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Sampling Strategy for the Genetic Analysis of Human Remains from Tepe Hissar

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Abstract

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Keywords

genetic analysis, mitochondrial DNA, human remains

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SAMPLING STRATEGY FOR THE GENETIC ANALYSIS OF HUMAN REMAINS FROM TEPE HISSAR

By

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Genetic testing has become a critical tool for the examination of ancient human remains. Earlier methods relied exclusively on observations and measurements. DNA analysis can date skeletal remains using radiocarbon dating, identify the sex of the individual, and determine the mtDNA and Y-chromosome haplogroups. Mitochondrial DNA and Y-chromosome haplogroups can be further used to understand kinship, burial practices, and other information about the civilization. For this study, I created a sampling strategy for the genetic analysis of a collection of 397 skeletons excavated during the 1930's from an Iranian Bronze Age site called Tepe Hissar. DNA analysis can be cost prohibitive and requires samples be extracted and destroyed. A successful sampling strategy mitigates these costs, balancing efficiency and effectiveness to create the smallest sample that remains representative for the entire population. Overall, preservation, location within the site and layers, and their status as an outlier should be considered as major determinants for the representative sample. Preservation is the most important factor followed closely by the status of skeletons as outliers in their respective research studies and the skeletons' relative geographic location. Although I ranked these factors, all three will be taken into account when determining the final representative sample.

Introduction:

Physical and biological anthropologists study human remains to better understand the origin, evolution, and diversity of *homo sapiens*. Human remains provide direct, tangible evidence of our ancestor's history, behavior, and biological adaptation to changing environments (Landau and Steele, 1996). Individual specimens not only provide information about their own health but indications of familial relationships and migration patterns among the larger population. Like humans themselves, the methods used by biological anthropologists continue to evolve with new technologies and advancements. Early researchers relied exclusively on observation and measurements of human remains. While many anthropologists will still examine the morphological characteristics of the human remains, they will also employ microscopic techniques and chemical analysis. More recently anthropologists have begun to utilize isotope and DNA analysis to further understand ancient civilizations. Research can yield improved findings by reviewing ancient samples with more modern methods.

DNA analysis of sex chromosomes, mitochondrial DNA, and autosomal DNA can provide information about the human remains at five different levels - individual, family, local, population, and species. Each level expands the number of people involved and potential implications. At the individual level, researchers can identify the number of unique skeletons and determine each skeleton's biological sex. These determinations are useful for population studies about human remains found at archaeological or historical sites (Kaestle & Horsburgh, 2002). For example, Pierce (2017) used genetic information to determine the sex of twenty sets of human remains from Tepe Hissar. The next level uses mitochondrial DNA (mtDNA) and Y-chromosomes to understand maternal and paternal lineage (Kaestle & Horsburgh, 2002). These lineages can provide further information about the society's social structure such as their

marriage and burial practices. Researchers examined the genetic information for thirty-four individuals from two Late Copper Age cemeteries in Southern Germany. Using mtDNA and Y-chromosomes, they created a family tree linking individuals to each other through first, second, and third-degree kinship (Sjögren et al, 2020). The local and population levels examine this information on larger scale to determine the movement or lack therefore of genes in smaller communities and throughout the entire population. The distinction between the family and local levels can be blurred because some societies are comprised of a relatively small number of families. Population genetics can majorly change over the course of time due to migration and population continuity/replacement (Kaestle & Horsburgh, 2002; Harney et al, 2018). For example, one of the Late Copper Age cemeteries had fourteen different mtDNA haplogroups among eighteen individuals showing this community might have participated in the exogamic marriage practice with women migrating into the community to marry (Sjögren et al, 2020). The species level is the last and highest level where researchers can identify and clarify relationships between modern humans and our hominid contemporaries and ancestors (Kaestle & Horsburgh, 2002). Researchers have sequenced the genomes of modern humans and have been able to identify genetic information from other hominids within these genomes. Neanderthal (Sankararaman et al, 2014) and Denisovan DNA (Reich, 2011; Sankararaman et al, 2016) is present in modern humans. Therefore, there must have been admixture between these groups and archaic *homo sapiens*.

DNA analysis of ancient human remains can have many implications for our current understanding of ancient populations and the evolution of humans. However, DNA analysis can prove cost prohibitive, hard to obtain, and destructive. Anthropologists try to prevent destroying any human remains but, unfortunately, the destruction of certain bones can be necessary to gain

useful data. The cochlea (i.e. part of the inner ear) must be exposed, removed, cleaned, and then turned into a fine powder. Therefore, this process destroys the cochlea from these sets of human remains and can potentially create small bone fragments. There are less destructive methods, such as the cranial base drilling method, but these can negatively impact the amount of DNA yielded from these samples and can still damage the bones (Reich et al, 2020; Sirak et al, 2017). Unless the human remains have been carefully preserved, the majority of ancient specimens subjected to DNA analysis fail to produce results. Researchers must be careful when selecting which ancient human remains to use within their genetic analysis. The purpose of this research is to help create a focused sampling strategy for an extensive DNA analysis of one such carefully preserved collection within the Penn Museum – the skeletal remains from Tepe Hissar.

Background Context:

Tepe Hissar is a Bronze Age archaeological site in northeastern Iran. It contains human remains and other archaeological artifacts from three distinct periods that date between the mid/late fifth and the early second millennium BCE. In the early 1930's, Erich Schmidt conducted an extensive excavation of this site. His findings, *Excavations at Tepe Hissar Damghan*, were published in 1937. Dr. Schmidt's expedition was partially sponsored by the University of Pennsylvania Museum of Archaeology and Anthropology and many of the skeletal remains and other Tepe Hissar materials he uncovered continue to be housed in the museum's collection storage. Researchers returned to Tepe Hissar in 1979, 1995, 2006, and 2010. The Penn Museum was associated with the 1979 re-investigation but has not been associated with the three most recent re-investigations (Afshar et al, 2018).

Dr. Schmidt's initial observations are well documented and other researchers have applied more recent techniques to analyze specimens from Tepe Hissar. Afshar et al. (2019) measured collagen and performed isotope analysis on the long bones to understand the place of origin and diet of this ancient population. Another research study examined mitochondrial DNA to identify the different haplogroups (Zargari et al, 2016). The sampling strategy and resulting DNA analysis to be undertaken in this research study is intended to build upon this research by examining morphological differences between individual Tepe Hissar skeletons on a genetic level. Samples will be taken from the petrous portion of the temporal bone to ensure that each is from a unique individual, avoiding costly duplications. If researchers cannot sample petrous bone, then they will turn to teeth or dense cortical bones as alternative areas to sample (Reich et al, 2020).

There are two categories of research questions for this project. The first type of questions can be answered with this project and relate to the sampling strategy. What are the most important factors to consider for this representative sample? Which skeletons should be chosen to be in this representative sample? The second type of questions only be answered after the genetic analysis is completed and are questions related to the completed genetic analysis. Are there genetic differences within the Tepe Hissar population? Do these genetic differences and or similarities connect to chronological, societal, or geographic factors? Do the mtDNA and or Y-chromosomes show first, second, or third-degree kinship among the Tepe Hissar samples? Is there any evidence from the mtDNA or Y-chromosome haplogroups that this society practices female or male exogamy? Do the amount or quality of burial goods correspond with genetic differences within the society?

Genetic testing has multiple uses. It can radiocarbon date the skeleton presenting a clearer timeline for when the individual lived and creating connections between the sets of human remains and other archaeological remains. Researchers used radiocarbon dating to identify the dates for human remains in the Iranian Zagros allowing researchers to identify contemporary animal remains (Meiklejohn et al, 2017) and understand site chronology (Becerra-Valdivia et al, 2017). It can also identify the sex of the human remains, mitochondrial haplogroups, and Y-chromosome haplogroups (Pierce, 2017; Reich, 2020; Sjögren et al, 2020). We can use this information to understand the selected human remains on an individual and family level. Information from the Y-chromosome and mtDNA can expose potential maternal and paternal relationships within a society and help to understand migration patterns in the surrounding area. Genetic samples have the potential to answer all of the questions in the previous paragraph and provide major insights into the prehistoric Tepe Hissar civilization.

This site is extremely important due to its long history of human occupation and its location. Tepe Hissar is located within the Middle East - an area with major cultural history and genetic diversity. The human remains from Tepe Hissar can present an opportunity to observe their cultural history through their mortuary practices and burial goods. Mortuary practices include significant group burials during the late second period and early third period along with less common vault burials. Many individuals are laid on their sides with a variety of grave goods surrounding them including weapons, ritual goods, pottery, jewelry, and other objects (Gürsan-Salzmann, 2016). The combination of these mortuary practices, grave goods, and the genetic analysis could help identify genetic trends between specimens from each of the site's three distinct chronological periods and

deepen our understanding of the people that once inhabited this ancient city-state. It could also reveal more information about the entire region.

Methodology:

This biological anthropology research project has two components. The first and main component is to create an efficient strategy to determine a representative sample of human skeletons collected from the Bronze Age archaeological site of Tepe Hissar for genetic analysis. This component is associated with the first category of research questions. The second will be completed in the future and is to test the effectiveness of this sampling strategy by conducting genetic analysis on ancient DNA extracted from the chosen human remains. This component is associated with the second category of research questions.

The population for this study is a collection of three hundred and ninety-seven human skeletons housed at the Penn Museum. These skeletons were exhumed in the 1930's as part of Erich Schmidt's excavation of Tepe Hissar, a Bronze Age archaeological site in northeastern Iran (1937). This collection contains human remains of both sexes, with a wide range of ages and degrees of preservation. They represent three distinct Bronze Age time periods: Period I (4300 – 3700 BC), Period II (3700 – 2900 BC), and Period III (2900 – 1800 BC). The number of specimens differ depending on the time period. The earliest period has the fewest skeletons while the latest period had the most, but all three periods are represented in the archaeological record. These skeletons already represent a smaller sample of the entire archaeological site. He and his archaeological team were able to excavate one thousand six hundred thirty-seven skeletons but only three hundred and ninety-seven skeletons are currently housed in the Penn Museum. Schmidt did not transport all of the skeletons from Tepe Hissar to the Penn Museum and,

unfortunately, the other skeletons are currently unaccounted for. These unaccounted for skeletons could have been reburied or moved to an unknown location in Iran (Afshar et al, 2018). Schmidt did not clearly explain his decision process on which human remains were brought to be studied and stored at the Penn Museum and which were not. The differing number of specimens per time period might be due to Schmidt's bias when he decided on which skeletons to bring back to the Penn Museum.

A successful sampling strategy will balance efficiency and effectiveness. The cost and destructive nature of DNA extractions encourage selecting as small a sample as possible. A representative sample, however, must be large enough to contain the characteristics of the broader population and not be skewed towards a smaller group within the population. To both draw comparisons and identify outliers, the sampling strategy developed as part of this project must be large enough to reflect the characteristics of the social structure present in Tepe Hissar throughout each of its three distinct time periods.

The first step in developing this strategy was to conduct an extensive literature review. I first turned to Eric Schmidt's field journal to begin this literature review. This field journal provided information that helped me understand the excavation of Tepe Hissar and gain further contextual information about the archaeological site. Next, I identified different journal articles and other sources that discussed Tepe Hissar. This resulted in a collection of seventeen different sources on a variety of topics related to Tepe Hissar. After closely reading all of these sources, I focused on the journal articles that discussed the human remains or the grave goods associated with these human remains. From there, I tried to compile the most relevant information from these sources into a couple documents. The main purpose of this literature review was to create

associations and discover differences between the individual skeletons and categorize the articles by content.

Previous research studies that utilized skeletal material from Tepe Hissar focused on one of four main areas: the ancestry/origin of Tepe Hissar (Zargari et al, 2016; Hemphill, 1998; Hemphill, 1999), paleopathology of the human remains (Krogman, 1940; Afshar et al, 2017; Afshar et al, 2018; Hemphill, 2008), testing different methods of analysis (Huan, 2000; Nowell, 1978), and morphological studies relating to race and sex (Pierce, 2017; Speakman, 2017; Krogman, 1940). In addition to the themes and findings of these studies, particular attention will be given to identify and record the catalog numbers and characteristics of previously studied remains. Wherever possible, previous researchers' observations will be linked to specific specimens. This data will be organized and maintained on a spreadsheet for further analysis during the sample selection process and attached as an appendix.

Having physical access to the collection will allow an opportunity to both compare previous observations with the actual specimens and to better determine their current state of preservation. These observations will be used to augment the information from the literature review and to help identify those remains with the highest potential to provide quality ancient DNA. This will need to be measured against the unique value of each individual specimen, because of the DNA extraction process' destructive nature.

After the representative sample has been selected, the process of genetic material collection and analysis can begin. Ideally, samples will be collected from the petrous part of the temporal bone of the selected human remains. Samples from these locations are taken and then ground down to a fine powder to be used in the DNA extraction (Pinhasi et al. 2015; Reich et al, 2020). If the temporal bone is missing or if the specimen is poorly preserved, samples might be

taken from the alternative areas. Teeth, especially large teeth with intact roots (Hansen et al, 2017), would be the next best alternative followed by dense cortical bones (Reich et al, 2020).

Unfortunately, the yield for ancient DNA from these alternative sites is expected to be reduced. The petrous portion of the temporal bone is preferred because this area usually has a higher endogenous DNA content than bones from other areas of the human remains. A higher endogenous DNA content increases the likelihood that the genetic analysis will be successful, which makes this location the optimal spot to take the genetic sample from (Pinhasi et al, 2015; Sirak et al, 2017). Once all of the genetic samples have been collected, then genetic analysis of these samples will be conducted at noted geneticist David Reich's lab at Harvard University. Reich has helped publish 232 research articles and books from 1998 to 2021 (Reich Publications, 2021). He and members of his laboratory have used over 12,000 prehistoric human samples to generate genome-wide data from over 6,000 different prehistoric individuals (Reich et al, 2020).

Once completed the genetic analysis conducted as part of this research will be used to study social and morphological differences between individual Tepe Hissar remains on a genetic level and to search for genetic trends between specimens from each of the three distinct chronological periods to help to better understand the population that once inhabited this ancient city-state.

Results:

The human remains from Tepe Hissar have been used in multiple different research studies. Some used Tepe Hissar as a point of comparison against other populations in the surrounding area or further out. I identified four main categories that used genetic or skeletal evidence: studies focused on the ancestry/origin of Tepe Hissar (Zargari et al, 2016; Hemphill,

1998; Hemphill, 1999), paleopathology of the human remains (Krogman, 1940; Afshar et al, 2017; Afshar et al, 2018; Hemphill, 2008), testing different methods of analysis (Huan, 2000; Nowell, 1978), and morphological studies relating to race and sex (Pierce, 2017; Speakman, 2017; Krogman, 1940). Researchers also examined the ceramics, lithics, and other goods surrounding the human remains as well as the burial positions of the remains (Gürsan-Salzmann, 2016). The most recent journal article about the Tepe Hissar human remains examined long bones to determine the amount of collagen and isotopes within these bones (Afshar et al, 2019).

Genetic information from human remains found in Tepe Hissar was used to determine the ancestry of Tepe Hissar and understand potential patterns of migration through the different time periods. This category of journal articles discussed the Tepe Hissar population in a broad sense both by discussing common haplogroups found in the Tepe Hissar population and by examining the genetic similarities and differences between Tepe Hissar and other Bronze Age archaeological sites. Zargari et al extracted (2016) ancient mtDNA from the skeletons and identified their haplogroups. A common haplogroup found was H32, which is a Eurasian haplogroup that probably originated in SW Asia and then migrated into Europe. Hemphill (1999) studied the craniometrics of the Tepe Hissar remains to theorize about possible migrations (especially from the Oxus civilization in central Asia) into Tepe Hissar. Compared to twelve other Bronze Age samples from surrounding areas, samples from the three time periods of Tepe Hissar are, as expected, closest to each other and another eastern Iran site called Shahr-I Sokhta (SHS). The third period of Tepe Hissar was genetically closer to SHS than the second period of Tepe Hissar (Hemphill, 1998).

Although Tepe Hissar was continually occupied, different populations could have migrated and lived there during the three different time periods. Evidence could provide support

for both sides. There were major cultural changes and societal unrest between the three periods (Afshar et al, 2018) but human remains associated with imported goods (Appendix 1) had a higher likelihood of being members of the Tepe Hissar elite rather than foreign elite (Hemphill, 1999). These potential differences between the three distinct time periods show that the representative sample must contain skeletons from each of the three distinct periods to compare and contrast with each other.

Multiple researchers looked at the paleopathology of Tepe Hissar to understand interpersonal violence, health, and the difference in paleopathology between time periods. Krogman (1940) identified dental, cranial, and long bone pathology for the human remains. Afshar et al. examined both the paleopathology (2017) and the connection between the prevalence of interpersonal violence and disease, stress, and changes within a society (2018). Signs of catastrophic events included charred human remains, mass burials, and the destruction of buildings (Afshar et al, 2018). Hemphill (2008) studied the connection between gender, wealth status (as determined by the items found in association with burials), and dental pathology. There was not a significant difference in wealth status for males and females, but the types of burial goods might differ. He examined seven different types of dental disease – pulp exposures, abscesses, hypoplasia, hypercementosis, alveolar resorption, antemortem tooth loss, and caries. The only significant differences in dental disease for males and females were caries, hypoplasia, and antemortem tooth loss with females being more likely to have all three types of dental disease. The results showed that the poorest and wealthiest individuals had equally good overall dental health. The two groups with more intermediate wealth (i.e. the near rich and affluent poor) were similar to each other but inferior to the other two groups. Men's dental health increased along with their social status while women's dental health declined as their social

status increased (except for the wealthiest women). Individuals especially women in lower statuses (i.e. affluent poor and near rich) were trying to increase their wealth leading to sacrifices in their dental health. Poor dental health, major cranial trauma, or extreme cases of long bone paleopathology could affect preservation and the ability to take usable genetic samples.

Tepe Hissar has also been used as a test population for a variety of different methods of analysis. Huan (2000) examined if the mandibular ramus flexure could be a single morphological indicator of sex. Nowell (1978) used the dental sample to evaluate the Miles method of aging. Unlike some of the other studies, these studies focused on the examination of morphological features rather than a genetic analysis. Both studies showed some success for their respective methods of analysis but there were limitations associated with both studies. These two methods of analysis do not apply to this project and neither identified specific skeletons.

The morphological features of the Tepe Hissar skeletons were also used to potentially identify the sex and ancestry of individual skeletons in this population. Pierce (2017) and Speakman (2017) both used the cranium or dental remains to determine sexual differences. Pierce (2017) compared her own morphological sex classification and genetic sex classification of certain skeletons. These two sex classifications frequently did not match up showing the difficulties of classifying sex from morphological features. Speakman (2017) also examined how stress can affect the different sexes differently through multiple types of dental lesions.

Krogman (1940) examined the morphological characteristics of the skulls and attempted to place them into different racial categories including Mediterranean, Proto-Nordic, Alpine, Armenoid, and Asiatic. He separated these groups by head shape; Mediterranean and Proto-Nordic were considered long-headed while Alpine, Armenoid, and Asiatic were considered round-headed. Krogman concluded that the Mediterranean cranial type was smaller and

smoother while the Proto-Nordic cranial type was larger and more rugged. He connected these two groups to more modern populations located in Southern and Northern Europe. He appears to use Negroid and "Pseudo-Australoid" as further classifications because certain crania that had already been identified as Mediterranean or Proto-Nordic had features from these two groups. Krogman categorized most of the Tepe Hissar remains as either Mediterranean or Proto-Nordic. He only identified three skeletons as Alpine and these three were the only "round-headed" crania found within Tepe Hissar. Krogman used the lack of "round-headed", "Negroid", and "Pseudo-Australoid" as evidence that these groups of individuals played a small or non-existent role in Tepe Hissar civilization and the few crania from these categories at Tepe Hissar were framed as concerning encounters. Krogman's racial categories have some similarities and differences to more modern racial categories.

Racial identification of human remains is a controversial topic within anthropological research. Race is a social construction. These "racial" categories have been used throughout history to justify racism and provide evidence for different racist theories of evolution and inherent differences between and among human populations. For example, Samuel Morton associated racial intellectual capacity with the size of the skull. He used his results as evidence for his racist theories. Unfortunately, these racial categories continue to persist in some areas of anthropology. Forensic anthropologists evaluate human remains for potential ancestry in an effort to identify an individual. To express this identification to the larger population, forensic anthropologists need to use terms that reflect local understanding of race. The evaluation of ancestry in biological archaeology can be extremely difficult. These categories are dynamic and constantly evolving. There is a noticeable overlap for most of the features that archaeologists use for morphological classifications providing some level of doubt for any classification (Cunha &

Ubelaker, 2020). I examined Krogman's *The Peoples of Early Iran and Their Ethnic Affiliations* (1940) to identify his perceived differences between the Tepe Hissar skeletons within the Penn Museum. These morphological differences could present possible ancestral differences and provide information about familial relationships and migration into Tepe Hissar.

I mainly examined the research articles that explicitly mentioned certain skeletons. These research articles included Afshar et al. (2018), Afshar et al. (2019), Krogman (1940), Pierce (2017), Hemphill (1999), and Gürsan-Salzmann (2016). Between these six sources and the previous Tepe Hissar DNA samples, one hundred and thirty-nine skeletons were identified and placed onto the spreadsheet.

The first category for this literature review used the Tepe Hissar population in an interesting way but these different methods of analysis were not applicable for this project. The third category helped with contextual information about previous genetic studies but failed to mention specific skeletons and mainly focused on the differences between Tepe Hissar and other Bronze Age sites rather than differences within the Tepe Hissar population. Therefore, the second and fourth categories were the most helpful categories for this project because the research studies in these categories were more likely to identify specific skeletons that were used in their projects. These journal articles also touched upon the differences within the Tepe Hissar population by identifying outliers for carbon and nitrogen isotopes and associated grave goods, morphological differences in crania, and cranial trauma. Pierce (2017) also conducted a genetic analysis on a smaller portion of the Tepe Hissar collection to determine the genetic sex of the human skeletons. This could indicate that this group of skeletons would be good candidates for further genetic samples and studies.

Zahra Afshar and her fellow researchers (2019) studied the collagen yield and the carbon and nitrogen stable isotope values in the long bones of Tepe Hissar. They examined the differences in these values between the three time periods and the two sexes within time periods. Researchers tried to analyze sixty-nine different skeletons but only sixty-eight provided collagen. This sample included eight skeletons from Hissar I, eleven from Hissar II, and forty-nine from Hissar III. She identified these skeletons by catalog number, so I was able to cross reference these numbers with the Tepe Hissar spreadsheet. Unfortunately, there were some discrepancies between the two lists. Afshar et al. (2019) listed some skeletons that were not on the spreadsheet and had more definite conclusions on age and sex of some of these skeletons. Overall, there were differences between the three periods and the two sexes, but these were insignificant. The isotope ratios for carbon and nitrogen increased in Hissar II and Hissar III.

Afshar et al. (2019) identified four outliers for the carbon isotope and nine outliers for the nitrogen isotope. All four of the carbon isotope outliers were from Hissar III. There were two males and two females. Of the nine outliers for the nitrogen isotopes, eight were from Hissar II and one was from Hissar III. From Hissar II, the nitrogen outlier isotopes also differed on if there was a high ratio of the nitrogen isotope or a low ratio of the nitrogen isotope. Three males and three females had high ratios of the nitrogen isotope. Two females exhibited a low ratio of the nitrogen isotope. There was one female outlier from Hissar III. There was not a connection between the carbon and nitrogen isotope outliers. They concluded that these outliers might differ in their diets to the other Tepe Hissar residents.

Ayşe Gürsan-Salzmann (2016) identified low and high outliers among the Tepe Hissar human remains. These graves were designated as outliers due to both the amount and the quality of their grave goods. The high outliers were associated with the largest amount and best quality

of grave goods. Some of the high outliers were given names such as “The Little Girl”, “The Dancer”, or “The Priest” that described the function or features of the associated grave goods. For example, “The Priest” was associated with grave goods that appeared to have a ritual/religious function. I attempted to coordinate the given burial site and skeleton number with the Penn Museum catalog numbers for the high and low outliers (Appendix 1). Unfortunately, not all of these outliers matched with the information from the Penn Museum.

Hemphill completed multiple studies that researched different topics. He examined the connection between wealth and dental pathology. In this research study, Hemphill (2008) studied eighty-eight skeletons but did not identify any of them. The skeletons were categorized by wealth score with similar amounts in four different groups. In a different research study, Hemphill (1999) mentioned that seven skeletons were directly associated with anomalous artifacts and provided identification for six of them.

Most of the human remains were only used for one research study as opposed to being used in multiple research studies. The outliers from these research studies usually did not match each other. For example, none of the individuals that Hemphill (1999) identified matched with Afshar’s (2019) isotope outliers. A couple of the low outliers for grave goods matched with Afshar’s (2019) isotope outliers (Appendix 1). The use of a spreadsheet provided the ability to easily determine how many studies certain skeletons had been used in and their status as an outlier in any of these research studies.

This group of genetic samples will be added to thirteen previous DNA samples of human remains from Tepe Hissar. Of the thirteen previous DNA samples, seven samples were taken from the petrous bone, four from the phalanx bone, and one from a molar. Three of the DNA samples were deemed as questionable due to damage or contamination of the genetic sample;

two of these questionable samples were from non-petrous bone while the other was from a petrous bone sample. Two other petrous bone samples were flagged for contamination but weren't categorized as questionable. This sample size is too small to create a connection between the type of bone of used for the sample and if the sample was deemed acceptable but previous research identifies petrous bone as the preferred area to gather a genetic sample from (Pinhasi et al., 2015; Sirak et al, 2017; Reich et al, 2020).

The sex composition of these thirteen genetic samples was almost even with seven females and six males being tested. Two of the female samples and one of the male samples were considered questionable. These skeletons were mainly from Period 1 and Period 3 but there is at least one skeleton in the grey area between Period 2 and Period 3 (Figure 1).

There also appeared to be potential familial relationships even within these thirteen samples. The male samples showed at least three different main Y-chromosome groups - T1, L2, and J2 (Figure 1). There were two sets of human remains (33-23-09 and 33-23-124) with identical Y-chromosome groups (J2a1a1b3) but didn't share the same mtDNA haplogroup. There also seemed to be slight distinctions between the two sets of human remains (33-16-118 and 33-23-73) that fall within the T1 group. Unlike the Y-chromosome groups, all of the samples will have an mtDNA haplogroup that they inherited from their mother. There was more variation in the mtDNA haplogroups than in the Y-chromosome groups. There were ten different mtDNA haplogroups between the thirteen genetic samples (Figure 1). Some of these haplogroups fell within the same larger branch but were connected to different sub-branches. The most common mtDNA haplogroup was W3b, which matched four of the genetic samples (two females and two males). The two males that had W3b did not share the same Y-chromosome group. There were also four genetic samples that fell underneath the U mtDNA haplogroup branch but were all

members of different sub-branches. These haplogroups didn't match with previous genetic studies that identified the H32 mtDNA haplogroup as a common haplogroup for Tepe Hissar (Zargari et al, 2016).

I then tried to delve further into the physical human remains. These first-hand observations helped to determine their preservation state, to ensure that the specific sets of remains are currently within the Penn Museum, and to locate the optimal location on them to take a genetic sample from.

Discussion:

Sampling strategies for any population-based studies center around the researcher's hypothesis. The number of individuals included in the sample can depend on multiple factors. Larger samples are usually considered more statistically significant and, therefore, more dependable when interpreting the data. Unfortunately, not all research studies can have large sample sizes due to potential costs and the number of eligible individuals. This sample will be much smaller due to the amount of available human remains and the cost of DNA analysis. The creation of a representative sample for the Tepe Hissar human remains will require a combination of information from the literature review and from first-hand observations of the human remains.

After examining the number of times these skeletons were cited in different journal articles, I have concluded that the number of times should not be used as the sole determinant of the representative sample. Most of the skeletons from this collection only appeared in one research study. The two skeletons (33-16-110 and 33-16-118) that were used in the most research studies have already been genetically tested and are, therefore, eliminated from

consideration for this project. Some of the Tepe Hissar skeletons have never been used in any research studies but could still be good candidates for genetic testing. Therefore other factors such as preservation, location within the site and the layers, and outliers should be considered as major determinants for the representative sample.

Preservation is extremely important to obtaining a usable DNA sample. A sample from the petrous portion of the temporal bone is optimal but different bones from individual sets of human remains can vary in their state of preservation. DNA can also be taken from an individual's teeth or the phalanx bone if these areas are better preserved. Unfortunately these other locations are less likely to provide a usable DNA sample as seen with the previous thirteen DNA samples. Preservation can also affect potential contamination of the DNA sample. DNA samples from ancient human remains can be contaminated from their burial environment, the excavations, and from the consequent research methods. If the human remains are not well preserved, then there is a higher chance for contamination. Therefore, preservation should be considered as a factor to distinguish if a set of human remains is a good candidate for genetic sampling or not. Candidates will be prioritized if their preservation status is either good or fair while their candidacy will be questioned if their preservation status is poor or very poor (Appendix 2). Many of the sets of human remains need to be further examined to fully determine their preservation status.

Similar geographic location could mean possible familial relationships. Researchers excavated multiple plots of land within the Tepe Hissar archaeological site with varying success on finding human remains. Some plots held human remains from each of the three distinct time periods separated from each other by multiple layers of soil. Schmidt (1937) drew detailed maps showing the relative depth and location of skeletons to each other in specific plots.

Archaeologists can use stratigraphy and the different layers to determine the relative age and associations between the remains. Human remains from the same plot or nearby plots could potentially have an increased likelihood of sharing a familial relationship. Evidence has shown that prehistoric societies could have their own complex burial customs. Multiple prehistoric and modern societies choose to bury their dead near their families, but these similarities in geographic location does not guarantee that the skeletons will share a familial relationship especially for larger populations. Genetic analysis of each skeleton's mtDNA or Y-chromosomes is needed to identify maternal or paternal lineage (Kaestle & Horsburgh, 2002). Skeletons that are from similar geographic locations to the previous thirteen tested or each other could potentially reveal relationships or the lack thereof between the skeletons. Unfortunately, Tepe Hissar had multiple burials that share a similar geographic location limiting the effectiveness of this strategy.

Each of the research studies had different research questions and hypotheses and, therefore, used the skeletons in a different way. Certain researchers such as Afshar et al. (2019) found outliers within their research studies. These outliers (Appendix 1) can be used as a point of comparison with the other samples. A genetic sample of these outliers and non-outliers from this population could show if these differences were genetic in nature or not. Overall, preservation, location within the site and the layers, and outliers should all be considered as determinants for the representative sample.

I ranked preservation as the most important factor followed closely by the status of skeletons as outliers in their respective research studies and the skeletons' relative geographic location. If the skeletons are not well-preserved, then there is a lower chance that the genetic sample will be usable. Without a usable sample, this skeleton cannot provide as much

information about the Tepe Hissar population. Outliers are skeletons that researchers identified as unique and different from the majority of the population. These potential differences are useful in understanding the Tepe Hissar population. Similar geographic location could help find potential familial relationships within the entire Tepe Hissar population and highlight connections or differences between the human remains.

Although I attempted to rank these factors relative to each other, all three are extremely important when determining this representative sample and all of these factors will be taken into account when determining the final representative sample. Individual sets of human remains could be eliminated as candidates or given special attention depending on any of the three previously identified factors.

Conclusion:

DNA analysis with ancient human remains was impossible when Erich Schmidt excavated Tepe Hissar in the 1930s. Modern advancements in DNA technology have helped researchers to amplify the limited amount of DNA found within these human remains allowing researchers to complete new research studies. Anthropologists currently use ancient DNA to understand individuals, smaller communities, populations, and species. This genetic analysis will provide individual characteristics such the biological sex of the human remains and familial/population data such as paternal and maternal lineage. This information can be used to identify potential genetic differences within the Tepe Hissar population and when interpreted help to better understand this prehistoric archaeological site.

The literature review highlighted about seventeen different research studies where human remains from Tepe Hissar were used. Cross-referencing the human remains with the different

research studies provided minimal information. Therefore, the sampling strategy for the human remains from Tepe Hissar will revolve around the preservation status of the remains, their distance from other remains especially the thirteen remains that have been analyzed, and if any research studies have identified them as outliers from their previous samples. Preservation will be the first step in evaluating the candidates for genetic analysis. Skeletons that have a well-preserved petrous part of the temporal bone will be prioritized. Information about the studies or lack thereof each skeleton was involved in and its' relative geographic location to other skeletons will also factor into the decision.

This sampling strategy can address questions surrounding family relationships, burial practices, and socio-economic divisions in the society. Multiple prehistoric and modern societies choose to bury their dead near their families, but this burial practice is not guaranteed. The genetic analysis could determine if family members were buried near each other through common mtDNA and Y-chromosome haplogroups. The lack of specific characteristics in this population could present information about migration and burial practices. For example, a gender or age imbalance could point towards a tendency to bury individuals with specific characteristics. Missing individuals could also point towards migration by individuals with specific characteristics away from Tepe Hissar. Isotopic outliers could show differences in diet (Afshar et al, 2019) or living environment (Sjögren et al, 2020). Individuals could have moved around altering their isotopic ratios providing potential migrations and movement within this community. Genetic analysis on outliers for burial goods could show if wealth status correlates with specific mtDNA and Y-chromosome haplogroups. These haplogroups can correlate with families and potential migrations into Tepe Hissar.

The next steps in this research project are to use the sampling strategy to determine which human skeletons should be genetically analyzed. When the number of possible genetic samples is determined, then the combination of these first-hand observations and information from the literature review (archaeological location, outliers, and previous use in research studies) will be used to decide which skeletons are the best candidates for this process. After the representative sample has been chosen, the genetic samples will be collected and then transported for analysis by Dr. Reich at Harvard University. It is hoped that this analysis will help us gain valuable insights into the genetic history and lives of the people who lived at Tepe Hissar thousands of years ago.

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Appendix 1: Spreadsheet with Tepe Hissar Data

Catalog #	Tested? Type of Sample?	Period	Afshar et al. (2019)	Krogman (1940)	Pierce (2017)	Afshar et al. (2018)	Hemphill (1999)	Gursan-Salzmann (2016)
33-16-02	Yes (Questionable); phalanx	I	Used					
33-16-04	Yes (Questionable); phalanx	I						
33-16-05		I	Used	Mediterranean				
33-16-07		I	Used					
33-16-09		I	Used					
33-16-11	Yes (Pass); phalanx	I						
33-16-12	Yes (Pass); phalanx	I			Used			
33-16-13					Used			
33-16-15		III		Proto-Nordic				
33-16-17		I	Used					
33-16-18		I	Used					
33-16-20		I	Used					
33-16-23		I	Used		Used			
33-16-29		II	Nitrogen isotope outlier (High)					
33-16-32		II	Nitrogen isotope outlier (High)					
33-16-33		II	Used					
33-16-34		II	Nitrogen isotope outlier (High)					
33-16-35		II	Used					
33-16-36		II	Used					
33-16-38		II	Nitrogen isotope outlier (Low)					
33-16-39		II	Nitrogen isotope outlier (High)					
33-16-40		II		Mediterranean				
33-16-42		II	Nitrogen isotope outlier (Low)					
33-16-44					Used			
33-16-45		III	Used					
33-16-47		III	Used					
33-16-50		III			Used			
33-16-51	Yes (Pass); petrous	III			Used			
33-16-52		III		Mediterranean				
33-16-56	Yes (Pass); petrous	III						
33-16-57		II/III						High Outlier
33-16-60		III	Carbon isotope outlier					
33-16-64		III		Proto-Nordic				Low Outlier
33-16-66		III	Used	Mediterranean				
33-16-67		III		Mediterranean				
33-16-68		II/III						Low Outlier
33-16-69		III		Mediterranean				
33-16-70		III	Used					Low Outlier
33-16-71		III	Used	Proto-Nordic				
33-16-74		III						Low Outlier
33-16-75		III						Low Outlier
33-16-78		III						Low Outlier
33-16-79		III	Used					Low Outlier
33-16-81		III	Used					Low Outlier
33-16-84		II/III						Low Outlier
33-16-87		III	Used					Low Outlier
33-16-88		III		Mediterranean				
33-16-89		III	Used					Low Outlier
33-16-92		II/III						Low Outlier
33-16-93		III		Mediterranean	Used			
33-16-94		III	Used					
33-16-95		III	Used					
33-16-97		III	Carbon isotope outlier					Low Outlier
33-16-98		III	Used					Low Outlier
33-16-99		III	Carbon isotope outlier	Mediterranean				
33-16-101		III	Used					Low Outlier
33-16-110	Yes (Pass); petrous	III	Used		Used			Low Outlier
33-16-112		III		Mediterranean				
33-16-114		II/III						Low Outlier
33-16-117		III	Used					Low Outlier
33-16-118	Yes (Pass); petrous	II/III	Used	Mediterranean	Used			Low Outlier
33-16-121		II	Nitrogen isotope outlier (High)					
33-16-123		II/III						Low Outlier
33-16-124		III	Used					
33-16-128		II	Nitrogen isotope outlier (High)		Used			Low Outlier
33-16-132		III		Mediterranean				Low Outlier
33-16-133		III	Used	Mediterranean				Low Outlier
33-16-135		III	Used					Low Outlier
33-16-136		III	Used		Used			Low Outlier

33-16-137		II/III						Low Outlier
33-16-139		II/III						Low Outlier
33-16-140		II/III						Low Outlier
33-16-141		III	Used					Low Outlier
33-16-142		III	Failure to analyze					Low Outlier
33-16-143		III	Used	Proto-Nordic	Used			Low Outlier
33-16-162		II/III					Used	
33-16-166		II/III						Low Outlier
33-16-167		III	Nitrogen isotope outlier					
33-16-181		III	Used					
33-16-182		III	Used					
33-16-196		III		Mediterranean	Used			
33-16-204		III	Used					
33-16-205		III	Used			Cranial trauma		
33-16-206		III	Used					
33-16-209					Used			
33-16-231					Used			
33-16-236		II					Used	Low Outlier
33-23-05					Used			
33-23-07						Cranial trauma		
33-23-09	Yes (Pass); tooth (molar)	I						
33-23-19		III		Mediterranean				
33-23-20		II		Mediterranean				
33-23-22						Cranial trauma		
33-23-26		II		Proto-Nordic		Cranial trauma		
33-23-35		II		Mediterranean				
33-23-36		III		Mediterranean		Cranial trauma		
33-23-48		III		Mediterranean				
33-23-58		III	Used					High Outlier
33-23-60		III		Mediterranean				
33-23-66		III		Mediterranean				
33-23-67					Used			
33-23-72					Used			
33-23-73	Yes (Pass); petrous	III						
33-23-74					Used			
33-23-76		III	Used					
33-23-79		III	Used					
33-23-80		III	Carbon isotope outlier					
33-23-94		III		Mediterranean				
33-23-96		III		Proto-Nordic		Cranial trauma		
33-23-101		III	Used					
33-23-102		III	Used					Low Outlier
33-23-103		III	Used					Low Outlier
33-23-104		III		Mediterranean				
33-23-106		III	Used	Mediterranean				Low Outlier
33-23-107		III		Proto-Nordic		Cranial trauma		
33-23-110		III	Used					Low Outlier
33-23-111		III	Used					Low Outlier
33-23-113	Yes (Questionable); petrous							
33-23-116		III	Used					
33-23-119		III	Used	Mediterranean				
33-23-120		III	Used	Mediterranean			Used	
33-23-122		III	Used					
33-23-124	Yes (Pass); petrous	III			Used		Used	
33-23-125							Used	
33-23-126							Used	
33-23-130		III		Mediterranean				
33-23-150		III		Mediterranean				
33-23-152						Cranial trauma		
33-23-158		III	Used					
33-23-168				"Negroid"				
33-23-178		III	Used					Low Outlier
33-23-179						Cranial trauma		
33-23-183		III		Mediterranean				
33-23-185		III	Used					Low Outlier
33-23-191		III		Mediterranean				
33-23-197						Cranial trauma		
33-23-205	Yes (Pass); petrous	III						
33-23-213		III		Proto-Nordic				
33-23-226		III	Used					Low Outlier

Appendix 2: Spreadsheet with Preservation Data

Catalog Number	Preservation Status
33-16-05	Fair
33-16-07	
33-16-09	
33-16-13	
33-16-15	
33-16-17	Poor
33-16-18	
33-16-20	Good
33-16-23	Poor
33-16-29	Good
33-16-32	Poor
33-16-33	
33-16-34	
33-16-35	
33-16-36	Poor
33-16-38	Fair
33-16-39	
33-16-40	
33-16-42	
33-16-44	
33-16-45	Fair
33-16-47	Fair
33-16-50	Good
33-16-52	Fair
33-16-57	Very Poor
33-16-60	
33-16-64	Fair
33-16-66	
33-16-67	Good
33-16-68	Fair
33-16-69	Fair
33-16-70	Fair
33-16-71	Good
33-16-74	Fair
33-16-75	Good
33-16-78	Fair
33-16-79	Poor
33-16-81	Fair
33-16-84	Fair
33-16-87	Fair
33-16-88	Fair
33-16-89	Good
33-16-92	Fair
33-16-93	Fair
33-16-94	
33-16-95	
33-16-97	Fair
33-16-98	Fair
33-16-99	
33-16-101	Fair
33-16-112	Fair
33-16-114	Good
33-16-117	Good
33-16-121	
33-16-123	Good
33-16-124	
33-16-128	Good
33-16-132	Good
33-16-133	Good
33-16-135	Good
33-16-136	Good
33-16-137	Good
33-16-139	Good

33-16-140	Good
33-16-141	Good
33-16-142	Poor
33-16-143	Fair
33-16-162	Good
33-16-166	Good
33-16-167	
33-16-181	
33-16-182	Poor
33-16-196	Good
33-16-204	
33-16-205	Good
33-16-206	Good
33-16-209	
33-16-231	
33-16-236	Good
33-23-05	
33-23-07	
33-23-19	
33-23-20	
33-23-22	
33-23-26	
33-23-35	
33-23-36	
33-23-48	
33-23-58	Good
33-23-60	Good
33-23-66	
33-23-67	
33-23-72	
33-23-74	
33-23-76	
33-23-79	
33-23-80	
33-23-94	
33-23-96	
33-23-101	
33-23-102	Good
33-23-103	Good
33-23-104	
33-23-106	Good
33-23-107	
33-23-110	Good
33-23-111	Fair
33-23-116	
33-23-119	Good
33-23-120	Good
33-23-122	
33-23-125	
33-23-126	
33-23-130	Good
33-23-150	
33-23-152	
33-23-158	Good
33-23-168	
33-23-178	Good
33-23-179	Fair
33-23-183	
33-23-185	Good
33-23-191	
33-23-197	Fair
33-23-213	
33-23-226	Good

Figures:

Skeleton Number	Period	Sex	mtDNA haplogroup	Y-Chromosome
33-16-02	I	F	U7	N/A
33-16-04	I	M	U1a'c	N/A (issues with contamination)
33-16-11	I	F	J1d	N/A
33-16-12	I	F	W3b	N/A
33-16-51	III	M	W3b	L2
33-16-56	III	F	U5b2	N/A
33-16-110	III	F	X2p	N/A
33-16-118	II (III)	M	HV	T1(xT1a1, T1a2b)
33-23-09	I	M	I1	J2a1a1b3
33-23-73	III	M	W3b	T1(xT1a, T1a2)
33-23-113	Unknown	F	W3b	N/A
33-23-124	III	M	T2h2	J2a1a1b3
33-23-205	III	F	U7a	N/A

Figure 1: Table with information about the 13 previous genetic samples