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Abstract

The health of humans is intricately linked to the substances - both food and non-dietary items - we ingest. Adverse health outcomes related to smoking of products like tobacco and other psychoactive substances are clearly established in modern populations but are less well understood for ancient communities. Grasping these dynamics is further complicated by the curative, religious, and medicinal context of many of these substances, which have often been commodified, refined, and altered in recent history. As part of a larger collaboration with the Muwekma Ohlone Tribe dedicated to understanding medicinal plant use among native Californians, we present a summary of new metabolomic data from three Middle and Late-period ancestral heritage Ohlone sites: *Thámien Rúmmeytak* (CA-SCL-128), *'Ayttakiš 'Éete Hiramwiš Trépam-tak* (CA-ALA-677/H/H), and *Sii Tuupentak* (CA-ALA-565/H/H). Using a UPLC-MS platform, we analyze chemical residues from 95 human dental calculus samples from 50 burials. Employing multivariate statistics, we co-analyzed demographic and skeletal pathology data with chemical residue profiles. We considered skeletal markers for a series of oral and postcranial health conditions. Results indicate sex and age biases in consumption patterns. Periodontitis stands out as the most significant local factor for changes in the oral metabolome. However, while chemical markers of oral diseases may be related to pathogen activity, associations between residues and postcranial conditions such as osteoarthritis suggest traditional curative practices and the ingestion of medicinal substances. Hence, our study yields new insights into the broader context of illness and healing in the past.

Introduction

To reconcile the concepts of "stress" and "health", Reitsema and McIlvaine (2014) argue that anthropologists are uniquely positioned to study the socioeconomic and environmental variables affecting human health. In the same volume, Klaus (2014) explains how pathophysiologic and molecular signaling mechanisms inform our understanding of skeletal lesions. Here, we offer new insights into the use of biomolecular signatures with a focus on the medicinal practices past societies employed to deal with adverse health conditions. More specifically, the metabolomic study of residues extracted from human dental calculus is an emerging field that joins approaches such as the analysis of the oral microbiome, the examination of enamel isotope ratios, and the observation of hypoplasia, wear patterns, and several pathologies to generate new data on past human health and life conditions based on preserved dentitions (Radini et al. 2017).

In a first untargeted study of the human oral metabolome, Velsko et al. (2017) extracted a series of compound categories including consumption-derived xenobiotics from dental calculus. Comparing present-day (n=5) and historic (n=12) European populations, the study demonstrated the effects of differential preservation among compound classes with lipids exhibiting a far greater presence in samples recovered from buried individuals. Despite the loss of metabolites due to degradation, multivariate statistics of historic profiles led to significant associations with sex, age, and health conditions, most notably periodontitis. Sørensen et al. (2021) on the other hand, stress that the extraction of over one hundred biomarkers of drugs from ten forensic calculus samples surpassed the metabolite recovery rate from blood samples of the same individuals. In sum, although taphonomic processes and historic time scales adversely affect chemical residues captured in dental calculus, preserved signatures can provide valuable insights into matters such as substance consumption and disease condition.

The most notable advantage offered by dental calculus studies is the potential to elucidate these phenomena on an individual level. Compared to artifacts which might or might not be associated with specific users, the analysis of plaque deposits allows us to examine the role of biocultural factors such as sex and age, status, or ethnicity. For Native California, for example, ethnographic and ethnohistoric accounts (e.g., Harrington 1932; Kroeber 1941) indicate that the Ohlone and their neighbors smoked tobacco (*Nicotiana* sp.) in pipes, but also chewed or ate tobacco combined with lime. Our team's previous research with clay and stone pipes shows that tobacco was smoked at least 1100 years ago in California (Tushingham et al. 2013; Tushingham and Eerkens 2016). Dental calculus studies, in turn, have produced evidence for a high proportion of female tobacco users, an unexpected result due to the ethnohistoric association of men with tobacco use (Tushingham, Damitio, et al. 2020; Eerkens et al. 2018; Tushingham, Eerkens, et al. 2020).

Here, we present the results of additional metabolomics-based analyses of human dental calculus from ancestral Muwekma Ohlone populations at three different settlements. The Muwekma Ohlone Tribe was designated Most Likely Descendant by the State of California Native American Heritage Commission for these projects. Tribal leadership then reviewed, recommended, and approved all analyses of ancestral remains including dental calculus analysis and partnered with the research team to conduct these investigations. Our study combines elements of biomarker analysis centered on a limited number of mind-altering substances with an untargeted approach exploring a broad range of potential non-food products by analyzing comprehensive chemical residue patterns and comparing them with osteological information. For the latter, we employ multivariate statistics to establish clusters of individuals with similar metabolomic profiles and examine covariation with demographic and pathological data gathered from preserved

skeletons. Subjecting hundreds of individual signal records to this kind of exploratory process leads away from an emphasis on the identification of specific substances and instead focuses on the possible impact of social and health conditions on individual ingestion patterns as evidenced by calculus deposits. Compared to Velsko et al. (2017), we expand the range of factors from oral pathologies to also include markers observed postcranially. This is particularly relevant as associations with the former might be related to the metabolic activity of pathogens (Warinner et al. 2014), or the ingestion of xenobiotics with healing or palliative properties. Covariance relationships between chemical residues and postcranial pathologies, on the other hand, should emanate more directly from ethnomedical practices.

Our working hypotheses predict the detection of nicotine in a subset of the analyzed samples. Following Tushingham et al. (2020) and Eerkens et al. (2018), we test the hypothesis that tobacco consumption is more widespread among men compared to women (e.g., Roulette et al. [2016]). Based on Robinson et al. (2020), *Datura* biomarkers scopolamine and atropine might be preferentially preserved in calculus samples belonging to young adult men. Furthermore, we believe the untargeted metabolomics approach allows for the observation of clusters of individuals reflecting endemic health conditions and related substance use patterns. Thus, we expect that individuals with more severe oral and postcranial pathologies would have been engaged in heavier use of traditional medicinal plants, and hence, to be characterized by specific metabolomic profiles. We also expect older individuals with aging immune systems to be plagued by a wider range of ailments, and hence, to be characterized by different suites of compounds than younger individuals. While our study goals do not include the detection of products beyond tobacco and datura, we point out how specific molecules driving such associations can be recognized, examined, and identified, thus laying the foundation for subsequent ethnopharmacological studies.

(FIGURE 1)

Study sites and population health

Síi Túupentak (ALA-565/H; "Place of the Water Round House Site") and *'Ayttakiš 'Éete Hiramwiš Trépam-tak* (ALA-677/H; "Place of Woman Sleeping Under the Pipe Site") are located in Alameda County, California, in the southeastern San Francisco Bay area. They are both ancestral Muwekma Ohlone villages with associated cemeteries which were excavated as a collaborative effort between Far Western Anthropological Research Group, Inc., and the Muwekma Ohlone Tribe of the San Francisco Bay Area. Extensive data recovery at both sites produced rich and diverse arrays of cultural materials with 60 features and 86 burials comprising 99 individuals (of which 18% are cremations) recovered from two phases of investigation at *Síi Túupentak* (Byrd et al. 2020; Byrd, Arellano, et al. 2022), and 5 features and 19 individuals from 16 inhumation burials at *'Ayttakiš 'Éete Hiramwiš Trépam-tak* (Byrd, Engbring, et al. 2022; Leventhal et al. 2017). Each ancestor was directly dated, and all dates presented below have been calibrated and the median intercept used to determine the age of individual burials and the occupation range. Radiocarbon analysis indicates that *'Ayttakiš 'Éete Hiramwiš Trépam-tak* dates from approximately 1190 Common Era (CE) to about 1435 CE (755 to 515 BP), while *Síi Túupentak* was established around 1315 CE and the primary occupation continued to around 1805 CE (635 to 145 BP), almost 30 years after the start of Spanish colonization and founding of nearby feudal missions, towns, and garrisons in the mid-1770s CE. There is also a reoccupation of the settlement in the 1830s after the collapse of the Spanish Mission colonization effort. Using the local chronological terminology (Groza et al. 2011), *'Ayttakiš 'Éete Hiramwiš Trépam-tak* contains components ranging from the

Middle/Late Transition into the Late 1 Period, and *Síi Túupentak* runs from the Late 1 period through the Late 2 period and into the Historic Mission Period.

Thámien Rúmmeytak (SCL-128; "Guadalupe River Site") is a large ancestral Muwekma Ohlone cemetery located in modern-day San Jose in Santa Clara County, California. The site spans several adjacent properties and has been impacted to varying degrees during several construction projects. Work associated with a parking garage in 1977 was initiated without archaeological oversight thus leading to the removal of approximately 2,000 m^3 of soil containing "thousands" of bones and artifacts (Winter 1978). Subsequent projects in 1987-88 (James et al. 1988), 1991 (Tannam et al. 1991), 2012 (Leventhal et al. 2015), and 2019-20 involved CRM specialists from the beginning and resulted in the controlled recovery of 81 burials. Before the latest intervention, DiGiuseppe et al. (2021) report on the extension of the cemetery with considerable historical impact including privy and trash pits as well as brick paving in close proximity to relatively shallow burials. Radiocarbon dates and temporally diagnostic artifacts place the 45 human interments recovered in 45 burials during the 2019-20 field season between 100 BCE and 1300 CE (2150 and 700 BP), or the Middle 1 Period through the Middle-Late transition (DiGiuseppe et al. 2021).

Osteological analyses of 76 ancestral Ohlone from the initial phase of investigations at *Síi Túupentak* (DiGiuseppe and Grant 2020) showed that the skeletal remains are generally in a fair to excellent state of preservation. The study revealed a high infant and subadult mortality rate. It also produced a distinct sexual dichotomy between survivorship rates starting at age 5, with females at around 85% and males at around 62% (Buonasera et al.). A sharp drop in survivorship for males between age 20 and 30 years culminates in a 30% difference by sex (DiGiuseppe and Grant 2020). The same analyses showed that while only 13% (n=10) of all individuals produced

evidence of blunt force trauma, surprisingly all but one of these cases are associated with women. The situation is quite different at *Thámien Rúmmeytak.* In a predominantly young population, a group of 16 individuals all dated within a hundred radiocarbon years of each other (roughly 650-750 CE) exhibits evidence of severe interpersonal aggression including missing limbs likely caused by trophy taking (DiGiuseppe et al. 2021). Even though other cases of multiple interments associated with violent events have been recorded in the study region (Eerkens et al. 2016; Byrd 2022), this case is anomalous both in terms of the number of affected individuals and the timing.

Returning to *Síi Túupentak*, 38% (n=29) of the analyzed burials exhibited developmental defects and congenital anomalies, an identical number were afflicted by non-specific infections, 41% (n=31) had osteoarthritis, and 26% (n=20) presented some indication of nutritional deficiencies with a third of those cases corresponding to children below the age of 12. Four individuals were diagnosed with tuberculosis based on osteological and aDNA analysis and one individual with treponemal disease, giving rise to the possibility of the spread of infections by early European explorers prior to Spanish colonization (DiGiuseppe and Grant 2020; Byrd, Arellano, et al. 2022).

(FIGURE 2)

Nutritional deficiencies were also recorded on preserved dentitions, with 23% (n=17) of individuals displaying linear enamel hypoplasia. With one exception, this subgroup's age at death was significantly below the population average. Dental analyses revealed low degrees of caries, yet a high frequency of open root canals (ORC) (average 2.9 per individual). Many open root canals are associated with tooth attrition which among native San Francisco Bay area communities

can be attributed in part to heavy fiber processing as evidenced by frequent slanted wear patterns (Grant 2010). Interestingly, there is no clear correlation between ORCs and abscesses (Figure 2) indicating that access to the circulatory and lymph systems did not automatically result in bacterial infection. While a strong immune system can suffice in containing abscesses, another possibility is intervention through medicinal plants with antimicrobial, anti-fungal, or antibacterial properties.

It stands to mention that willow (*Salix* sp.) was not only a prime source of basketry material whose processing might have contributed to tooth attrition, but it is also well known for the antimicrobial properties of salicylic acid (Griffin 2014). Even though their origin is unclear, salicylates were identified by Velsko et al. (2017) in historic samples from western Europe. Important medicinal plants for indigenous San Francisco Bay area inhabitants include bay laurel (*Umbellularia californica*), coffeeberry (*Frangula californica*), California poppy (*Eschscholzia californica*), yerba buena (*Clinopodium douglasii*), and yarrow (*Achillea millefolium*) (Salima Bennadja et al. 2013; Carranza et al. 2015; Garcia and Adams 2012).

Lastly, we acknowledge that the time spans during which these ancestral sites in central California were inhabited were characterized by notable changes in population density, and socio-political interaction and organization at the regional and intra-community scale, as well as by fluctuations in climatic conditions (Milliken et al. 2007; Byrd et al. 2017; Leventhal 1993). Mortuary practices are among the facets of ancestral settlements that have been explored to better understand the nature of these developments that led to extremely high cultural and linguistic diversity and some of the highest population densities in North America at the time of European colonization in the late 1700s CE (Cook 1976; Golla 2011; Heizer 1978). Diachronic trends in intra-community mortuary practices have been discerned in a variety of ancestral settlements indicative of increasing socio-political and ceremonial complexity (e.g., Atchley 1994;

Bellifemine 1997; Byrd and Rosenthal 2016; Leventhal 1993; Luby 2004). There is also recognition that there was rarely a simple direct relationship between mortuary practices or offerings and the status or wealth of an individual. The public nature of mortuary events and their role in constructing and reinforcing community-wide identities also must be considered, along with a recognition that a variety of social categories (including group affiliation, sex, and age) played a role in mortuary practices (Byrd and Rosenthal 2016; Reddy 2015; Byrd, Arellano, et al. 2022). Hence, we decided to limit our analysis to direct associations between chemical residues and demographic and pathological data at the scale of the individual, while the impact of social variables such as wealth, status, and burial disposition remain under consideration for future studies.

Materials and methods

Materials

This study is based on a grand total of 95 human dental calculus samples (Supplementary Online Materials [SOM1]). Eighteen samples belong to 11 burials recovered at *'Ayttakiš 'Éete Hiramwiš Trépam-tak*; 11 samples are associated with 5 *Síi Túupentak* burials; and 66 samples correspond to 34 burials from *Thámien Rúmmeytak* (Byrd, Arellano, et al. 2022; Byrd et al. 2023; DiGiuseppe et al. 2021). When more than one option was available for calculus recovery, samples were preferentially gathered in complementary fashion from anterior vs posterior, and maxillary vs. mandibular teeth. Analyzed samples range in weight from 1.1 to 44.5 mg. Prior osteological analyses provided demographic (sex, age) information for each burial, as well as pathologies observed both within the oral cavity (e.g., caries, abscesses, periodontitis), and the complete skeleton (e.g., trauma, infections, degenerative conditions).

Osteological analyses

Estimation of sex followed a three-pronged approach. Osteological analyses of all skeletal remains were followed by DNA analysis of all directly dated burials. Finally, several mostly subadult individuals which lacked a clear sex determination based on the initial two lines of evidence were selected for amelogenin protein analysis sex estimation (Buonasera et al. 2020; Parker et al. 2020). During age assessment, dental development was considered to present a more accurate determination for aging subadults than long bone development (Richards and Anton 1991).

Skeletal observations of pathologies were grouped into seven categories featuring degenerative diseases, infectious diseases, non-specific infections, developmental defects and congenital anomalies, neoplasms, nutritional deficiencies, and activity markers. Caries, tertiary dentin and calculus deposits, abscesses, open root canals, and periodontal disease are included among the oral indicators of health. The latter was assessed by measuring the recession from the cementum enamel junction to the alveolar bone. Similar classificatory approaches with grades from 0 (absence) to 5 (severe) have been applied to other populations of human remains in the study area (Grant 2014; Grant et al. 2019).

Metabolomic analysis

Sample processing started with grinding the dental calculus fragments inside of their respective vials using a stainless-steel blunt-tipped spatula. The resulting sediment was covered with 0.2 mL of formic acid (FA) and sonicated for 10 min. After sonication samples were centrifuged at 3,300 × g for 10 min, and 0.15 mL of the supernatant was transferred to a second microvial. Both the extract and the remaining sediment were air-dried. Dry extracts were resuspended with 0.1 mL of

0.1% formic acid/50% aqueous acetonitrile and centrifuged at 21,000 × g for 10min at 4°C. Lastly, 0.075 mL aliquots were transferred to amber vials for ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) analysis.

FA and ethanol (EtOH) blanks were included in the analysis batch. For these, empty microvials were filled with 0.2 mL of each reagent to then undergo the extraction sequence parallel to the archaeological samples. While the FA blank allows to control for impurities from the solvent and microvials, the EtOH blank was added to ensure contamination was not introduced during the extraction process. In addition, during analysis resuspension solvent blank was injected every 5 samples. Finally, chemical standards for nicotine, caffeine, scopolamine, and atropine were prepared and co-run with the sample batch to allow for biomarker analysis. The UPLC-MS protocol followed Brownstein et al. (2020) with the only differences being the scan range – set to 50-1200 m/z – and the acquisition time - set to 13min (SOM2). This configuration is suitable for detecting molecules of smaller weights in the alkaloid, peptide, or amine classes (in contrast to long-chain primary metabolites such lipids, or sugars). Sample order was randomized for analysis.

We used Progenesis QI (Nonlinear Dynamics, Milford, MA) as well as TargetLynx (Waters, Milford, MA) software for raw data processing. For long-term storage, .raw files were uploaded to the MetaboLights repository (www.ebi.ac.uk/metabolights/MTBLS5658) (Haug et al. 2019). Given the culturally sensitive nature of our data, the corresponding profile is kept private, and access needs to be authorized by the Muwekma Ohlone Tribe of the San Francisco Bay Area via the corresponding author. Rather than preventing the democratization of research resources, this protocol aims to prioritize Indigenous data sovereignty (Tsosie et al. 2021).

Data processing

To prepare the multivariate analyses characterizing the untargeted metabolomics-based approach, we decided to reduce the list of all detected chemical features (n=3317) to a compilation of "true" signals. For this, the first filter targeted contaminants excluding signals whose highest abundance among the archaeological samples was not at least 10 times stronger than the maximum observed among the blanks. We then standardized peak area values by sample weight and isolated signals with significant variance. Thus, all signals with a coefficient of variation (CV) below 100 were also eliminated from further consideration. We recognize this procedure removes many features associated with the human oral metabolome. However, for this study's purposes our emphasis fell on diagnostic markers that distinguish between samples rather than uniting them. Signals significantly affected by instrument noise at the start and the end of the run were also excluded from multivariate analysis, as were features whose highest abundance value among archaeological samples did not surpass a minimum threshold of 100 (arbitrary intensity units established by Progenesis QI). In the end, the retention time interval between 1.34 min and 9.88 min contains a concise list of 305 "true" signals.

As mentioned above, the availability of multiple samples for most of the individuals allows for two sets of comparisons between chemical residue and osteological data. Provenience variables such as tooth position (anterior vs. posterior) or type (incisor, canine, premolar, molar), tooth row (mandibular vs. maxillary), and side of calculus deposit (lingual/buccal vs. labial) can be examined on a per-element basis. To avoid introducing incremental biases caused by burials with well-preserved residues, demographic and pathological data should be contrasted by individual. For the latter assays, the highest abundance value among samples belonging to the same individual was retained for each chemical feature when reducing the number of cases from 95 (calculus samples) to 50 (burials).

Multivariate statistical analyses were conducted in the R environment. We used the *prcomp* function from the 'stats' package for principal components analysis (R Core Team 2013), and the *plsda* function from the 'caret' package for partial least squares – discriminant analysis (Kuhn et al. 2016). To visualize outputs, we employed the *biplot* function from the 'stats' package, as well as the *ggplot* function from the 'ggplot2' package (Wickham 2016).

Results

Data analysis started with a scan for the above-mentioned diagnostic biomarkers for tobacco (*Nicotiana* sp.) and datura (*Datura* sp.). In contrast to previous studies from the same area (Eerkens et al. 2018; Tushingham, Damitio, et al. 2020; Tushingham, Eerkens, et al. 2020), none of the 95 calculus samples from *Síi Túupentak*, *'Ayttakiš 'Éete Hiramwiš Trépam-tak*, or *Thámien Rúmmeytak* yielded a confirmed nicotine signal. However, this does not mean that the sites' inhabitants were not consuming tobacco. We detected peaks which match nicotine standards in both retention time and molecular mass (± 5 ppm). Yet, their abundances fell within background noise levels (Figure 3). Given the broader artifactual and chemical residue evidence for indigenous tobacco consumption in the San Francisco Bay region, this suggests that local taphonomic factors led to rates of degradation inhibiting nicotine detection under the current protocols.

(FIGURE 3)

Scopolamine and atropine were absent from the dental calculus samples. Direct evidence for the consumption of *Datura* in North America thus continues to be limited to quids (Robinson et al. 2020) and residues extracted from ceramics (King et al. 2018). Caffeine, however, is present

in trace amounts in all archaeological samples and the FA and EtOH blanks. As a result, we cannot determine if this compound is original to the calculus samples or is a contaminant, though we suspect the latter. In either case, we removed caffeine from further analysis.

(FIGURES 4A AND 4B)

To reduce dimensionality in the residue dataset without imposing an additional matrix in a second step, multivariate statistical processing began with a principal components analysis (PCA) featuring the totality of true signals. A per-element calculation aimed to explore differences in residue deposits between anterior (incisors and canines) and posterior (premolars and molars) teeth. Prior studies had shown residue detection not to be consistent among different samples from the same individual, with modes of consumption – tobacco smoking vs tobacco chewing – among possible causal factors (Tushingham, Damitio, et al. 2020). Figure 4A indicates that chemical residue signatures tend to be more diverse in calculus recovered from premolars and molars. However, it also exemplifies the issue of within-mouth variation as Samples #374 and #375 both correspond to *'Ayttakiš 'Éete Hiramwiš Trépam-tak* Burial 1-20. Focusing on burials (n=50) instead of calculus samples (n=95), three individuals separate as outliers while the majority remain bound to a single large cluster centered on the graph's point of origin (Figure 4B).

While PCA is an efficient tool for exploring variance within a sample population, an evaluation of associations between chemical residues on the one hand, and demographic and pathological variables on the other, benefits from statistical processing centered on covariance. Given the former are of a continuous and the latter mainly of a categorical nature, we employ partial least squares – discriminant analysis (PLSDA). Initial results (SOM3) confirm the weight

of the three outliers detected through PCA, thus leading us to remove the corresponding data from a second round of PLSDA aimed at an in-depth look at the remaining 47 individuals. Before moving ahead, we present a brief description of the three outliers that were cut:

(FIGURES 5A AND 5B)

'Ayttakiš 'Éete Hiramwiš Trépam-tak Burial 1

This interment was in good/excellent condition and corresponds to a male young adult radiocarbon dated to the Late 1 Period and determined through strontium isotopes to be a lifelong local at the site. The skeleton exhibits evidence of degenerative conditions through vertebral osteoarthritis, laminal spurs, and Schmorl's nodes. The spinal column includes a sixth lumbar vertebra. The individual survived a left radius fracture, but signs of periosteal reactions were noted on the left ulna and right tibia. The dentition retains 33 teeth (including a supernumerary like an LI2) with moderate wear and calculus formation. The RM_1 occlusal surface was destroyed by a huge caries with two thirds of the crown gone and two active buccal abscesses, one for each root. Both LM^1 and LM^2 display large, active abscesses which led to root rotting and broken alveolar bone. Moreover, the maxillary contains a large bony bulbous formation that nearly fills the left sinus. The etiology of this pathology is unknown, but it is dramatic and unusually large.

Síi Túupentak Burial 73A

This burial belongs to a middle adult woman dated to the Late 2a Period determined to have been a lifelong local by strontium isotope analysis. Like the *'Ayttakiš 'Éete Hiramwiš*

Trépam-tak outlier, her skeleton produced evidence for laminal spurs and Schmorl's nodes, as well as an enthesopathy on the left patellar ligament. The mandible preserved all 16 teeth exhibiting modest wear and moderate to heavy calculus deposits. While no open root canals or abscesses were observed, the degree of periodontitis was scored as severe.

Síi Túupentak Burial 75

The third individual with exceptional residue preservation is also a middle adult woman and lifelong resident, dated to the Late 1 Period. The only degenerative condition observed was arachnoid fovea. Periosteal reactions were noted across the skeleton's right side on the clavicle and all long leg bones. The right hand was affected by an osteochondroma on the fourth metacarpal. Both the mandible and maxillary were recovered with 14 remaining teeth. Wear is modest on the lower and heavy on the upper row. RM^2 is the only tooth with an open root canal. Caries is absent, but periodontitis was noted as Grade 3.

(FIGURE 6)

Figure 6 integrates the scatterplots resulting from ten separate PLSDA which help to visualize the possible associations between the chemical residue data and two demographic factors (sex; age), three postcranial conditions (infections; trauma; osteoarthritis), and five oral pathologies (periodontitis; caries; open root canals; tertiary dentin; abscesses). While the population without outliers still gravitates toward the point of origin, the spread of the respective central clusters allows for the observation of meaningful tendencies.

Albeit several exceptions, when sex estimation is reduced to male, female, and sex-indeterminate individuals, notable differences arise between the first two categories (Figure 6A). Most males cluster centrally indicating a low degree of variation related to chemical residues. Females on the other hand present a much larger spread and higher values on both components. The panorama is different for age categories. While case numbers are too low for children, it can be observed that the confidence ellipses for juveniles and young adults are overlapping. Middle adults, in turn, are also scarce but older individuals are summarized by an ellipse running perpendicular to the first two categories (Figure 6B). Scored as binary variables, records of trauma and infections are somewhat less insightful. However, in both cases there appears to be a trend for pathologically affected burials to cluster more tightly than healthy individuals (Figures 6C/D).

Even though there are again several crossovers, the panorama for osteoarthritis is the most heterogenous. Most individuals with some degree of osteoarthritis cluster on the positive side of Component 2, while unaffected burials almost exclusively register with negative values. The considerable number of categories however leads to a strong draw generated by somewhat outlying samples thus rendering the windwheel shape of the confidence ellipses (Figure 6E). With respect to oral pathologies, the similarity between Figures 6H, 6I, and 6J is striking. If it were not for the missing single negative outlier on Component 1, even the caries plot (Figure 6G) would be part of a near identical group in terms of spread. In conjunction, there is a slight tendency for individuals with the corresponding dental health problems to set themselves apart from their unaffected counterparts on both components. Nonetheless, periodontitis yields a significantly clearer picture. Unaffected individuals display almost exclusively positive values for Components 1 and 2, while the majority of those with some degree of the disease cluster in the third quadrant. In addition, the

confidence ellipses for more severely affected individuals tend to be wider due to the weight of Component 2 (Figure 6F).

Lastly, multivariate statistics such as PCA or PLSDA also allow for an assessment that weighs the impact of specific variables on the newly established components. Figure 7 exemplifies this aspect by highlighting the chemical features which significantly affect the sample spread after a PLSDA that factors in degrees of osteoarthritis. Component 1 scores are positively correlated with values for a signal with a retention time of 2.96 min and mass-charge ratio of 261.130, while the 5.62_214.119 feature represents an opposing force. The panorama for Component 2 is more complex. Paired features separate on its positive (2.25_239.174; 3.06_253.190) as well as its negative (8.12_311.268; 7.07_295.237) side. However, here the relative distances to the central cluster and thus weights are smaller compared to their Component 1 counterparts.

(FIGURE 7)

Our attempt to calculate possible elemental compositions for these key signals via Progenesis QI using a CHNO configuration was not successful. Unfortunately, this situation remains common in untargeted metabolomics-based analyses. However, over the last decade social molecular networking efforts have made considerable progress in rendering LC-MS data comparable across different systems. With Muwekma tribal approval, we aim to employ these resources on a case-by-case basis in the near future to evaluate if the same features have been detected in other studies.

The fact that most databases curated as part of such networks do not originate from archaeometric projects but rather studies pertaining to the realm of food science, pharmacology,

or bioengineering, to name only a few, carries the advantage that the organisms subjected to analysis are known. Thus, specific molecules might be assigned to certain products even without being individually identified. On the other hand, to remedy the effects of a common focus on organisms that are currently exploited for economic purposes, we also keep expanding our reference database for culture-specific resources. With regard to the plants used by ancestral Ohlone populations, this work benefits from the detailed ethnobotanical accounts compiled by John Peabody Harrington in the early 20th century (Bocek 1984). His data covers, among others, dietary, medicinal, and psychoactive species, and preparations. Interestingly, macrobotanical analyses carried out on samples from *Síi Túupentak* and *'Ayttakiš 'Éete Hiramwiš Trépam-tak* indicate minor but notable differences in resource acquisition (Wohlgemuth 2020, 2022) that aid in explaining site-dependent differences in metabolomic signatures observed before filtering "true" signals. Unfortunately, a similar comparative dataset is not yet available for *Thámien Rúmmeytak*.

Discussion

While the lack of confirmation for diagnostic *Nicotiana* and *Datura* biomarkers is lamentable, consistently detecting these and other specific molecules in human dental calculus remains a methodological challenge as evidenced by the varying recovery rates associated with different substances, instruments, and extraction protocols (Eerkens et al. 2018; Velsko et al. 2017; Sørensen et al. 2021). Nonetheless, the presence of smoking paraphernalia and other artifacts and ecofacts associated with psychoactive plants in pre-Contact contexts from the southern Northwest Coast and central California, including their presence as a mortuary offering at *Síi Túupentak*,

gives testimony to the consumption of such products by local Native groups (Nelson 2000; Robinson et al. 2020; Eerkens et al. 2012). Ongoing studies of dental calculus collections from present-day users, as well as past populations of historical and archaeological time scales will help gain a better understanding of factors such as consumption-to-deposit ratios, degradation over time, and specific taphonomic agents.

For the time being, untargeted metabolomics-based approaches produce meaningful data suited to explore an array of subsistence and health-related questions. With regard to overall variation in preserved chemical residues, our results mirror the general observations made by Velsko et al. (2017). Most samples share a substantial number of metabolites, while only a relatively small number of individuals stand out due to the particular chemical makeup of their calculus deposits. One such case is Burial 1 from *'Ayttakiš 'Éete Hiramwiš Trépam-tak*, which confirmed its outlier characteristics identified through chemical residue analysis with osteological examination. The severity of oral pathologies, including several active abscesses and a not yet fully understood bulbous growth into the sinus, must have altered metabolite production significantly. Despite being afflicted by a series of health conditions, the two outliers from *Síi Túupentak* do not present a similarly striking pathological profile. Representing two out of only five available burials from *Síi Túupentak*, their metabolomic profiles might have been affected by site-dependent factors. Further experimental work is necessary to control for such possibilities.

After the removal of said outliers, statistical processing via PLSDA led to the observation of a series of tendencies of covariance between chemical residues and osteological reports.

Age and Sex Consumption Patterns: While men and women do not appear to split dichotomously per se, male individuals tend to cluster while samples from female burials display much more internal variation. The same effect had been observed in stable isotope studies of bone

collagen at *Síi Túupentak*. Eerkens et al. (2020) argue that males are much less variable than females because of a cultural preference for patrilocality. Age-based dynamics also seem to have an impact on the corresponding deposits. Specifically, the clustering tendencies of old adults versus younger individuals might reflect social norms surrounding the consumption of non-food items such as psychoactives or medicines. It stands to mention that our sample is biased, with larger numbers of male individuals among young adults, and a dominance of women among older adults. Thus, it might be possible that older women were socially encouraged to consume a larger variety of non-dietary substances. As mentioned initially, this argument has previously been made by Eerkens et al. (2018) for the case of tobacco among Native Californians. Yet, without confirmed nicotine positives, the observed patterns might also be an outcome of other social norms such as the gendered labor and the use of teeth in the production of traditional basketry (O'Neale 1932).

Oral Health: Our results suggest that higher rates of dental pathologies, in particular periodontitis, affect dental calculus residue loads, thus confirming a pattern observed in modern populations (Velsko et al. 2017). However, making firm conclusions about associations between dental pathologies and metabolomic profiles was somewhat hindered by the small number of cases with higher counts of caries, open root canals, or tertiary dentin deposits. Nonetheless, the persistence of this association through archaeological time scales is intriguing. Notably, dental caries rates are lower among many pre-Contact communities particularly before agriculture, and studies of Native populations have shown that changes in diet can significantly alter oral health outcomes (Walker and Erlandson 1986). Moreover, our results indicate that as an endemic condition among native Californians, periodontitis can be traced through both osteological and metabolomic approaches. While there are linkages between smoking and/or tobacco use and

periodontal disease among modern populations (Watts and Addy 2001; Shariff et al. 2017), almost nothing is known about how psychoactive substances may affect oral health in ancient peoples.

Post-Cranial Pathologies and Medicinal Practices: The detection of associative tendencies between chemical residue data and postcranial pathologies is very promising. While the ultimate causes of the observed metabolite profiles are still elusive, the recognition of such relationships opens the door for in-depth studies of medical practices and pathogenic outcomes among past human populations. Working with records of specific diseases rather than aggregate data for classes such as trauma, infections, or degenerative conditions, future analyses can aim at the identification of specialized regimes of xenobiotics administered to relieve patients from their pain or illness. Despite the untargeted approach of many ancient metabolomics studies (Brownstein et al. 2020), multivariate statistics provide effective tools for filtering signals with greater weight, thus facilitating subsequent efforts at identifying the associated medicinal substances. This does not mean that available databases such as the Global Natural Products Social Networking (GNPS) (Nothias et al. 2020), KNApSAck (Afendi et al. 2012), or KEGG (Okuda et al. 2008) will automatically yield matches. The nature of the archaeological record always requires an understanding of taphonomic processes leading to degradation and derivatization of the original compounds. Nonetheless, experimental studies (e.g., Chovanec et al. 2012) can lead to a more efficient tracing of metabolic cycles and the derivative byproducts of known xenobiotic biomarkers that might preserve in samples hundreds or even thousands of years of age.

Conclusion

We detected significant variation in patterns of chemical residues preserved in the dental calculus of Native Californians. Signals likely corresponding to secondary metabolites, and alkaloids more

specifically, show associations with demographic and health-related variables observed during osteological analyses. Metabolomic profiles varied between males and females, as well as across age groups. In addition, we show that chemical residues in dental calculus are not only related to oral diseases alone, but archive information concerning curative practices associated with health problems located far from the mouth. The former confirms the relationship between substance use and oral health outcomes among present-day populations, while the latter opens a window into (self)care and medicine in ancient societies. Untargeted metabolomic analyses specifically grant the opportunity to move beyond the biomarkers of well-studied and often commercialized psychoactive substances such as tobacco and instead lay the groundwork for much broader studies of ethnopharmacology. While complex, potential connections between non-dietary substances and skeletal observations of trauma, infections, and degenerative disease reinforce the sophistication of traditional ecological knowledge among the indigenous societies of California and elsewhere.

Acknowledgments

As many of us are affiliated with public institutions of higher education, we want to assert that our work is carried out on the traditional homelands of Native American communities, and in this case specifically, the historic Muwekma Ohlone Tribe of the San Francisco Bay Area. We acknowledge their presence since time immemorial as well as our responsibility to establish and maintain relationships of inclusion and reconciliation. We also acknowledge that our work can violate communities in ways we may not fully understand. Harms may be inadvertent, may occur in alignment with practices of our time, or may have been chosen as the best outcome by all parties using their best judgment at the time but nonetheless cause harm. Recognizing this reality, we pledge to engage in inclusive, respectful, and responsive collaborative practices. We also extend

gratitude to our respective academic units for their continuous support. This list includes the WSU Dept. of Anthropology and Inst. of Biological Chemistry, the UCD Dept. of Anthropology, and the SJSU Dept. of Anthropology. Lastly, we want to thank our sponsors. Analyses were made possible by funding provided by Far Western Anthropological Research Group via archaeological mitigation contracts with the San Francisco Public Utilities Commission for ALA-565/H and with California Department of Transportation. BFB thanks the SFPUC's Kimberly Stern Liddell, Bryan Deassaure, and Deborah Craven-Green, SFPD's Adrian Praetzelis, Sally Morgan, and Allison Vanderslice, and Caltrans's Todd Jaffke, Kristina Montgomery and Kathryn Rose for their support. Additional funding for this project was awarded to ST and DRG by the National Science Foundation (Grant #1918966).

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Figure captions

Figure 1: Location of study sites

Figure 2: Relation between two oral health indicators at *Síi Túupentak*: Open root canals and abscesses

Figure 3: Chromatogram and mass spectrum corresponding to Burial 34 from *Thámien Rúmmeytak*. Possible nicotine signal is masked by larger coeluting peaks.

Figure 4: Scatterplots featuring values for Principal Components 1 and 2 based on PCA of 305 chemical features. A) Individual dental calculus samples (n=95) labeled by tooth position within the oral cavity B) Individual burials (n=50) labeled by sex assignation (F1 = Female; F2 = Probable female; F3 = Possible female; Ind = Indetermined; M1 = Male; M2 = Probable male; M3 = Possible male)

Figure 5: Dental pathologies observed in *'Ayttakiš 'Éete Hiramwiš Trépam-tak* Burial 1-20: A) Active abscesses under maxillary molars; B) Formation of bony tissue protruding from the maxillary into the sinus (Photos by David Grant)

Figure 6: Scatterplots featuring values for PLSDA Components 1 and 2. A) Sex assignation; B) Age (C = Child; J = Juvenile; YA = Young adult; MA = Middle Adult; OA = Old adult); C) Trauma; D) Postcranial infections; E) Osteoarthritis (by degree); F) Periodontitis (by degree); G) Caries (by number); H) Open root canals (by number); I) Tertiary dentin (by number); and J) Abscesses (by number). The ellipses represent 95% confidence intervals.

Figure 7: PLSDA biplot featuring component and variable scores for the factorization of osteoarthritis (Y labels are composed of rounded retention time and m/z values)

Figures (in alphanumerical order)