


Mycobacterial and Plasmodium ovale-associated destruction of the jaw bones

Xianrui Yang¹ | Adam Yongxin Ye² | Negin Katebi¹ | Vladimir Volloch¹ | Shelley M. Khullar^{1,3} | Vinod Patel^{4,5} | Bjorn R. Olsen¹ 

¹Harvard School of Dental Medicine, Boston, MA, USA

²Harvard Medical School, Boston, MA, USA

³Drammen Specialist Senter, Drammen, Norway

⁴Oral Surgery Dept, Guy's & St Thomas NHS, London, UK

⁵Foundation Trust, London, UK

Correspondence

Bjorn R. Olsen, Hersey Professor of Cell Biology, Harvard Medical School and Professor of Developmental Biology, Harvard School of Dental Medicine, 188 Longwood Avenue, Boston, MA 02115, USA. Email: bjorn_olsen@hms.harvard.edu

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Abstract

Objectives: The project aims were to identify infectious mechanisms responsible for an extreme form of mandibular osteonecrosis and osteomyelitis in West African populations and test the hypothesis that Mycobacterium tuberculosis plays a pivotal role.

Materials and Methods: DNA was extracted from mandibular fragments of 9 of 19 patients previously included in a prospective study leading to the mycobacterial hypothesis. Amplified DNAs were used for preparing libraries suitable for next-generation sequencing. For comparison of the whole-genome sequencing data of the 9 patients with DNAs of both microbiota and human tissues, DIAMOND v0.9.26 was used to align sequencing reads to NCBI-nr database and MEGAN 6 for taxonomy binning and identification of Mycobacterium tuberculosis strains.

Results: The data show that mandibular bone fragments of all 9 patients not only contain Homo sapiens and Mycobacterium tuberculosis DNAs; they also contain DNAs of Plasmodium ovale wallikeri, Staphylococcus aureus, Staphylococcus hominis, and Prevotella P3-120/intermedia; as well as large numbers of DNAs from other infectious components.

Conclusions: The data obtained provide direct evidence to support the conclusion that combinations of Mycobacterium tuberculosis, Plasmodium ovale wallikeri, and other oral bacteria are involved in this particular type of mandibular destruction in West African individuals of many ages.

KEYWORDS

M. tuberculosis, mandibular necrosis, P. ovale wallikeri

1 | INTRODUCTION

Osteonecrosis of the jaws (ONJ) is a severe and complex multi-factorial condition often with recalcitrant properties. The underlying causes are frequently associated with complications following medical procedures aimed at treating other chronic disorders, such as head and neck radiotherapy for cancer and the use of bisphosphonates and denosumab for osteoporosis. Other more rare causes have also been identified (Gadiwalla & Patel, 2018). Eight years ago,

Khullar et al. (2012) reported a retrospective case series of extreme ONJ and osteomyelitis in a West African population. Shared characteristic features among the patients included a unilateral gross facial swelling, extraoral discharging fistulae, scarring of the angle region of the mandible and vertical splitting of the ramus, and micro-pitting of the condylar head.

A subsequent prospective study of mandibular tissue fragments, collected over a 3-month period from 19 patients who voluntarily attended the Mercy Ships Dental Clinic for surgical treatment while the ship was stationed in Sierra Leone or the Trinity Dental Clinic in

Liberia, led to the histological finding that some of the collected tissues were positively stained with antibodies against *Mycobacterium tuberculosis* (TB) proteins. This raised the possibility that TB may have a role in this jaw bone destruction (Khullar et al., 2016).

Although the lungs are the most common site, extra-pulmonary TB (EP-TB) accounts for 15%–25% of all reported cases of TB. Within EP-TB, 35% of cases are related to bones and joints, often bearing joints (Grosskopf et al., 1994; Watts & Lifeso., 1996). The jaws, including the condylar joint, is a rare site for EP-TB and accounts for less than 2% of all skeletal cases (Sheikh et al., 2012). This rarity was highlighted by Andrade and Mhatre (2012) in a series of 33 cases over 16 years in an endemic region. Within the jaws, condylar TB is so uncommon that public reports have been only single case reports (Assouan et al., 2014; Gandhi et al., 2011; Helbling et al., 2010; Kumar et al., 2015; Park et al., 2014; Patel et al., 2012; Ranganathan et al., 2012; Soman & Davies, 2003; Towdur et al., 2018; Wu et al., 1998). The extent and severity of the cohort presented by Khullar et al. (2012) was not considered as being associated with *Mycobacterium tuberculosis* until data in support of that possibility were published (Khullar et al., 2016).

Although bone loss is expected in osteonecrosis of jaw bones, the extreme nature of the mandibular destruction evident in the West African cohort was unusual. Such a radical bone loss is not commonly seen in oral and dental pathology except in cases of malignancy, odontogenic tumors or cysts and in the genetic disorder cherubism. In cherubism, mutations in SH3BP2 play a key role in disrupting the equilibrium of jaw bone homeostasis (Ueki et al., 2001). Overactivity of SH3BP2 leads to hyperactivity of macrophages and osteoclasts and results in inflammation and maxillary and mandibular bone loss (Ueki et al., 2007). The current study was aimed at testing the hypothesis that the mandibular bone destruction in the West African patients (Khullar et al., 2016) is indeed associated with inflammatory hyperactivity of macrophages and osteoclasts as seen in cherubism and a pivotal *Mycobacterium tuberculosis* infection. To accomplish this, DNA was extracted from formaldehyde-fixed tissue

fragments of most of the 19 patients included in the previous analysis (Khullar et al., 2016).

2 | MATERIALS AND METHODS

2.1 | DNA preparation and sequencing

The samples used for sequencing of DNA came from the surgical removal of mandibular fragments of the 9 patients listed in Table 1 below:

DNA was extracted from formaldehyde-fixed surgical materials (50–100 mg bone) using the CryoPrep (Covaris, Inc.) extraction system; the rationale being that dry pulverization increases the tissue surface area, breaks up the extracellular matrix, and improves extraction efficiency of target biomolecules. Liquid nitrogen flash-frozen samples were pulverized in a CryoPrep device (set at number of hits 2 with level 4 power). Pulverized tissue samples were transferred into glass tubes, and extraction buffer containing Proteinase K was added. The prepared samples were incubated overnight at 56°C. DNA was extracted using an extraction kit (Sigma-Aldrich) the following day. Using NanoDrop spectrophotometer, DNA concentrations and sample purity (260/280 ratio) were measured. The quality of extracted DNAs was checked by running samples on electrophoresis gels to make sure that there was no degradation.

For library formation, the concentrations of DNA samples from 9 different patients were measured (NanoDrop) and the solutions were diluted in TE buffer (10 nM Tris pH 8.0, 1 mM EDTA) to final DNA concentrations between 300 µg/µl and 500 µg/µl. Fragmentation of the DNA was done at 37°C for 17 min with NEBNext Ultra II FS reaction buffer and enzyme mixture (NEB #7805). Adaptor ligation was accomplished with NEBNext Ultra II ligation master mix, ligation enhancer and adaptor for Illumina (NEB #7335) at 20°C for 15 min in a thermocycler with the

Patient Number (Khullar et al., 2016)	Gender	Age at presentation	Specified mandibular location
8	M	32	Anterior mandible
9	M	41	Right ramus/ coronoid
11	F	70	Left body
13	M	42	Right ramus/condyle
14	F	22	Right body/ramus/ condyle
15	M	17	Left body/ramus/ condyle
16	F	21	Bilateral body
17	F	27	Bilateral body/ condyle
19	F	30	Right body

TABLE 1 Identification numbers, genders, ages at presentation, and mandibular locations of tissue fragments available for DNA extraction

TABLE 2 Potential *M. tuberculosis* strain DNAs in different patients

Patient Number (Khullar et al., 2016)	Possible <i>M. tuberculosis</i> strains
8	RGTB327
9	RGTB423, RGTB327, KZN 605
11	str. Haarlem, KZN 605
13	RGTB327, 7199-99
14	CCDC5180, DK9897, EAI5
15	KZN 605, str. Haarlem
16	DK9897, M0018684-2
17	DK9897, CCDC5180, str. Haarlem
19	SCAID 252.0, str. Haarlem, M0002959-6

heated lid off, followed by addition of USER enzyme to the ligation mixture and incubating at 37°C for 15 min. Size selection of adaptor-ligated DNA was carried out using NEBNext sample purification beads with 0.4X for the 1st bead selection and 0.2X for the 2nd bead selection. After selecting the size of the nucleotide fragments, between 270 and 370 bp, PCR enrichment was done with NEBNext Ultra II Q5 Master mix, universal PCR primers, and different index primers for different patients (NEB #E7335). Five cycle steps, each step consisting of initial denaturation at 98°C for 30 s, denaturation annealing at 98°C for 10 s and 65°C for 75 s, were followed by a final extension at 65°C for 5 min. The products from the PCR reactions were cleaned with NEBNext sample purification beads with 0.9X, followed by 80% freshly prepared ethanol washing and 0.1X TE buffer elution. The final sequence libraries were diluted, and samples were run on a DNA high sensitivity chip bioanalyzer for quality check. Next-generation sequencing was carried out at the biopolymer facility of Harvard Medical School (<https://genome.med.harvard.edu>).

2.2 | Metagenome analysis

To analyze the whole-genome sequencing data of the 9 samples with both microbiota and human tissue DNAs, we used DIAMOND v0.9.26 (Buchfink et al., 2015) to align reads to NCBI-nr database and MEGAN 6 for taxonomy binning (Huson et al., 2016). To estimate the proportion of human DNA, we also used bowtie2 (Langmead & Salzberg, 2012) to align reads to the human hg19 genome. To identify possible *Mycobacterium tuberculosis* strains in each sample, we used MEGAN 6 to extract reads aligned to *M. tuberculosis*, and then used tblastx (BLAST 2.9.0+) to realign them to the genome sequences of 28 *M. tuberculosis* strains recorded in the BioCyc database. The results are listed in Table 2 below:

3 | RESULTS

The DNA isolated and amplified from the tissue fragments of all 9 patients contained a large number of bacterial and other sequences in addition to human sequences (Figures 1-9). About 50% of the DNAs represented a spectrum of sequences that differed between different patients. Analysis of the remaining 50% showed that bone fragments from all 9 patients contained DNA sequences of *Homo sapiens*, *Mycobacterium tuberculosis*, *Plasmodium ovale wallikeri*, *Staphylococcus aureus* and *hominis*, *Prevotella P3-120*, and *intermedia* (Figure 10). In addition, DNA from a group of four other bacteria, *Pseudomonas aeruginosa*, *Streptococcus thermophilus*, *Bordetella bronchiseptica*, and *Shigella sonnei*, was found in fragments from patients 9, 11, 15, and 19 and sequences from another group of four, *Tannerella forsythia*, *Treponema denticola*, *Porphyromonas gingivalis*, and *Fusobacterium nucleatum*, known for their association with periodontal damage, were present in material from patients 11, 13, 14 and 16 (Figure 10). As a consequence, the bone fragments from all 9 patients shared DNA of 10 species, except in the case of patients 8 and 17, who shared only 9 or 8. The DNA levels in fragments of all the 9 selected patients covered a range from 2% to 26% for *H. sapiens* DNA, 1 to 9% for *M. tuberculosis*, and 4 to 15% for *Plasmodium ovale wallikeri*. The range of *S. aureus* was 0.4%-2%, *S. hominis* 0.3%-1.9%, and *P. sp.* (*P3-120* and *intermedia*) 0.3%-4% (Figure 10).

In considering potential roles of the different bacteria in the distraction of jaw bones among the patients, we compared the levels of *Mycobacterium tuberculosis* and *Plasmodium ovale wallikeri* DNAs and found that they (with the exception of patient 11) could be clustered into two groups. In Group 1, consisting of patients 13, 14, 16, and 17 with ages ranging from 21 to 42 years, the *M. tuberculosis* DNA levels were 1%-3%. In Group 2, consisting of patients 8, 9, 15, and 19 and with ages ranging from 17 to 41 years, the *M. tuberculosis* levels were 5%-9%. Patients in the two groups had overlapping levels of *Plasmodium ovale wallikeri* DNA (6%-15% in Group 1 and 4%-9% in Group 2). Interestingly, when *M. tuberculosis* DNA levels in Groups 1 and 2 were compared with levels of *Staphylococcus aureus/hominis* DNA, tissues of Group 2 patients, with the highest levels of *M. tuberculosis* DNA, also had the highest levels of *S. aureus/hominis* DNA (Figure 11). DNA levels in tissue from patient 11 were unique in that *Plasmodium ovale wallikeri* DNA was at a relatively low level (4%), but *M. tuberculosis* DNA was among the highest (9%) among all 9 patients.

Bone fragments from Group 1 patients all had 1% *S. aureus* DNA, but no DNA for *P. aeruginosa* (except for patient 13), *S. thermophilus*, *B. bronchiseptica*, and *S. sonnei*. In contrast, patients in Group 2 and patient 11 had 2% *S. aureus* DNA as well as DNAs for *P. aeruginosa* (except for patient 8), *S. thermophilus*, *B. bronchiseptica*, and *S. sonnei*. Furthermore, material from patients in Group 1 and patient 11 contained DNA for *T. forsythia* (except for patient 17), *T. denticola*, *P. gingivalis*, and *F. nucleatum* (except for patient 17). Although this last group of bacteria (*T. denticola* - *F. nucleatum*) may represent late invaders into tissue that was already much destroyed, the mandibular

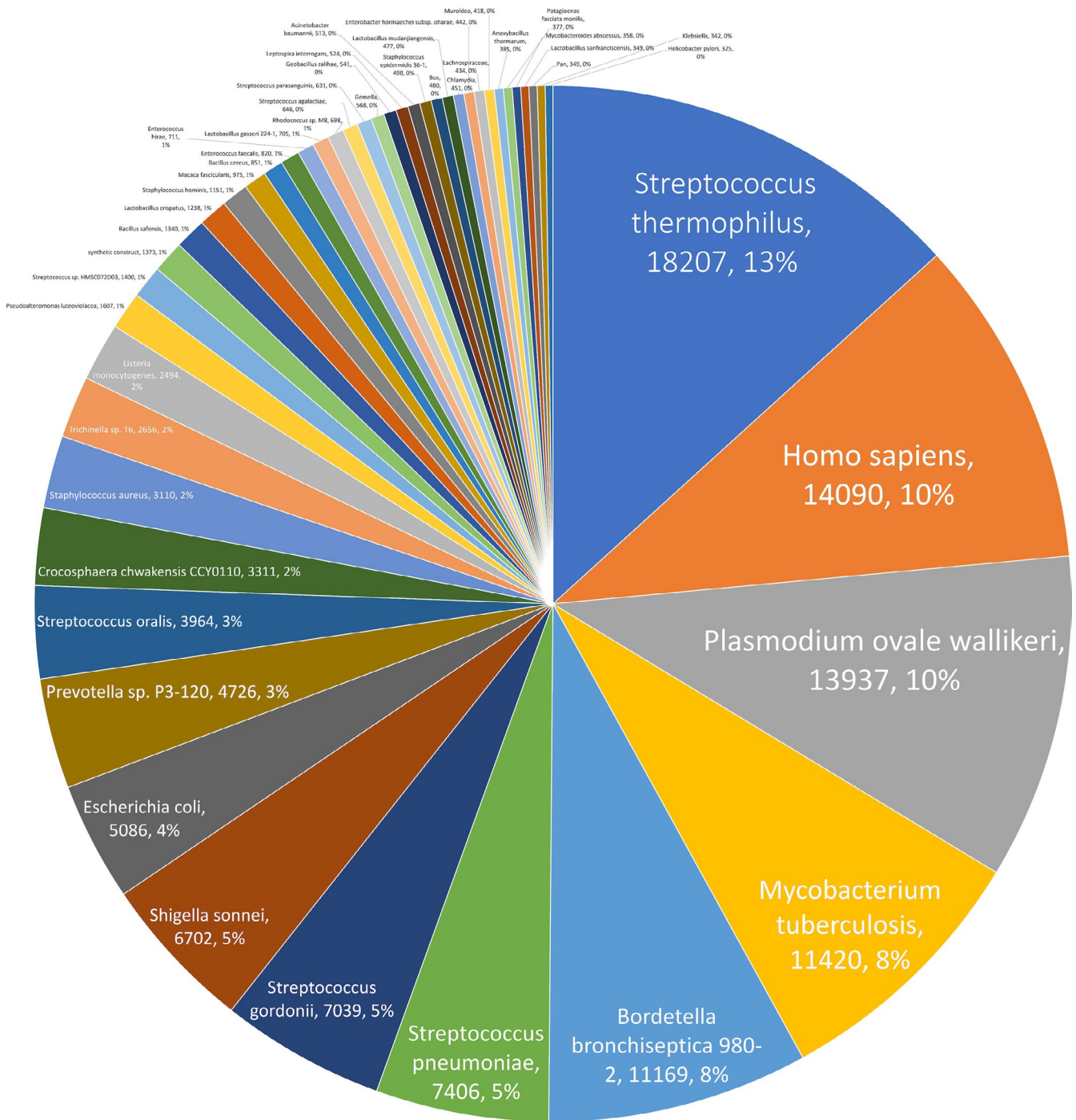


FIGURE 1 DNA data obtained from patient 8. The large number of sequences makes it difficult to read names of low-level components in Figures 1–9. Bar diagrams showing percentage levels of infectious elements from the top-down to not more than 1% of DNA from mandibular fragments of all 9 patients investigated are therefore provided in Supplemental Figures S1–S9. Vertical red arrows point to the 6 sequences shared by all patients. In addition, taxa data on all 9 patients with sequence reads are available upon contact with the corresponding author

bone of patient 11 clearly presented the most severe destruction and loss of human DNA among the 9 patients examined.

Plasmodium ovale, utilizing humans as the only natural host, causes a form of malaria that is endemic to western Africa, but rarely causes severe illness or death (Oguike et al., 2011). The prevalence is relatively low. Malaria and TB co-infections are also rare.

Based on recent data from the National Tuberculosis Treatment Centre in Uganda, co-infections are only at the level of 2.2% (Baluku et al., 2019). Therefore, the presence of *Plasmodium ovale* DNA (4% to 15%) in bone fragments of all 9 patients and the correlation between the levels of *M. tuberculosis* and the *Plasmodium ovale wallikeri* DNAs in samples from Groups 1 and 2 patients raise the question of

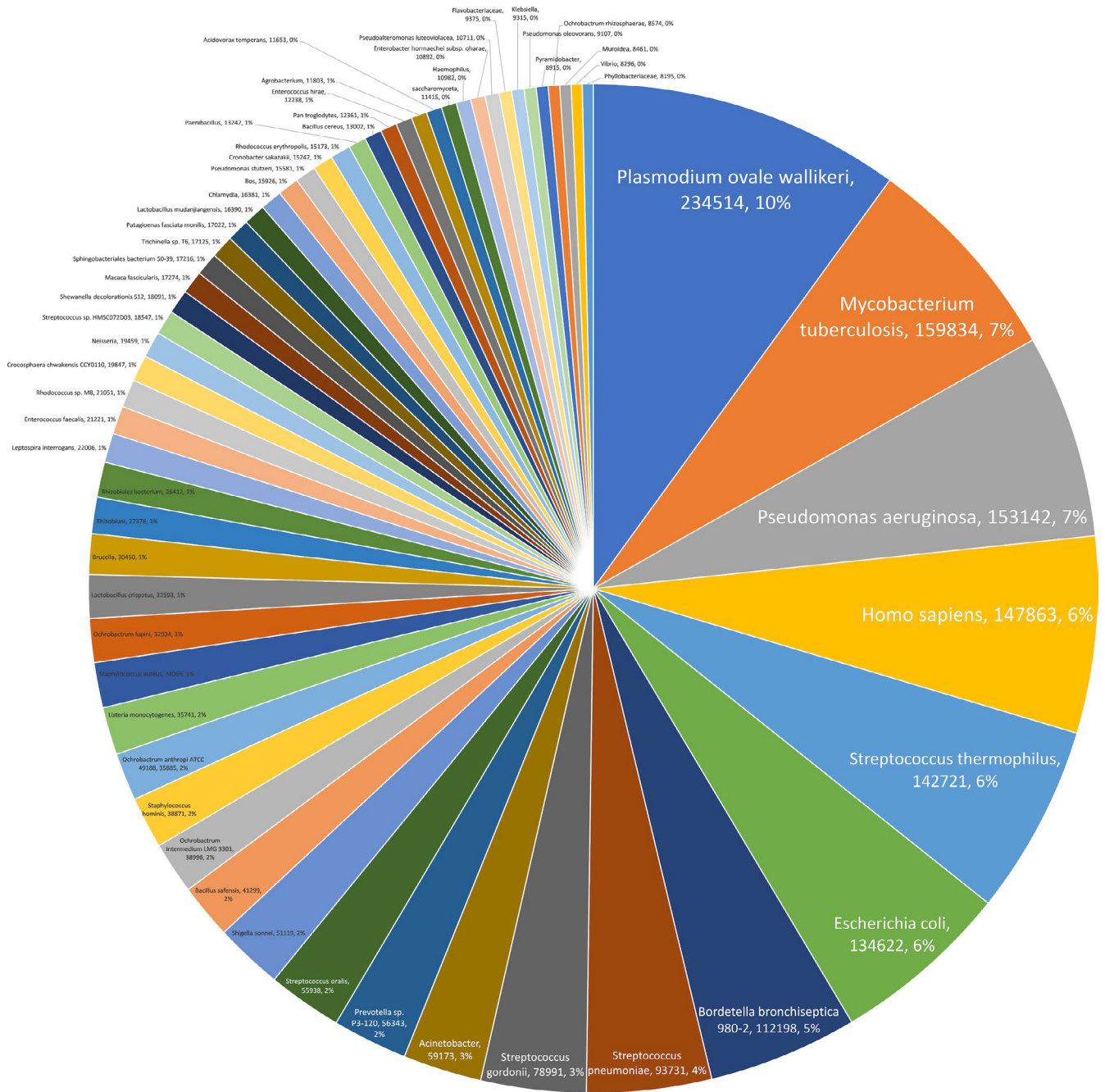


FIGURE 2 Data obtained from patient 9

whether interactions between the two could have a role in this type of jaw bone destruction.

One possibility may be related to *Plasmodium ovale wallikeri*-dependent destruction of capillaries in jaw bones infected by *M. tuberculosis*. Another possibility is based on the finding that *P. falciparum*-derived hemozoin-laden monocytes may contribute to exacerbation of mycobacterial infection both in vitro and in vivo (Hawkes et al., 2010). With only the *wallikeri* strain detected in the DNA sequences of all 9 patients, a recent study of the genes encoding the *circumsporozoite protein (ctsp)*, the *circumsporozoite surface protein (csp)*, and *merozoite surface protein1(msp1)* of *P. ovale wallikeri* and

curtisi is relevant. The data reveal a clear difference between Th2R and Th3R haplotypes in the two strains (Saralamba et al., 2019). This raises the possibility of strain-specific immune cell effects between the two strains, such that *P. ovale wallikeri* may specifically stimulate TB-based bone destruction.

Compared with other types of Mycobacterial-dependent bone loss, a combination of *M. tuberculosis* and additional factors is also consistent with the severe destruction of mandibular bones in the patient cohort. In addition to the data described above, histopathological features of extensive areas of empty lacunae in mandibular bone fragments indicated osteocyte destruction beyond the macrophage- and

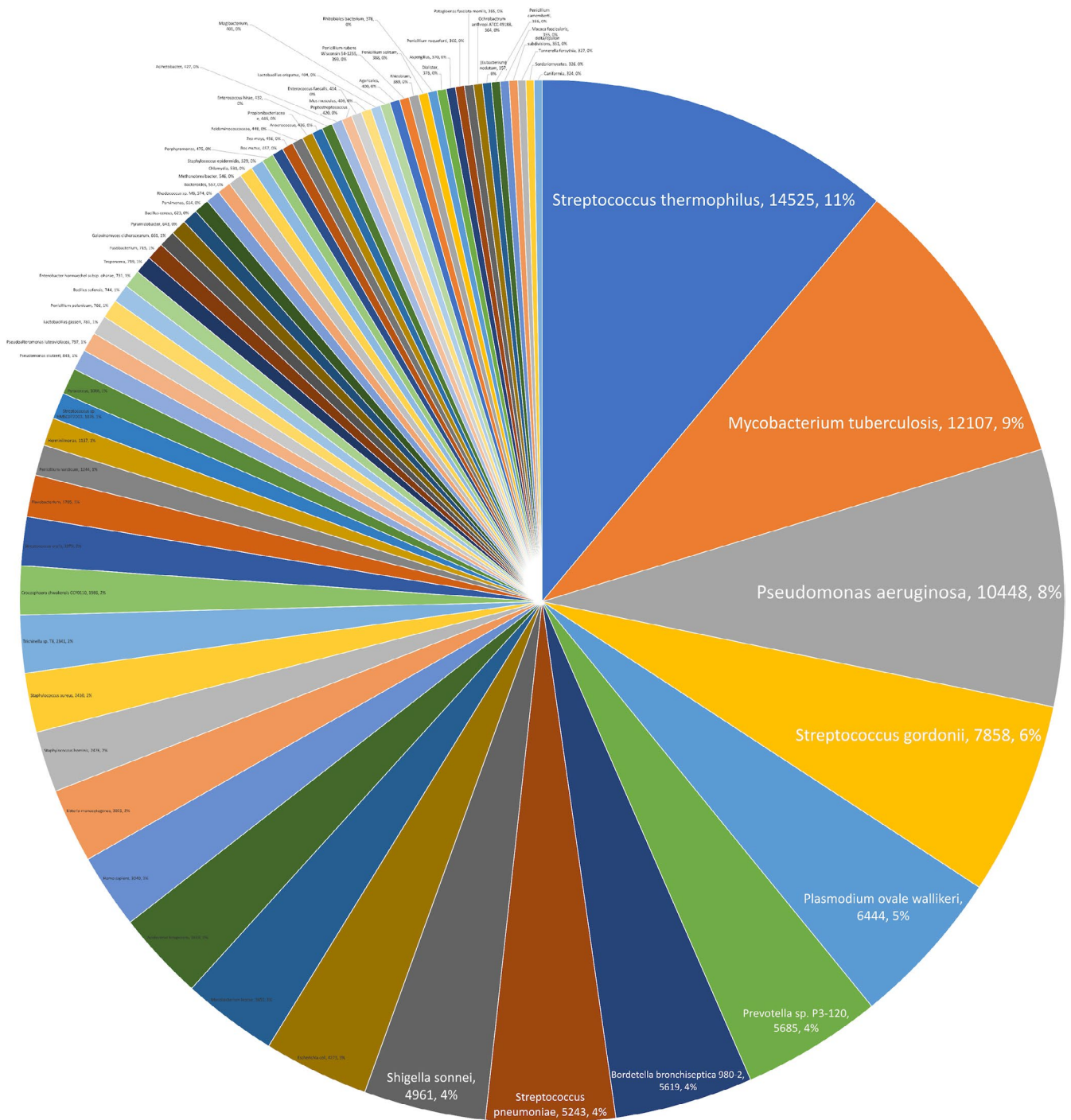


FIGURE 3 Data obtained from patient 11

osteoclast-induced surface damage caused by *M. tuberculosis* alone (Khullar et al., 2016). The presence of relatively high levels of *S. aureus* DNAs in the bone from patients in Group 2 and in patient 11 and the highest levels of *M. tuberculosis* DNA (Figure 11) suggest that a correlation between the two bacteria may be a significant contributor to the damage. This is strongly supported by a statistical test using the Pearson correlation coefficient ($p = .000588, r = 0.913$). Finally, the levels of DNA from *Prevotella species* in fragments from all 9 patients are also correlated with levels of *M. tuberculosis* (Figure 12), as supported

by the Pearson correlation coefficient ($p = .00112, r = 0.895$), suggesting that *Prevotella sp.* may also be contributors.

These data are consistent with the possibility that mandibular bone destruction may have been accelerated by *S. aureus* and *Prevotella sp.* Being part of the normal microbial flora in 20%–50% of the population and usually existing in harmony on the skin or mucous membranes of the host, *S. aureus* may enter into blood vessels following a breach of the epithelial barrier in jaw bones, attach to exposed vulnerable surfaces and work with *M. tuberculosis* to destroy

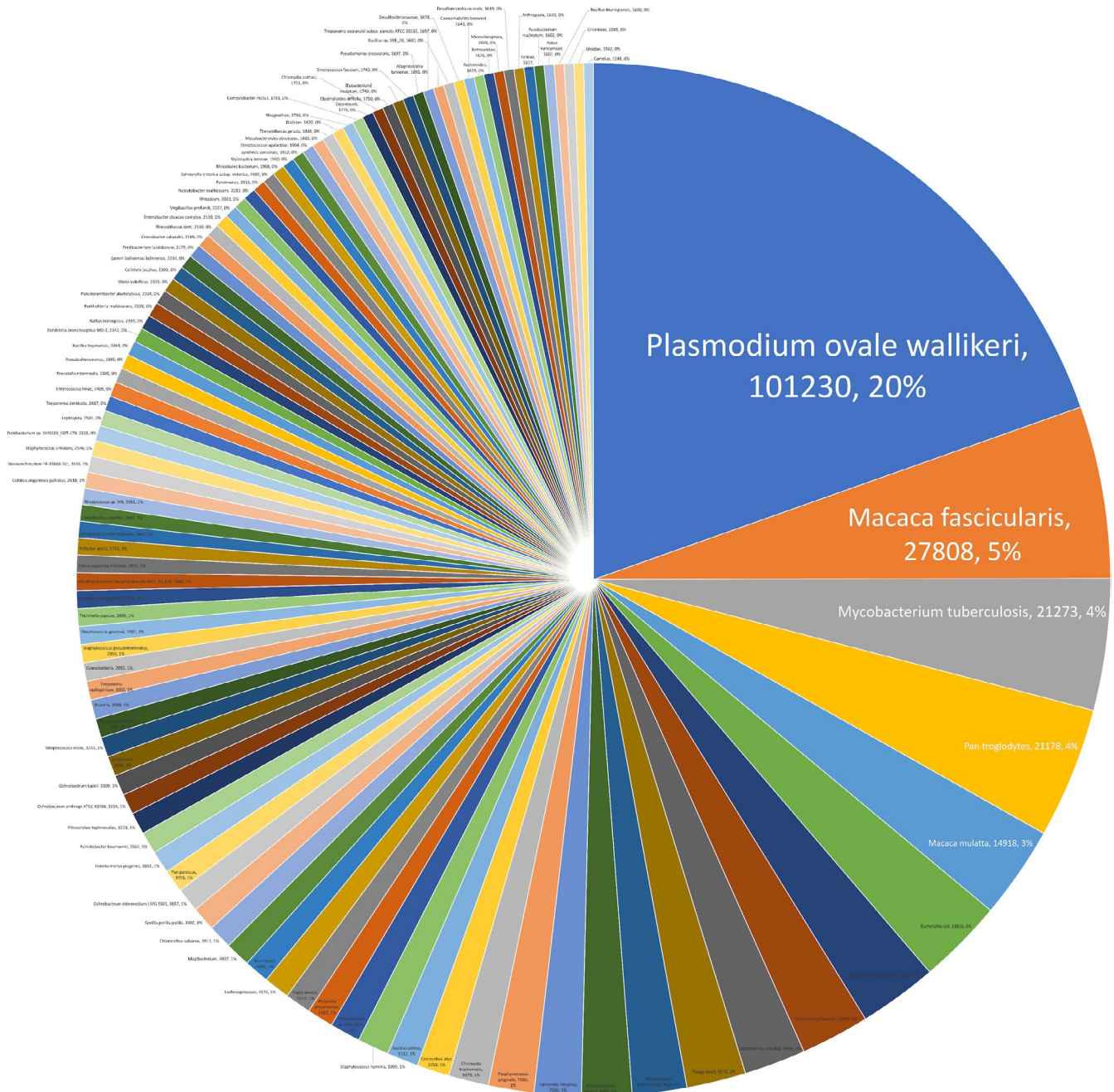


FIGURE 4 Data obtained from patient 13

bone (Figure 13). Binding to fibronectin in the extracellular matrix and interacting with integrins on cell surfaces allow *S. aureus* to enter into osteoblasts and osteocytes, in which it can survive for extended periods of time.

This resident behavior has been examined both experimentally and in patients (Proctor et al., 2006; Yang et al., 2018). *S. aureus* induces osteoclast formation by stimulating expression of *NFATc1*, *RANKL*, the *MCSF* receptor, and *MCSF* (Brandt et al., 2018; Krauss et al., 2019), and it can induce osteoblast and osteocyte apoptosis; thus leaving empty lacunae in the bone similar to what we have detected in sections of bone fragments from the patients.

4 | DISCUSSION

The data obtained in this study provide direct evidence to support the conclusion that *M. tuberculosis* plays a major role in this particular type of jaw bone destruction (Khullar et al., 2016). The finding that DNAs from five different sources, *M. tuberculosis*, *P. ovale wallikeri*, *S. aureus*, *S. hominis*, and *Prevotella P3-120/intermedia*, in addition to *H. sapiens*, are present in bone fragments from all 9 patients, indicates that several pathological elements contribute to this unique oral disorder. The data do not provide sufficient evidence to fully explain how the process was initiated, but one possibility is that it may have started with *M. tuberculosis* entering the mandibular bone

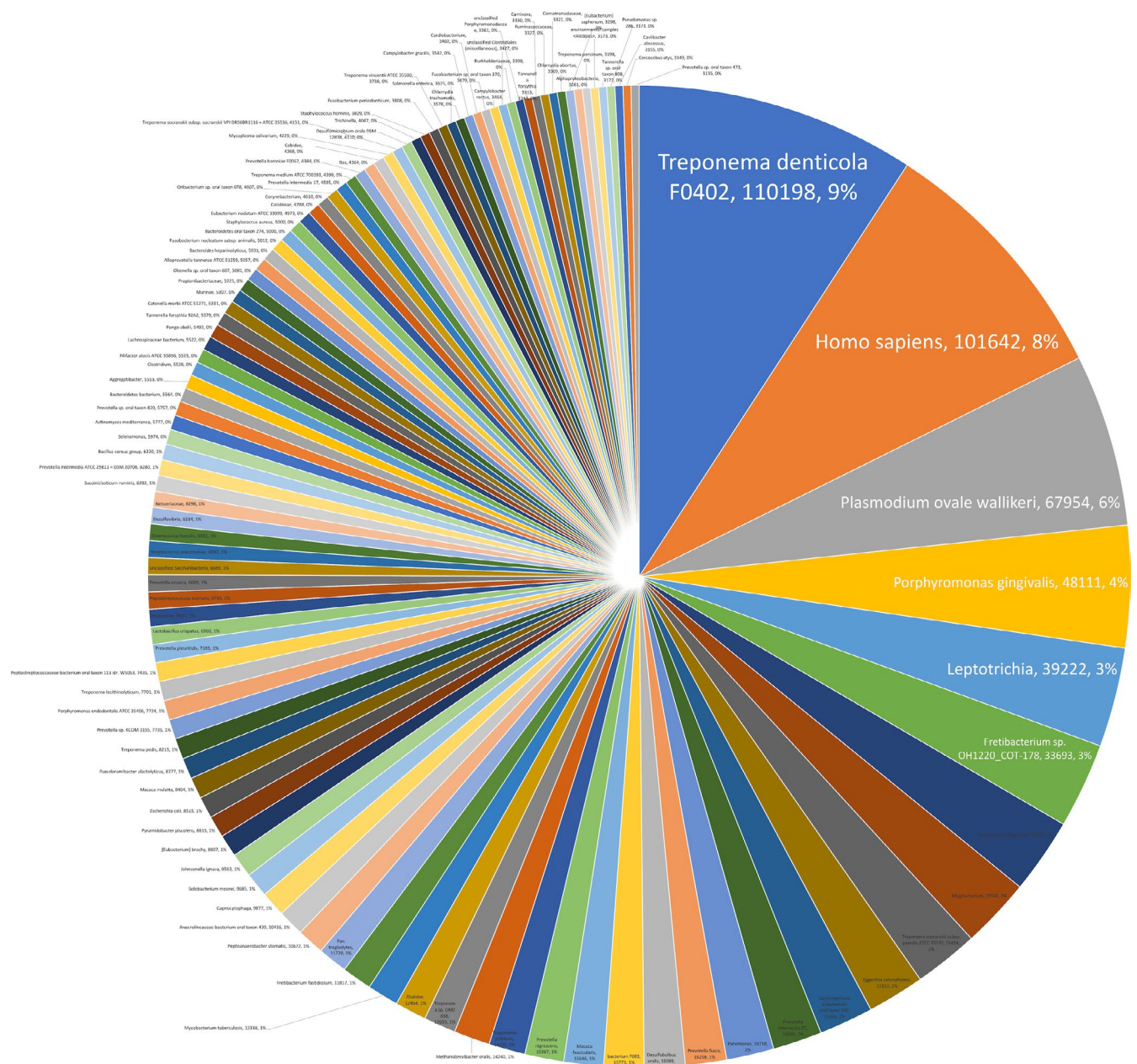


FIGURE 5 Data obtained from patient 14

via a hematogenous or lymphatic route. The mandible is known to have poor blood supply, while the condyle, with its superior blood supply, is often spared. Thus, hematogenous spreading of mycobacteria, leading to mandibular bone degradation, is a reasonable hypothetical mechanism to consider for initiation of bone loss (Anatomy & third edition, 1987; Castelli, 1963; Lanigan & West, 1990; Lownie et al., 1980). The spreading to cervical lymph nodes and then to the mandible from the hilar lymph nodes is made possible via the lymphohematogenous route (Heney et al., 1988). A similar involvement of *M. tuberculosis* in osteoarticular regions and in long bones is most commonly found in children and the elderly and is generally secondary to hematogenous seeding. However, the most frequent sites of bone involvement are the vertebrae (Pott's disease)

and the proximal extremities of long bones (Gunasekera et al., 2008; Tuli, 2002).

Although interesting, these possibilities do not provide an explanation for why the target for severe bone destruction by *Mycobacterium tuberculosis* was the mandible in the cases described. They also do not explain the high levels of *Plasmodium ovale wallikeri* DNA in the affected bone targets in all the patients. Not only are the patients infected with *Plasmodium ovale*, but they all have the *wallikeri* strain and not *curtisi*. *P. ovale* malaria is a clinically mild disease compared to that of *P. falciparum*, but it may still be able to induce dysregulation of inflammatory mechanisms that exacerbates proliferation and the effects of *M. tuberculosis*, similar to what may happen with *P. falciparum* (Hawkes et al., 2010).

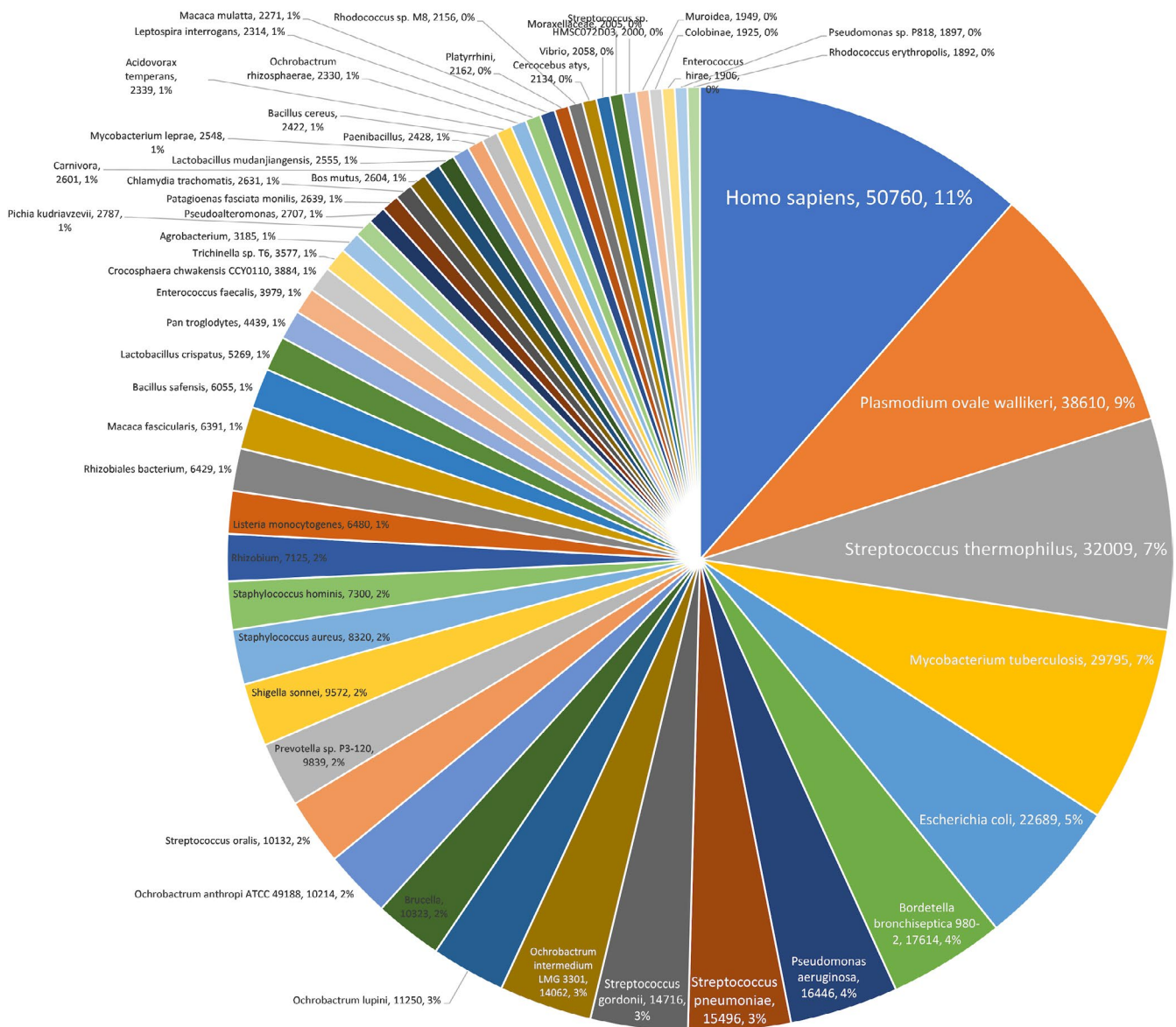


FIGURE 6 Data obtained from patient 15

Plasmodium ovale malaria rarely causes severe illness or death (Mace et al., 2018), but the erythrocytic changes elicited by *P. ovale* merozoites are somewhat similar to those of sickle cell anemia and may promote avascular osteonecrosis. This raises the possibility that the initiation of the unusually severe phase of this mandibular bone loss disorder was caused by the combination of *P. ovale wallikeri* and *M. tuberculosis* infections in patients that at the start may have had periodontal inflammation with activation of *S. aureus/hominis* and *Prevotella P3-120/intermedia*. In the West African countries from which the patient cohort was collected (Khullar et al., 2016), *P. ovale* and *M. tuberculosis* infections are unfortunately relatively common. Thus, an early mandibular periodontal inflammation may have provided conditions for the local capillary accumulation of *P. ovale* merozoite-containing reticulocytes and the local growth of *M. tuberculosis* bacteria. Infected reticulocytes would have started an erythrocytic cycle in which merozoites are released and invade

other erythrocytes (Collins & Jeffery, 2005). One of the consequences may have been packing of infected red blood cells in bone capillaries and promotion of avascular osteonecrosis. When considered in conjunction with the *P. ovale wallikeri* strain-specific effects on immune cells that in turn stimulate *M. tuberculosis*-dependent bone destruction, described in the Results section, we propose that *P. ovale wallikeri* specifically helped stimulate the bone destruction together with *M. tuberculosis*.

The negative effects on bones are also associated with inflammatory responses that include TNF- α and IL-1 (Kodaira et al., 2000). Linked to localized and systemic mucosal diseases, *Prevotella* activates TLR2 in dendritic cells, promotes Th17 immune responses, and stimulates bone loss and tissue destruction (Larsen, 2017). *Prevotella intermedia* has also been considered by some investigators to play a key role in noma lesions (Baratti-Mayer et al., 2003; Enwonwu et al., 2000; Fieger et al., 2003; Huyghe et al., 2013).

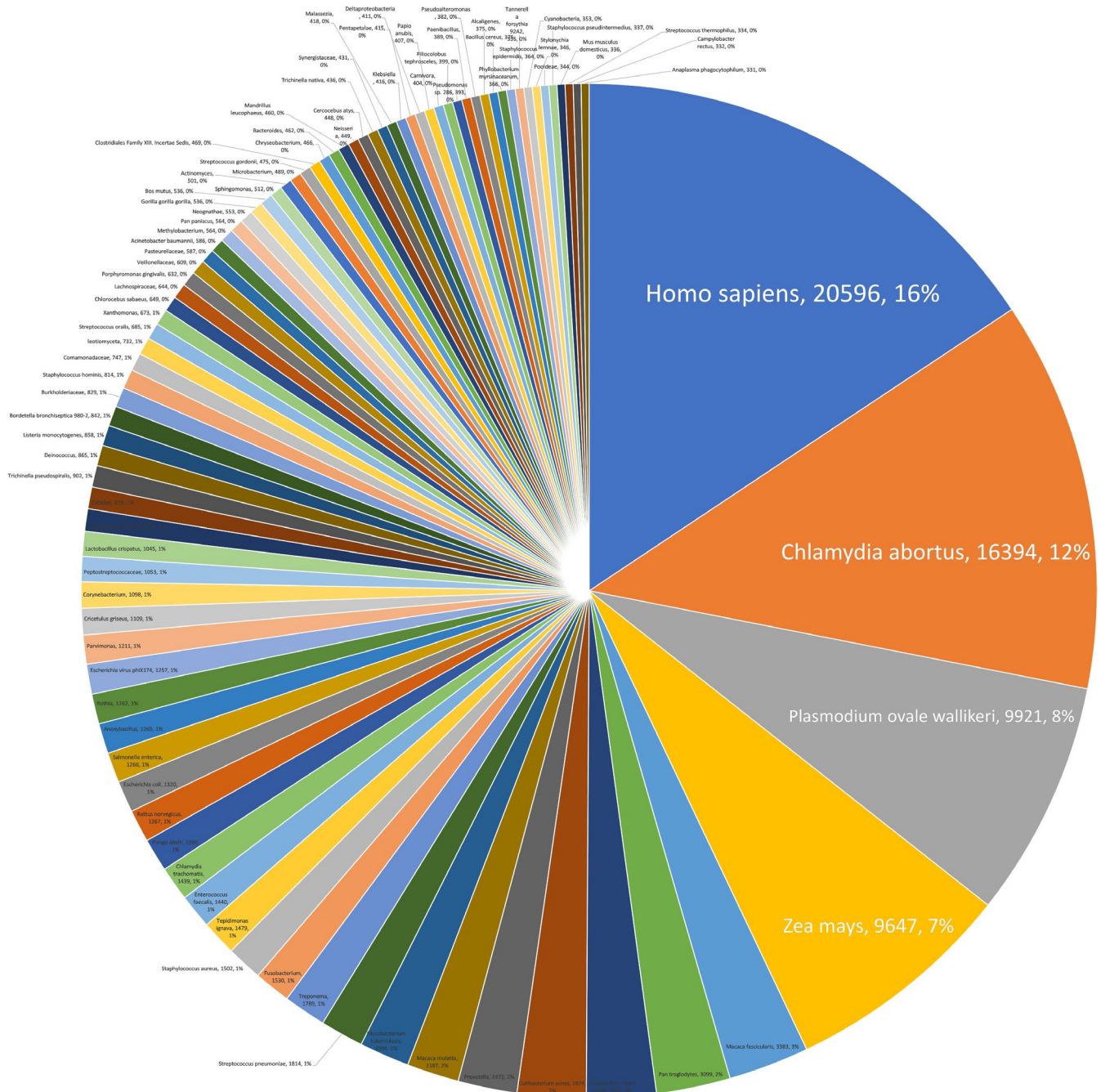


FIGURE 7 Data obtained from patient 16

The bone fragments of patient 11 and patients in Group 2 (8, 9, 15, and 19) contained DNAs for *P. aeruginosa*, *S. thermophilus*, *B. bronchiseptica*, and *S. sonnei*. The fragment of patient 8 had DNA from only three of the four bacteria, but the other four patients had relatively high levels of all four (Figure 10). Interestingly, together with patient 11, all samples from this group contained DNAs for *S. thermophilus*, but they ranged from 13% in patient 8 to 3% in patient 19.

Although these differences could reflect stages in the destructive process, a more likely explanation is probably that it may be a consequence of different bone fragments available for DNA extraction.

Images of patient 9 at the time of dental care demonstrate severe inflammation and substantial loss of mandibular bone on his right side, consistent with high levels of bacterial and plasmodium DNAs and low levels of *Homo sapiens* DNA (Figure 14).

Bone fragments of patients in Group 1 (13, 14, 16, and 17), with lower levels of *M. tuberculosis* DNA, had no DNA from *P. aeruginosa*, *S. thermophilus*, *B. bronchiseptica*, and *S. sonnei*. Instead, they had DNAs for *Tannerella forsythia*, *Treponema denticola*, *Porphyromonas gingivalis*, and *Fusobacterium nucleatum*. The levels of these DNAs varied from less than 1% up to more than 9% (in the case of *Treponema denticola* in patient 14). The data provide no obvious reason for these

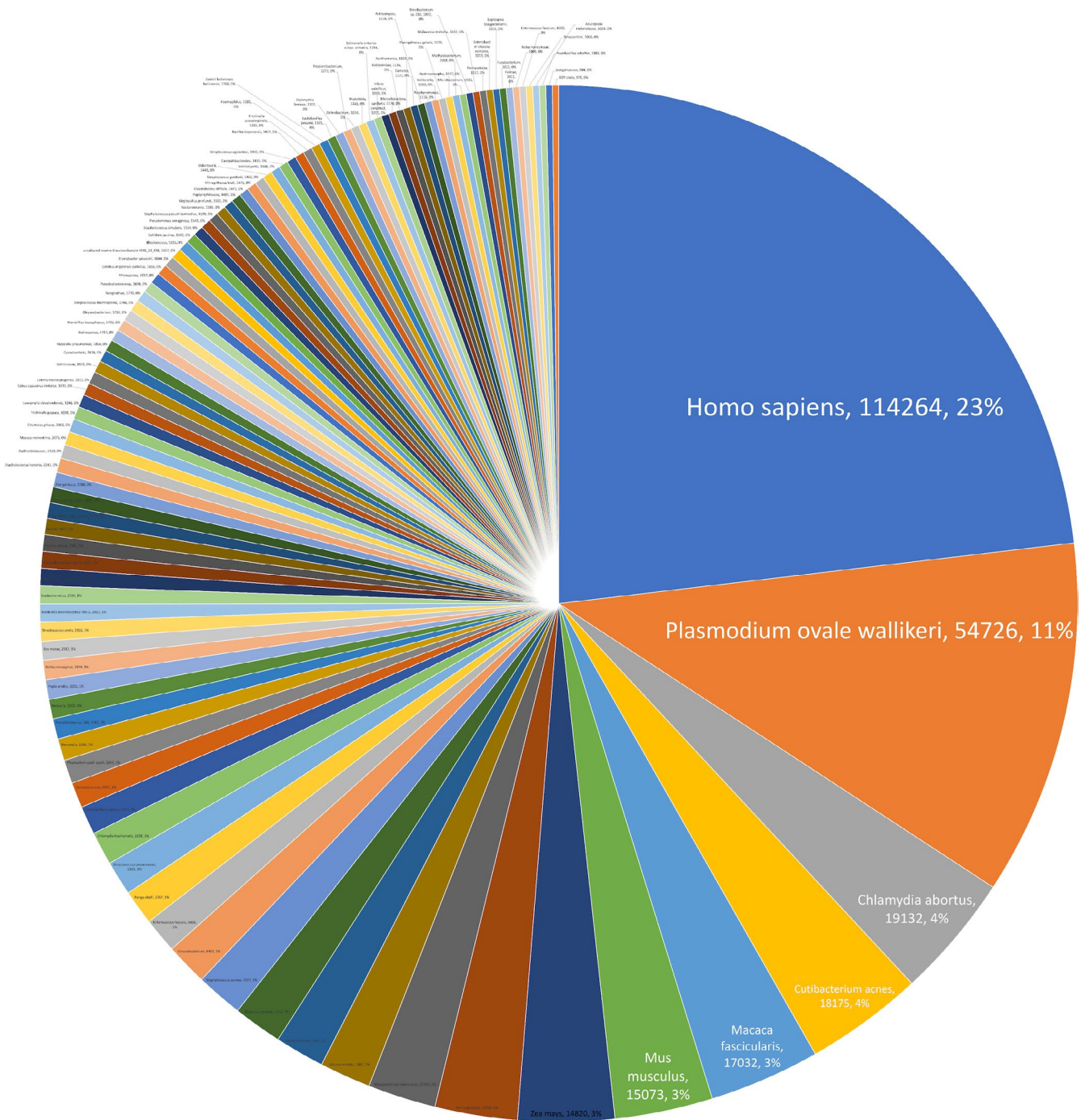


FIGURE 8 Data obtained from patient 17

differences between groups of patients, but the mandibular destruction process in patients of Group 1 appears to be as efficient as what happens in Group 2 patients.

Although infection of bone secondary to *Fusobacterium nucleatum*, identified in the lesions of patients 11, 13, 14, and 16, is rare, there are reports describing hematogenous osteomyelitis due to *F. nucleatum* in vertebrae (Freedman & Cashman, 1979; Ramos et al., 2013), pelvis (Beauchamp & Cimolai, 1991), and long bones (Lee et al., 2012). DNA-DNA hybridization performed on deep bony

lesions showed anaerobic bacteria with a predominance of *P. gingivalis*, as well as *Actinomyces* species, *Prevotella* species, and *F. nucleatum*. *F. nucleatum* has also been identified in osteoradionecrosis bone lesions (Støre et al., 2005).

Periodontal damage associated with *T. forsythia*, *P. gingivalis*, and *T. denticola*, together with *P. intermedia* and *F. nucleatum*, can degrade the basal lamina between the junctional epithelium and the tooth (Fouillen et al., 2019; Ksiazek et al., 2015; Sharma, 2010). This may allow access of *S. aureus* and *M. tuberculosis* to mandibular

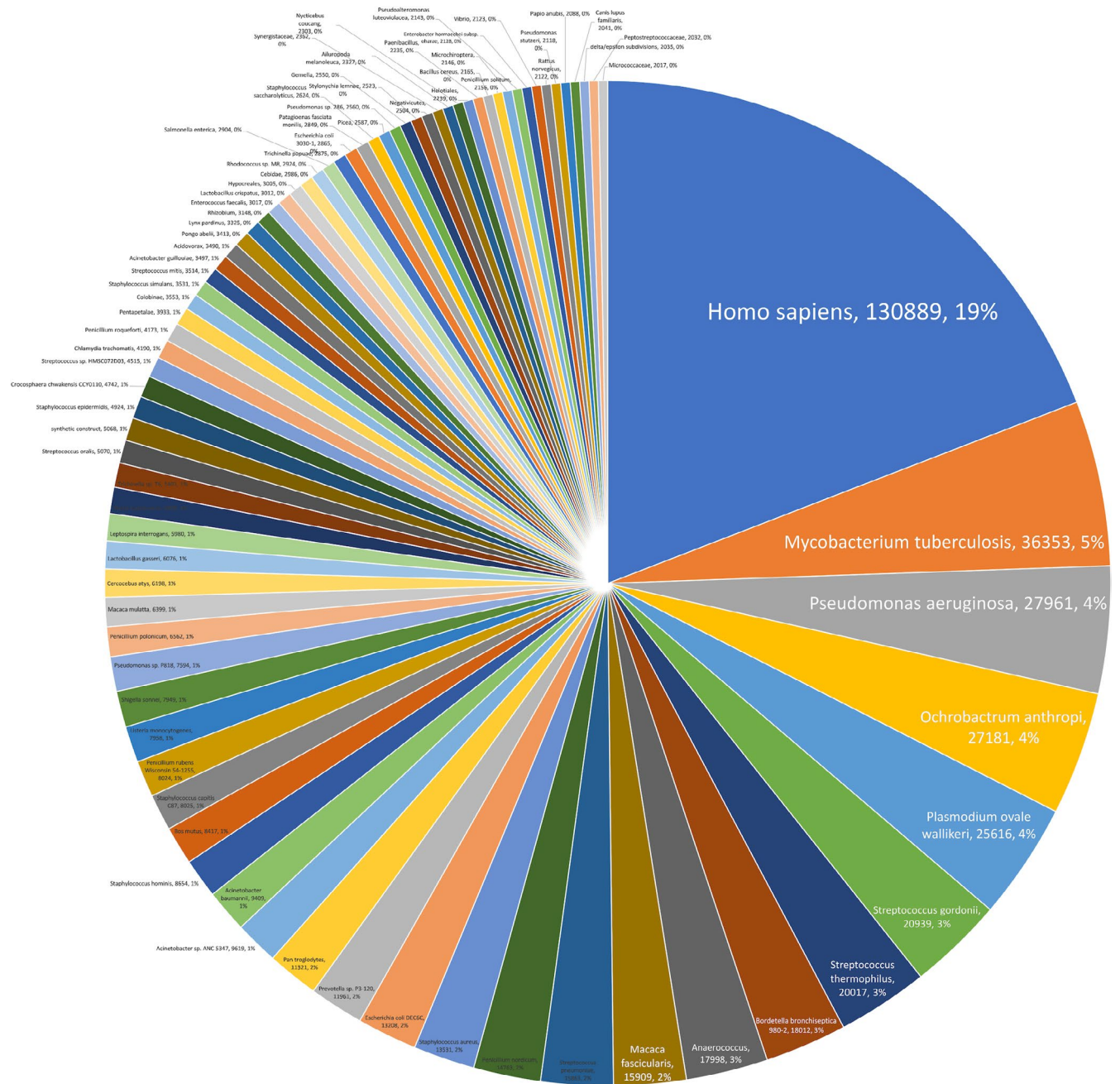


FIGURE 9 Data obtained from patient 19

and maxillary bone. Numerous other bacterial factors have similar effects via a variety of mechanisms. For instance, proteins from *P. gingivalis* directly regulate production of RANKL and osteoprotegerin in human periodontal ligament cells and gingival fibroblasts, increasing stimulation of osteoclastogenesis (Belibasakis et al., 2007). Destructive periodontitis is associated with Th1-Th17 immune responses and activation of RANKL-induced osteoclasts. Polymicrobial intraoral inoculation of *P. gingivalis*, *T. denticola*, and *T. forsythia* has been shown to cause high levels of bone resorption (Mysak et al., 2014).

Therefore, although the initiation of the mandibular bone loss in the patients described in this case may have included *M. tuberculosis* and *P. ovale wallikeri*, multiple bacteria appear to have contributed to the subsequent severe destruction. This possibility is consistent with the images of patient 14 at the time of dental care, demonstrating a dramatic purulent destruction of the mandible on the right side (Figure 15). This destruction also explains the low DNA levels of *M. tuberculosis*, *P. ovale wallikeri* in bone fragments of this patient.

During the early stages of the disorder, *M. tuberculosis* would have stimulated synthesis and secretion of TNF- α and IFN- γ by

Patients	<i>Homo sapiens</i>	<i>Mycobacterium tuberculosis</i>	<i>Plasmodium ovale wallikeri</i>	<i>Staphylococcus aureus</i>	<i>Prevotella hominis</i>	<i>Prevotella P3-120, intermedia</i>	<i>Pseudomonas aeruginosa</i>	<i>Streptococcus thermophilus</i>	<i>Bordetella bronchiseptica</i>	<i>Shigella sonnei</i>	<i>Tannerella forsythia</i>	<i>Treponema denticola</i>	<i>Porphyromonas gingivalis</i>	<i>Fusobacterium nucleatum</i>
8	10%	8%	10%	2%	0.8%	3%	0%	13%	8%	5%				
9	8%	9%	13%	2%	1.7%	3%	8%	8%	6%	3%				
11	2%	9%	5%	2%	1.9%	4%	8%	11%	4%	4%	0.25%	0.55%	0.36%	0.54%
13	26%	3%	15%	1%	0.7%	0.3%	2%	0%	0%	0%	1.09%	0.44%	1.05%	0.23%
14	8%	1%	6%	0.4%	0.3%	2%	0%	0%	0%	0%	0.45%	9.19%	0.64%	0.42%
15	11%	7%	9%	2%	1.7%	2%	4%	7%	4%	2%				
16	16%	2%	8%	1%	0.6%	2%	0%	0%	0%	0%	0.27%	1.36%	0.48%	1.16%
17	23%	2%	11%	1%	0.5%	0.7%	0%	0%	0%	0%		0.23%	0.20%	
19	19%	5%	4%	2%	1.3%	2%	4%	3%	3%	1%				

FIGURE 10 Percentage levels (in black) of DNAs from *Homo sapiens* to *Prevotella P3-120,intermedia*, are shared by all 9 patients; DNAs from *Pseudomonas aeruginosa* to *Shigella sonnei* are shared by some patients, 0% indicates that levels were below 1%; percentage levels (in blue) DNAs from *Tannerella forsythia* to *Fusobacterium nucleatum*, also shared by some patients (empty boxes indicate that DNA was not detected). Note that DNA from patient 11 (framed by red lines) covered all the bacteria in the figure. Percentage levels (in black) of DNAs from *Homo sapiens* to *Prevotella P3-120, intermedia*, are shared by all 9 patients; DNAs from *Pseudomonas aeruginosa* to *Shigella sonnei* are shared by some patients, 0% indicates that levels were below 1%; percentage levels (in blue) DNAs from *Tannerella forsythia* to *Fusobacterium nucleatum*, also shared by some patients (empty boxes indicate that DNA was not detected). Note that DNA from patient 11 (framed by red lines) covered all the bacteria in the figure

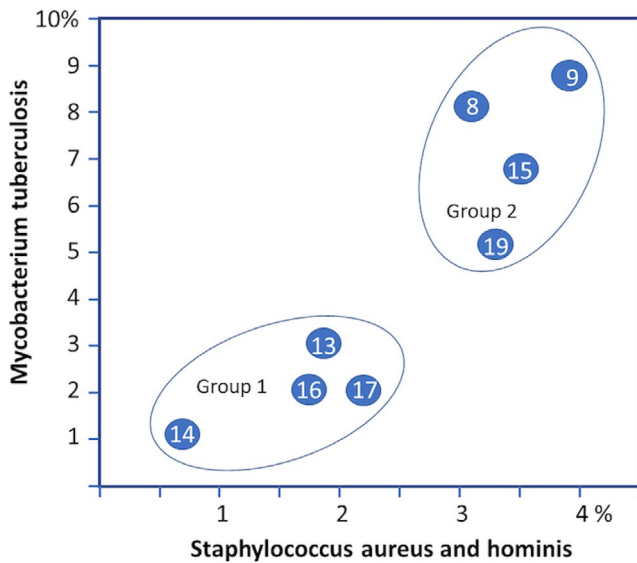


FIGURE 11 Diagram showing the percentage levels of *M.tuberculosis* and *S.aureus* and *hominis* in Group 1 and Group 2

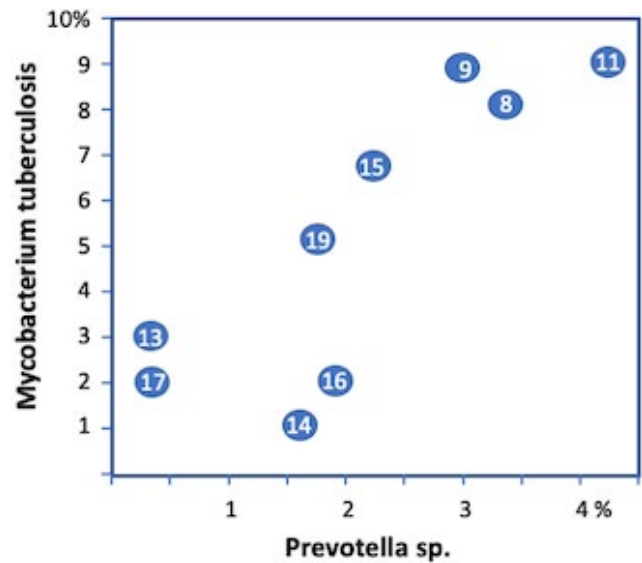
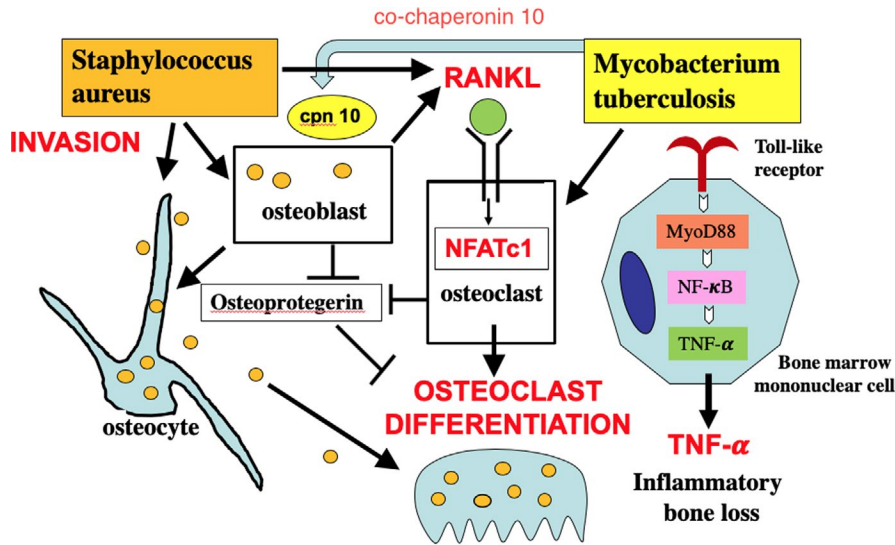


FIGURE 12 Diagram showing the percentage levels of *M.tuberculosis* and *Prevotella P3-120/intermedia* for all 9 patients

bone macrophages as part of the host defense against the invader. The subsequent increased levels of RANKL and decreased osteoprotegerin expression by osteoblasts would result in increased expression of NFATc1 and differentiation of osteoclasts. These mechanisms (Li et al., 2016) have a stark similarity to the pathogenetic dysregulation underlying loss of jaw bones in the genetic disorder known as cherubism (Ueki et al., 2007; Yoshitaka

et al., 2014). However, since this infectious jaw bone destruction is not a genetic disorder, an early identification of the underlying mechanisms, such as the results of a combination of *M. tuberculosis* and *P. ovale wallikeri* infections, would have provided an outstanding opportunity for preventing the subsequent destructive outcomes that made life difficult and painful for the patients involved.



Osteoblast, osteocyte and osteoclast destruction by *S. aureus* and *M. tuberculosis*

FIGURE 13 Diagrams showing how *M. tuberculosis* and *S. aureus* can induce inflammatory bone loss, increase osteoclast differentiation, and destroy osteoblasts, osteocytes, and osteoclasts



FIGURE 14 Images of patient 9, obtained with patient consent at the time of dental care, showing severe inflammation combined with loss of mandibular bone



FIGURE 15 Images of patient 14, obtained with patient consent at the time of dental care, demonstrating a dramatic purulent destruction of the mandible on the right side

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R. Olsen came from the NIH-NIAMS grant AR072192 to Bjorn R. Olsen.

Keith Chapman and Dag Tvedt have given their consent to be acknowledged:

(1) Sun 5/2020 12:25 p.m.

Dr. Olsen, Thank you so much for your continued work on this research. I give my full consent for acknowledgement in your upcoming manuscript. Please let me know if anything else is needed. Thanks again. I will be in Boston in September so I will reach out to you before then and see if I might pay you a short visit and meet face to face. Maybe then we'll all feel comfortable shaking hands again!

All the best,

Keith Chapman

(2) Sun 5/10/2020 6:59 a.m.

Dear Prof Olsen. I am happy to learn that the next article on our osteo patients from West Africa is about to be published.

According to Shelly you need my consent. I do consent to being acknowledged in your manuscript for "Oral Diseases."

Regards,

Dag Tvedt.

CONFLICT OF INTEREST

All the authors state that they have no conflict of interests.

AUTHOR CONTRIBUTIONS

Xianrui Yang: Data curation; Formal analysis; Investigation; Methodology; Software; Visualization; Writing-original draft; Writing-review & editing. **Adam Yongxin Ye:** Data curation; Methodology; Resources; Software; Writing-review & editing. **Negin Katebi:** Investigation; Methodology; Writing-review & editing. **Vladimir Volloch:** Investigation; Methodology; Writing-review & editing. **Shelley M. Khullar:** Investigation; Resources; Visualization; Writing-review & editing. **Vinod Patel:** Writing-original draft; Writing-review & editing. **Bjorn R Olsen:** Conceptualization; Funding acquisition; Investigation; Project administration; Resources; Supervision; Visualization; Writing-original draft; Writing-review & editing.

ETHICAL APPROVAL

Approval regarding the use of patient material for the previous 2016 study of 19 patients, from which samples of 9 patients were used for DNA sequencing in the current project, was negotiated by Mercy Ships with the host countries. Ethical exemption for laboratory examination of the 19 tissue samples was given by Harvard University Faculty of Medicine, Committee on Human Studies (CHS study number M21590-101). Signed patient consent was obtained for all 19 individuals participating in the original 2016 study.

PEER REVIEW

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ORCID

Bjorn R. Olsen  <https://orcid.org/0000-0001-6070-231X>

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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