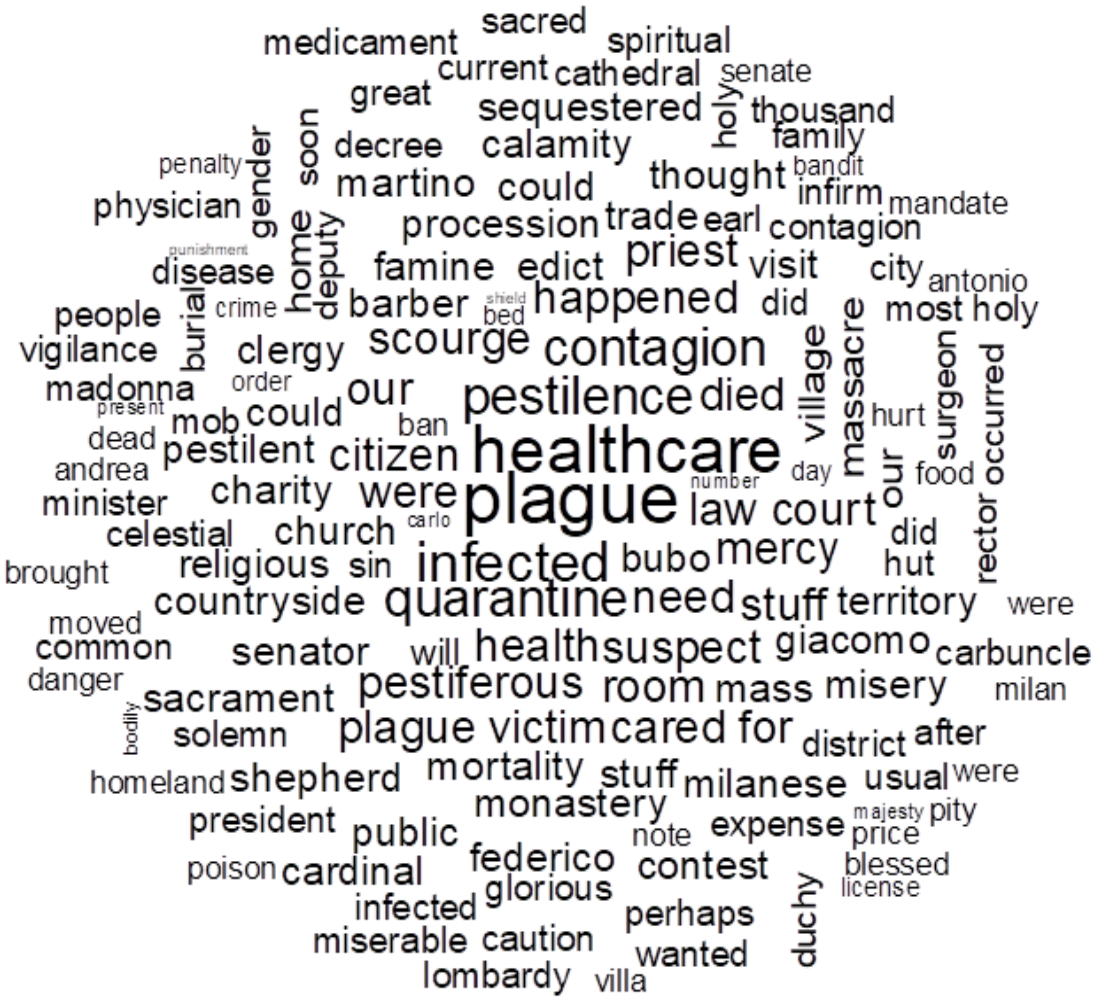

Fig. 5. WordCloud displaying the words overrepresented in plague-related texts from Northern Italy (adjusted P value <=0.05 and log2fold change >0). The size of the words represents their adjusted P enrichment in plague-related texts compared to controls.

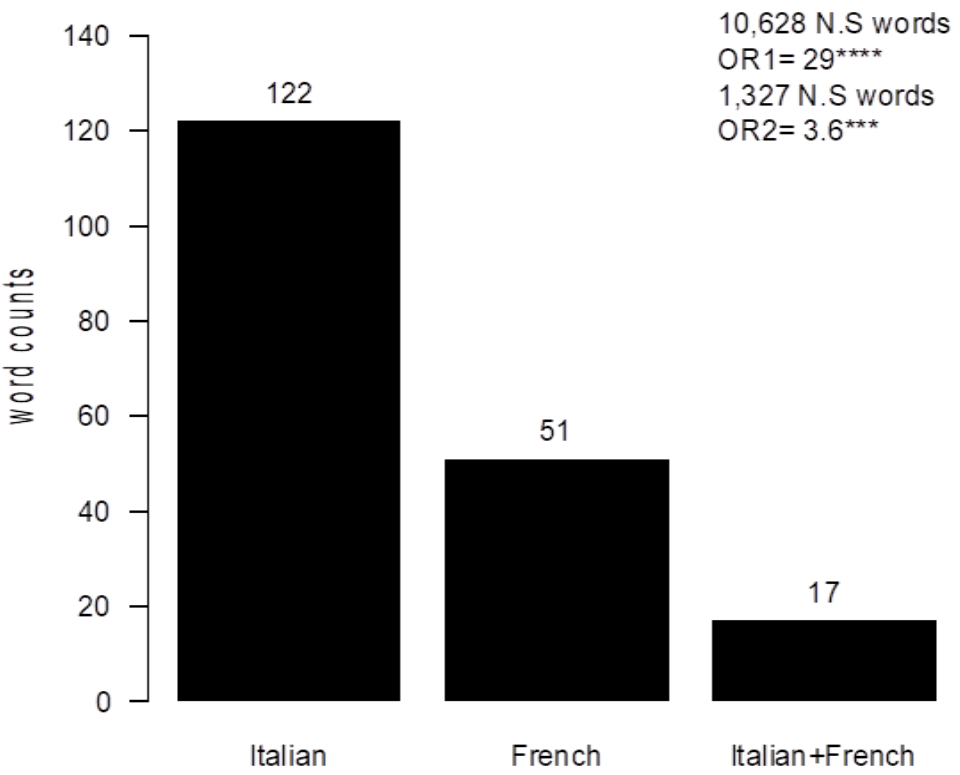

Fig. 6. Barplot displaying the numbers of unique and significantly overrepresented words from Italian (IT) or French (FR) sources or shared between both. The overlap of significantly overrepresented words between Italian and French texts was tested using a Fisher test on all nonsignificant words (OR1) or nonsignificant words with a baseMean > than the lower quartile of significant words (OR2). P val <0.001***; P val <0.00001****.

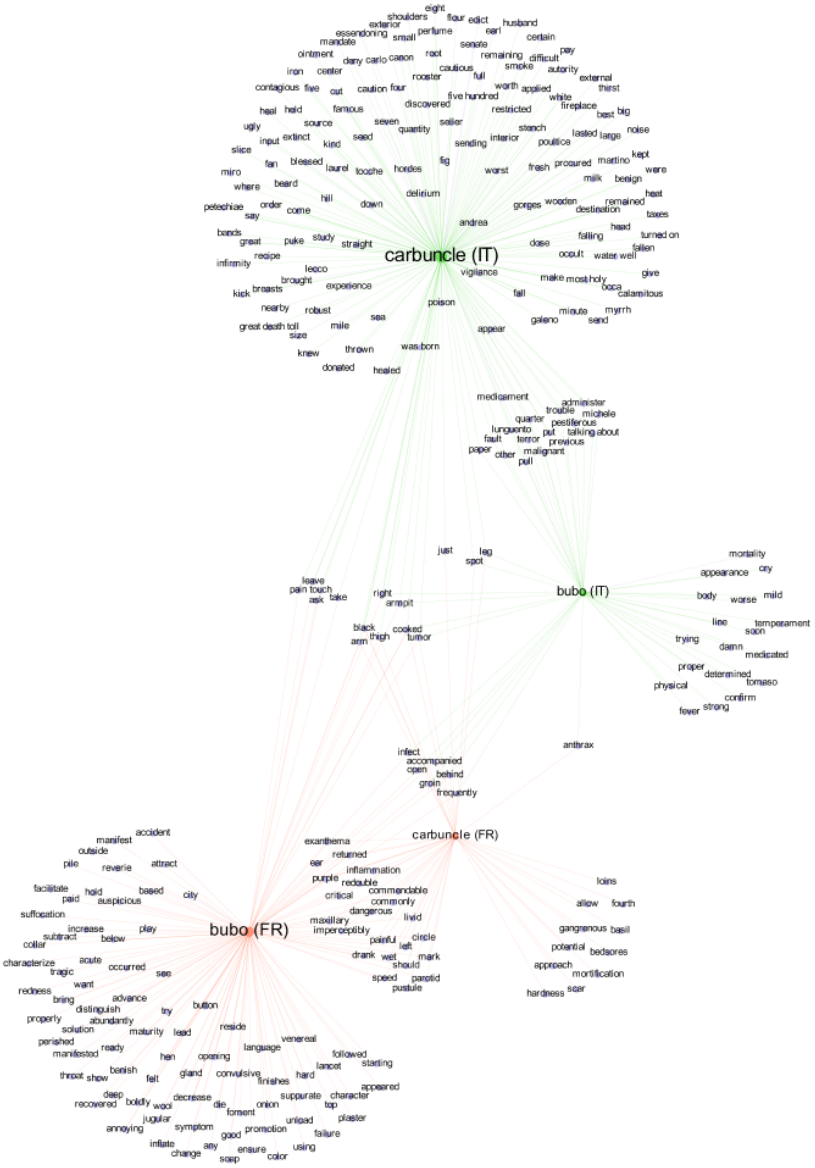

Fig. 7. Word network representation of the words related to two plague symptoms, “carbuncle” and “bubo”, from the Marseille plague (displayed in green) and the Northern Italian plague (displayed in orange). Network generated with Gephi software, based on the adjusted P values from the nested analysis (P adj<0.01). Word label dimensions are proportional to the number of links with other words.

2.2 Manuscript n°2

The plague of 1630 in Milan: had the procession with the body of San Carlo a role in the spread of the epidemic?

Manuscript in preparation

Title: The plague of 1630 in Milan: had the procession with the body of San Carlo a role in the spread of the epidemic?

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Abstract

Background

Milan suffered in 1630 the last and perhaps most devastating plague epidemic. The chronicles attributed to the procession of June 11 of that year a decisive role in the spread of the disease. Analysing the information contained in the registers of *Mortuorum liber*, preserved in the *Archivio di Stato* of Milan, it was possible to reconstruct the epidemic evolution before and after the procession in different part of the city.

Methods

The contents of the registers have been digitized and the parishes existing in 1630 were geolocalized. A clustering analysis has been performed on the dynamics of the epidemic observed in each parish.

Findings

Two clusters of parishes were identified showing different epidemic pattern. Parishes of the centre of the city were only marginally hit by plague before the procession and suffered a huge increase of mortality thereafter. On the contrary, in the densely populated parishes of the peripheral part of the city the disease peaked in the days around the procession, declining thereafter.

Interpretation

The results of this study support the historical testimony depicting a role of the mass gathering procession of San Carlo in the spreading of the epidemic, especially in the central areas of the city. However, it should be defined how a large concentration of people could increase the spread of an infection mainly transmitted by ectoparasites.

Research into context

Evidence before this study

To our knowledge, the only information on the plague epidemic occurred in Milan in 1629-1631 was that provided by the chroniclers of the time, in which the epidemiological aspects are based on authors impressions and gross estimates. In particular no epidemiological reconstruction has been performed on the basis of detailed epidemiological records. One of the few sources of raw data on which it is possible to base epidemiological studies are the death registers of the city of Milan. The analysis of data reported in the 1630 registers offered us the opportunity to study in detail the spatio-temporal dynamics of this plague epidemic, allowing for the first time an interpretation based on real data.

Added value of this study

This study presents in detail the spatio-temporal pattern of one of the most renown plague epidemic of XVII century, and allow to discuss the role of a mass event, in this case a religious procession, in increasing the spread of the epidemic.

Implications of all the available evidence

The plague deaths occurred in the city before, at the time and thereafter the procession evidence two different spatial patterns, suggesting a spreader effect of this mass gathering event. Questions remain open on how a concentration of people could increase the transmissibility of plague in conditions in which a role of pulmonary plague could not be supported.

Introduction

The plague that hit Northern Italy in 1630 left a profound mark on the country's demography, economy and culture, and represented a relevant factor of the Italian economic decline in the XVII century [1]. The epidemic, that struck both cities and rural communities, was brought in Italy in 1629 by a war, while a famine was ravaging the country [2,3]. Mortality would have been 30-35%, with about two million victims [2]. In 26 main cities of northern Italy, it was estimated a median mortality of 40% [1]. Milan, at that time the capital of a vast duchy under Spanish rule, was hit hardly [4–6]. After the first cases officially recorded in October 1629, the epidemic seemed to disappear during the winter and then re-emerged in March 1630 [4,5]. At the end of March in the Lazzaretto were reported to be no more than three hundred inmates [7], but at the beginning of June the private houses closed by the Sanità (the health authority) were between 300 (Archive of the State of Milan 8 June 1630) and 500[6] and the guests of the Lazzaretto were already thousands (Archive of the State 2 June 1630). The contemporary chroniclers affirmed that the solemn procession on June 11, with the body of St. Carlo Borromeo carried through the streets of the city centre, was the cause of a marked increase in the plague cases [4,5]. Till now, no epidemiological reconstruction has been performed on the basis of real epidemiological records. The Milan *Mortuorum Liber* (MML), collecting the deaths occurred in the city from 1450 [8] offers the opportunity to study the spatio-temporal dynamics of 1630 epidemic. In the present paper are reported the results of an investigation on the impact of the St. Carlo Borromeo procession on the epidemic, the first based on real data.

Materials and methods

Death registers (*Mortuorum Libri*) digitalization

The information used for all the analysis were obtained from the Milan *Mortuorum Liber* (MML), i.e. the Book of Dead, which consists of a corpus of more than three hundred registers stored at the National Archive in Milan, where the deaths occurred in the City from 1452 to 1801 were recorded (Atti di Governo, 118-119.). Each death report included the demographic information (name, surname, age, gender, titles or particular status of the deceased person) the parish and the Sestiere

(sixth part of the city) of the city where the person lived, the main cause of death and other contributing illnesses, and often the duration of the last disease, as certified by the city's appointed medical officers, as well as the name of the certifying person. For the purposes of the present study the three registers available for 1630 were consulted and digitalized. One register of 1630 could not be found in the public archive and the relative records between 4th August 1630 and 31th August 1630 were missing. An expert palaeographer (LF) translated from Latin and transcribed the text of each death certificate and all the data were transferred into a database. The medical terminology of the time was interpreted according to the definitions reported in the medical treatises of Sennert [9] and Borsieri [10].

Parishes geolocalization

The location of each parish was determined at the position of the respective church. A digitized historical map of Milan (dated at 1629[11]), reporting the positions of most of the churches included in the registers, was georeferenced to the actual map of Milan (OpenStreetMap 2008) using the QGIS software. The GPS positions of the parishes and the distance of each parish from the centre of the city (defined as the centroid of the area delimited by the medieval walls of the city) were obtained considering the georeferenced map and the information reported in historical texts [12].

Parishes clustering and statistical analyses

The spatio-temporal dynamics of the pandemic was firstly investigated computing the median distance of the recorded deaths (referred to their relative parishes) over time, week by week. The median distance versus time regression was computed using the Spearman test in R.

Furthermore, the parishes with more than 30 registered deaths were selected for clustering analysis. For each of these parishes, the cumulative relative frequency curve of plague deaths was computed. Parishes were then grouped on the basis of the curves, using the fuzzy k-means clustering algorithm implemented in the FKM function of the R library "fclust" (Ferraro 2019), which automatically identifies the most suitable number of clusters considering from 2 to 6 clusters. We

performed a further clustering analysis using the hierarchical clustering algorithm implemented in the “hclust” R function, with Manhattan distance and ward.D2 method.

The dates at which the cumulative relative frequency curve of plague deaths reached 25%, 50%, 75%, 100% and the inflection point of the curves were computed for each parish. For each of these parameters, the dates were compared among the parishes of the identified clusters, using the Mann-Whitney test.

Results

MISFORE data

The digitized death registers have been analysed to obtain relevant demographic information from the records. The deaths reported as caused by plague was 5,261 (64·5%) out of 8,152 total cases found. Excluding three isolated cases between January and March 1630, the deaths from plague occurred starting from the 24th February to the 30th December (71·2% of the deaths reported in that interval time). The six Sestieri (the relative gates) had different amounts of deaths related to plague, ranging from the 452 of Porta Nuova to the 1193 of Porta Romana. Similarly, the number of plague deaths varied largely among parishes and the one with the most recorded deaths was S. Stefano in Brolo with 592 cases.

Parishes geolocalization

As described before, the recorded death cases were geolocalized on the basis of the parishes position. A total of 91 parishes, accounting for the 93% of all the plague deaths reported in the registers for 1630, has been geolocalized and their data used in the present analysis. It was not possible to geolocalized the remaining 37 parishes mentioned in the registers, which accounted for a total of 52 plague cases (0·99% of the total plague deaths). Lastly, for 316 deaths (6%) the parish was not reported in the registers.

Epidemiological curve

The daily plague-related or not plague-related deaths have been graphically reported in Figure1_figure histogram. No deaths are reported in Figure 1 for the period of time between 4th and 30th August, graphically highlighting the incompleteness of MISFORE records likely due to the absence of at least one register.

To better visualize the spatio-temporal dynamics of the epidemic, the deaths were plotted on a georeferenced 1629 historical map of Milan. The frequency of plague deaths per parish over time and the relative absolute number of plague and not-plague deaths are shown in VideoS1_video_peste.

Figure 2 shows the weekly trend for the median distances of plague-related deaths from the center of the city.

Parishes clustering and statistical analyses

To study the spatio-temporal spreading of the epidemic in the city we clustered the parishes on the basis of their epidemiological curves. We focused on the 37 parishes for which more than 30 plague-related deaths had been recorded in the MISFOREs. The fuzzy k-means unsupervised clustering algorithm and the hclust hierarchical clustering algorithm coherently clustered the parishes in two groups named “cluster 1” and “cluster 2” (Figure3a). The heatmap showing the epidemiological curves and the result of the hclust hierarchical clustering analysis is reported in Figure3a.

Then we studied these two clusters, comparing the date at which their parishes reached the 25%, 50%, 75%, and 100% of plague deaths, as well as the date at which their epidemiological curves reached the inflection point. The parishes of cluster 1 reached 25%, 50% and 75% of the deaths earlier than the parishes of cluster 2 (Mann-Whitney test, p-value <0·0001 for all three percentages, Figure3b). Coherently, the cumulative relative frequency curves of plague death of cluster 1 parishes reached the inflection point earlier than those of cluster 2 (Wilcoxon test, p-value <0·0001, Figure3b).

In Figure4, the spatio-temporal distribution of the plague and not-plague deaths of the two parish clusters is shown: the 91 georeferenced parishes of the two clusters have been plotted on the

historical map (Figure4a) and the incidence histogram of plague and not-plague deaths of the two clusters are reported (Figure4b).

In order to assess the differences between the two clusters the distance from the centre of the city for each parish grouped by cluster has been evaluated (Figure S1). The median distance of parishes of cluster 1 from the centre of the city was 809 meters and for parishes of cluster 2 it was 492 meters. The difference between the two median distance is slightly significant (p-value = 0·09). Moreover, parishes of cluster 1 showed a 2·5-fold increase of plague-related deaths after the procession of 11th June, while parishes of cluster 2 showed a 10-fold increase (Wilcoxon test, p-value <0·0001).

Discussion

In 1630 northern Italy was the scene of the War of Succession of Mantua, an episode of the Thirty Years' War. Plague was brought into Milan duchy by Aleman soldiers who passed the Alps directed to besiege Mantua [5]. The first cases of plague reported on the territory of the duchy date back to July 1629 in Chiavenna, at the end of the Tellina valley, along the path the imperial troops used to descend from the Alpine passes, while the first officially recognized case in the city dates back to 22 October. Although, it is not unlikely that there have been some cases also in the previous weeks (handwritten note of 1632 by Senatore Settala [13]). Despite this, no death was attributed to plague in the register of 1629, completely digitalized and analysed by us (Galli et al, manuscript in preparation). The epidemic remained apparently silent until January 30, when a case, occurred in a thirteen years old girl, was reported in the registers. After that, no other death from plague was registered until February 26th. Between 6 and 19 March only about fifteen admissions to the Lazzaretto were reported and seven plague deaths were registered from March 15 to March 29. Even considering a possible underestimation of the cases of plague due to a reticence of health officers in diagnosing plague for political reasons, in fear that other Italian states could decide to stop the contacts and ban Milan from trades, it seems evident that the spread of the infection remained limited at least until spring. Contemporary chroniclers attributed to the great procession of June 11 the trigger role for the further spread of the epidemic. San Carlo Borromeo (1538-1584)

was the archbishop of Milan during the great plague of 1576. His canonization in 1610 was promoted by the role attributed to him in the recovery of Milan from plague. In 1630 he was considered a patron saint against the plague. With the aim to stop the epidemic, the archbishop in charge in Milan at that time, who was Federico Borromeo, cousin of the saint, took the decision to promote a procession with the saint's body, still preserved in the Milan cathedral. A decision made by the archbishop not without reluctance, and only after repeated insistence also by some of the health officials [4]. The procession started at 7 a.m. from Duomo square, [4,14] crossed all the main central streets of the city and came back to Duomo at 7 p.m. [4] and was attended by anyone in Milan who could stand. The body of San Carlo was exhibited thereafter for 8 days and 8 nights in the Cathedral where "the people flocked to it, begging, with tears and prayers, for that help which, due to the inscrutable divine decrees, was now inexorably denied " [4]. According to the contemporary chroniclers, the death cases rose after the procession from about 100 to about 1700 per day [4,6]. Our study could be based on complete data individually recorded in more than 5,000 persons who died of plague in Milan in 1630. It is therefore the first investigation on this issue that can be based not on estimates, but on real data. Contemporary authors reported that the epidemic peaked in the summer months. Accordingly, 4,204 (79·9%) out of the total 5,261 plague deaths reported in the available registers occurred between the beginning of June and the end of July. Our analysis evidenced that the number of registered deaths was sensibly different in each *Sestiere*, with an apparently higher mortality in the south-eastern ones. A more in-depth assessment of mortality in relation to the estimates of population density of the various districts of Milan in 1630 is beyond the scope of the present study. However, it is conceivable that some areas of the city were actually more affected than others, as suggested by the mortality observed in the individual parishes, that makes it possible to more precisely locate each deceased person on the city map. In fact, about 60% of the deaths were recorded in 10 parishes out of the 126 mentioned in the registers. The observed differences could be at least in part justified by the high variability in the territorial extension and population size of each. Moreover, our data show a significant relationship between time and distance. In fact, the epidemic moved from the peripheral areas of the city towards the centre, with a very high proximity to the city centre after the procession, indicating the inward movement of the epidemic due to this mass gathering of people.

However, our data show a clear difference in the progress of the spread of the infection among the parishes that, according to the date of restart of the plague deaths observed after the winter break, could be divided in two clusters. Interestingly, the parishes included in each cluster were not randomly distributed in the city, being most of those with an earlier restart of the epidemic closer to the city's walls, while those showing a delayed restart were mostly located in the central part of the city (p-value = 0·09). Among the 14 parishes of cluster 1, only 4 are localized near the centre of the city and all 4 with few plague-related deaths: of the total of 1813 deaths from plague in parishes of cluster 1 (average deaths 129·5), these 4 contain a total of 165 deaths (9·1%). Removing these four outlier parishes from the analysis, the difference in the median distance between the two clusters became highly significant (p-value = 0·00026). Moreover, while in the parishes with an earlier restarting of the epidemic plague deaths dropped down from mid-June, in those of the cluster with later restarting plague mortality drastically increased from the end of that month. Thus, it could be inferred that something happened in the middle of June that influenced the dynamic of the epidemic, extending it into the central area of the city. Is it possible to argue, as claimed by contemporaries, that the procession was actually the trigger for the increase in the epidemic? The "split" of the dynamic of plague deaths between the two parish clusters occurred about 1-2 weeks after the procession. Furthermore, the deaths' median distance from the city centre drastically dropped down two weeks after the procession, suggesting that this event had a role in the epidemic movement from peripheral areas to the centre of the city. This is also shown by the different increase in number of plague-related deaths before and after the procession date between the two observed clusters: before the procession, in the parishes of cluster 1 were recorded double the cases with respect to the parishes from cluster 2. After the procession, this ratio completely overturned. In fact, in cluster 2, 90% of the cases were recorded after the procession (232 cases before and 2019 cases after), while for cluster 1 only 68 % were recorded after the procession (481 cases before and 1040 cases after).

That of 1630 was clearly a bubonic plague epidemic, as can be easily deduced from the chronicles of the time, and confirmed with data from 21 death certificates, that clearly report the presence of a pestiferous bubo, often specifying its anatomical site. To justify this limited number of reports, it

is probable that the various figures entrusted with the task of registering the deaths, including, at the moment of maximum spread of the epidemic, numerous barbers, limited as much as possible the contact and inspection of the corpses and greatly simplified the registrations. In bubonic plague, the illness onset generally follows 2 to 6 days after the bite of the infected flea [15], and during the second pandemic in lethal cases of bubonic plague the time from symptoms onset to death rarely exceed 6-8 days [16]. In the three registers of 1630, the duration of illness before death was reported in 47 cases of plague. The median time from onset to death was 4 days (interquartile range 3-6). In three of these cases, in which death occurs within five days, the presence of buboes is specified. It is therefore conceivable that the death took place on average between seven and twelve days after the infection occurred. A period of time not incompatible with the occurrence of the increase in cases observed in the parishes of the city centre after the procession with the body of the Saint. However, during the winter, the numerous religious processions and various events with large popular participation that were promoted in December to celebrate the birth of the Infant of Spain did not cause the resumption of contagion. Similarly, the Carnival celebrations at the end of January did not cause an immediate spread of the disease. Therefore, it seems evident that a concentration of individuals was not in itself sufficient to restart the epidemic and that climatic factors played a decisive role. The prevalent mode of transmission of bubonic plague is through the rat - flea - human chain [17] even if various evidences also support an important role of the human louse in the transmission of Yersinia pestis [1,18–22]. A resurgence of the epidemic as a consequence of a procession with great participation of people would suggest a transmission by air, which is described in case of pneumonic plague. This rare clinical form affected in the most recent epidemics - those starting from the end of the 19th century - no more than 2-3% of the cases [23], with some exceptions, such as the recent epidemic of predominantly pneumonic plague observed in Madagascar [24]. However, the droplet transmission occurs only in the final stage of the disease - when the patients are so severely ill that their mobility and consequently the possibility to participation into events such as a long procession are strongly reduced - and only through very close contact for a prolonged period of time [25,26]. Moreover, it was estimated from eight documented pneumonic outbreaks that a pneumonic plague patient can infect an average of 1·3 other persons [27]. After excluding a predominant role of airborne transmission, to support a crucial role of the procession in the further spread of the epidemic, it should be supposed a relevant

exchange of infected ectoparasites among the participants. Even if it would be not impossible in a massed crowd, this type of transmission more likely occurs in a domestic context. Nevertheless, it would be possible that the dynamic of the plague epidemic in Milan was the consequence of a more conventional transmission of the disease throughout the classic zoonotic chain, with the passage of the Yersinia pestis infection from the rats of the poorest and most populous neighbourhoods, to the rats of most central areas of the city, in climatic condition facilitating flea activity. Consequently, the role of the procession in causing the spread of the epidemic may have been only marginal.

Our study has some limitations. In particular, it includes only a minority of the plague deaths occurred in Milan during 1630. Data of thousands of deaths occurred in the great Lazzaretto of Saint Gregorio [4,5] and those occurred in hospitals and convents were not preserved or were unavailable for our investigation. However, it was possible to analyse almost all the deaths registered at home or on the city streets, at least until the health authorities were able to do it, with the exception of those of a single register. Giuseppe Ferrario, who in the 19th century probably had access to all the registers, including the one that we suppose that have been lost or displaced, reported for 1630 a total of 13,350 deaths [28], that is 5,181 more deaths for any cause than those available for our analysis. Since the missing data concern the period of maximum intensity of the epidemic (mainly August and September 1630), it is possible that the percentage of deaths due to plague was comparable to that observed in July 1630 in the available registers (32%). Despite this, we believe that the large sample of cases included in the present study is sufficient to describe the dynamics of the epidemic in the city from its resumption, at the end of March, to the end of July.

In conclusion, the last great plague epidemic that devastated Milan resumed in spring and reached its peak in the summer months. The resurgence of the epidemic started from the most peripheral and densely populated neighbourhoods in the south-eastern part of the city. There are no data that can exclude a new introduction of the plague from the outside, but it is also possible that the infection could have passed the winter, transmitted by human hosts, not necessarily killed by the disease through their lice, not hindered in their activity, like the flea, by low temperatures [29]. Our results show how, the path and the timing of the 11th June Procession together with the different epidemic dynamics showed by the clustered parishes support a role of said procession in the further

spread of the disease and the rapid increase in mortality from plague in the central parishes of the city.

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Figures and Tables

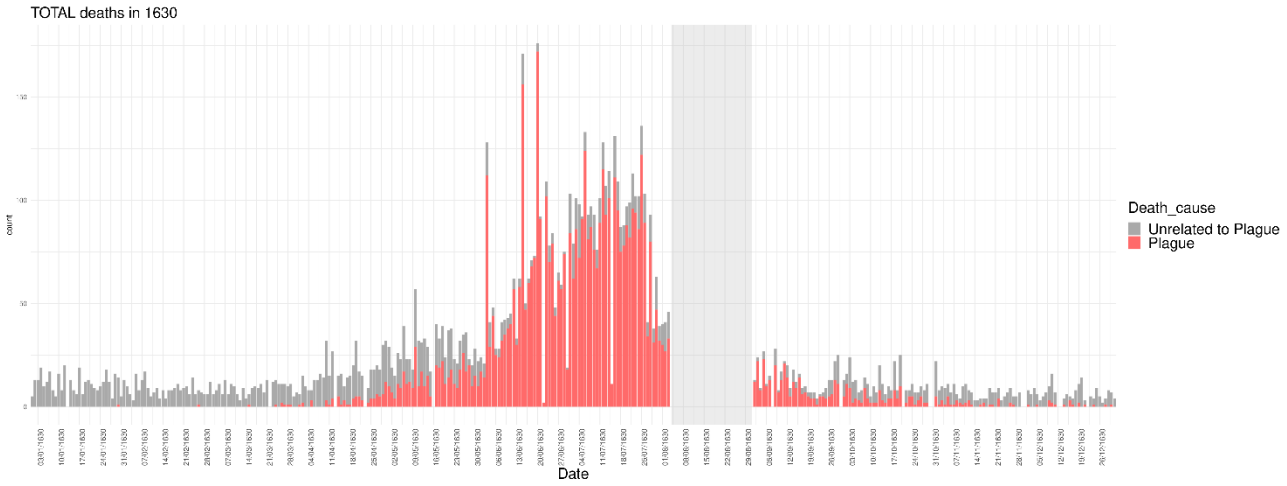

Figure 1. Barplot of the total deaths per day during 1630 in the city of Milan.

Plague deaths are reported in red and all the others in grey. The shaded area (August 1630) indicates the period not covered by the death registers (register missing).

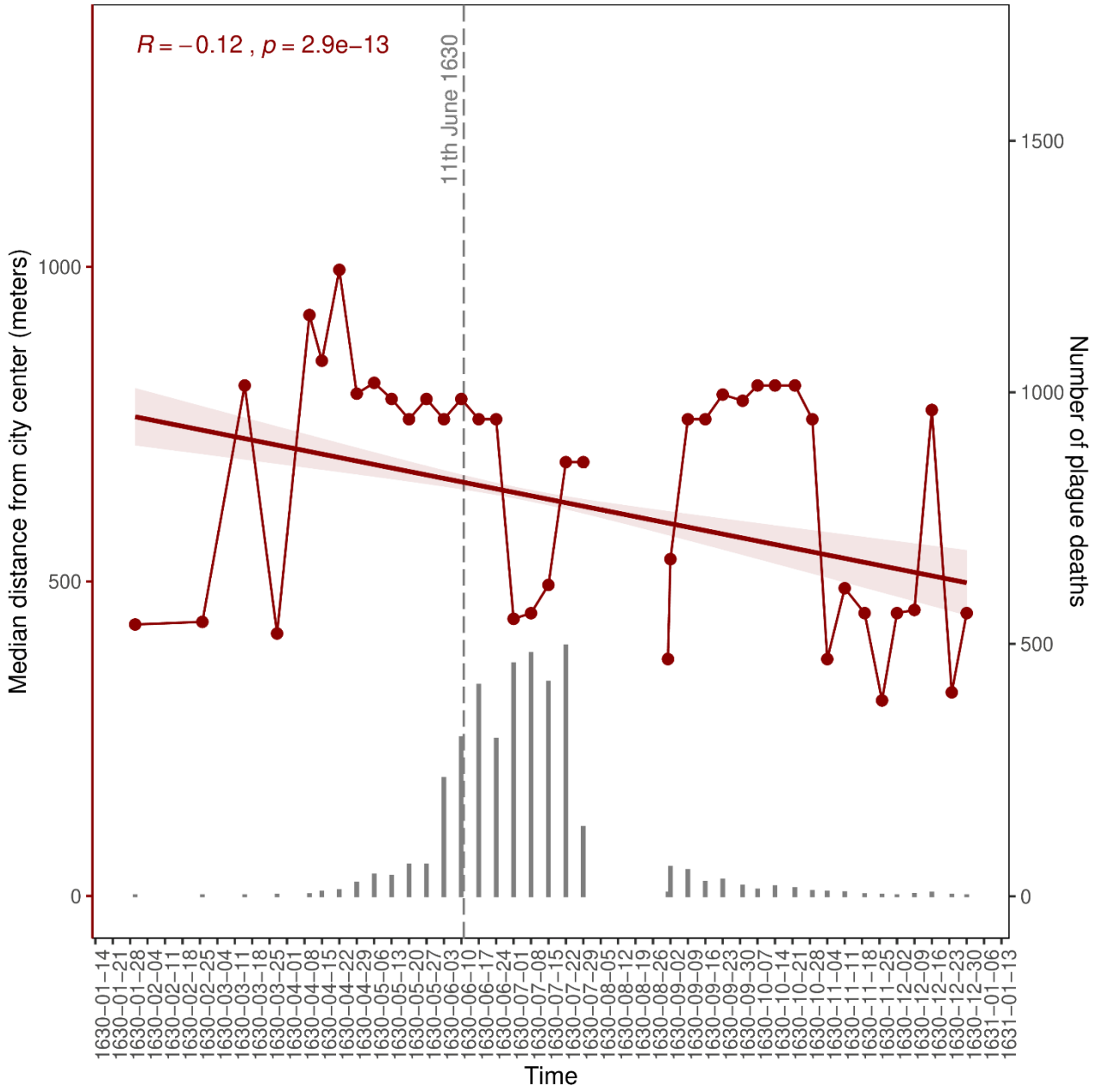

Figure 2. Spatio-temporal dynamics of the plague epidemic.

In red, Scatter plot of the weekly median distance of plague deaths from the city centre of plague deaths (left y-axis). The relative regression line and the p-value of Spearman test are also reported. At the bottom, the amount of plague deaths is also reported as grey weekly histograms (right y-axes).

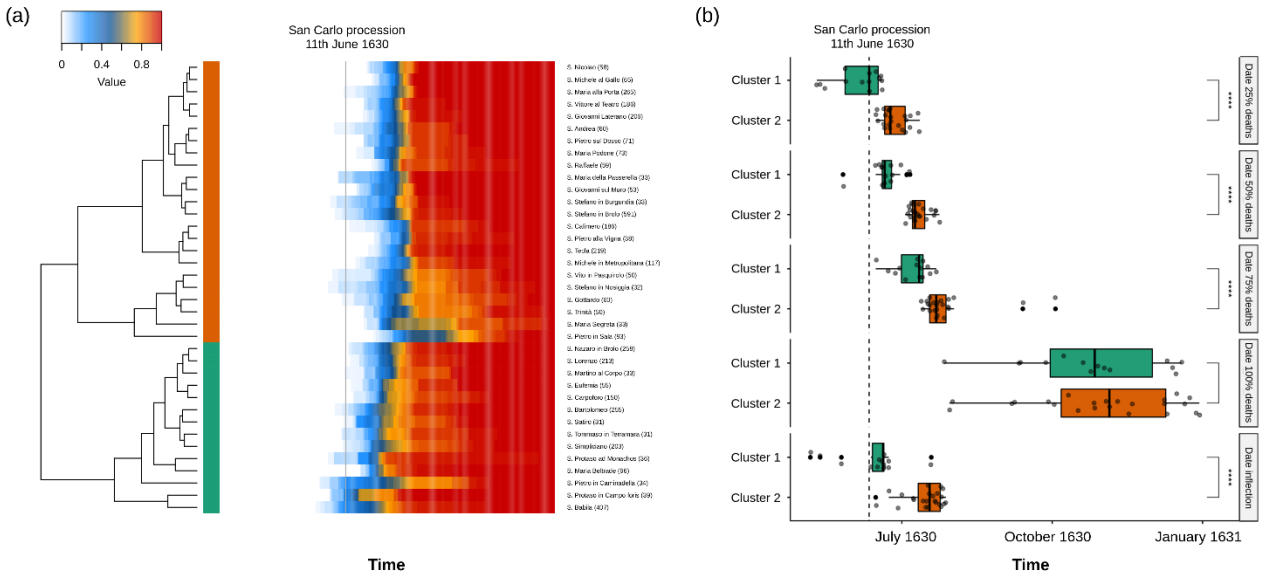

Figure 3. Clustering of the parishes dependent on epidemic progress.

Analysis performed on the cumulative relative frequency curves of plague deaths relative to 37 parishes for which more than 30 plague-related deaths had been recorded during 1630 in the death registers of Milan. a) Heatmap of the cumulative percentage of plague deaths in parishes. On the left, the dendrogram obtained by hierarchical clustering. Orange and green tiles indicate the cluster attributed by fuzzy clustering to each parish. On the right, the cumulative percentage of deaths from plague over time. The gray vertical line shows when the procession of San Carlo took place (11th June 1630). b) Boxplots of the dates when 25%, 50%, 75%, 100% of plague deaths were reached in the parishes of the two clusters. The two boxplots in the bottom show the dates of the inflection point of the cumulative curve of plague deaths for the parishes in the two clusters. The dotted line shows the date of the procession of San Carlo, 11th June 1630.

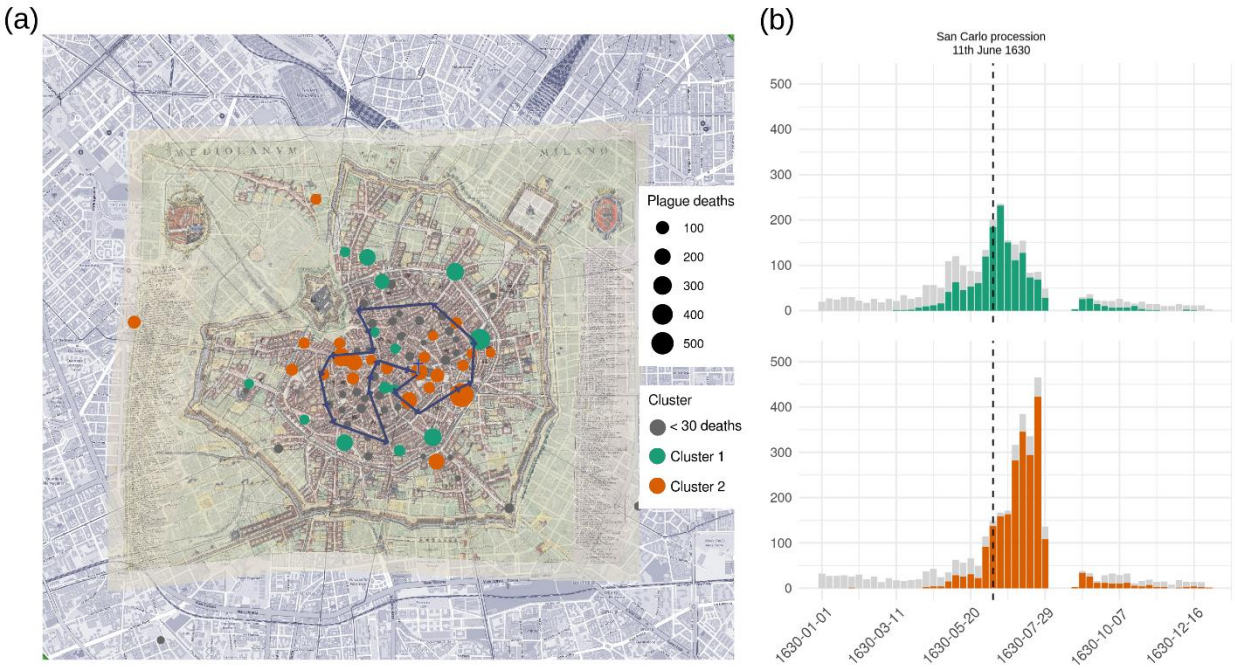

Figure 4. Spatio-temporal characteristics of the two identified clusters.

a) Spatial localization of the parishes of the two clusters. b) Daily deaths for cluster 1 (top-right) and cluster 2 (bottom-right. Plague related deaths are in green (for cluster 1) and orange (for cluster 2), while deaths not related to plague are in light-grey. The dotted line shows the date of the procession of San Carlo, 11th June 1630.

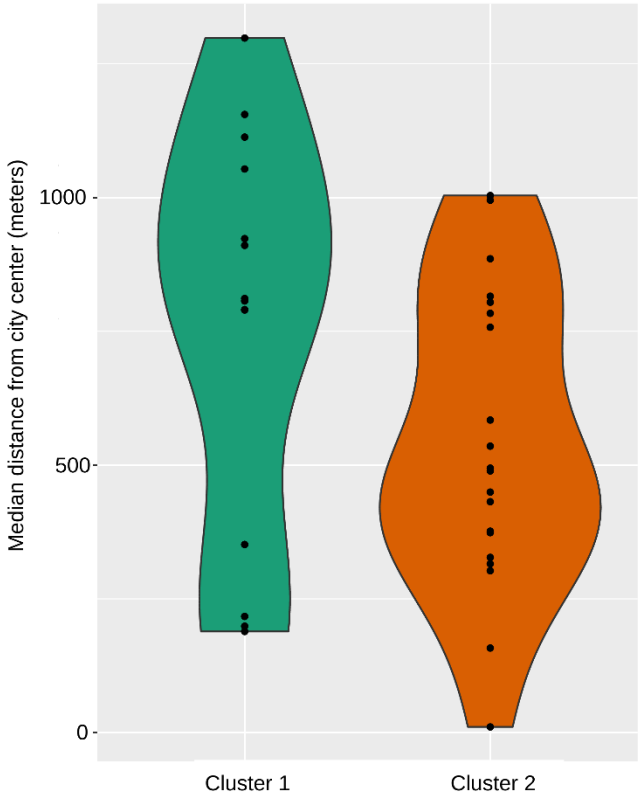

Figure S1. Violin plots of the median distance of each parish of the two clusters from the centre of the city.

The city centre has been defined as the centroid of the area delimited by the medieval walls of the city. The difference between the two clusters is quasi-significant (Mann-Whitney test, p-value = 0·09).

2.3 Manuscript n°3

Paleomicrobiology of the human digestive tract: a review.

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Abstract

The microbiota is a hot topic of research in medical microbiology, boosted by culturomics and metagenomics, with unanticipated knowledge outputs in physiology and pathology. Knowledge of the microbiota in ancient populations may therefore be of prime interest in understanding factors shaping the coevolution of the microbiota and populations. Studies on ancient human microbiomes can help us understand how the community of microorganisms presents in the oral cavity and the gut was shaped during the evolution of our species and what environmental, social or cultural changes may have changed it. This review cumulates and summarizes the discoveries in the field of the ancient human microbiota, focusing on the remains used as samples and techniques used to handle and analyse them.

Keywords

Coprolite; dental calculus; microbiome; microbiota; mummified tissue; paleomicrobiology; paleogenomics; paleoproteomics.

1. Introduction

In the last 20 years, there has been an exponential increase in interest in the microbiome and its effect on human health. We currently know that the microbiome plays a fundamental role in the host via the development, maintenance and priming of the immune system [1–3], defense against pathogens [4,5], metabolism, digestion and the production of vitamins [6]. Furthermore, slight modification of the bacterial equilibrium of the microbiome (dysbiosis) due to changes in diet, physical exercise or antibiotic treatments can lead to major consequences for the host [7–11]. Recent studies have focused their attention on the composition of the microbiome of our ancestors and its evolution throughout history. Particular attention was given to the periods of major revolutions in human lifestyles, such as the Neolithization process (debuted 12,000 years BCE [12]) and the industrialization period (1760-1840 CE). With the Neolithization process, we observed the transition from a nomadic hunter-gatherer diet characterized by the consumption of only wild and unprocessed food foraged and hunted from the environment [13] to a sedentary one based on agriculture and livestock. Then the industrialization period arose the advent of modern sanitation and Western diet, characterized by food that has been processed and modified and has a low quantity of vegetables and fibers [14–16].

In the literature, there is some confusion about the definition of the terms “microbiota” and “microbiome” and are often used interchangeably. The term “microbiota” refers to all the microbial taxa associated with the host [17], while “microbiome” can refer to “the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space and have been all but ignored as determinants of health and disease” [18] or to “the collective genome of our indigenous microbes” [19].

Diets of past and present human populations are very variable and mostly depend on resource availability and ecological settings [20]. Comparative studies on the microbiota among human populations with different dietary habits have shown how differences in diet are reflected in differences in the microbiota composition [14,16]. For instance, De Filippo and colleagues [21] showed how children from urban Europe, with a modern Western diet, and children from rural Africa, with a diet very high in fibers, present differences in microbiota composition that can be attributed to an evolutionary adaptation to the diet composition, allowing each population to

maximize the nutritional value of each diet. Other studies on mouse models have shown how changing diet correlated with changes in the microbial composition of the gut microbiota [22]. Moreover, Groussin et al. [23] demonstrated how microbiota composition reconstructed from ancient mammal remains could be used to reconstruct ancient diets of these animals and that information about dietary shift can be recorded by the gut microbiome. For these reasons, ancient microbiota studies are not restricted to the microbial diversity and composition of the ancient remains but also to the dietary information that is preserved inside these specimens. The first study to target the ancient DNA of multiple species of bacteria on human remains was conducted by Ubaldi et al, analyzing the gut microbiome from the colon of a mummy recovered in Cuzco, Peru, that dated back to the 10th-11th century CE [24]. Following by many other PCR-based studies on coprolites and mummified samples [25–27]. In 2008, Tito et al. performed the first study on an ancient microbiota using NGS techniques, and from that point, the number of ancient microbiome analyses multiplied [28].

Retrieving information on ancient human microbiomes is not easy and is strictly dependent on the availability of suitable samples for the analysis. Dental calculus and coprolite are the only two human remains that persist long enough to be used for these studies, with the exceptions of frozen and mummified tissues [29]. In this review, we will present the remains used as starting samples for ancient human microbiota studies and the methods used to retrieve this information.

2. Literature search strategy and terminology use

All the papers used for this review were obtained from PubMed and Google Scholar. The keywords used alone or in combination for the search were “paleomicrobiology”, “ancient”, “human”, “paleo”, “microbiota”, “microbiome”, “oral”, “dental calculus”, “gut”, “intestinal”, “feces”, “faeces”, “paleofeces”, “paleofaeces”, “coprolite”, “latrines”, “mummy”, “mummified”, “tissue”, “metagenomic”, “shotgun”, and “proteomics”.

Only studies written in English and on samples of human origin that targeted multiple species from the digestive tract microbiota of the host were considered. The results of this bibliographical research are shown in Table 1 (last search March 2021).

As described in the introduction section, the two terms “microbiota” and “microbiome” are often used interchangeably, and since a discussion on the terminology was not one of the purposes of this review, we used the two terms as synonyms, following the definition given by Lederberg [18].

Contamination prevention and authentication

Paleomicrobiological studies on ancient molecules must follow standard protocols to avoid modern exogenous contamination and ensure the authenticity of the results [30]. Contaminating molecules can come from the burial site, handling, transport, storage, and laboratory environments; from the materials and instruments used during the analysis; and from the operators at each step of the study [31,32]. Regardless of the starting sample type, all the steps of the analysis of ancient molecules must be performed in a dedicated clean hood and laboratory, ideally exclusively dedicated to work on ancient remains, excluding any positive controls, and including several negative controls that should be handled strictly in parallel with investigated samples [31]. Authentication of ancient DNA can be performed by checking for fragmentation (ancient DNA rarely exceeds 200-300 bp length) [33,34] and degradation (e.g. assessing the PCR success related to the size of the amplicon of a selected DNA sequence) [33]. Multiple bioinformatics tools can contribute to the authentication of ancient molecules. Software like MapDamage 2.0 [35,36] quantify the patterns of damage in NGS datasets generated from ancient DNA and therefore help differentiate endogenous from exogenous/contaminating DNA. Other tools like SourceTracker [33,37] estimate the proportion of DNA sequences that come from the source environments to determine whether the reconstructed microbial communities observed are endogenous to the samples. Moreover, directly comparing the DNA sequences obtained from different tissues with those from soil from the site where samples have been collected through bioinformatic analysis may facilitate the interpretation of the data [34].

3. Ancient Oral Cavity Microbiota.

3.1. Dental calculus

Dental calculus, or tartar, is a mineralized bacterial biofilm that forms on the teeth as a result of the action of bacteria and saliva in the oral cavity and is very common in adults without active dental hygiene [29]. Dental calculus presents some characteristics that make it the most reliable source for information about the oral microbiota. It is common and relatively abundant in archaeological sites; it has the capability to preserve ancient biomolecules, avoiding environmental contaminants; and it contains a high quantity of DNA originating from the oral microbiome [29,38,39]. In fact, it can be found on teeth in all past and present human populations and can survive for thousands and even millions of years. Accordingly, dental calculus remains were obtained from a 12-8 million-year-old fossil of the ape *Sivapithecus* [40].

Geographical distribution of the specimens analyzed for the oral microbiota are represented in Figure 1.

3.1.1. Analysis of ancient DNA from dental calculus.

After the recent confirmation of the presence and abundance of DNA in dental calculus [41], multiple studies have successfully extracted and analyzed DNA sequences from this type of sample. Different protocols have been applied for DNA extraction in the past. In general, it is very similar to apply the extraction methods used for other types of samples, such as bones and teeth, with a “lysis step” followed by a “purification step”. The lysis step usually relies on decalcification through ethylenediaminetetraacetic acid (EDTA) with or without Tris-HCl and protein digestion using proteinase K with or without sodium-dodecyl-sulfate (SDS), with a variable incubation time and temperature (usually 24 h at 56 °C) [38,42–46]. Occasionally, in the lysis step, Tris-HCl and SDS are added to the sample to improve decalcification and protein digestion [38,47]. After the lysis step, purification of DNA is performed using the phenol:chloroform:isoamyl alcohol (25:24:1) method [47] preferentially followed by Qiagen MinElute column purification [38,43,45,46] or using commercial kits for DNA purification, such as the EZ1 DNA tissue kit (Qiagen) [44,48] and QIAamp DNA Investigator Kit (Qiagen) [42]. It is also possible to use an in-house silica-based method for the extraction and purification of ancient DNA [20].

Polymerase chain reaction (PCR) amplification and subsequent sequencing can be used to analyze DNA sequences specific for microorganisms from the oral microbial community. PCR primers were specifically designed for five species of bacteria, *Actinomyces naeslundii, Fusobacterium nucleatum, Porphyromonas gingivalis, Streptococcus gordonii* and *Streptococcus mutans*, by De La Fuente et al. [47], which were detected in dental calculus from 4,000-year-old to modern samples. Specific primers are useful for the identification of single species of microorganisms, such as pathogens or common microbes of the oral microbiota; to determine the conservation of DNA in the sample; and to study specific genes [49].

The amplification of a conserved bacterial region of the 16S ribosomal RNA (16S rRNA) gene and subsequent high-throughput sequencing yields information about the diversity of bacteria present in the samples, describing the bacterial community of the ancient oral microbiota [20,38,42,50,51]. Using this method, it is possible to identify hundreds of species with a single PCR amplification [49]. Adler et al. [42] used the results of high-throughput sequencing to compare the bacteria present in the oral cavity of Mesolithic, Neolithic, Bronze Age, medieval and modern individuals, reporting a reduction in the bacterial diversity and a shift toward cariogenic bacterial dominance after medieval times, concomitant with the industrial revolution [42]. Following the same principle, regions conserved among any organism of interest can be amplified. PCR sequencing of 16S rRNA and methyl coenzyme M reductase (*mcrA*) genes was used to detect and describe the methanogen community present in the oral human microbiota, allowing the identification of multiple archaea, such as *Candidatus Methanobrevibacter* sp. N13, *Methanobrevibacter oralis* and *Methanoculleus bourgensis* [44]. Unfortunately, this approach has limited applicability when the aDNA is known to be highly degraded and fragmented or when the targeted region contains extensive length polymorphisms that induce a bias toward taxa with the shortest amplicon lengths [43].

In contrast to targeted gene amplification, shotgun sequencing allows the sequencing of random DNA fragments present in the sample independently of their origin (eukarya, bacteria, archaea, and viruses), overcoming targeted sequencing problems [38,43]. This approach allows the analysis of all the sequences inside the sample at the same time without any prior knowledge, providing both taxonomic and functional information [49]. Shotgun metagenomics applied to ancient human dental calculus allowed the characterization of the oral microbiota from multiple archaeological

sites [20,38,39], providing information about antibiotic resistance and metabolic functions of ancient bacteria [20,38]. Data obtained with this approach showed a possible correlation between behavior, diet, and health and the oral microbiota composition of two populations of European Neanderthals [20]. This finding suggested a central role of meat consumption in early hominid microbiota composition and that the repertoire of microorganisms preserved in dental calculus could be used to derive the dietary behavior of ancient populations by comparing the shotgun data obtained to those obtained for other ancient calculus specimens from populations with a known diet. Moreover, the shotgun metagenomic approach also allowed the reconstruction of draft genomes of organisms present in ancient dental calculus, such as the bacterium *Tannerella forsythia* [38] and the archaeon *Methanobrevibacter oralis* [20].

3.1.2. Analysis of ancient protein from dental calculus.

Similar to shotgun metagenomics for nucleotides, shotgun proteomics allows the detection and study of extracted peptides from ancient human dental calculus [52]. Despite the better preservation capability of proteins than DNA molecules, proteomic approaches are relatively new in the field of paleomicrobiology, providing valuable information about the diet, microbiota function and composition, host health and immune response [53,54].Warriner and colleagues showed how tandem mass spectrometry (MS/MS) used for shotgun proteomics can produce information about the presence of bacterial species-specific proteins, virulence factors, and antigenic proteins, as well as human immune response proteins (antimicrobial gene products) [38,55]. In addition, it has been shown that mass spectrometry for proteomic analysis can be used to describe the oral human metabolome (the collection of all the low- weight metabolites produced by metabolism [46]), providing important information about salivary and microbiome-related proteins [46] and dietary information from ancient populations [56,57].

3.1.3. Microscopical analysis on dental calculus.

Microscopic analysis of dental calculus allowed the detection of aliments and the presence of calcified microorganisms [58,59]. Examination through scanning electron microscopy (SEM) can be

performed without treating the sample, allowing complete preservation [60], while optical microscopy and transmission electron microscopy (TEM) need a pretreatment to be performed that partially or completely disrupts the sample [60]. For SEM, a preliminary step for the decalcification of the sample is performed using hydrochloric acid (HCl) at different concentrations [38,61]. For TEM, ancient samples follow routine procedure, with treatment with osmium tetroxide fixative and epoxy resin embedding [41]. Optical microscopy can be used to distinguish between gram-positive and gram-negative bacteria [38,60,62]. Optical and electron microscopy usually do not allow the identification of species but can provide important pieces of information about the presence, dimension and shape of microorganisms in the samples [59]. Microscopic analysis can also be used for biomolecular investigations, using gold-labeled antibody TEM to detect the presence of DNA [41]; fluorescence microscopy and polyclonal antibodies for the identification of specific bacteria, such as *Streptococcus mutans* [62]; and fluorescent in situ hybridization (FISH) to reveal archaea, such as *Candidatus Methanobrevibacter sp.* [44].

3.2. Other sources of ancient oral microbiota.

Despite the vast majority of studies on ancient oral microbiota have been performed on dental calculus, some authors tried to think outside of the box obtaining interesting results. In 2019, Jensen and colleagues analyzed a chewed birch pitch from Denmark dated to 5,700 years ago and revealed the presence of preserved DNA material. Starting from this specimen, they reconstructed a complete ancient human genome and identified microbial DNA sequences that reflected the oral microbiome of the person who chewed the pitch [63]. This work highlighted the potential utility of chewed birch pitch in the field of paleomicrobiology for the study of ancient microbiota.

Moreover, the analysis of buccal swabs from Inca mummies through shotgun proteomics analysis was used to describe active states of infection on an individual at the time of death by providing information on the immune response and potentially on the pathogens present when combined with DNA amplification [64].

4. Ancient Gut Microbiota.

4.1. Coprolites and palaeofaeces.

The primary sources for information about the ancient gut microbiota are coprolites, which are “any formed fecal mass remains, including mineralized, desiccated, or frozen feces and even the intestinal contents of mummies” [65]. These remains can be preserved for thousands or even millions of years [66], providing information about the gut microbiota components, the presence of pathogens and the diet of past human and animal populations [67]. In fact, traces of aliments consumed by the host, such as bones of small animals, feathers, seafood shells, and plant seeds, are commonly found in coprolites [65,68–71]. Moreover, it is possible to correlate the presence of animal parasites with the diet of an ancient population [70,72,73].

4.1.1. Molecular analysis on coprolites.

DNA analysis can potentially produce additional information about the complete set of species inside the coprolite and the evolution of these organisms. To reduce environmental contamination, extraction of DNA is performed on the core of the coprolites after the removal of a few millimeters (3 mm - 1 cm) of coprolite surface. Multiple protocols and commercial kits are available for DNA extraction from fecal and soil samples and are also used on ancient samples, e.g., UltraClean Fecal DNA Isolation Kit (MOBIO) [33] and PowerSoil® DNA Isolation Kit (MOBIO) [70,72–74]. Protocol specific for coprolites that does not require commercial kits have also been developed, such as the one from Kuch *et al.* [75]. In 2019, Hagan and colleagues tested the efficacy of 5 different methods for DNA extraction from ancient fecal material [36], indicating that despite every tested method being valuable, methods used for modern microbiota analysis recovered less DNA than methods developed and optimized for ancient bone material [39,76].

Massive sequencing of specific amplified regions of the DNA, such as 16S rDNA, provided information about the prokaryotic community inside the coprolite [34,70,74]. The same approach can be used for the eukaryotic community, e.g., by using primers that target the 18S ribosomal DNA (18S rDNA) or Internal transcribed spacer (ITS) region for fungal diversity [70,74]. Whole-genome shotgun sequencing does not require previous amplification of the DNA and gives information on the entirety of DNA sequences present in the sample, allowing both taxonomic and functional

characterization [28,34,73,77]. Furthermore, pathogen DNA sequences, including those of *Clostridium botulinum, Trypanosoma cruzi* and human papillomaviruses (HPVs) [34], as well as drug resistance genes [34,77], are detected. Moreover, viral metagenomes from coprolites have been described [72,78].

4.1.2. Microscopic and immunoassay analysis on coprolites.

Rehydration of samples is the preliminary step for subsequent microscopic analysis. Different protocols have been developed for coprolite rehydration [79]. These protocols all rely on using 0.5% trisodium phosphate (Na3PO4) aqueous solution sometimes complemented with 5% glycerinated water and formalin (Le Bailly 2010) or hydrochloric acid (HCl) to dissolve the calcium carbonates [80]. This step can last from 24 h to one week [81,82] and can be combined with a sedimentation step [68,73,77]. Microscopic analysis allows the identification of multicellular parasites, such as helminth eggs [73,77,83]; protozoan cysts [84–86]; and food traces [87,88]. Parasite identification relies on morphometric characterization with light and electron microscopy, which is based on the experience and ability of the operator. Cases have been reported in which pollen grains, which are morphologically similar to pinworm eggs, have been confused for parasitic eggs [89]. The use of confocal laser scanning microscopy (CLSM) has been revealed to be a powerful tool for the identification of parasites in coprolites. This technique allows better morphological identification, detects intrinsic autofluorescent molecules and has the great advantage of being less destructive to the specimen, allowing further analyses to be performed (e.g., molecular analysis) on the same specimen [90]. Moreover, immunofluorescence assays (IFAs) or enzyme-linked immunosorbent assays (ELISAs) have been used for the detection of intestinal protozoa, such as *Giardia spp.* [84,91–95], *Cryptosporidium spp.* [84,95,96], and *Entamoeba histolytica* [93–95,97,98].

4.1.3. Culture-dependent analysis on coprolites.

Finally, a culture-dependent investigation was performed on a 14th-century coprolite found in Belgium in parallel to a metagenomic analysis [77]. A portion from the inner part of the coprolite was inoculated into Schaedler and R2A liquid and solid culture media and maintained for several

days at 30 °C under various atmospheres created using gas-generating pouch systems (BD Gas PakTM EZ). Small colonies were observable after 10 days of incubation, and ten different bacterial species (*Paenibacillus macerans, Bacillus jeotgali, Staphylococcus pasteuri, Staphylococcus epidermidis, Staphylococcus cohnii, Micrococcus luteus, Pseudomonas geniculate, Stenotrophomonas maltophilia, Bacillus horti,* and *Clostridium magnum*) were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and 16S rRNA gene sequencing. Additionally, a portion from the inner part of the coprolite was cultured in BD BACTEC, Lytic/10 Anaerobic and Aerobic bottles enriched with 7 mL of defibrinated sheep blood and incubated at 37 °C. After two days, cultures were diluted and plated onto COS culture plates (bioMérieux) and reincubated at 37 °C under different atmospheres. Eight different bacterial species, *Rhodanobacter sp.* organism, *Paenibacillus sp.* organism, *Paenibacillus macerans, Paenibacillus thiaminolyticus, Paenibacillus ehimensis, Staphylococcus arlettae, Propionibacterium acnes,* and *Enterobacter cloacae*, were isolated and identified from the blood-enriched cultures using MALDI-TOF-MS and 16S rRNA gene sequencing.

4.2. Mummified tissues.

Other than coprolites, natural and artificial mummified tissues are also valuable sources for ancient human microbiota studies. DNA, proteins, and even entire parasites (eggs, larvae, and adults) have been found in mummified tissues. Well-preserved mummies offer the rare possibility to study microbiota both inside and outside of the digestive tract, allowing the identification of specific pathogens of certain organs, i.e., *Helicobacter pylori* [99], and even compare the microbial communities present in different organs from the same individual [25]. Mummified tissues from the digestive tract are used for the identification of the microbial community of the gut, stomach, and oral cavity. Similar to the methods used for coprolite analysis, mummified tissues can be analyzed for parasite and protozoan detection through microscopy. The rehydration step can be performed following the same methods used for coprolites with trisodium phosphate [79] or with a solution of potassium hydroxide at 4-7% [79,100].

Techniques of 16S rRNA gene high-throughput sequencing and metagenomics performed on mummified digestive tract tissues were used for the characterization of ancient microbiota. The presence of DNA sequences homologous to many organisms of medical interest were also detected, such as *Clostridium botulinum, Trypanosoma cruzi*, HPVs, *Pseudomonas spp.,* and *Leishmania donovani* [24,101,102]. The genomes of 5,300-year-old *Helicobacter pylori* [99], *Clostridium perfringens, Clostridium sp. Ade.TY, Clostridium algidicarnis*, and *Pseudomonas veronii* [102] were reconstructed and used for phylogenetic analysis to study the evolution of these organisms. Moreover, metagenomic analysis allowed the identification of sequences homologous to bacteriophages [103], metabolic profiles [101], multiple sequences associated with antibiotic resistance and virulence-related genes in old mummified gut tissues [34,102,104]. Data obtained from mummified gut remains could also potentially be used to infer ancient dietary habits [104].

Organs connected to the digestive tract have been studied using microscopy or DNA-related techniques for the presence of pathogens and parasites: studies on gallbladder samples detected *Clonorchis sinensis* [105], *Mycobacterium tuberculosis* [106]; studies on liver samples detected hepatitis B virus [107].

Geographical distribution of the specimens analyzed for the gut microbiota are represented in figure 1.

5. Perspectives

We have just begun to understand the relationships between diet, health, pathological conditions, and the composition of our microbiota, but it is clear that the gut microbiota plays a role in the health and disease conditions of humans. This explains the strong interest in medical research for this subject. Combining modern techniques and protocols used in the field of paleomicrobiology for the analysis and the extraction of data from ancient human remains, we can study the past of humankind and its microbiota. An evolutionary framework that leads us to understand how these changes in human lifestyle have influenced changes in the microbiota can be built through the comparison of the microbiota composition among different eras and with data on the microbiota in the modern world. Studies on the microbiota of our ancestors can help us describe the evolutionary

path that led humans and their microbiota to where we are now, revealing how changes in diet, innovations in modern medicine, hygiene, and antibiotics have changed the composition and, therefore, how the characteristics of our microbiota have shaped humans as a species.

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Declaration of competing interest

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Figures and Tables

Table 1. Scientific studies performed on ancient human digestive tract microbiota catalogued per archaeological site, datation, sample type, and method used for the analysis. Only studies written in English, published before March 2021, and on samples of human origin that targeted multiple species from the digestive tract microbiota of the host were considered.

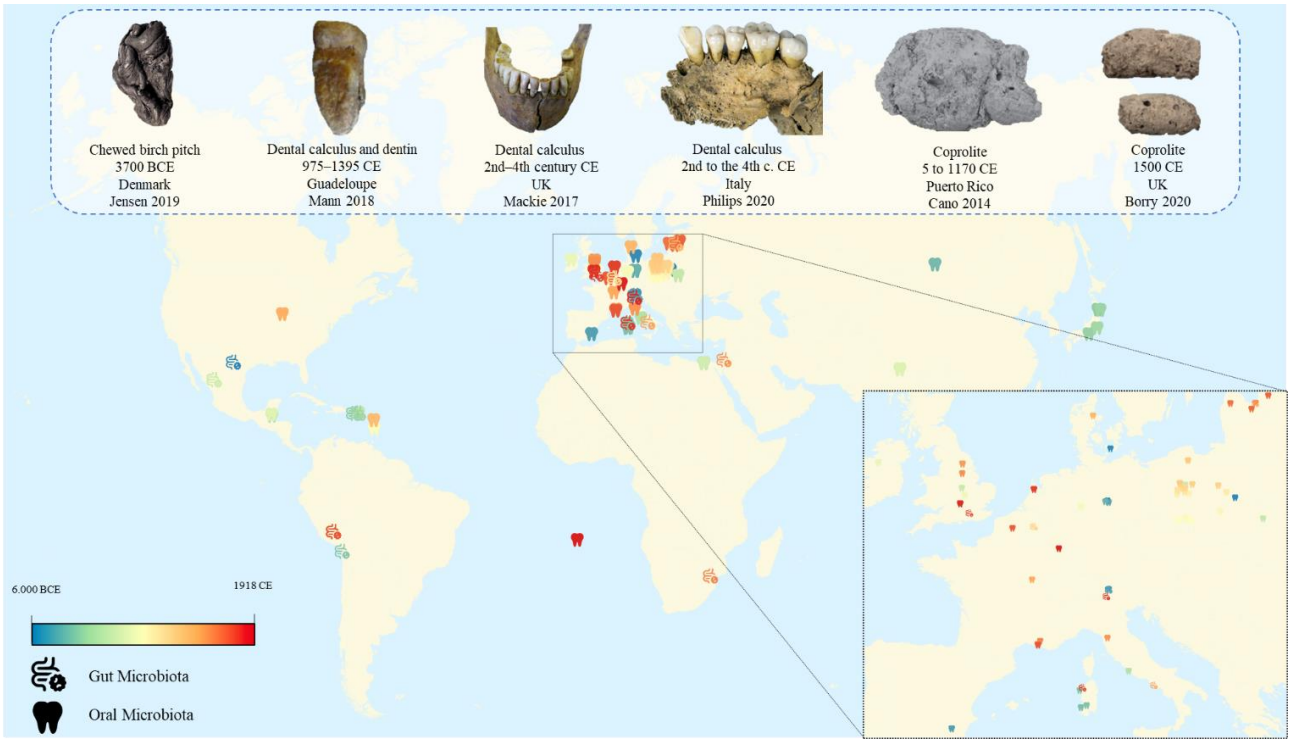
Archaeological site	Present-day country	Datation	Sample type	Method	References
Cuzco	Peru	10th-11th century CE	Mummified colon	PCR-based techniques	[24]
Similaun mountain	Italy	3400-3100 BCE	Mummified stomach and colon	PCR-based techniques	[25]
Cuzco	Peru	980-1170 CE	coprolites	PCR-based techniques	[26]
Forni glacier (Alta Val di Pejo, northern Italy)	Italy	1918 CE	Mummified colon	PCR-based techniques	[27]
Similaun mountain	Italy	3400-3100 BCE	Mummified colon	PCR-based techniques	[27]
La Cueva de los Chiquitos Muertos'', Rio Zape, Durango Durango	Mexico	600 - 700 CE	coprolites	Shotgun metagenomic	[28]
Caserones	Chile	400 CE	coprolites	16S rRNA Gene High-Throughput Sequencing	[33]
Hind's Cave	Texas	6000 BCE	coprolites	16S rRNA Gene High-Throughput Sequencing	[33]
La Cueva de los Chiquitos Muertos'', Rio Zape, Durango Durango	Mexico	600 - 700 CE	coprolites	16S rRNA Gene High-Throughput Sequencing	[33]
Benzingerode-Heimburg	Germany	2150-1600 BCE	dental calculus	16S rRNA Gene High-Throughput Sequencing	[42]
Dudka	Poland	5550-3450 BCE	dental calculus	16S rRNA Gene High-Throughput Sequencing	[42]
Halberstadt-Sonntagsfeld	Germany	5400-4725 BCE	dental calculus	16S rRNA Gene High-Throughput Sequencing	[42]
Northamptonshire	England	900-1150 CE	dental calculus	16S rRNA Gene High-Throughput Sequencing	[42]
Quedlinburg XII	Germany	2450-2000 BCE	dental calculus	16S rRNA Gene High-Throughput Sequencing	[42]
York	England	1250-1350 CE	dental calculus	16S rRNA Gene High-Throughput Sequencing	[42]
York	England	1000-1600 CE	dental calculus	16S rRNA Gene High-Throughput Sequencing	[42]
Yorkshire	Engalnd	Bronze age	dental calculus	16S rRNA Gene High-Throughput Sequencing	[42]
Sorcé, island of Vieques	Puerto Rico	180 - 600 CE	coprolites	16s rRNA gene and ITS region PCR-based techniques	[74]
Tecla, Guayanilla	Puerto Rico	180 - 600 CE	coprolites	16s rRNA gene and ITS region PCR-based techniques	[74]
Namur	Belgium	14th-century	coprolites	shotgun metagenomic	[78]
Namur	Belgium	14th-century	coprolites	culture-base method	[77]
Namur	Belgium	14th-century	coprolites	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[77]
Sorcé, island of Vieques	Puerto Rico	5 to 1170 CE	coprolites	16S rRNA Gene High-Throughput Sequencing	[70]
Tecla, Guayanilla	Puerto Rico	5 to 1170 CE	coprolites	18S rRNA Gene High-Throughput Sequencing	[70]

Similaun mountain	Italy	3400-3100 BCE	gingival tissue biopsy	PCR-based techniques	[108]
Similaun mountain	Italy	3400-3100 BCE	Mouth swab	PCR-based techniques	[108]
St. Petri church and convent complex in Dalheim	Germany	950-1200 CE	dental calculus	mass spectrometry (MS)-based proteomics profiling	[38]
St. Petri church and convent complex in Dalheim	Germany	950-1200 CE	dental calculus	Shotgun metagenomic	[38]
Cuzco	Peru	11th century CE	paleofaeces	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[34]
Cuzco	Peru	11th century CE	mummified colon	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[34]
Anse à la Gourde	Guadalupe	975–1395 CE	Dental calculus	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[43]
Camino del Molino, Caravaca de la Cruz, Murcia	Spain	2340–2920 BCE	Dental calculus	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[43]
Lavoutte	St. Lucia	990–1255 CE	Dental calculus	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[43]
Middenbeemster	Netherlands	1611–1866 CE	Dental calculus	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[43]
Rupert's Valley	St. Helena	1840–1872 CE	Dental calculus	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[43]
Samdzong	Nepal	400–650 CE	Dental calculus	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[43]
Tickhill, Yorkshire	UK	1450–1600 CE	Dental calculus	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[43]
Avosnes	France	1300 CE	dental calculus	PCR-based techniques	[44]
Douai	France	1710-1712 CE	dental calculus	PCR-based techniques	[44]
Forbach	France	1813 CE	dental calculus	PCR-based techniques	[44]
Les Fedons	France	1590 CE	dental calculus	PCR-based techniques	[44]
Martigues	France	1720-1721 CE	dental calculus	PCR-based techniques	[44]
Saint-Mitre-les-Remparts	France	1500-1700 CE	dental calculus	PCR-based techniques	[44]
Cuzco	Peru	980-1170 CE	Mummified descending colon	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[103]
Cuzco	Peru	14-15th century CE	Mummified abdominal viscera	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[103]
Cuzco	Peru	1410–1530 CE	Mummified transverse colon	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[103]
Cuzco	Peru	980-1170 CE	Mummified descending colon	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[101]
Cuzco	Peru	14-15th century CE	Mummified abdominal viscera	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[101]
Cuzco	Peru	1410–1530 CE	Mummified transverse colon	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[101]
Similaun mountain	Italy	3400-3100 BCE	Mummified distal gut	shotgun metagenomic	[102]
Leicester, UK (NGR SK 586038), Oxford Street	UK	2nd–5th century CE	dental calculus	mass spectrometry (MS)-based proteomics profiling	[109]
Rome, Isola Sacra	Italy	1st–3rd century CE	dental calculus	mass spectrometry (MS)-based proteomics profiling	[109]

York, UK (NGR: SE 59324510 & SE 59285095), Driffield Terrace	UK	2nd–4th century CE	dental calculus	mass spectrometry (MS)-based proteomics profiling	[109]
Sorcé, island of Vieques	Puerto Rico	10 - 385 CE	Dental calculus	16S rRNA gene high-throughput sequencing	[50]
Cuzco	Peru	980-1170 CE	Mummified descending colon	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[104]
Cuzco	Peru	14-15th century CE	Mummified abdominal viscera	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[104]
Cuzco	Peru	1410–1530 CE	Mummified transverse colon	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[104]
Naples, Basilica of St. Domenico Maggiore	Italy	15-16th century CE	Mummified colon and abdominal viscera	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[104]
Radcliffe Infirmary Burial Ground collection	England	1770-1855 CE	Dental calculus	mass spectrometry (MS)-based metabolites characterization	[46]
Tjærby, Jutland, Denmark	Denmark	1100–1450 CE	dental calculus	mass spectrometry (MS)-based proteomics profiling	[55]
Anse à la Gourde	Guadalupe	975-1395 CE	dental calculus	shotgun metagenomic	[39]
Arbulag Soum, Khövsgöl,	Mongolia	930-1650 BCE	dental calculus	shotgun metagenomic	[39]
Camino del Molino, Caravaca de la Cruz, Murcia	Spain	2340-2920 BCE	dental calculus	shotgun metagenomic	[39]
Kilteasheen	Ireland	600-1300 CE	dental calculus	shotgun metagenomic	[39]
Middenbeemster	Netherlands	1611-1866 CE	dental calculus	shotgun metagenomic	[39]
Norris Farms	Illinois (USA)	1300 CE	dental calculus	shotgun metagenomic	[39]
Samdzong	Nepal	400-650 CE	dental calculus	shotgun metagenomic	[39]
Anse à la Gourde	Guadalupe	975-1395 CE	dental calculus	shotgun metagenomic	[110]
Arbulag Soum, Khövsgöl,	Mongolia	930-1650 BCE	dental calculus	shotgun metagenomic	[110]
Camino del Molino, Caravaca de la Cruz, Murcia	Spain	2340-2920 BCE	dental calculus	shotgun metagenomic	[110]
Kilteasheen	Ireland	600-1300 CE	dental calculus	shotgun metagenomic	[110]
Middenbeemster	Netherlands	1611-1866 CE	dental calculus	shotgun metagenomic	[110]
Norris Farms	Illinois (USA)	1300 CE	dental calculus	shotgun metagenomic	[110]
Samdzong	Nepal	400-650 CE	dental calculus	shotgun metagenomic	[110]
site of Syltholm on the island of Lolland	Denmark	3700 BCE	chewed birch pitch	shotgun metagenomic	[63]
San Pietro di Pozzeveri	Italy	11th to 19th century CE	Dental calculus	16S rRNA gene High-Throughput Sequencing	[51]
La Cueva de los Chiquitos Muertos'', Rio Zape, Durango Durango	Mexico	700 CE	coprolites	Shotgun metagenomic	[111]
Similaun mountain	Italy	3400-3100 BCE	intestinal mummified tissue	Shotgun metagenomic	[111]
Radcliffe Infirmary Burial Ground collection	England	1770–1855 CE	dental calculus	mass spectrometry (MS)-based proteomics profiling	[112]
Radcliffe Infirmary Burial Ground collection	England	1770–1855 CE	dental calculus	shotgun metagenomic	[112]
La Cueva de los Chiquitos Muertos'', Rio Zape, Durango Durango	Mexico	700 CE	coprolites	Shotgun metagenomic	[113]
Surrey	UK	Post medieval	coprolites	Shotgun metagenomic	[113]
palazzo ducale Sassari	Italy	1800-1850 CE	cesspit sediment	16S and 18S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[114]
La Cueva de los Chiquitos Muertos'', Rio Zape, Durango Durango	Mexico	700 CE	coprolites	Shotgun metagenomic	[36]

Abusir	Egypt	340-395 CE	Dental calculus	Shotgun metagenomic	[115]
Abusir	Egypt	138-200 CE	Dental calculus	Shotgun metagenomic	[115]
Abusir	Egypt	2196-2045 BCE	Dental calculus	Shotgun metagenomic	[115]
Abusir	Egypt	109-40 BCE	Dental calculus	Shotgun metagenomic	[115]
Abusir	Egypt	369-211 BCE	Dental calculus	Shotgun metagenomic	[115]
Brzeg	Poland	900-1400 CE	teeth	shotgun metagenomic	[116]
Dziekanowice	Poland	900-1400 CE	teeth	shotgun metagenomic	[116]
Gniezno	Poland	900-1400 CE	teeth	shotgun metagenomic	[116]
Gołuń	Poland	900-1400 CE	teeth	shotgun metagenomic	[116]
Końskie	Poland	900-1400 CE	teeth	shotgun metagenomic	[116]
Kowalewko	Poland	100-400 CE	teeth	shotgun metagenomic	[116]
Ląd	Poland	900-1400 CE	teeth	shotgun metagenomic	[116]
Łęgowo	Poland	900-1400 CE	teeth	shotgun metagenomic	[116]
Markowice	Poland	900-1400 CE	teeth	shotgun metagenomic	[116]
Masłomęcz	Poland	100-400 CE	teeth	shotgun metagenomic	[116]
Niemcza	Poland	900-1400 CE	teeth	shotgun metagenomic	[116]
Obłaczkowo	Poland	900-1400 CE	teeth	shotgun metagenomic	[116]
Opole	Poland	900-1400 CE	teeth	shotgun metagenomic	[116]
Ostrów Lednicki	Poland	900-1400 CE	teeth	shotgun metagenomic	[116]
Płońsk	Poland	900-1400 CE	teeth	shotgun metagenomic	[116]
Poznań–Śródka	Poland	900-1400 CE	teeth	shotgun metagenomic	[116]
Sowinki	Poland	900-1400 CE	teeth	shotgun metagenomic	[116]
Warszawa	Poland	900-1400 CE	teeth	shotgun metagenomic	[116]
Bushman Rock Shelter (BRS) in Limpopo Province	South Africa	1460 CE	paleo-faecal sample	Shotgun metagenomic	[117]
western Belize: Chan Chich	Belize	713-885 CE	Dental calculus	Shotgun metagenomic	[118]
western Belize: Chan	Belize	170 BCE-50 CE	Dental calculus	Shotgun metagenomic	[118]
Lu Maccioni (Alghero)	Italy	1126-825 BCE	Dental calculus	Shotgun metagenomic	[118]
Capo Pecora (Arbus)	Italy	1384-936 BCE	Dental calculus	Shotgun metagenomic	[118]
Perdalba (Sardara)	Italy	1400–850 BCE	Dental calculus	Shotgun metagenomic	[118]
Riga	Latvia	1356 CE	Latrine sediment	Shotgun metagenomic	[119]
Jerusalem	Israel/State of Palestine	1400-1500 CE	Latrine sediment	Shotgun metagenomic	[119]
Ebishima shell mound in Iwate prefecture	Japan	1000-400 BCE	Dental calculus	Shotgun metagenomic	[120]
Ikawazu, Aichi prefecture,	Japan	1000-400 BCE	Dental calculus	Shotgun metagenomic	[120]
Miyano, Iwate prefecture	Japan	1000-400 BCE	Dental calculus	Shotgun metagenomic	[120]
Ikenohata-Shichikencho site, Taito-ku, Tokyo	Japan	1000-400 BCE	Dental calculus	Shotgun metagenomic	[120]
Riga	Latvia	1500-1700 CE	Dental calculus	Shotgun metagenomic	[121]
Cesis	Latvia	1600-1700 CE	Dental calculus	Shotgun metagenomic	[121]
Kuldiga	Latvia	1500-1700 CE	Dental calculus	Shotgun metagenomic	[121]
Jelgava	Latvia	1500-1700 CE	Dental calculus	Shotgun metagenomic	[121]

Figure 1. Geographical localization of ancient human remains analyzed for microbiota studies in the World. Colour indicate the datation of the analyzed samples from 6000 BCE to 1918 CE. Oral microbiota and gut microbiota studies are indicated with two symbols representing a tooth and the gut respectively. The corresponding references are listed in Table 1. Map generated with QGIS software [122]. In the upper part of the figure, 6 pictures representative of the original samples used to study the digestive tract microbiota [39,63,70,109,113,116].



3. Conclusions

With the Neolithic revolution (approximately 10,000 BCE) human lifestyle changed dramatically (Eddine et al., 2020); from small, nomadic bands of hunter-gatherers, humans started to live in larger, sedentary groups of people sustained by agriculture and animal husbandry (Vigne, 2011). All the changes associated to this new lifestyle (e.g. larger group of people living in small areas, cohabitation with domestic animals) favoured the emergence of what became the main antagonist for the survival of our species: infectious diseases. Mostly of animal origin, infectious diseases like pneumonia, tuberculosis, plague, malaria, typhus, cholera, are just few of the many terrible "enemies" that humans had to face during their history. Recent estimates suggest that life expectancy in pre-modern times was around 30 years (Roser, Ortiz-Ospina, et al., 2013), with a mortality in children below 5 years of age (defined as child mortality) of 50% (Roser, Ritchie, et al., 2013). Life expectancy and child mortality stayed fairly stable till the beginning of XIX century (Roser, Ortiz-Ospina, et al., 2013) when progress in medicine and public health, such as antibiotics, vaccinations, and access to safe drinking water, have greatly reduced the impact of infectious diseases on human lives. The 43% child mortality estimated for 1800 has been reduced to less than 5% nowadays (Roser, Ritchie, et al., 2013) (https://data.unicef.org/topic/child-survival/under-five-mortality/) with a consequent increase in life expectancy from 30 years in pre-modern times to an average of 70 years nowadays (Roser, Ortiz-Ospina, et al., 2013). Although with notable differences between the developed countries and the so-called least developed countries, all countries in the world have benefitted from this progress (Roser, Ortiz-Ospina, et al., 2013). Even in disadvantaged areas of the globe, such as Africa, life expectancy increased, reaching an average of 63 years (Roser, Ortiz-Ospina, et al., 2013), despite the high impact of malaria and other diseases especially on children (World Health Organization, 2021).

Despite these amazing improvements, infectious diseases continue to pose a threat to human health. The recent coronavirus pandemic (COVID-19), which continues to devastate people's health and also world economy, has evidenced the vulnerabilities of our species to such communicable diseases. This disease is just the last of an endless array of potentially pandemic diseases that we have had to deal with just in last decades. SARS, bird flu, MERS, Ebola, Zika and Nipah are some of the diseases that resurged in the course of the twenty-first century and that were considered to have pandemic potential (Devnath & Masud, 2021; Lynteris, 2019). The reasons why a disease such

as COVID-19 has managed to cause a global pandemic while the other diseases listed above have not reached such high numbers of infected people and geographical extension can be manifold. The fact is that to date no pathogen has been predicted prior to its appearance (Morse et al., 2012; Murphy, 1998). Nonetheless, the analysis of past epidemics has led to a better understand of diseases and epidemic dynamics. For example, studying how and where pathogens emerged in the past helped identify the causes for pathogens emergence (Keesing et al., 2010; Morse et al., 2012). The identification of hotspots for emerging pathogens is essential for epidemic preparedness, so that surveillance and monitoring programs can be improved (Allen et al., 2017; Morse et al., 2012). The application of knowledge derived from the study of past epidemics is not limited to pathogen emergence, but can be used to understand different aspects of human-pathogen-environment interactions.

The main objective of this thesis was to highlight how past microorganisms and epidemics can be studied and what information can be derived from these events, so that information from the past can help us to face present and future challenges. The attention was primarily focused on a specific epidemic episode: the plague epidemic that ravaged northern Italy and Milan during the XVII century, which is considered one of the deadliest epidemics of that century (Alfani, 2020).

The first part of the project was focused on the paleomicrobiological analyses of teeth recovered in an archaeological site situated in Milan and dated back to the period of interest (Caruso et al., 2013). As already explained in the introduction, the search for *Y. pestis* traces was performed on the dental pulp. Extracted DNA was analysed through PCR for the detection of the two markers of choice: *caf1* and *pla* (Schuenemann et al., 2011; Spyrou et al., 2019). In parallel to the extraction and analysis of DNA, the same samples were then analysed for the protein content through a proteomic analysis. The proteins extracted from the dental pulp of the samples were subjected to a paleoproteomic analysis through mass spectrometry (Barbieri et al., 2017). Up to now, the paleomicrobiological analysis of the samples gave inconclusive results. In fact, the genes used as marker for *Y. pestis* presence were not found in the samples. At the same time the protein analysis found only few proteins of human origins. The absence of PCR and proteomic positive results cannot be considered as absence of *Y. pestis;* in fact, the possible explanations for these results are multiple. The most plausible one is that the samples were too much degraded for the detection of the pathogen of

interest. The analysis of the complete DNA content of the samples can inform on the effective presence of *Y. pestis* or other pathogen traces. Therefore, aDNA extracted from the same samples, was also used for the preparation of NGS libraries (Kircher et al., 2012; Meyer & Kircher, 2010) for a subsequent metagenomic analysis through Illumina platform. Unfortunately, the pandemic emergency to which we have been subjected in the last two years has heavily influenced the progress of this portion of the project. As a result, the metagenomic analysis of the samples is still in progress and the results will be available in the next few months.

Greater efforts were thus devoted to the historical and epidemiological part of the project. Two main studies were performed on historical records. The first one, described in manuscript n° 1 (pg. 45), is a study on the application of a novel quantitative method for the automatic analysis of ancient plague texts. In particular, the words contained in texts written by people who have directly witnessed plague epidemics were analysed and compared with "negative" control texts written in the same period as the texts of interest but not related to the plague epidemic. This method has been tested on two epidemic episodes: the great plague of Marseille of 1720-1722 and the plague of northern Italy of 1629-1631. Among the results produced by the analysis of the texts associated to these two epidemics the most interesting ones were on what historical data can potentially suggest about pathogen sources and transmission. Differently for what has been described during the XX century, there were only marginal and non-specific mentions of rats for both epidemic (<5 total mentions out of more than 3 million words total extracted from the 32 analysed texts). On the contrary, contemporary people saw clothes, merchandise, and movables as potential sources of plague. These results support the hypothesis proposed by previous epidemiological (Dean et al., 2018, 2019), historical (Davis, 1986) and archaeological evidence (Hufthammer & Walløe, 2013), of a role of human ectoparasites during the second plague pandemic (Cohn, 2008; Dean et al., 2018, 2019; Houhamdi et al., 2006). Moreover, it was observed that words that were overrepresented in the French texts had a higher probability to be overrepresented also in the Italian texts. This result opens up to a potential future application of this method for the identification of pathogen responsible for a described disease in ancient text and help uncover information about the dynamic of past epidemic events, as performed in this study.

In the second historical study, described in manuscript n° 2 (pg. 75), the detailed information contained in the registers of the deaths of Milan (the date of death and the parish of residence) were used to reconstruct the spatio-temporal progress of the epidemic that hit the city in the year 1630. The study also considered the historical testimony of contemporary people that directly observed the epidemic and that noted a heavy increase in plague cases after a religious event that was carried out in early June: "The St. Carlo procession". Chroniclers of the time, in fact, attributed to this procession an important role in the spike of cases observed during summer of 1630. In this study we performed for the first time an investigation on the effective impact of the procession on the epidemic based of real data and not only on personal deduction of past chroniclers. The analysis of the recorded plague-related cases showed that the parishes of the city were not hit in the same way by the epidemic. In fact, the epidemic began in the periphery and then moved to the centre of the city. Interestingly, the timing at which the epidemic moved from these two areas of the city was consistent with the procession. In the peripheral areas of the city, plague-related deaths declined after the procession, while in the central areas it was registered a strong increase in plague-related deaths starting from the days immediately following it. These results open-up questions about how high contacts and concentrations of people could increase the transmissibility of plague, which is considered primarily a vector-borne disease transmitted by rat fleas. This should be considered in the debate around the role of human-to-human transmission of plague during past pandemics.

Finally, in the last part of the project, described in manuscript n° 3 (pg. 96), studies performed on the ancient human microbiota were collected and summarized. Since the microbiota reflect the lifestyle of its individual, ancient microbiota can be used to study the evolution of human lifestyle throughout history. The review is focused on the paleomicrobiological techniques and the different samples that can be tested to analyse the ancient microbiota of human digestive tracts. An extensive bibliographical research on papers publish on these subjects was performed and the results are available in an easy-to-consult table, which summarizes basic information of the papers, such as archaeological site, dating, sample type, and method used for the analysis.

Considering the never-ending threat that infectious diseases pose for human health, it is important to exploit every single piece of information that can help us to better understand how pathogens

evolve and interact with humans and the environment. In this, past epidemics, that are too often forgotten, offer us the chance to study the “big picture” around epidemic events and therefore should be considered as lessons from which we all can learn.

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Sitography

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