Native North American Resistance and Susceptibility to Infectious Diseases: An Anthropological Approach

By

Linda Anne Larcombe

A Thesis submitted to
the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

Department of Anthropology
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Abstract

Infectious disease agents have a profound effect on human biological and cultural evolution. Understanding the processes involved in the biological response to pathogens and the genetic factors that contribute to disease resistance and susceptibility, are integral to understanding the adaptation to diseases by New World Aboriginal populations. Complex historic and evolutionary events led to the immunogenetic profile of the North American indigenous population, and it is only within an evolutionary context that we can make sense of the differential rates of morbidity and mortality that occurred and continue to occur between ethnic groups. This research compared the frequency of single nucleotide polymorphisms (SNPs) in the promoter region of cytokine proteins (key regulators of the immune system) in a contemporary Aboriginal and Caucasian study population and established that a significant difference exists. Results suggest that the contemporary Aboriginal study population differs from Caucasians in their ability to express cytokines that are effective against infectious pathogens that require a cell-mediated immune response. The analysis of the cytokine SNP frequencies maintained by the contemporary Aboriginal population within a functional, adaptive context suggests substantially more complex pattern of evolution.

To explore the observed immunogenetic profile of Aboriginals within an evolutionary framework, a novel technique for detecting SNPs in the cytokine promoter regions of ancient human remains from Manitoba was developed. The development of new molecular methods for detecting SNPs which have been shown to be linked to disease resistance and susceptibility, is the first step towards understanding the role of genetic factors in the human immune response to infectious disease. This will contribute to understanding why certain infectious diseases continue to profoundly impact contemporary North American Aboriginal populations.

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Archaeologists in Manitoba have worked for years to establish a working relationship with First Nations communities with respect to the analysis of archaeologically recovered human remains and it is certainly as a result of their efforts that I was able to conduct DNA analysis on the human remains. I would like to express my appreciation to the Manitoba First Nations communities that supported this research and for their interest in the work.

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What I will take into the future from this research is the pleasure I had learning aspects of new disciplines, and the enjoyment I had working with old and new colleagues, and friends.

Chapter 1 Introduction

> *The fine detail of the living past, rather than supporting one or other unequivocal pattern, is pointing up the diversity and contingency of past life. When we project back from the diversity of the present to find its roots in the distant past, the simple explanations are the favoured ones. When, however, we inject a rich and detailed body of evidence from the past itself, perhaps it is not surprising that those simple explanations become less persuasive, and the past becomes as diverse and intricate as the present* (Jones 2001:108)

For some time researchers have observed a differential pattern of resistance and susceptibility to infectious diseases between populations (Stead 1992). In the case of tuberculosis for example, contemporary populations clearly display differential susceptibility and resistance to this infectious disease. In Manitoba, 43% of all new cases of tuberculosis, occur among Aboriginals, 37% occur among foreign-born individuals and 20% among Canadian born non-aboriginals (FitzGerald et al. 2000; Grzybowski and Allen 1999). Infectious diseases like tuberculosis have, in the past and continue to have, a devastating effect on certain North American populations as a result of a combination of biological, genetic and social factors (Grzybowski and Allen 1999; Smeja and Brassard 2000). Studies of infectious disease among Aboriginal populations have focused on socio-economic factors contributing to disease resistance and susceptibility (Ferguson 1928; Herring 1992; Hrdlička1909; Waldram et al. 1995). Some studies acknowledge that genetic differences between populations are a contributing factor in disease susceptibility and resistance, however the basis for this disparity remains largely unexplored (Bellamy et al. 1998; Clark et al. 1987; Greenwood et al. 2000; Hill 1998a; Stead 1992). This research was undertaken to test the hypothesis that at the time of European-Aboriginal contact, the immunogenetic program of Aboriginal people could not efficiently mount an effective immune response against certain infectious diseases. Further, these differences in the immunogenetic programming are still present in contemporary Aboriginal and Caucasian populations (Nickerson et al. 2000).

Infectious disease agents have a profound effect on human biological and cultural evolution and have repeatedly influenced the course of history in the Old World (Crosby 1976; McNeill 1998; Ramenofsky 2003). A similar situation should be expected in the New World; however, much of what we know about disease on this continent comes from the historic period when written records chronicled events from the point of European contact to the present. For the most part, these records speculated that the pre-contact Native North Americans were healthy and disease-free prior to European contact (Crosby 1972). The assumption that the pre-contact Aboriginals lived in a disease- free and static environment without change, in terms of exposures to pathogens, is not only false but creates an image of a fragile population. Recognition that pre-contact Aboriginal populations did not reside in a disease-free environment is essential for understanding the health and disease status of these people. Identifying the presence of diseases throughout the pre-contact period is an important step toward exploring the biological and cultural adaptation that occurred in conjunction with diseases. The archaeological, osteological and molecular evidence does not support the idea that Aboriginal people lived in a disease free state before contact. Native North Americans, like the Europeans, experienced different pathogens and were equally influenced, culturally and biologically, to a specific albeit distinctive disease environment (Buikstra and Cook 1980; Cohen 1989; Pfeiffer 1984; Waldram et al. 1995).

The first migrants, who came across the Bering Strait into North America, had a common hunting and gathering lifestyle with those they left behind in Asia. These groups came from broadly similar nutritional and socio-economic environments, and they had experienced similar pathogenic environments. When the small migratory groups traveled to the New World, they left behind a range of infectious diseases that were "filtered" out by the arctic climate or by the bottleneck effect and they broke contact with the larger ancestral population in Asia. After Native North Americans established themselves in the New World, contact with people from the Asian and European continent ceased for thousands of years (Turner 1986; Wright 1995). It is generally accepted that the New and Old World populations were separated for a period of time that was long enough for each of the populations to adapt to differential environmental and pathogenic conditions (Peschken and Esdaile 1999; Ramenofsky 2003; Sousa et al. 1997; St. Hoyme 1969).

Chapter 2 of this thesis reviews the archaeological, osteological and molecular anthropological literature to explore and highlight the types of diseases that may have been present among pre-contact Aboriginal populations.

Chapter 2 also examines the events that occurred at the time of contact when the indigenous people of North America suffered a severe demographic collapse (Verano and Ubelaker 1992). The effects of contact between Old and New World populations is seen in the demographic changes that occurred among many Aboriginal groups in the 16th and 17th centuries (Pfeiffer 1984; Saunders et al. 1992). The high rate of mortality that occurred is usually attributed to the introduction of new diseases among the immunologically naïve Aboriginal populations -- a phenomenon referred to as the "virgin- soil" effect (Cockburn 1971). Virgin-soil epidemics are characterized by high mortality in all age groups because of the population's lack of acquired immunity to a disease (Crosby 1976). The idea that Aboriginal people had no immunity against the new infectious diseases introduced by Europeans is certainly not new, and in fact it was an idea that was consistent with some long held ideas about racially based biological inferiority or eugenics (Cunningham 1926). More recently, it has become apparent that tuberculosis, like other infectious diseases, continues to have a devastating affect on certain Native American populations as a result of a combination of biological, genetic and social factors (Canada 1999; FitzGerald et al. 2000; Hurtado et al. 2003; Smeja and Brassard 2000). The unequal risk of disease between different Native American populations may in part, be explained by environmental and socio-economic differences; however, the immunogenetic composition of a population, in relation to disease resistance and susceptibility, has been indicated (Cuenca et al. 2001; Hoffmann et al. 2002). Hurtado et al. (2003) recently demonstrated that the Aché experienced immunologic naïveté against tuberculosis. The fact that Native North Americans were not able to successfully battle smallpox, measles or influenza seems to support the concept that these people were immunologically naive however, archaeological and molecular evidence suggests a much more complex situation. The rates of morbidity and mortality among Native North Americans at the time of contact and during the post-contact period were geographically and temporally varied. It is hypothesized that, as a group, Native North Americans were adapted to a disease environment that inhibited their ability to

mount an effective immune response against new infectious diseases that were encountered a the time of contact with European populations. However, we also see geographic and temporal differences in mortality and morbidity rates among New World groups that may be a function of the fact that these populations did not follow a single subsistence and settlement pattern, nor was their risk of exposure to pathogens equal.

The fields of molecular biology and genetics have made exponential strides over the past five decades in unlocking the human genome. Within the past fifteen years, the analysis of DNA from ancient materials has become possible. Specifically, the Human Leukocyte Antigen (HLA) region which codes for an individual's immune response against infection has been extensively studied in relation to population relationship and disease associations (Braun et al. 1998; Kolman and Tuross 2000; Lawlor et al. 1991; O'Rourke et al. 2000). Chapter 3 discusses the adaptation of the human immune response to infectious pathogens and examines how anthropologists have used immunogenetic information to study migration patterns, ethnic associations with diseases, and genetic markers and disease. The identification and understanding of the genetic traits that influence a host's resistance or susceptibility to infectious diseases may provide new insights into disease transmission. Chapter 4 of this thesis presents the methods and results of a study in which differences in the frequency of specific immunogenetic markers were identified in a contemporary Aboriginal population as compared to a Caucasian population. In the context of their adaptation to different cultural histories and pathogen environments, the observed differences in the immunogenetic program of the Aboriginal group inspired the question -- what did their immunogenetic code look like in the past and why did the observed differences occur?

The advancements that have recently been made in the fields of molecular biology and genetics have contributed to the development of ancient DNA techniques and research. Consequently, the analysis of DNA from ancient materials has become possible, and molecular techniques are available to examine nuclear DNA target regions. Chapter 5 reviews the development and status of molecular analysis of ancient DNA derived from archaeologically recovered tissue. The methods for extraction and amplifying DNA from the mitochondria of ancient human cells are well developed. Less well developed, however, are techniques for detecting polymorphisms in the genetic

material from ancient nuclear DNA. Immunogenetic analysis of contemporary individuals is a standard clinical procedure, but this type of analysis has never been conducted on ancient human remains. Chapter 6 presents the methods and results of the mitochondrial DNA analysis in the skeletal remains from eleven archaeological sites in Manitoba. This chapter also reports the results of the development and application of a novel method for amplifying and detecting target regions in the nuclear chromosomes of fourteen ancient individuals. The assessment of some of the similarities and differences between modern and ancient individuals at targeted nuclear sites will expand our understanding of the evolution of disease susceptibility and resistance among Aboriginal populations.

The contemporary immunogenetic differences between the Aboriginal and Caucasian populations suggest that these populations are dissimilar with respect to their ability to defend against infectious diseases. Since the time of Aboriginal contact with Europeans, selective pressures and genetic admixture may have lessened the degree of immunogenetic differences between contemporary Aboriginal and Caucasian populations. The analysis of cytokine genotypes of ancient populations will enable the investigation of the extent of this change and will contribute significantly to our understanding of the influence that genetic traits have on a host's resistance or susceptibility to infectious diseases. This research will contribute directly to our understanding of the selective pressures that served to influence the immunogenetic program of contemporary Aboriginal populations (Hill 1998a; McGhee 1994; Thornton 1997). The comparison of the cytokine genotypes of ancient and contemporary Aboriginal populations will assist in understanding the evolutionary processes regarding biological and cultural response to infectious diseases. The holistic nature of anthropology allows for a multifaceted approach to determine the factors that served to shape the human immunogenetic program. The identification and understanding of evolutionarily-derived genetic traits that influence a host's resistance or susceptibility to infectious diseases may provide new insights into the evolution of diseases among Aboriginal populations.

Chapter 2 Dispelling the Myth: Disease in the New World

2.1 Introduction

Human populations adapt biologically and culturally to disease-causing pathogens found in their environments. The first migrants into North America had a common ancestry with those they left behind on the Asian continent; therefore, they shared a heritage in terms of their adaptation to pathogens that afflicted mobile hunter-gatherer populations. After Native North Americans moved across the Bering Strait and established themselves in the New World (ca 12,000 years before present), contact with

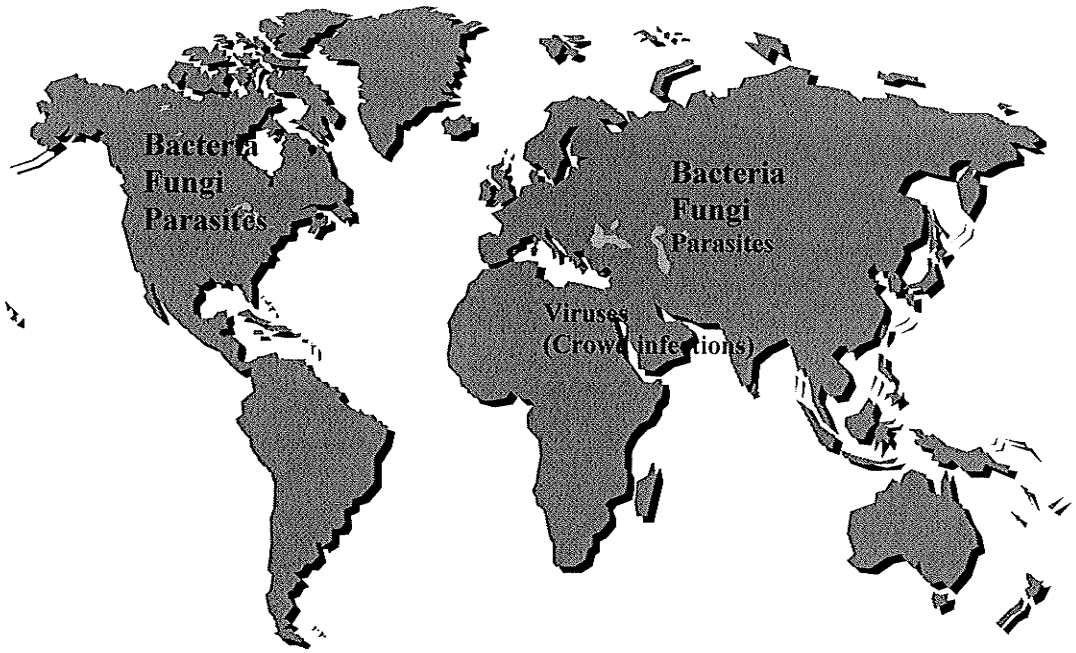


Figure 1. Disease distribution

people from Asia and Europe ceased until no earlier than ca. 1000 years ago. During that period, Native North American populations adapted to the new environmental conditions (including the pathogenic environment) they encountered in the New World. Meanwhile in the Old World, after 12,000 years before present, populations traded their hunting and gathering lifestyle for one that included the domestication of plants and animals, sedentism and subsequently, urbanization. These changes in subsistence and settlement resulted in profound changes in the pathogenic environment in the Old World. The so-called "crowd infections" associated with urbanization became prevalent, and, in order to survive, the Old World populations adapted to these novel infections both biologically and culturally. At the time of contact between North American Natives and those of European descent, the populations were therefore adapted to distinctly different disease-causing pathogens. This chapter explores the archaeological and historical records to reveal the types of pathogens that may have been prevalent in the New World before the contact period.

2.2 Disease in the New World

Some historic accounts of North American Aboriginal people at the time of European contact recount a handsome, strong healthy indigenous population. Brebeuf, for example, described the Huron as being "more healthy than we" (Thwaites 1896-1901:257). These glowing reports, however, do not match the osteological evidence, which shows that severe, and chronic disease conditions were not uncommon in the New World. Contrary to the myth that the New World people were disease-free, the osteological record of human remains demonstrates that these people were acquainted with a range of disease pathogens, which caused specific and non-specific infections. Merbs (1992:6) contends that migrants into the New World brought with them from Asia, a variety of disease-causing infectious organisms. The bacteria *Staphylococcus aureus,* for example, has a well-established parasitic relationship with human hosts, and tapeworms of the genus *Echinococuss* may have found transportation to New World populations via the dogs that accompanied the people. Hookworms are common to both the New and Old World, as were a variety of intestinal worms and parasites (Cockburn 1971:53). McNeill (1998:52) suggested that parasites like yaws or lice could survive

within small migratory hunting communities so long as the human hosts were not severely incapacitated by the pathogen. In essence, equilibrium between the pathogens and the human hosts was established and mutual survival was possible. The migrants also likely left behind certain infectious diseases that were "filtered" out by the arctic climate (i.e. malaria, leishmaniasis) or by the bottleneck or founder effect of small groups breaking contact with the larger ancestral populations.

The first New World inhabitants were exposed to a host of new disease-causing agents that resided in the Americas. The environment, into which these people came and adapted, contained its own resident pathogens, some of which were not found in the Old World. Animal reservoirs were a likely source of disease-causing bacteria in the New World as they were in the Old World. Lyme disease, which is caused by *Borrelia burgdorferi*, has been identified as having New World origins, as has Rocky Mountain spotted fever caused by the bacteria *Rickettsia rickettsii* (Steere 1989). These infectious disease-causing bacteria are both carried by wood ticks, and they have a particular geographic correlation in North America. Domesticated animals are reservoirs of viral and bacterial infection, which are potential pathogens for humans. The relative absence[1] of domesticated animals in the New World likely reduced the range of infectious diseases that could potentially infect pre-contact populations (Cohen 1989). However, St. Hoyme (1969) noted that the small, scattered human groups in the New World were in contact with large populations of wild animal populations that may have acted as reservoirs for endemic diseases (i.e. brucellosis and tuberculosis). In addition, New World populations were exposed to environmental pathogens such as soil fungi that can cause specific infectious diseases such as blastomycosis (Buikstra 1976).

An assumption that the New World people lived in a static environment without change or adaptation in terms of exposures to pathogens is not only false but creates an image of a fragile population. Recognition that pre-contact Aboriginal populations did not reside in a disease-free environment before contact is essential for understanding the health and disease status of these people (Saunders et al. 1992). Identifying the presence

[1] McNeill (1998: 210) contends that in South America the wild ancestors of the domesticated guinea pigs, alpacas and llamas were too few and too isolated to sustain infections within their populations, so that they were not a source of infection for humans.

of diseases throughout the pre-contact period is an important step toward exploring the biological and cultural adaptation that occurred in conjunction with diseases

2.3 Archaeological and palaeopathological evidence for disease in the New World

Osteological remains are the only source of information from which a pre-contact epidemiological profile can be derived. However, not all diseases that were present in the New World will be represented in the osteological remains. Sampling biases, differential preservation of remains and the fact that not all infectious diseases are represented on the hard tissue, result in an incomplete record of the disease status of the past populations. The historic records can be relied upon to a certain extent to document the health and disease status of Aboriginal populations, but they contain inherent biases. It is difficult to make valid comparisons between the pre- and post-contact Aboriginal populations using written accounts because they can only report on the post-contact populations. A comparison of infectious diseases among pre- and post-contact populations can make use of historic documentation, but it needs to be supported using the osteological record.

Evidence of the pathogens that afflicted the early North Americans have been diagnosed from mummified and skeletal remains dating to the pre-European contact period. An underlying premise of palaeopathology is that illness and injury will to a varying extent, cause changes in the bone morphology. Ortner (1991) pointed out that the infectious disease process work slowly on bone so that an individual must survive with the infection, perhaps chronically, in order for it to have an affect on bone morphology. If the disease is so virulent that the infected host dies quickly, then evidence of the infection will not be manifest on the bone. Recorded history provides documentation of plagues and epidemics that played havoc among populations. Although the Black Death killed one quarter to one third of the European population in the fourteenth century, palaeopathological evidence of this and other rapidly killing diseases such as small pox and measles leave no skeletal lesions. Evidence of non-specific infectious diseases that might compromise the health of individuals leading to reduced life-expectancy, or increased morbidity of a population, are identified by periosititis or osteomyelitis. Further, lesions of cribra orbitalia, porotic hyperostosis and enamel hypoplasia among

infants and children suggest that the individuals were stressed either because of malnutrition, and/or from parasitic infection. Although it is impossible to identify or classify the specific disease that may have caused these lesions, they can indicate a depressed state of immunity.

The use of molecular analysis of pathogens preserved in skeletal remains provides a means by which the presence of a limited number of infectious diseases such as tuberculosis and treponema can be confirmed in pre-contact populations. The analysis of the ancient DNA of disease pathogens gives us the ability to shed light on the evolution of pathogens. This avenue of research will contribute to a better understanding of the diseases that existed in the New World and their affect on human populations.

2.4 Non-specific indicators of infection and metabolic diseases

Many infectious diseases that cause death do not leave evidence on hard tissue. Viruses, plague, cholera, meningitis and dysentery all can cause acute infection and death yet leave no evidence on skeletal remains. Similarly, many types of infections or metabolic conditions can cause hard tissue pathologies but are virtually impossible to diagnose specifically.

Infections resulting from trauma, stress or the chronic presence of certain bacteria can cause bone lesions. Birkett states that,

> *The term [non-specific infections] is of course a misnomer, for every infection is a specific infection, but the more interesting infections due to leprosy, tuberculosis, and syphilis are traditionally described as specific, leaving the others as an undistinguished non-specific group (Birkett 1983:100).*

Non-specific infections and metabolic conditions can range in degree of severity from acute causing death to a long-term chronic infective state. The chronic conditions can cause gross morphological changes of hard tissues and compromise the immune system of an individual. Bone has only a limited gamut of reactions to any stress. Bone density may change, the contour of the hard tissue may undergo alteration with either erosion or reactive new bone formation, or reaction of the periosteal or endosteal membranes may be present in varying degrees. Osteomyelitic infections can cause dramatic bone pathologies, and before the development of sulphamides the mortality rate

from this disease was 30% (Birkett 1983). Osteomyelitis describes infection of the bone marrow cavity, which is produced by various kinds of micro-organisms and generally includes periostitis and osteitis. Osteitis is an inflammation of the bone and periostitis is an inflammation of the tissue covering the bone -- the periosteum.

Although the aetiology of non-specific infections is often not discernable from the skeletal remains, it is reported that 90% of the cases of bone inflammation are caused by the invasion and proliferation of the bacteria *Staphlylococcus aureus* into the blood stream and bone (Birkett 1983). The primary locations that osteomyelitic infections may occur are at the long bone metaphysis (Steinbock 1976). Acute infection will occur most frequently at the location on the long bones where the rate of growth and the size of the bone are the greatest. The focus and acuteness of the resulting infection is dependent upon the individual's ability to mount an effective immune response. Infection may be chronic as opposed to acute and may cause bone pathologies that may not necessarily directly result in the death of an individual.

Individuals who suffer from iron deficiency leading to anaemia may manifest the condition in certain bone structures. Cranial lesions on both the skull vault (porotic hyperostosis) and on the orbital plates indicate iron deficiency anaemia during childhood (Stuart-Macadam 1992). Cribra orbitalia is a term used to describe lesions on the orbital plates of the frontal bone. The lesions typically occur symmetrically on both orbital plates and the condition was linked with individuals and populations where anaemia may have existed (Cybulski 1977). Cribra orbitalia was first reported as a racial characteristic found more frequently among Mongoloids than Caucasoids (El-Najjar et al. 1976). The reports of the bony changes in living individuals with Thalasemia Major identified the relationship between iron deficiency anaemia and cribra orbitalia (El-Najjar et al. 1976). It is now generally agreed that cribra orbitalia and porotic hyperostosis are manifestations of acquired iron-deficiency anaemia which is caused by systemic stress resulting from malnutrition or pathogens (Stuart-Macadam 1992). Porotic hyperostosis is a condition where small holes of variable size occur on the surface of the skull vault.

Iron deficiency anaemia is a metabolic condition found in populations throughout the world although there are certain groups that are more likely to develop the condition

than others are. Diets that are low in iron content or high in foods that inhibit iron absorption (maize, rice and wheat) are implicated in the prevalence of iron deficiency anaemia during childhood. Weaning and parasites may also contribute to anaemia in children. Women of childbearing age, children and individuals suffering from chronic bleeding caused by parasites, ulcers and gastrointestinal cancer are particularly likely to develop anaemia. Among children, malnutrition is the leading cause of anaemia.

Although the exact causes of the stresses that caused lesions such as cribra orbitalia or porotic hyperostosis may not clearly defined from the analysis of skeletal remains, the presence of these lesions can indicate a depressed state of health and immunity of an individual. Evidence of non-specific infections and metabolic conditions that might compromise the health of individuals leading to reduced life-expectancy, or increased morbidity of a population are identified by periostitis, osteomyelitis, lesions of cribra orbitalia, and porotic hyperostosis.

2.5 Evidence of non-specific infections and metabolic conditions in North America

The osteological record provides evidence of non-specific and metabolic conditions that were present in the pre-contact indigenous populations of North America. Among the Alaskan Eskimos and Aleuts, Keenleyside (1998) reported that these people were afflicted with a number of infectious diseases including respiratory, ear and fungal infections and parasitic diseases. Active periostitis on the pleural surfaces of five ribs of an adult male individual from Alaska suggested that the individual had an inflammation of the pleura. Symptoms of this type could have been caused by pulmonary tuberculosis, although there is insufficient evidence to make a definitive diagnosis, and other causes are possible (Keenleyside 1998). Although rib periostitis was identified in the remains of individuals who had died of tuberculosis in the Hamann-Todd and Terry Collection, other conditions such as pneumonia and trauma can also produce rib lesions (Pfeiffer 1991).

A number of chronic lung conditions have been reported in frozen and mummified remains of pre-contact Eskimos and Aleuts (Keenleyside 1998). Emphysema, fibrosis and anthracosis resulting from the inhalation of smoke from seal oil lamps and cooking fires have been identified. From the mummified remains of a Thule child dating

to 800 years ago Zimmerman et al. (2000) identified extensive damage to the lungs as a result of pulmonary anthracosis. Damage of this type is frequently found in ancient Alaskan remains although the degree of destruction varies. The child was buried in a circular depression that was lined with seal and whale oil and may have once served as a meat cache in the floor of a semi-subterranean dwelling. A differential diagnosis was made which concluded that the child likely suffered respiratory disease and starvation. This diagnosis is supported by the association of the individual with a poorly ventilated dwelling and the lack of food in the meat cache (Zimmerman et al. 2000).

Pfeiffer (1984) found that periostitis, osteitis and osteomyelitis frequencies were surprisingly low among the Iroquois from the Uxbridge Ossuary. It was assumed that the incidence of non-specific infection might be high given the crowded living conditions in the Iroquoian longhouses. Evidence of non-specific infections among the Iroquois, in combination with archaeological information about settlement patterns and living conditions, can assist in the interpretation of the health status of the population or individuals. Pfeiffer suggests that immunity to staphylococci may have been acquired early in life among these Iroquoian individuals because of the living conditions (1984:187).

Evidence of cribra orbitalia and porotic hyperostosis has been found in a number of individuals from Manitoba. Finch and Waddell (1996) made this unexpected find in the pre-contact collection of remains from southern Manitoba and the Red River region. Six children from a collection recovered from mound burial sites yielded this evidence of anaemia (Finch and Waddell 1996). Finch and Waddell (1996) noted that anaemia is rarely found on the northern plains. The identification of this pathology in southern Manitoba may have implications for the interpretation of the local subsistence patterns and/or the prevalence of parasitic infections. Currently there is little archaeological evidence to indicate heavy reliance on maize horticulture in Manitoba although it may not have been entirely absent (Garvie 1993). In addition, the consumption of wild rice which is high in carbohydrates, may have been associated with increased incidence of dental caries (Ens 1998). Periostitis is also indicated in some individuals from the analyzed collections of human remains from Manitoba, although it is considered to have a low degree of expression (Finch and Waddell 1996).

Cybulski (1977) identified cribra orbitalia from 25 different historic Native localities on the coast of British Columbia. The high incidence of the condition among the Haida (52.9%) suggests that the pathology may have been a combination of iron-deficiency anaemia caused by disease or nutritional stress, and an inherited disorder. The higher frequency of cribra orbitalia among adult women and children (ages 6-18) in the historic sample is consistent with the contemporary expectations of the occurrence of iron-deficiency anaemia. Factors that would affect iron intake among the historic population would likely include parasites and diseases such as small pox, measles, and influenza and tuberculosis. The historic and pre-contact diet of fish and seafoods would potentially provide a rich source of iron, although disruption in acquisition of these resources may have occurred particularly during the historic period. Iron-deficiency anaemia therefore might have played an important role in the occurrence of cribra orbitalia among the coastal populations. The higher frequency of cribra orbitalia among the Haida who likely were affected by similar environmental factors for iron-deficiency anaemia may be explained by an inherited disorder that made this population more susceptible (Cybulski 1977).

Although cribra orbitalia and porotic hyperostosis are not necessarily life-threatening conditions themselves, they are indicators that an individual suffered from compromised health during childhood. The causes of these episodes, whether it was dietary deficiency, parasitic infections or the result of other undetermined diseases, cannot be determined from the lesions alone. However, these skeletal changes indicate biological and/or immunological stress, related to living in the New World environment.

2.6 Specific diseases

The hard tissue transformation caused by disease is complicated by the fact that the pattern, distribution and the degree of impact may vary. A single disease may look quite different on the remains of different individuals because each persons experience with the same disease will differ. In addition, skeletal remains do not typically display the full range of disease symptoms, which can further hamper an accurate diagnosis. In order to facilitate making a precise diagnosis, a researcher must make a differential diagnosis from a database of potential diseases and their related pathologies.

2.6.1 Arthritis

Arthritis occurs at the synovial joints (hips, knees, shoulders and elbows) where the articular surfaces of the bones are covered with cartilage and are held in place by the fibrous capsule and ligaments (Steinbock 1976:278). Rheumatoid arthritis (RA) is an autoimmune disease affecting symmetrically the joints of the arms and legs, particularly the hands and feet. New bone growth at affected sites is minimal. Additionally, the sacroiliac joints and the postcranial spine are not generally involved (Rothschild 1995:659). RA is difficult to diagnose in skeletal remains since it is difficult to distinguish between the arthritic conditions of osteoarthritis, septic arthritis, osteoarthropathies (ankylosing spondylities, psoriatic arthritis) and DISH (diffuse idiopathic skeletal hyperostosis) (Merbs 1992:33). A compounding difficulty in diagnosing RA is that an individual may have suffered from RA in addition to other arthritic conditions. RA usually affects the bones symmetrically, which is an aspect of the disease that makes it unique among the arthritic conditions. From a population perspective, RA usually affects women more frequently than men and has an onset period between 35 to 45 years of age. The lack of involvement of the spine in RA and the symmetry of bone erosion on the joints are indicators used to distinguish RA from other arthritic conditions.

The diagnosis of rheumatoid arthritis has been made for numerous specimens from the United States where its initial appearance was geographically confined during the Middle Pre-contact period to the Illinois area (Rothschild et al. 1992). In an examination of a range of Archaic, Mississippian and Woodland sites from central North America, Rothschild et al. (1992) documented a spread of the disease from a core region north and eastwards. In addition, the researchers illustrated that the European exploration of this core RA area was not undertaken until the mid-18th century and that this might explain why RA does not show up as a recognized disease in the Old World until after this time.

Lyme disease is vector-borne and therefore the occurrence and distribution of the disease within a population would be quite different from RA however, lyme disease produces arthritic lesions that can be mistaken for RA. In 1977, Lyme disease was diagnosed in children who had originally been diagnosed with juvenile arthritis, and it

was quickly determined that the disease was transmitted via an insect vector (Merbs 1992). In its most advanced stages, Lyme disease causes a chronic inflammation of one or a few large joints such as the knee (Steere 1989). Differentiation between Lyme disease and RA would likely come from the analysis of the pattern of bone lesions in an individual and from the pattern of distribution of the disease in a population.

2.6.2 *Treponema*

Evidence of treponemal disease found in human osteological remains in the New World has occupied palaeopathologists in considering its origins, manifestations and spread into the eastern hemisphere. The four syndromes of treponemal disease have geographic distributions that are correlated to climatic conditions. Yaws is found in humid, tropical climates and bejel (also called endemic treponema or nonveneral syphilis) in arid zones north and south of the humid zone (Hackett 1983:111). The distribution of venereal syphilis is not directly correlated to climatic conditions but is typically associated with urban centres in more temperate climates where clothing might prevent body-to-body contact (Steinbock 1976:92). Hackett (1983) asserted that yaws and pinta originated in the tropical or temperate zone of the Americas where it persisted and was spread by casual skin-to-skin contact. Bejel is commonly found in populations that inhabit more temperate regions where clothing is still somewhat optional and skin-to-skin contact is still frequent, again particularly among children (Hudson 1965). At Moundville Illinois, pathologies found on the skeletal remains matched the lesion morphology, prevalence and age distribution of modern treponemal disease more closely than any other disease (Powell 1988:175). Although the lesionous pattern was not identical to either yaws or modern non-venereal syphilis, "researchers have noted that treponemal non-venereal cases in locales climatically intermediate between the moist tropics and the dry subtropical or temperate regions often display clinical pictures intermediate between yaws and endemic syphilis" (Powell 1988:175).

The ability to discriminate genetically and biologically between the organisms that are potentially responsible for the four syndromes continues to elude researchers. The four different infections might be caused by different species of *Treponema* (*T. carateum*, *T. pertenue*, and two subspecies of *T. pallidum*) (Baker and Armelagos

1988:705). Rothschild and Rothschild (1996:560) like Hacket (1983) argued that bejel, yaws and syphilis are distinctly different diseases and not just different manifestations of the same disease. However, DNA analysis indicates that *T. pertenue* (yaws) and *T. pallidum* (endemic and venereal syphilis) may in fact have homologous sequences and therefore may represent a single species (Baker and Armelagos 1988; Hudson 1965). Three of the treponemal syndromes, yaws, endemic syphilis and venereal syphilis, can cause osseous changes. The fourth type of treponema infection, pinta, affects only the skin and so is not observable on skeletal remains.

In an extensive review of osteological evidence for treponema, Baker and Armelagos (1988) investigated the origins of the disease and contended that treponema (the non-venereal form) originated in the tropical or temperate areas of the Americas and that Columbus and his crew took the non-venereal disease to the Old World. In doing so, they passed on a pathogen that caused the epidemics of venereal syphilis in Europe as documented in the 1500's. "We do not claim that venereal syphilis existed in the New World; rather, we argue that the endemic non-venereal treponemal infection was present and was transformed into a sexually transmitted disease following its spread to Europe" (Baker and Armelagos 1988). Baker and Armelagos (1988) base this hypothesis on the osteological evidence from the New World where treponematosis is known to have a pre-contact distribution among Aboriginal populations. The non-venereal form of syphilis was documented in populations from numerous archaeological sites in the eastern United States including Illinois, Florida, Georgia, Alabama and California, which are well dated to the Pre-contact period. Evidence for treponema is lacking in the north-western portion of the United States although it has been identified in a Middle Pre-contact population in Manitoba (Waddell 1994).

It was only at the time of European exploration in the America's that Columbus's crew contracted the non-venereal infection and took it with them to Europe. The social and environmental conditions in Europe encouraged the transformation of the non-venereal disease to the venereal form in urban areas (Baker and Armelagos 1988:719). The "virgin-soil" phenomenon accounts for the rapid and virulent spread of the disease in the Old World during the 1500's. Additionally, this Columbian hypothesis is based on the presumption that the venereal and non-venereal syndromes of treponema are similar

enough to allow one syndrome to transform to another although the mechanism for this change is unknown. Baker and Armelagos (1988) support this argument by comparing the plethora of skeletal remains diagnosed with treponemal disease in the New World with the apparent lack of such remains in the Old World.

Barker and Armelagos's (1988) noted that there is extensive evidence of treponema in the New World, however, there is relatively little published evidence for this condition in Canada. Saunders (1988:727) noted that a recent recovery of 16th century remains that were potentially diagnosed as syphilic, prompted her to re-examine some 15th century southern Ontario evidence. "It is possible that treponemal disease became prevalent in this region as settlement intensified and population increased in the 15 th century" (Saunders 1988:727). Geise (1988) also reported that Cybulski (per. comm. in (Geise 1988) identified treponemal infection at two sites on the northwest coast of British Columbia. The sites have radiocarbon dates of 2,325 ± 90 B.P. and 3,490 ± 125 B.P. It is suggested that the four individuals had endemic non-venereal syphilis and that it was only after contact that venereal syphilis became more prevalent in this region (Geise 1988:722).

Possible treponematosis was identified in skeletal remains from sites in southern Ontario (Lennox and Molto 1995). One individual from the E.C. Row Site (ca. A.D. 1200 - A.D. 1400) had a systemic infection suggestive of tremponematosis. Chronic infection at the elbow was evident from the reactive periosteal tissue at the distal end of the humerus and the thickened proximal ends of the ulna and radius. In addition, the individual had thickening of the distal half of the tibia. The researchers considered treponemal disease to be a possibility for the observed lesions (Lennox and Molto 1995:23). Bone infection in the form of periostitis and dental pathologies was reported in 40 % of the individuals from that site, which suggested that the population experienced considerable nutritional and/or disease stress (Lennox and Molto 1995:23).

It is interesting to note that Saunders (1988) did not consider treponemal infection to be a potential diagnosis of pathologies found on human remains from the pre-16th century southern Ontario sites because treponema had never been identified in this area or time period. However, once the disease was identified and researchers considered it as

a potential diagnosis, the frequency of the disease increases. As difficult as it is in some cases to make an accurate diagnosis, it is even more difficult to declare that a disease was not present. Saunders et al. (1992) argued that knowing the non-biological cultural factors that existed in the past were important for identifying the diseases. The conditions within the 14th and 15th century Ontario Iroquoian villages, for example, likely increased the opportunity for infectious pathogen survival. The close and crowded living conditions in the long houses would have facilitated the transmission of disease from person to person or through animal vectors. The scattered refuse dumps would have attracted mosquitoes, ticks and midges that are known disease carriers, as well as dogs and rodents, which are carriers of a variety of zoonoses like rabies and typhus (Saunders et al. 1992).

2.6.3 Tuberculosis

Before 1957 is was thought that tuberculosis did not exist in pre-contact New World populations. Lichtor and Lichtor (1957) and Morse (1960) reported the occurrence of tuberculosis in skeletal remains from ancient individuals in the Americas and changed the way that researchers regarded the status of the disease in the New World. Tuberculosis has been identified from skeletal remains dating to as early as 1000-2000 BC in Egypt and in Europe 5,000 BC which coincides with the Neolithic period (Steinbock 1976). The high rates of morbidity and mortality among Aboriginal populations caused by tuberculosis at the time of contact with Europeans suggested that tuberculosis was new in America at the time of contact (Cockburn 1963). However, pre-Columbian evidence of tuberculosis in the New World now comes from a variety of locations in both South America and North America. Although Hrdlička (1909) recognized that cultural and environmental factors influenced the spread of tuberculosis, he also cited lack of immunity and contact with Caucasians as important causes for the spread of the disease among Aboriginal people. Between the time of Hrdlička's (1909) report on the status of tuberculosis among contemporary Aboriginals and Morse's (1960) review of tuberculosis in the New World, a number of palaeopathology reports presented diagnoses of tuberculosis primarily involving extreme cases of spinal pathologies (Ritchie 1952). The extent of tuberculosis in pre-contact America was underestimated because the disease was only identified in specimens with acute pathologies. Interestingly, the geographic concentration of tuberculosis was in eastern North America

with very few cases reported from the Southwest (Buikstra 1981). This may have represented the true distribution of tuberculosis, or it might have been an artifact of a sampling bias on the part of the researchers.

Evidence of tuberculosis has been found in mummified remains in Peru that date to 700 AD (Allison et al. 1981) and in central Illinois where tuberculosis bone lesions are found among Mississippian groups (Buikstra and Cook 1980). In addition, Pfeiffer (1984) identified skeletal tuberculosis in remains from a Pre-contact Iroquoian ossuary in southern Ontario. Each of these reports suggested that the presence of tuberculosis, while tenuous if based on skeletal analysis alone, was possible given the cultural context (sedentary horticulturalists) in which the individuals lived. Saunders et al. (1992) pointed out that the socio-demographic factors of pre-contact Iroquoian population made it possible for infectious diseases to proliferate. Longhouse villages occupied by 200-300 individuals predisposed these people to infectious disease as compared to single-family house dwellers.

Hartney (1981) and Pfeiffer (1984) considered tuberculosis as a reasonable diagnosis of the pathologies on the remains from several ossuary sites in southern Ontario. Hartney (1981) found that a sample of 1523 individuals from ossuaries dating between A.D. 1000 and A.D. 1650, 3% had resorptive lesions. Pfeiffer (1984:188) found that lytic lesions of the vertebra affected too many of the individuals from the Uxbridge ossuary (ca A.D. 1450) for the pathology to be explained by any rare disease or neoplasm. Rather, Pfeiffer (1984:188) considered the resorptive lesions on the vertebra, and at the articular surfaces of the long bones to be indicative of tuberculosis. The frequency and distribution of lesions in the lower thoracic and lumbar regions of the spine and on the pelvis were consistent with tuberculosis. A potential diagnosis of fungal diseases such as blastomycosis was considered, but it was unlikely that a soil-born fungal infection would be found as frequently as the observed lytic lesions at Uxbridge (Pfeiffer 1984). At the time that Uxbridge was occupied, between A.D.1000 and A.D. 1650, large villages with multi-family dwellings were used, and a subsistence base of hunting, fishing, gathering and horticulture encouraged sedentism. The occurrence of tuberculosis among pre-contact populations may have been correlated with the increased population density and sedentism. Tuberculosis requires a person-to-person transmission, and would

cause higher frequencies of lytic lesions in the vertebrae than would infection resulting from local fungi. The crowded living conditions, shared eating utensils and lack of sanitation would promote the transmission of tubercular infection. In addition, Pfeiffer (1984) suggested that poor-quality cortical bone might point to nutritional deficiencies that may have weakened the immunity of the individuals making them more susceptible to infectious diseases.

Among northern populations of Aleuts, Eskimo and Caribou Inuit, evidence for pre-contact and post-contact tuberculosis from skeletal remains is sparse. Keenleyside (1998) found rib lesions on one Alaskan Eskimo individual, which may have been caused by tuberculosis although other sources of infection were not ruled out. The archaeological evidence indicated that crowded living conditions, such as those found among the Aleuts who lived in large semi-subterranean dwellings with as many as 40 people may have facilitated the spread of infectious disease. Buikstra (1976) evaluated the frequency of rib lesions, the age distribution and socio-economic conditions of a Caribou Inuit skeletal population to differentially diagnosis tuberculosis among that historic population. Although the population base was low, evidence showed that human reservoirs of the disease could be maintained within small populations because of the long infectious period (Black 1975).

The presence of tuberculosis among pre-contact Aboriginal populations raises questions about the evolution of the disease and human biological and cultural adaptation to the disease. In 1909, Hrdlička concluded that based on the mortality and morbidity rates among the Aboriginal populations attributable to tuberculosis, the populations lacked immunity to the disease because they lacked experience with the disease (Hrdlička1909). However, pre-contact evidence of tuberculosis in the New World now comes from a variety of locations in both North and South America including Peru (Allison et al. 1974) central Illinois, and from the Mississippi area (Buikstra and Cook 1980). In addition, skeletal tuberculosis has been identified in the human remains from a pre-contact Iroquoian ossuary in southern Ontario (Pfeiffer 1984). The existence of tuberculosis in the New World prior to European contact as indicated by the osteological record required some rethinking regarding the origins and nature of the disease. It is apparent that pre-contact populations supported a form of tuberculosis despite the fact

that they were not living in dense urban environments. Black (1975:517) noted that among the small contemporary tribes of Amazonian Indians, tuberculosis could maintain itself contrary to the notion that the disease is considered a "crowd infection". Tuberculosis is a disease most often associated with a certain crowded living conditions, poor sanitation and consistent and sustained contact between humans. The reservoirs of mycobacteria that exist among plants and animals had the potential for infecting humans.

The diagnosis of *Mycobacterium tuberculosis* among ancient populations has been greatly enhanced by DNA probes for the direct detection of the pathogen (Braun et al. 1998). The presence of tuberculosis cannot be reliably diagnosed through visual inspection of bone alone; therefore, the identification of the disease through DNA analysis can help confirm its presence. Braun (1998) confirmed the occurrence of *Mycobacterium tuberculosis* from vertebral lesions in two pre-contact specimens from North America by testing lesions for a known modern sequence of *Mycobacterium tuberculosis.* Bone specimens were examined for the presence of two specific human mtDNA fragments and for the mycobacterial IS6110 genomic element (Braun et al. 1998). Braun et al (1998) used small cloned 92-bp and 123-bp regions of ancient DNA to compare to modern mycobacterial IS6110 genomic element. The results indicated that the ancient genomic sequence from their samples matched the modern mycobacterial DNA sequence although the full extent of the genomic similarity between modern and ancient tuberculosis is yet to be determined (Braun et al. 1998). Baron et al. (1996) also detected *Mycobacterium tuberculosis* in bone specimens that had no visible tubercular lesions. Using autopsy specimens from a historical pathological collection, the researchers were able to amplify the IS6110 sequence from the bone samples and therefore confirm the presence of tuberculosis from visually uninvolved bone. In theory, it is plausible that *M. tuberculosis* bacteria may have been present in the bone without causing lesions. Additionally, Mays et al.(2001) confirmed the presence of *M. tuberculosis* using DNA probes in a bone specimen where osteological indicators led to an ambiguous differential diagnosis of as many as three different causes.

Recent characterization of the genomic deletion events of mycobacteria suggested that it is *M. bovis* rather than *M. tuberculosis*, which has undergone extensive adaptation. Tuberculosis would therefore be a more ancient strain of mycobacteria than *bovis*. The

fact that the *M. bovis* genome is much smaller (because of genomic deletions) than that of *M. tuberculosis,* suggests the human form of the pathogen is older (Brosch et al. 2002) Brosch et al. (2002) identified a "modern" strain of *M. tuberculosis* that is recognized by the deletion of "TbD1". In order to assess whether or not the TbD1 strain was present prior to human migration into the New World, Brosch et al. (2002) noted that it would be helpful to know whether the Egyptian and South American mummies (Arriaza et al. 1995) carried the TbD1 deleted gene. The resurgence of tuberculosis as an epidemic disease among Aboriginal populations could be explained by the introduction of a "modern" *M. tuberculosis* strain with the TbD1 deletion into a population who had survived with an older endemic form of the disease.

2.6.4 Dental disease

Diseases of the teeth and gums were present among the pre-contact North American populations. The investigation of dental diseases and pathologies can provide information about the health and nutritional status of a population (Waldram et al. 1995). Researchers use changes in the prevalence and incidence of dental pathologies to compare the health and nutritional status of various groups. Teeth are sensitive indicators of disease and health status of individuals and populations. Developmental disruption because of episodes of disease or malnutrition that occurred during childhood may affect the development of the tooth enamel. Increased prevalence of dental disease would lessen a population's resistance to other infectious diseases because of a suppressed immune response. Therefore, while dental diseases they may not directly contribute to mortality their presence reflects compromised immunity and an increase in the host's susceptibility to infectious diseases.

Tooth wear and dental diseases are typically related to age. The probability of having infectious oral pathologies increases in relation to the amount of tooth wear, which in turn is related to the age of the individual. The prevalence of dental caries and plaque related diseases among pre-contact populations has a strong correlation with diet – particularly with the amount of carbohydrates. The introduction of maize, which is high in carbohydrates, was accompanied by changes in oral health. The health of the Mississippian populations and the Iroquoian groups changed when they began to rely

intensively on maize between 900 and 1200 A.D. (Schwarcz et al. 1985). This change was reflected in the increased rate of dental diseases caused by the high carbohydrate content of maize. Cook (1984) indicates that periodontal disease was related to tooth wear and age but these affects were more pronounced during the Mississippian period when maize was more intensively cultivated as compared to earlier Woodland periods. The Indian Knoll Site mirrors this pattern in the Ohio valley where, during the Archaic period, Cassidy (1984) noted that dental caries were rare to absent. During the later Fort Ancient phase represented by the Hardin Village site where maize was intensively cultivated, dental caries were common in all age classes after infancy.

Antemortem tooth loss among individuals can be attributed to dental abscesses from trauma, caries and attrition. Costa (1980), Merbs (1983) and Keenleyside (1998) identify attrition as the primary cause of antemortem tooth loss among Eskimo and Aleut populations given the almost complete absence of caries among the studied groups. Keenleyside (1998) identified only 1 of 1,840 teeth from Pre-contact Eskimo and Aleut populations that had carious lesions. Merbs (1983) identified a number of behaviours that may contribute to antemortem tooth attrition and loss particularly of the anterior teeth. Typically among the Inuit populations the anterior teeth were used as vices or tools for holding or pulling other materials such as hides, twine, drills, or meat (Merbs 1983). Tooth wear might also account for the observed frequency of periodontal disease among the Eskimo and Aleut. Approximately 43% of the Eskimo sample and 47% of the Aleut sample had periodontal disease (Keenleyside 1998). Costa (1980) found similar frequencies among the Eskimos from Point Hope Alaska. Both studies found that periodontal disease was more frequent among older individuals.

Specific and non-specific infections as well as nutrient deficiencies can cause defects such as pitting or scoring of the enamel. Enamel hypoplasia and the microscopic enamel defects called Wilson's bands are enamel defects resulting from the disruption in the metabolic processes of the ameloblasts which form the enamel (Lim et al. 2002; Powell 1988:68). Changes in the prevalence and incidence of enamel hypoplasia can be indicative of systemic stress in a population. The aetiology of this stress can have multiple sources however; enamel hypoplasia is not a definitive indicator of infection, disease or malnutrition. Episodes of nutritional stress are believed to be the primary cause

of hypoplasia particularly in children whose teeth are particularly sensitive. Hypoplasia was found more often among the early horticulturalists than among hunters and gatherers. Cook and Buikstra (1979) noted that horticulturalists experienced less frequent, but more severe nutritional stress and starvation than did earlier hunters and gatherers who likely suffered more frequent but less severe seasonal hunger. Among the Eskimo and Aleut populations, Keenleyside (1998) found that the Eskimo had a significantly higher frequency of enamel hypoplasia than did the Aleut. Both of the pre-contact populations likely suffered periodic food shortages, but it is suggested that the Eskimo group may have sustained longer more intense shortages than did the Aleuts (Keenleyside 1998:65).

2.7 Culture change and adaptation to disease during the pre-contact period

Many pre-contact Aboriginal people continued to be mobile hunters and gatherers until well into the 20th century while others developed subsistence strategies that involved horticultural activities and increased sedentism. Cohen (1989) identified and discussed changes in the health and disease status of populations in the Old World that occurred in association with the advent of agriculture. Drawing upon research from the American Midwest, Cohen (1989) used osteological and epidemiological evidence to demonstrate that increased cultural complexity, sedentism and agriculture (or "civilization") did not lead to improved health and nutrition. The types of infectious disease and the amount of immunological stress placed on certain populations by disease changed because of alterations in subsistence and settlement patterns. The resulting metabolic stresses are indicated on skeletal remains by a change in the frequency of osteological and dental stress markers including enamel hypoplasia and dental pathologies.

Dietary changes are implicated in the decline of health status in general among horticulturalists as compared to hunters and gatherers. In both the New and Old Worlds, the change in diet from one of meat and a variety of vegetables to a diet that relied heavily on starch, is implicated in the higher prevalence of dental caries (Larsen 1995). In During the Early Woodland period of the Ohio River valley for example, the rate of dental diseases increases as a result of the high carbohydrate content of maize which reflects a shift in subsistence (Cassidy 1984). Increased prevalence of dental disease would lessen a population's resistance to other infectious diseases by suppressing or

overloading the immune response. Powell (1988), Cook (1984) and Cassidy (1984) found that the increased prevalence of dental caries among the Mississippian and Woodland peoples of Illinois and Ohio was related to the increased dependence of maize horticulture. In these studies, the frequencies of dental caries among early horticulturalists were compared to the relatively low frequency of caries found among earlier hunters and gatherers. Goodman et al (1984) demonstrated that the introduction of maize in the Lower Illinois valley coincided with the worsening of health in children. The authors contended, however, that the decline in health during the Mississippian period was the result of increased population density, and increased long-distance trade, which occurred along with the intensification of maize agriculture (Goodman et al. 1984).

Changes in specific endemic disease patterns coincided with the alteration of subsistence and settlement patterns of horticultural populations. Pfeiffer (1984), Powell (1988) and others have identified an increased prevalence of tuberculosis and treponema among the early horticulturalists that was likely related to changes in subsistence and cultural practices and the subsequent decline in overall health. Cook (1984) compared the indicators of health stresses among the early horticultural Late Woodland Illinois Valley population with the well established Mississippian horticulturalists and found that the change in diet alone could not explain the identified pattern of ill health among these groups. Cook (1984) demonstrated that while Late Woodland populations suffered nutritional stress that was evident in the apparent childhood health problems, the later Mississippian populations suffered less from nutritional stress but experienced increased density-dependent infectious diseases that were absent among the earlier populations. Cook (1984) concluded that health changes during the incipient and later horticultural phases of the North American pre-contact period were a complex interaction between ecological and cultural changes.

2.8 Culture change and adaptation to disease at the time of contact.

The effects of contact with Europeans by New World populations had direct consequences on the mortality rate of the native population. The extent to which epidemics caused New World depopulation is largely evaluated through population estimates before and after contact. Estimates of pre and post-contact populations rely on

data from historical and ethnohistorical accounts, archaeological surveys, computer simulations and epidemiological theory, all of which contribute to understanding the New World demographic profile around the time of contact. Demographic profiles of pre and post-contact Native Americans shed some light on the effect of diseases in the New World, however, the historic, ethnohistoric and archaeological records are incomplete and/or biased sources of information that make reconstructing population profiles difficult. The reaction to disease by New World populations at the time of contact is difficult to evaluate because the effects of indirect contact and of the local or regional variable responses to infectious pathogens. However, it is from this time that we have the most archaeological and historical documentation regarding demographic decline, primarily as a result of mortality due to disease.

The effect of European contact on the health of Native Americans was wide-ranging and varied. It cannot be assumed that Native Americans experienced a homogenous response to the infectious diseases at the time of contact with Europeans or that population decline was uniform from region to region (Joraleman 1982). The diverse responses and adaptations to European contact both biologically and culturally ensured the survival of the Aboriginal population in the New World. Herring (1994) and Trimble (1994) use historic information to demonstrate how cultural and behavioural factors influence a population's response to epidemics. It is evident that cultural behaviours and patterns played a significant role in disease transmission and in the ability of populations to respond to epidemics. The use of more recent historic information for the development of models regarding transmission and population responses to disease are important techniques for understanding the consequences of initial Aboriginal contact with Europeans. The simplistic virgin-soil model has limited application for understanding the complex biological and cultural responses to disease.

North American Aboriginal demography was influenced by infectious disease through direct and indirect contact with Europeans from the seventeenth century onward (Waldram et al. 1995). There is significant variability in the amount and quality of demographic and epidemiological information from the time of Aboriginal-European contact; however, Ubelaker (1992) has estimated a pattern - if not actual numbers – of significant population decline among Aboriginals. The decline of the New World

population after the time of contact and the subsequent population stabilization and increase, demonstrates the impact of a novel disease on a population and a degree of long-term successful adaptation. The evaluation of the impact of disease beginning at the time of contact on Native Americans rests on estimates of population size pre- and post-contact.

It is evident that the transmission of novel diseases likely preceded the arrival of Europeans in many regions of the New World. McGhee (1994) and Ramenofsky (1987; Ramenofsky 2003) used the archaeological information to demonstrate that the effects of infectious disease caused depopulation and culture change before direct European contact. A simplistic model of a catastrophic demographic decline and the resulting cultural response, cannot adequately explain the varied consequences of contact. It is only through the exploration of both the biological and cultural responses to disease that enable researchers to develop more complex models regarding the impact that European contact had on the New World populations.

2.8.1 *Disease and population estimates at the time of contact*

The extent of the Aboriginal population decline at the time of contact was typically underestimated until Dobyns (1966), demonstrated that the impact of new disease pathogens resulted in demographic collapse. Dobyns (1983) used two methods for calculating the population density of Native Americans at the time of contact. The Malthusian theorem was based on the assumption that human beings tend to increase in population to the limits imposed by key food resources. Dobyns (1983) also used historic population estimates, which were historically enumerated. These sample censuses were then used to estimate a continental population of approximately 18 million (1.4 persons per km^2) Native Americans in the early sixteenth century (Dobyns 1983:42). This estimation was significantly higher than the figure calculated by the U.S. Census bureau in the nineteenth century of 0.77 persons per km^2. Dobyns (1983) contended that Native American populations could not have survived the numerous epidemic events and subsequently increase in population size if their numbers had not been so great prior to contact.

The rapid spread of disease and the high rates of mortality among Aboriginal groups as a result of measles, small pox and influenza and then later tuberculosis, follow the pattern of a virgin-soil epidemic. The virgin-soil effect describes a populations inability to mount an immune response against an infectious disease that it has never previously experienced (Crosby 1972). Virgin-soil epidemics are characterized by high mortality in all age groups because of the population's lack of acquired immunity to a novel disease. Survivors of a virgin-soil epidemic likely had some biological or cultural advantage, and, over time, this advantage was reproduced within the population. The more detailed analysis of the depopulation events in the New World demonstrate that the virgin-soil epidemic model represents an overly simplistic model of the effect of contact between Aboriginal and European populations. Thornton et al. (1991), for example, demonstrated that the various depopulation rates estimated by Ubelaker (1988) and Dobyns (1983) present different versions of Aboriginal depopulation after 1500 A.D. Ubelaker (1988) estimated a depopulation rate that gradually increased from 1500 to 1800 A.D. (Figure 2).

In contrast, Dobyns (1983) showed a gradual decrease in the rate of population decline from 1517 to the mid -1500's and then a gradual increased rate of decline into the 1600's (1983). Thornton et al. (1991) use this information to support a model of the initial response and diffusion of the smallpox epidemics. The model does not support a sudden pandemic outbreak of smallpox throughout the southeast United States. Although the estimated rate of depopulation during the 1500's is far from adequate, the model suggests that either isolated episodes of small pox occurred where populations were buffered by limited contact, or that a series of slowly developing epidemics occurred throughout the region or parts of it.

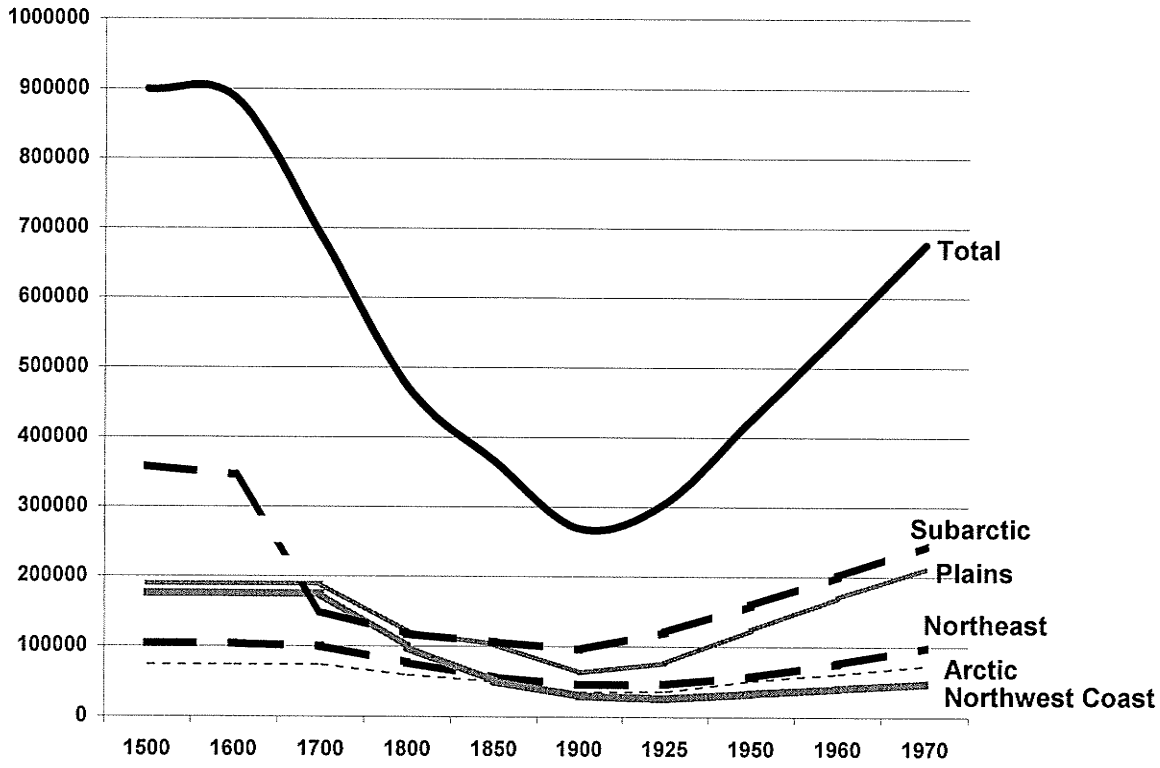


Figure. 2 Estimates of Native North American Population Size by Region

(Adapted from (Ubelaker 1988)

Settlement patterns, trade and social networks and subsistence patterns all played a significant role in the transmission of disease in the New World at the time of contact with Europeans. A simplified model suggests the largest and densest Native American populations that were in contact with Europeans, experienced the earliest and most intense depletion. However, trade routes served as vectors for infectious diseases and the affects of contact were therefore not necessarily related to geographic position or the extent of direct contact (Trimble 1985). Morbidity was not the only factor that contributed to Aboriginal population decline at the time of contact. Thornton (1997) pointed out that decreased fertility rates, which are associated with increased mortality would have been important in long term population depletion. In addition, the mortality rates cannot provide details regarding the other ramifications of an epidemic.

Herring (1994) used archival data from parish registers, Hudson's Bay Company journals and the Provincial Archives to demonstrate the differential impact that the Spanish Flu epidemic of 1918-1919 had on Aboriginal communities in northern Manitoba. Contact with fur traders had occurred for some time at Norway House before the epidemic, and the community was an important trade focus for other northern centres. At Norway House, eighteen percent of the people died as a result of the 1918-1919 flu epidemic–a number far in excess of the estimated three percent mortality rate for Canadians (Herring 1994:96). In addition, Herring (1994) noted that while Norway House suffered extensive losses, Oxford House and God's Lake escaped the epidemic altogether. Norway House's key position as a fur trade centre for the north, and the frequent contact between traders with locations to the south, brought residents of this community into more frequent contact with micro-organisms than was the case for other communities. Although eighteen percent of the population at Norway House died of the flu, the population recovered within five years due to increased rate of births after the crisis. This differential occurrence and impact of an epidemic in a relatively small region serves as a model for the early historic disease transmission.

Trimble (1994) examined the response to the 1837-1838 small pox epidemic among Northern Plains populations. Differential mortality and morbidity rates were historically observed between the horticultural communities of the Mandan, Hidatsa and Arikara and the mobile hunter-gatherers—the Sioux, Iowa and Otoe. The greater freedom of movement maintained by the mobile Sioux, Iowa and Otoe allowed for fewer contacts with potential pathogens, less crowded living conditions and the ability to disperse to avoid contact with infected individuals. The semi-sedentary Mandan, Hidatsa and Arikara on the other hand resided in crowded semi-permanent earth lodges that facilitated the spread of pathogens and fixed individuals to an infected community (Trimble 1994). Trimble (1994) explains that the mobile tribes that experienced the fewest losses were able to sustain traditional life ways and eventually became the most powerful tribes on the Plains.

Herring (1994) and Trimble (1994) used historic information to demonstrate how cultural and behavioural factors influence a population's response to epidemics. While mortality rates are often the most dramatic response to a virgin-soil epidemic, the impact

of disease played a significant role in the history of these populations, and they can provide models for understanding the complex interaction of disease and cultural adaptation during the pre-contact period. It is evident that cultural behaviours and patterns play a significant role in disease transmission and in the ability of populations to respond to epidemics. The use of more recent historic information for the development of models regarding transmission and population responses to disease are important techniques for understanding the consequences of initial Aboriginal contact with Europeans. The simplistic virgin-soil model has limited application for understanding the complex biological and cultural responses to disease. Palaeo-epidemiological models need to include a detailed understanding of the long and short-term human cultural responses to disease in combination with knowledge of the biological factors that facilitated resistance and susceptibility to certain pathogens.

2.9 Summary

The New World was home to numerous resident pathogens that did not distinguish between animal and human hosts. The skeletal remains of the North American Aboriginal population show that these people were not disease free and that parasitic, fungal and bacterial infections were present before historic contact with Old World populations. The analysis of pathologies on the skeletal remains from pre-contact archaeological sites has resulted in the identification of a number of diseases including treponema, rheumatoid arthritis, fungal infections, a range of non-specific infections, and possibly a strain of tuberculosis, all of which would have compromised the health of past populations. New World populations resided in the Americas for thousands of years, subsisting, for the majority of that time, as mobile hunters and gatherers. Changes in the settlement patterns, subsistence strategies and social organization occurred during the pre-contact period in the New World, and these changes resulted in alterations in the frequency and prevalence of some diseases. Among the few horticultural groups that appeared after ca. 1200 A.D., the frequency of osteologically identifiable disease changed in comparison to the hunter-gatherer groups. These changes caused swift biological responses to changes in the pathogen environment. The pre-contact New World Aboriginal populations were distinctive in terms of their geographic distribution and

temporal placement and were therefore hosts to a range of differing pathogens depending upon climate, population size and subsistence pattern. It is not surprising therefore, that at the time of contact with Europeans, diseases affected North American Aboriginal populations in a non-uniform manner.

New World pre-contact populations were neither disease-free nor were they unchanging in their cultural or biological adaptation to their environment. The human response and adaptation to disease pathogens is a major factor in human history, one that is true of both the Old and New Worlds. There is substantial archaeological, osteological, and genetic evidence to suggest that many pre-contact populations in the New World experienced mortality and morbidity due to disease conditions related to population density, subsistence and cultural behaviours. The documented presence of infectious diseases such as tuberculosis, treponema and rheumatoid arthritis illustrate that the pre-contact New World populations lived with endemic diseases in addition to parasitic and some bacterial infections that were brought from the Old World. While it is currently not possible to know the full extent of the disease load that the pre-contact populations carried, it is certainly evident that New World populations were acquainted with, and adapted to, resident disease pathogens. The archaeological and osteological evidence supports the contention that the New World populations were adapted to a disease environment that was distinctive compared to that of the Old World. The North American Aboriginal populations sustained primarily a hunting and gathering lifestyle that was conducive to the maintenance of diffuse settlements with low population densities. This New World lifestyle and the isolation from Old World pathogens were influential factors in the Native North American Aboriginals biological adaptation to disease.

Chapter 3 Human adaptability to infectious disease

3.1 Introduction

The most dramatic evidence of the Native North American's adaptation to the New World pathogen environment was the demographic disaster that occurred when Old World explorers, merchants and military introduced novel pathogens into the Americas. The virgin-soil epidemic model is a useful tool for understanding the overall effect of new diseases such as measles, small pox, and flu among Aboriginal populations, however it fails to explain the varied population and individual response to these diseases. The Old and New World populations shared a common biological heritage yet; within the past 15,000 years, biological and cultural changes occurred. The virgin-soil epidemic model does not adequately explain the biological processes that occurred, at the time of contact, nor does it take into account the adaptability of the human immunogenetic program. The human immune response to foreign pathogens that invade the body is a complex system that has evolved to allow individuals to survive exposure to infectious agents. Understanding the processes involved in the biological response to pathogens and the genetic factors that contribute to disease resistance and susceptibility are integral to understanding the adaptation to diseases by New World Aboriginal populations.

This chapter will examine how the human host responds to infectious agents at the cellular level and investigate how the immune system has adapted to the vast array of disease-causing pathogens. The adaptiveness of the human immune system and the variation that exists between individual's immune response to pathogens are features that are directly related to variability in the structure of the genes that function to keep the body clear of infection. The level of immunologic diversity that exists between populations and individuals is evident in blood group analyses, the serological and genetic analysis of the Human Leukocyte Antigen Complex (HLA) and in the proteins that influence the immune response. The identification and analysis of immunogenetic polymorphisms has exploded with the comparatively new and emerging technologies.

New molecular technologies allow for the detection of single nucleotide polymorphisms (SNPs) that affect the immune response by influencing the protein expression and in turn influences resistance and/or susceptibility to infectious pathogens. Anthropologists have focused on the polymorphisms in the HLA complex because it holds information about population relationships and ancestry. However, the occurrence and maintenance of particular SNP polymorphisms within populations is related primarily to functional adaptiveness, and it is this relationship that is of interest here.

3.2 Anthropology and immunology

The affiliation between anthropology and immunology is a comparatively recent development with respect to the discipline's expansive multidisciplinary collaborations. One of the most famous stories in anthropological genetics is Frank Livingston's analysis of the relationship between the HBB*S allele and endemic malaria in Africa (Livingstone 1983; 1984). The hemoglobin beta locus polymorphism (HBB) was one of the earliest blood proteins studied, and it is a classic example of evolution defined as "allele frequency change" and the maintenance of balanced selection within a population (Williams 2003). The HLA-A locus is relatively new to human genetics but has been intensively studied over the past three decades. One of the primary characteristics of the HLA loci is its allelic diversity and variation. As of 2002, 237 DNA alleles have been defined at the HLA-A locus (Williams 2003). The number of HLA allelic variants is so great that the chance of finding two unrelated individuals with identical HLA is very small. The diversity and specificity[2] of each individual's immune system became clear in the late 1960's and 1970's when organ and tissue transplantation posed difficult genetic problems. Tissue rejection was determined to be genetic because transplantation between monozygotic twins who had identical genotypes was always successful, whereas transplantation between one individual and another chosen at random from the population was usually not (Bias 1981). Many different genes participate in the immune response, and this diversity creates the incompatibility in transplanted organs. It is this diversity that has intrigued anthropologists, not so much for its functional significance with respect

[2]The ability of the immune response to identify and respond to a specific antigen.

to the immune response, but because the genetic markers (polymorphisms) can be used to investigate the historical relationships between different human populations.

3.3 Human Leukocyte Antigen polymorphisms and variation in disease susceptibility

Interest has increased in the past ten years in both Major Histocompatibility Complex (MHC) and non-MHC genes that function to direct the mammalian immune system against infectious pathogens. The MHC complex, or more specifically for humans, the Human Leukocyte Antigen (HLA) complex is responsive to the countless array of pathogens and to the mutable nature of those pathogens. The role of the HLA complex is to provide individuals with protection against pathogens that invade the body. The HLA complex encodes the genes of many proteins (e.g. cytokines – see below) that are involved in the immune response. An intricate relationship exists between a population's ability to resist diseases and their HLA composition, both of which are a function of the population's disease history and evolutionary forces. The HLA complex continues to be the focus of attention concerning disease outcome, resistance and susceptibility, and population studies. However, the capacity to take a genome-wide approach to mapping and subsequently identifying new resistance and susceptibility genes has made been made possible by the identification of a large array of new polymorphic candidate genes (Hill 1998a).

The importance of HLA genes and gene products was first recognized in tissue transplantation experiments with mice (Benjamini et al. 2000). Within the last 25 years, it was recognized that the molecules coded for by the HLA genes are critical to the development of activated T- cells and for their response to foreign pathogens. The most significant aspect of HLA genes is the fact that different individuals within a species can have slightly different forms (or alleles) of each HLA genetic loci. The HLA class I and class II genes have over 200 possible alleles and are known to be highly mutable (Eren and Travers 2000). HLA is the most highly polymorphic gene system in the body and in a population and are considered to be powerful markers for studying the genetic relationship between human populations. A HLA haplotype is characterized by a specific set of alleles, which occur more frequently than expected from a random combination of

individual genes (Tokunaga et al. 1996). Each HLA haplotype is considered to have a single origin; therefore, if two populations share a characteristic haplotype, then to some extent the two should share a common ancestry (Tokunaga et al. 1996).

The evolutionary development of the polymorphisms of HLA alleles is the result of selective pressures related to encounters with pathogens that mutate rapidly in order to avoid detection (Bontrop 2000). The evolution of HLA polymorphism is largely due to the combined selective forces of disease pathogens and pre- and post-reproductive mechanisms (Meyer and Thomson 2001). Pathogen driven selection occurs when specific alleles are favoured because of their ability to provide resistance to a pathogen. Studies of HLA polymorphism demonstrate HLA heterozygosity is favoured by both pre- and post-reproductive mechanisms. Hutterite populations, for example, display on average a greater degree of HLA homozygosity, which has negative consequences for pregnancy and fetal viability. The greater the number of HLA alleles that a couple shares, the higher the risk of fetal loss (Meyer and Thomson 2001). It is therefore evident that genetic drift and gene flow (migrations and/or cultural isolation) can influence the evolution of HLA polymorphism and genetic resistance and susceptibility to pathogens. The polymorphic nature of MCH/HLA alleles provides individuals and populations with a significant selective advantage for resisting infectious pathogens.

It has been established that there is a correlation between specific HLA alleles and the occurrence of certain infectious diseases such as malaria, rheumatoid arthritis, tuberculosis and spondyloarthropies, among others (Glynne and Price 2000). In addition, differential HLA allele frequencies exist between populations (Bias 1981; Suarez et al. 1985). The HLA complex is itself highly mutable and variable so that it can adapt effectively to pathogens that mutate rapidly and vary from region to region. The human penchant for migration, and their involvement in events of genetic admixture and/or isolation, served to influence the differential distribution of HLA haplotype frequencies in New World populations (Blanco-Gelaz et al. 2001). The ability for individuals and populations to respond to infectious pathogens is therefore the product of a complex evolution of genetic, biological and cultural events and its diversity confers a selective advantage in a changeable pathogenic environment.

The clinical significance of HLA alleles and haplotypes has resulted in the collection of large sets of population data on the distribution of HLA allele frequencies (Chen 1999). Specific HLA alleles have been shown to vary in frequency between populations and in association with certain diseases. Studies of the association of specific HLA alleles and disease show that pathogens have shaped the distribution of HLA alleles in human populations (Meyer and Thomson 2001; Tiwari and Terasaki 1985). In the case of malaria, a high frequency of certain class I and class II HLA alleles among African individuals that are resistant to severe malaria compared to other racial groups indicates selection favouring individuals carrying the resistance alleles. The non-random distribution of HLA haplotype frequencies between populations and an equally non-random distribution of diseases has been identified and is beginning to be explored among Aboriginal North American and Inuit populations (Blanco-Gelaz et al. 2001; Suarez et al. 1985; Tokunaga et al. 2001).

HLA alleles are known to have an association with a variety of diseases including leprosy, tuberculosis, rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and others (Lechler and Warrens 2000). HLA-B27, for example, has a relatively high frequency in all contemporary Aboriginal North American populations, the highest frequency being among the Inuit-Aleut groups (Peschken and Esdaile 1999:368). In a small control population of Inuit-Aleut subjects, 37% tested positive for HLA-B27, and 87% with spondyloarthropathies were HLA-B27 positive (Peschken and Esdaile 1999). Investigations of allele frequency among numerous Aboriginal populations demonstrated that susceptibility to RA and the virulence of the disease might be related to the prevalence of HLA-B27 and a number of other shared relevant genes. Certain HLA-DRB1*0401 alleles, for example, are associated with both chronic Lyme disease and an increased risk of rheumatoid arthritis (Glynne and Price 2000:318). HLA genes or their products are not solely responsible for RA susceptibility because factors such as geographic isolation and socio-economic disadvantage complicate the roles of environmental and genetic factors, which may influence the emergence of disease.

Studies of infectious disease among New World Aboriginal populations have focused on socio-economic factors contributing to disease resistance and susceptibility. In most of these studies genetic differences between populations are acknowledged as

contributing factors in disease susceptibility and resistance; however, the basis for this disparity remains largely unexplored (FitzGerald et al. 2003; Long et al. 1999). The unequal risk of disease between different North American populations may be partly explained by environmental and socio-economic differences; however, the immunogenetic program of a population in relation to differential disease resistance and susceptibility has been indicated but has not yet been fully explored. Susceptibility to tuberculosis among the relatively isolated South American Aché is not surprisingly high; however, individuals with high nutritional and socio-economic status within this group are equally at risk as those with low nutritional and socio-economic status (Hurtado et al. 2003). It is suspected that the high rates of antibody production and T_h2 mediated activation by Aché individuals competes with the T_h1 mediated defenses required to effectively fight against infectious diseases such as tuberculosis and malaria. The South American Indians in fact produce the highest levels of IgE[3] antibody of any population in the world although they do not generally experience asthma, allergies, or more severe reactions such as anaphylaxis (Hurtado et al. 2003). High levels of IgE have also been shown to occur during infections with ascaris (a roundworm) (Benjamini et al. 2000).

It has been shown that tuberculosis is associated with certain negative and positive associations with specific HLA class II alleles (McKinney et al. 1998; Miller 1991). The association between tuberculosis and genetics is thought to be reflected in differential ethnic susceptibility. In a comparison of African American and Euro-American individuals, it was found that Blacks had a higher prevalence of tuberculosis than Whites and "unequal partitioning of disease burden along racial lines was evident when socially and economically similar groups were compared" (McKinney et al. 1998:93). Stead (1992) showed that there was a correlation between a person's resistance to tuberculosis and their ancestry. Those who were the most susceptible to infection came from places that were once free of tuberculosis. Stead observed that, among North America Natives, there is a high risk for developing clinical tuberculosis (1992). In addition, twin studies have shown a greater concordance of risk for tuberculosis among monozygotic than among dizygotic twins (Comstock 1978), which also suggests that

[3] A specific class of antibody produced in the lungs, skin, and mucous membranes in response to allergens.

tuberculosis susceptibility is related to genetic programming. Recently, it has become clear that a single nucleotide base substitution (polymorphism) of the natural resistance-associated macrophage protein 1 (NRAMP1) gene can affect the level of expression of that gene (Bellamy et al. 1998). Bellamy (1998) reported that specific allelic variations in the NRAMP1 gene found on chromosome 2 are associated with susceptibility to tuberculosis, and one of these polymorphisms is much more common in Africans than in Caucasians. In addition, Greenwood (2000) demonstrated that in a large Aboriginal family, NRAMP1 and/or genes closely linked to it, had a role in the rate at which individuals progress from being infected to having active tuberculosis.

The brilliance of the HLA complex is its polymorphic nature, which allows the immune system to be responsive to a pathogen environment that is itself dynamic. Natural selective pressures favour heterozygosity in the HLA promoter regions because of the flexibility that this confers on an individual in terms of their capacity for mounting a T_h1 or T_h2 immune response (Mitchison et al. 2000). The maintenance of polymorphisms in HLA proteins is only a small part in the broader immune response that is comprised of immune activation, proliferation and recruitment of immune effectors that ultimately kill the pathogen. The array of effector proteins that actually constitute the immune response are the product of many gene loci that themselves have been found, through the human genome project, to be polymorphic.

3.4 Innate and acquired immune responses

The investigation of the human immune system is a relatively new science dating back to the late 1700's when cowpox or "vaccinia" was introduced by Edward Jenner as a protective device again human smallpox (Janeway et al. 1999). It was not until the 19th century however, that Robert Koch proved that micro-organisms were the cause of disease and that different micro-organisms exist and caused various pathologies (Benjamini et al. 2000). Since that time, the sciences of cellular biology and chemical immunology have revealed the diverse and complex mechanisms that interact to confer host immunity from infectious agents. Extensive clinical and experimental research in cellular and chemical immunology has led to an in-depth understanding of the large and diverse array of chemical responses to foreign agents that occur in the human body. More

recently, molecular biology and genetics have explored the exact genetic mechanisms that allow for the specificity and variety in immune responses.

At the most fundamental level, the human immune response enables the body to distinguish between what is "self" and what is "non-self". Two genetically similar sponge colonies, for example, when placed close together, will tend to grow toward each other and fuse into one large colony. This demonstrates a mechanism of identifying self. In contrast, unrelated colonies when placed in proximity to each other, will destroy cells that come into contact and leave a zone of rejection between the two colonies (Benjamini et al. 2000). Immunity refers to the mechanisms in the body that protect it against environmental agents that are foreign or that are "non-self".

The human body maintains a protective stance against a plethora of harmful foreign elements that might be encountered. Physical barriers such as skin, cells walls, nostril hair, mucous-membrane barriers and coughing and sneezing reflexes serve to prevent foreign organisms from invading the body. If a micro-organism manages to penetrate the physical barriers (i.e. skin, mucous membrane) the next line of defense are specialized cells whose primary purpose is to kill the foreign agents (Janeway et al. 1999) (Figure 3). The innate immune response relies on invariant receptors that are immediately available to combat a wide range of pathogens without requiring prior exposure. Their primary purpose is to identify and destroy invading micro-organisms either through phagocytosis[4] or through the activation of killer cells such as natural-killer cells or cytotoxic T-lymphocytes (Benjamini et al. 2000). Inflammation is an important aspect of both the innate and the later acquired response, and is a complex process that typically functions as a protective reaction to injury and infection (Benjamini et al. 2000).

[4] The engulfing and ingestion of bacteria or other foreign bodies by white blood cells called phagocytes

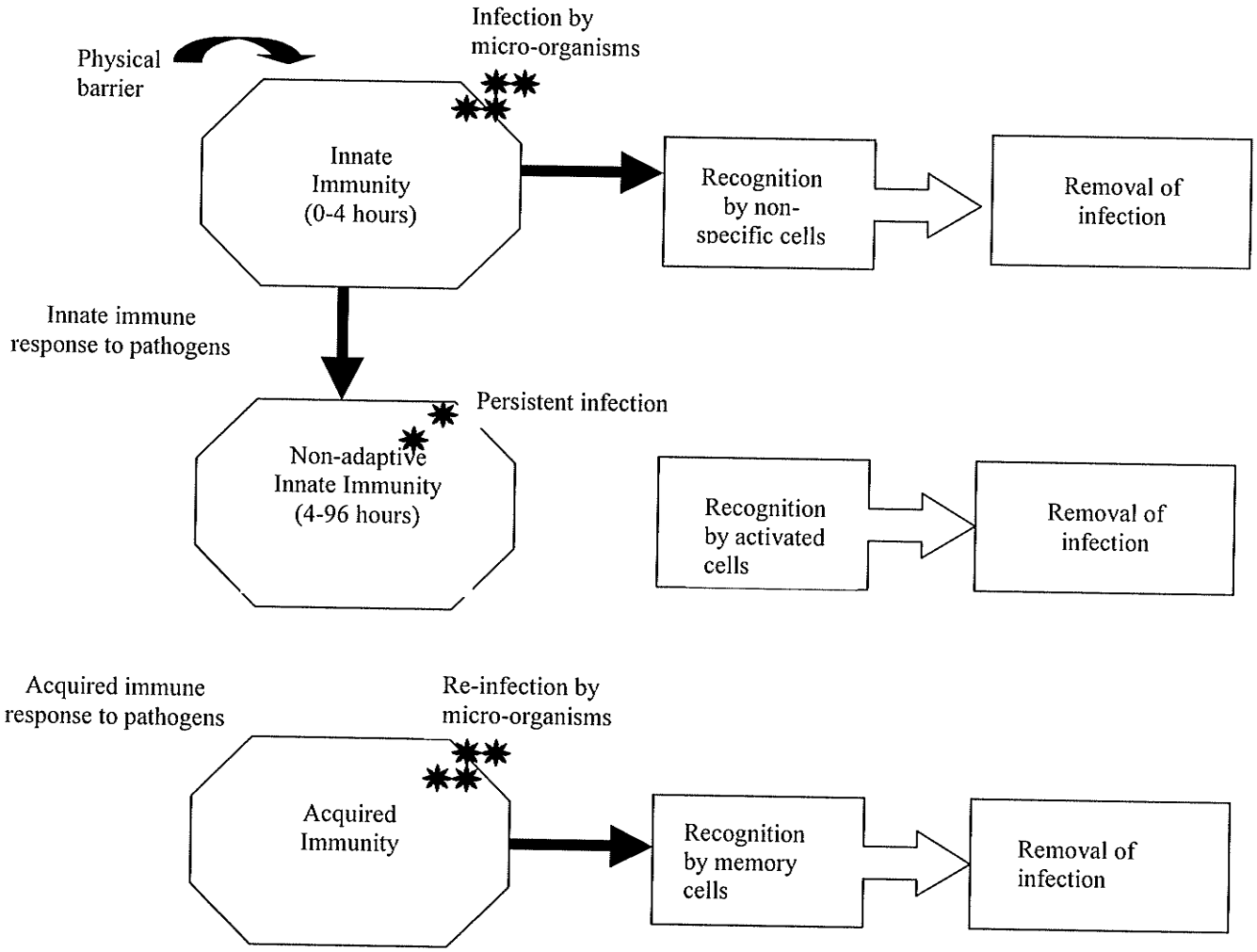


Figure 3. The innate and acquired immune responses to infection

Cytokines, which are activated by macrophages at the site of injury or infection, play a key role in the inflammatory response. Cytokines are a diverse collection of soluble proteins made by cells that affect the behaviour of other cells. Macrophages are phagocytic cells that function to engulf and degrade agents such as bacteria or parasites from the blood.

An infection cannot always be eliminated by the innate immune response, and there are many antigens that the innate immune cells cannot recognize. Consequently, an additional level of immunity has evolved that provides an increased level of protection

and subsequent re-infection from the same antigen. The cells of the innate immune system play a key role in the subsequent direction that the acquired immune response will take. The balance and level of cytokines secreted at every stage of the battle against the foreign substance affect the outcome of the immune response. The innate immune response serves as a front line of defense against infection but it lacks the ability to recognize certain pathogens, and it cannot provide specific immunity to prevent re-infection. The innate immune response is non-adaptive and cannot confer protection from novel pathogens.

The acquired immune response is activated by an encounter with a foreign substance, which induces an immune response specific against that foreign substance. Although individuals are genetically capable of mounting an immune response against the foreign substance, acquired immunity is exhibited only after an initial encounter with that substance. Acquired immunity therefore occurs only after an initial exposure to, or immunization, with a given foreign substance (Benjamini et al. 2000; Janeway et al. 1999). The acquired immune response differs from the innate response in that it can confer protection from new pathogens and it can lead to long-term protective immunity (Janeway et al. 1999).

The *Mycobacterium tuberculosis* bacterium for example, will battle with the innate immune response for several weeks before the acquired immune response is induced. It is the acquired immune response where specific cytokines, the key regulators of the immune system, are differentially produced to illicit either a T-Helper cell 1 (T_h1) or a T-Helper cell 2 (T_h2) immune response (Flynn and Chan 2000). T-cells identify the presence of intracellular pathogens when infected cells display a peptide fragment from the pathogen's protein that is bound to a HLA molecule on the surface of the cell (Figure 4). Different cytokines are required to activate different arms of the adaptive immune response either through antibodies or cell-mediated responses (Janeway et al. 1999).

Figure 4. Human immune response and cytokine synthesis

All nucleated cells (almost every cell in the body) express HLA class I molecules on their cell surfaces. HLA class I molecules on the surface of a virus-infected cell, presents peptide fragments of the viral protein to cytotoxic T-cells that are pre-programmed to kill cells. In contrast, MHC class II molecules are restricted to a few specialized cell types (macrophages, B cells, activated T cells), which present peptides that are recognized, by T_h1 and T_h2 cells. When T_h1 cells recognize an antigen on an antigen presenting cell, the T_h1 cells are activated to recruit macrophage, which leads to the destruction of the intracellular bacteria. T_h2 cells recognize antigen on B cells, resulting in the proliferation B cells and antibody- producing plasma cells (Janeway et al. 1999). The T_h1 and T_h2 cytokine responses are mutually inhibitory. IL-10 for example inhibits the production of T_h1 cytokines and down regulates the MHC II expression. The cytokine response to infection is complex, but in general, a strong T_h1 (cell-mediated immune response) is required to combat bacterial and viral infections. In contrast, a T_h2 (antibody mediated immune response) is effective against parasitic and fungal infections.

Two major categories of white blood cells or lymphocytes are responsible for the acquired immune response, B- and T-cells. B-cells rapidly respond to extra cellular micro-organisms (parasites and viruses that spend part of their life cycle in extra cellular fluid) by producing soluble factors known as antibodies. T-cells coordinate the acquired immune response through the production of a wide variety of cytokines and T-Helper cells recognized by the MHC II, which are only found on antigen presenting cells. The function of MHC gene products is most importantly, to process and present foreign antigens to T-cells that function to clear infection. T-cells recognize antigens in the form of peptides bound to highly polymorphic cell surface molecules (MHC molecules) encoded by the MHC system (Eren and Travers 2000). The MHC molecules bind and present peptides of bacterial fragments to T-lymphocytes. The cytokine environment is an important regulator of the immune system and is integral in determining the type (e.g. cell-mediated or antibody immune response) and effectiveness of the immune response.

It is descriptively convenient to subdivide the immune system into innate and acquired immune responses (as described above) and to further subdivide acquired immunity into cell-mediated or antibody mediated responses. However, these artificial divisions mask the complex interaction at and between the cell-mediated and antibody-

body mediated immune responses. In addition, genetic variation in the promoter regions of HLA (MHC) and the cytokine promoter regions can effect immunogenetic expression and immunological responsiveness (Mitchison et al. 2000). Figure 5 summarizes the innate and acquired immune response to an infectious pathogen and illustrates the involvement of the cytokines and how they enhance and direct the immune response.

3.5 Cytokine promoter gene polymorphisms

The relative success of an individual's immune response to pathogens is a function of the variation that is characteristic of the genes that respond to infection. Cytokines are integral in signaling the cellular response to a foreign antigen, and genetic variability in the promoter regions of the genes that control cytokine production, can influence the quality of the immune response. Nearly all cells of the innate and adaptive immune systems produce cytokine proteins. Cells are controlled by the quantity and types of cytokines they are exposed to and by the levels of cytokine expression. The cytokine cascade that is induced by the host's immune response to pathogens is a maze or network of activity specifically aimed at eradicating the foreign agent. Mutations in the coding regions of genes can result in a change in protein structure and the resulting protein expression (Kwok and Chen 2003). Polymorphisms in the cytokine promoter region can result in varied levels of cytokine expression and therefore, and consequently, differential success in a host's defense against certain pathogens (Frank 2002).

Comparison between two human genomes reveal that they are 99.9% identical; however, each person has as many as 3.2 million differences in their diploid genome within the complete 3.2 billion base pair genome (Kwok and Chen 2003). The simplest type of polymorphism, or nucleotide change, results from a single base mutation where one nucleotide is substituted for another. The locus of such a change has recently been termed a "single nucleotide polymorphism" (SNP) (Kwok and Chen 2003; Schork et al. 2000). SNPs are found throughout the genome in both coding (exon) and non-coding regions (intron) including cytokine promoter regions, and are therefore more likely to have a functional or physiologically relevancy than other sorts of polymorphisms (Figure 6) (Lazarus et al. 2002; Schork et al. 2000). In the majority of cases, these single base pair substitutions have no biological consequence. A small portion of the substitutions,

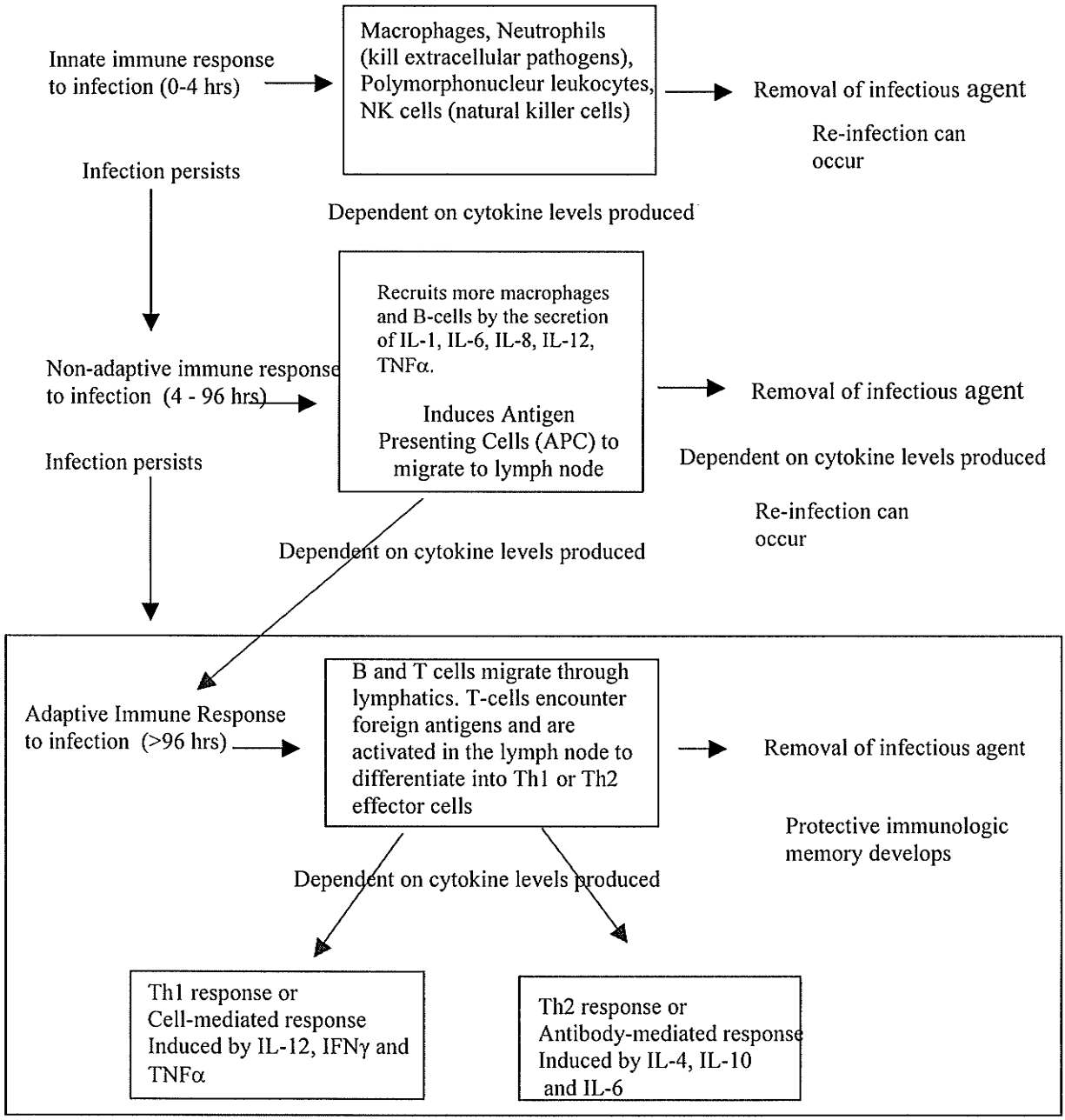


Figure 5. Summary of the innate and acquired immune response to infection

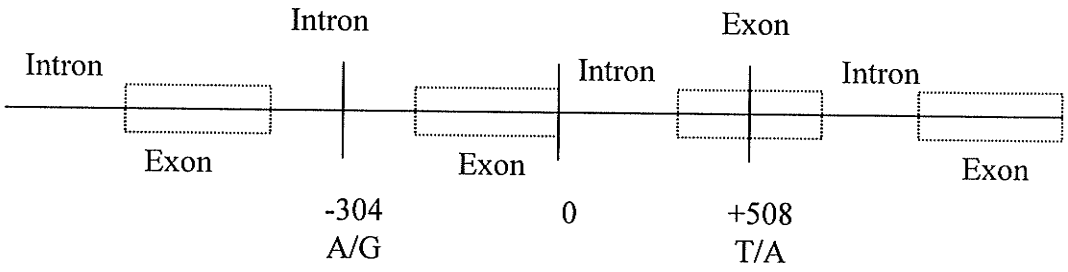


Figure 6. Cytokine promoter gene showing the position of introns and exons and two SNP loci

however has functional significance and is the basis for diversity among humans. A SNP in the coding region may impact a relevant protein and in the cytokine promoter region, a SNP can influence gene expression (Bayley et al. 2004; Fishman et al. 1998).

Significant advances in molecular genetic technologies in the last decade have contributed towards understanding the complex association between the genes of the immune system and disease (Schork et al. 2000). Studies of inbred mice have clearly demonstrated that the genetic background plays a key role in determining the quality of the cytokine response (e.g. T_h1 vs. T_h2). These factors, in turn, affect the host's ability to eradicate an infectious agent e.g. *Leishmania major, Toxoplasma gondii, Mycobacterium tuberculosis* (Nagabhushanam et al. 2003; Nishikomori et al. 2001; Reiner and Locksley 1995; Scharton-Kersten et al. 1996; Von Stebut et al. 2003). In the context of the host's immune response to an infectious agent, the identification of differences in cytokine (key regulators of the immune system) SNP profiles may have important implications for the host's resistance or susceptibility to that particular pathogen.

Indeed, single nucleotide polymorphisms in the regulatory regions of cytokine genes can influence gene transcription levels, and they have been associated with susceptibility to and/or severity of certain diseases and autoimmune disorders (Fishman et al. 1998; Westendorp et al. 1997). *In vitro* studies of cytokine polymorphism have documented variation between individuals in cytokine expression, and numerous studies have identified a genetic basis for this variation in cytokine levels (Fishman et al. 1998; Olomolaiye et al. 1998; Turner et al. 1997; Wilson et al. 1997). *In vivo* studies have linked cytokine genotypes to disease associations through research that attempts to

identify immunogenetic markers for a given disease. Studies have identified statistically significant associations between cytokine alleles, disease and certain autoimmune disorders (Eskdale et al. 1997). Ethnic differences in SNP allelic frequencies and genotypic frequencies of certain cytokines have been found in a number of studies. Hassan (2003) identified differences in the allelic frequencies of interleukin-6 (IL-6) and interferon gamma (IFNγ) in a study of Afro-American and Euro-American women. In a separate study, it was found that Afro-American, Hispanics and Asians had IL-6 and IL-10 genotypes that resulted in the high expression of these cytokines as compared with Euro-Americans (Hoffmann et al. 2002).

Cytokine interaction, or the cascade effect of gene expression and suppression, may influence the predominance of T_h1 orT_h2 response, thereby directly influencing the clinical outcome of exposure to infectious pathogens. Therefore, it is important that cytokine polymorphisms not always be studied in isolation. The identification of population differences of genotype frequencies in the cytokine promoter regions for IL-6, IL-10, tumor necrosis factor alpha (TNFα), IFNγ, and transforming growth factor beta (TGF-β) has important implications for a population's resistance and susceptibility to infectious disease.

TNFα, IL-6 and IFNγ facilitate innate immune responses and activate functions of inflammatory cells. TNFα is a pro-inflammatory (T_h1) cytokine that has been implicated in the severity of different diseases including autoimmune diseases, rheumatoid arthritis and malaria (Bayley et al. 2004; Feldmann and Maini 2001; McGuire et al. 1999). The cytokine TNFα is initially produced by macrophages at the site of infection. The local production of TNFα encourages other immune cells (neutrophils and macrophages) to move to the site of tissue damage or infection and activates phagocytes to engulf and clear infectious agents and cellular debris (Hajeer and Hutchinson 2000:220). The TNFα gene is located on the human chromosome 6p21.3 in a locus that is highly polymorphic. To-date eight DNA variants have been identified in the TNFα promoter (-1031 T/C, -863C/A, -857C/T, -575A/G, -376A/G, -308A/G, -244A/G and –238A/G) and certain of these polymorphisms have been linked to ethnicity and disease susceptibility and outcome (Bayley et al. 2004; Cuenca et al. 200; Hajeer and Hutchinson 2000; Hoffmann

et al. 2002). Certain SNPs in the TNFα promoter region have been implicated in the pathogenesis of infectious diseases such as malaria. For example, cerebral malaria has been associated with the TNFα (-308) "A" allele while severe malarial anemia was associated with the TNFα (-238) "A" allele (McGuire et al. 1999). The results of this study in Gambian children suggest that the outcome of malaria is influenced by SNPs situated in the cytokine promoter regions.

The combined production of TNFα, IL-6 and IL-1 leads to elevated body temperature, sleepiness, and inflammation that have a short-term value of fighting certain infections. Although the acute reaction is advantageous for clearing infection, long-term exposure to high levels of circulating TNFα is associated with toxic shock. TNFα has been extensively studied in conjunction with tuberculosis research and is believed to play roles in immune and pathologic responses to tuberculosis including the formation of granuloma in tuberculosis and other mycobacterial diseases (Flynn and Chan 2000). Specifically, treatment with anti-TNFα therapy has been associated with the occurrence or reactivation of tuberculosis, thereby confirming the important role of TNFα as an immune modulator or neutralizer (Lim et al. 2002).

IFNγ has a clear important role in a host's resistance to infectious diseases although IFNγ production cannot adequately control *M. tuberculosis* infection without the presence of other critical cytokines (Flynn and Chan 2000). Although there is no evidence to date from population studies that common variants in the IFNγ receptor genes affect susceptibility to tuberculosis, there are cases of inactivating mutations in the IFNγ receptor, which were associated with susceptibility to usually nonpathogenic mycobacteria (Ottenhoff et al. 2002). IFNγ enhances expression of MHC class I and class II molecules and modulates the expression of other molecules in antigen presentation (Ottenhoff et al. 2002). The term "interferon" was coined when it was discovered that this cytokine interferes with viral replication and blocks the spread of viruses to uninfected cells. IFNγ is a T_h1 cytokine, and it has been reported that high IFNγ production is correlated with the presence of allele 2 (12 CA repeats). It was also reported that there is a positive correlation between allele 2 and the presence of a "T" allele at the SNP at

position +874 in the first intron and that his polymorphic site may affect IFNγ gene expression (Pravica et al. 1999).

Interleukin-6 is a multifunction cytokine, which stimulates the growth and differentiation of human B cells and antibody production. IL-6, secreted by helper T cells, contributes to B-cell activation (Janeway et al. 1999) and is important in the acute-phase immune response where a shift in the proteins secreted by the liver into the blood plasma is the result of the action of IL-1, IL-6 and TNFα (Janeway et al. 1999). The over-expression of T_h2 type cytokines (IL-6 and IL-10) induces hyperactivity of B-cells which is characteristic of systemic lupus erythematosus (Linker-Israeli et al. 1999). The gene encoding IL-6 is located on chromosome 7p21 and the SNP of interest is at position –174 and the SNP at IL-6 (-174) was implicated in the age of onset of rheumatoid arthritis (Pascual et al. 2000). Conversely, studies of families with *Shistosoma mansoni* infection have confirmed the protective role of T_h2 type cytokines IL-4, IL-6 and IL-13 in protection against parasitic infection (Marquet et al. 1999).

Interleukin-10 (IL-10) is generally described as an anti-inflammatory cytokine and has a mainly stimulatory effect on human B-cells (Benjamini et al. 2000). IL-10 inhibits the secretion of TNFα, IFNγ and other pro-inflammatory cytokines such as IL-6 and IL-8 among others. Interleukin-10 plays a crucial role in regulating the balance between inflammatory and humoral immune responses and inhibits or down regulates the production of T_h1 cells and macrophage function (Gibson et al. 2001). IL-10 is an anti-inflammatory (T_h2) cytokine that may antagonize the effects of T_h1 cytokines such as TNFα. The gene encoding for IL-10 is located on chromosome 1q31-q32 (Turner et al. 1997). Three SNPs have been confirmed in the IL-10 gene promoter region in addition to two C/A repeat micro satellite regions (Gibson et al. 2001). The presence of the G allele at position –1082 is associated with the higher IL-10 production, while the A allele is associated with lower IL-10 production (Turner et al. 1997). Polymorphisms at the position –819 and –592 do not independently influence IL-10 production. High levels of IL-10 production have been associated with autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus (Eskdale et al. 1997; Fishman et al. 1998; Flynn and Chan 2001; Gibson et al. 2001). Variability in the expression of IL-10 is

associated with genetic variation (Westendorp et al. 1997), and studies have associated IL-10 polymorphisms with a diverse range of diseases including asthma, systemic lupus erythematosus and arthritis (Gibson et al. 2001; Lim et al. 1998; Martinez et al. 1997; Turner et al. 1997). IL-10 is central in the suppression of cytokine-mediated inflammatory processes and is of current interest for addressing the prevention and management a wide range of diseases (Lazarus et al. 2002).

Transforming Growth Factor-beta (TGF- β) is an inhibitory cytokine recognized as a key regulator of immunological homeostasis and inflammatory responses. The TGF-β gene is located on chromosome 19q13.1 and controls the proliferation and differentiation of many cells types, and variability in the production of this cytokine has been linked to atherosclerosis (Blobe et al. 2000).

Research is ongoing regarding the functional action and interaction of cytokines. Indeed, these proteins rarely if ever act alone. Thus, cells are exposed to an array of cytokines that can have an additive, synergistic, or antagonist affect. In addition, polymorphisms in the promoter regions of certain cytokines cause variation in the level of expression and may affect the severity or outcome of disease (autoimmune/infections).

3.6 Summary

The genetic diversity in the MHC in all mammals and in the HLA in humans is necessary for a species to respond effectively to the barrage of pathogens that they can potentially experience. This genetic variation is expressed in an individual's ability to mount an effective immune response against pathogens and to respond to novel pathogens. During the past 40 years, the use of cellular biological techniques has resulted in significant advances in understanding the human immune response to pathogens and in illuminating the complex interaction of the innate and acquired immune responses. Typing for the HLA was conducted using serological techniques and was difficult, imprecise and liable to errors (Doxiadis and Claas 2003). Despite these difficulties, HLA research has contributed a database of knowledge regarding population variation and the disease association related to varied expressions of HLA alleles and disease (Lechler and Warrens 2000).

The development of molecular genetic technologies and the completion of a map of the entire human genome have caused an explosion of research directed towards identifying, cataloguing and mapping SNPs throughout the genome. It is evident that most phenotypes are determined by a multitude of genetic and non-genetic (or environmental) factors. However, it is also apparent that SNPs in the promoter regions of HLA and cytokine genes influence a host's ability to mount an effective immune response and may play a key role in a host's and a population's ability to adapt or survive. There is an expectation that studies of cytokine SNPs and gene variants that play a significant role in pathology will result in a greater understanding of the regulatory mechanisms in both health and disease (Mitchison et al. 2000).

The knowledge that the immune response is dependent upon the cooperative effort of multiple cellular pathways that are the product of complex interactions between variable gene loci seriously complicates the simplistic anthropological definition of evolution. The notion that evolution is "a change in allele frequency" as a result of random mutation, natural selection, gene flow and genetic drift is foundational for understanding the genetic response to selective pressures. However, in the context of modern genetics, our capacity to explore both contemporary and past populations at a molecular level will demand an approach that is beyond that of neo-Darwinism. In many respects, the rapid application of complex technology towards unraveling human DNA has resulted in a vast array of data about human diversity that has outstripped any interpretive framework. Molnar likens the data overload to "doing a survey of a forest for marketable timber content by counting the leaves instead of whole trees" (2002:178). Nevertheless, human immunogenetic diversity will likely only be understood within a revised evolutionary and historical framework that incorporates more fully the nature of the relationship between human biology and behavior and the pathogen environment.

Chapter 4 Cytokine genotype frequencies among contemporary North American Aboriginal and Caucasian populations

4.1 Introduction

Genetic diversity related to the human immune response is a key factor in individual and population survival and adaptation throughout human history (McNeill 1998). Cytokines are key regulators of the immune system and their functional activities are involved with the regulation, development and behaviour of immune effecter cells. With the completion of the human genome project, it has become apparent that single nucleotide polymorphisms (SNPs) are common in the human genome and depending on their location, they may affect gene transcription levels (Kwok and Chen 2003). The current study explained in this chapter describes the frequency of SNPs, which are known to correlate with differential gene expression for the IL-6, IL-10, TNFα, IFNγ and TGF-β_1 genes, in Caucasian and Canadian Aboriginal populations. The following study found that the population of Canadian Aboriginal patients has a higher frequency of cytokine single nucleotide polymorphisms favoring a low production of TNFα, IFNγ and IL-10 and high production of IL-6 as compared to a Caucasian population.

4.2 Materials and methods

4.2.1 Study population

End-stage renal disease (ESRD) patients were initially enrolled, with informed consent and with approval of the University of Manitoba Internal Review Board, into a study to explore the relationship between cytokine genotypes and inflammatory events (e.g. infection). However, it was noted that cytokine genotype profiles appeared to correlate with ethnicity, so the following groups were identified to formally evaluate this relationship between cytokine SNPs and ethnicity further: North American Aboriginal with ESRD (n=78), Caucasian with ESRD (n=217) and Caucasians disease free and >40

years old (n=92)[5]. The North American Aboriginal cohort with end-stage renal disease was composed of self-proclaimed Status Indian individuals. Given the geographical location of the study centre in relation to the surrounding Aboriginal populations, the study group is likely Ojibwa or Cree. They are non-Caucasian and their respective heritages are considered to be native to North America. In contrast, the Caucasian patients are of European descent. These ethnic classifications are based on self-identification with certain socio-cultural constructs that are not well defined, and have no defined biological basis (Collins 2004). However, health disparities exist between ethnic groups therefore "ethnicity" may serve as proxy indicator for ancestral geographic origin "which in turn is a surrogate for genetic variation across an individual's genome" (Collins 2004:S14).

4.2.2 Cytokine genotyping

Genomic DNA was extracted from buffy coat cells by absorption onto QIAamp silica-gel following QIAGEN protease digestion (Qiagen, Mississauga, Canada). After column elution the purity and concentration of extracted DNA was determined by UV spectroscopy (BioRad, Mississauga, Canada). Single-nucleotide polymorphisms for IL-6, TNF-α, IFN-γ, IL-10, TGFβ_1 codon 25 and TGFβ_1 codon 10 were determined using a commercial Polymerase Chain Reaction – sequence specific primers (PCR-SSP) kit (One Lambda, Canoga Park, USA). The PCR-SSP method uses primers designed to have perfect matches only with a single allele or group of alleles. The primers will amplify and give a positive result if the primers match the target sequence, but they will not amplify if the primers are mismatched, giving a negative result. The specific gene polymorphisms probed for were as follows: -174 (G/C) in the IL-6 promoter, -1082 (A/G), -819 (T/C) and –592 (A/C) in the IL-10 promoter, -308 (A/G) in the TNFα promoter, codon 25 (G/C) and codon 10 (T/C) of the TGFβ_1 signal sequence, and +874 (T/A) of intron 1 of IFNγ.

Each PCR reaction contained pre-optimized sequence specific primers, 100ng of genomic DNA, and 0.25U Taq polymerase (PE Biosystems, Mississauga, Canada). Following the initial denaturation steps, samples were subjected to an initial 9 rounds of

[5] Individuals were required to be over 40 years of age to be considered disease free.

PCR consisting of 96°C for 10 sec, 63°C for 60 sec followed by 20 rounds of PCR consisting of 96°C for 10 sec, 56°C for 30 sec and 72°C for 30 sec. To visualize the PCR products, 10 μl of the amplified reaction was run in a 2.5% agarose gel containing 0.5% ethidium bromide at 150V for 5 minutes. DNA bands were then visualized with UV light on a transilluminator and photographed for subsequent analysis. Patients were classified into the predicted high, intermediate or low producer phenotypes according to their genotypes (Table 1).

Table 1. Reporting for cytokine genotypes and phenotypes

Cytokine Polymorphisms	Genotype	Phenotype Producer
TNFα (-308)	A/A	High
	A/G	Intermediate
	G/G	Low
$TGF\beta_1$ (codons 25)	G/G	High
	G/C	Intermediate
	C/C	Low
$TGF\beta_1$ (codons 10)	T/T	High
	T/C	Intermediate
	C/C	Low
IL–10 (-1082)	G/G	High
	A/G	Intermediate
	A/A	Low
IL-10 (-819)	C/C	High
	C/T	Intermediate
	T/T	Low
IL-10 (-592)	C/C	High
	C/A	Intermediate
	A/A	Low
IL-6 (-174)	G/G	High
	G/C	Intermediate
	C/C	Low
IFN-γ (+874)	T/T	High
	T/A	Intermediate
	A/A	Low

4.2.3 Statistical analysis

Statistical analysis was performed using SAS software (SAS Institute, Cary, NC). The null hypothesis in the statistical analysis of this data is that there is no difference between the observed cytokine genotypes of Aboriginal and Caucasian populations. The Fisher Exact Probability test was to test the null-hypothesis that there is no difference between the observed Aboriginal and Caucasian cytokine SNP frequencies. The Fisher Exact test was used to compare the two categorical variables rather than chi-square because some of the values of the samples were less than five. This test calculates the exact probability of obtaining the observed results if they were to occur simply by chance. A "P" value of <0.01 was reported as significant which means that the chance that the observed numbers would occur by chance is 1 in 100 or less. The higher degree of stringency was used because multiple comparisons, as in this test, frequently lead a result to be significant at the $P<0.05$ levels.

4.3 Results

Cytokine genotypes of North American Aboriginals with ESRD were compared to the Caucasian cohort and found to have the following phenotypic differences (Table 2). All cytokine SNPs were in Hardy-Weinberg (H/W) equilibrium within the Caucasian and Aboriginal cohorts.

The cytokine phenotype frequencies of the two Caucasian cohorts (those with end-stage renal disease (ESRD) and normal controls) were not significantly different from one another strongly suggesting that ESRD as a broad classification is not a determining factor in favour of a given cytokine phenotype (Table 2). A diversity of aetiologies led to ESRD in the Caucasian cohort and in the other ethnic groups, therefore it is not surprising that is not a determining factor in the cytokine phenotypes. Because cytokine genotype frequency of the two Caucasian categories were not found to differ significantly, they were combined and compared to Aboriginal cohort.

Table 2. Cytokine phenotypes by percent

Cytokine	Caucasians Normal Control (N=92) (H, I, L)	Caucasians ESRD (N=217) (H, I, L)	Caucasians Combined (N=309) (H, I, L)	Aboriginal ESRD (N=78) (H, I, L)
TNFα (-308)	2, 29, 69	3, 29, 68	2, 29, 69	1, 10, 88*
IFNγ(+874)	25, 45, 30	19, 46, 35	21, 45, 34	6, 24, 69*
IL-10 (-1082)	21, 54, 25	20, 47, 33	20, 49, 31	9, 37, 53*
IL-10 (-592)	63, 32, 5	58, 32, 10	59, 32, 9	37, 42, 20*
IL-10 (-819)	63, 32, 5	58, 32, 10	59, 32, 9	37, 42, 20*
IL-6 (-174)	41, 45, 14	38, 45, 17	39, 45, 17	82, 17, 1*
TGFβ Codon 25	85, 15, 0	87, 12, 1	86, 13, 1	97, 3, 0*
TGFβ Codon 10	36, 53, 11	35, 50, 15	35, 51, 14	33, 49, 18

(*P<0.01 when compared to Caucasian population (either ESRD, Normal or combined)).

As compared to the Aboriginal cohort, Caucasians maintain a higher frequency of the TNFα (-308) and IFNγ SNPs whose phenotypic expression is associated with the production of high levels of these cytokines (Figure 7 and 8).

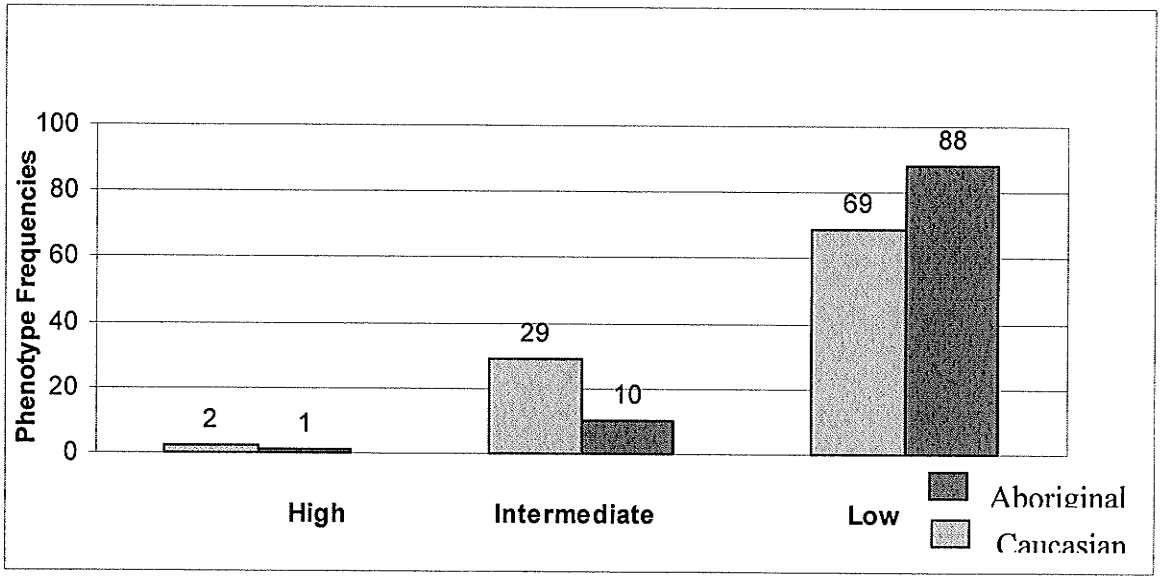


Figure 7. TNFα (-308) Phenotype frequencies for Aboriginal and Caucasian populations

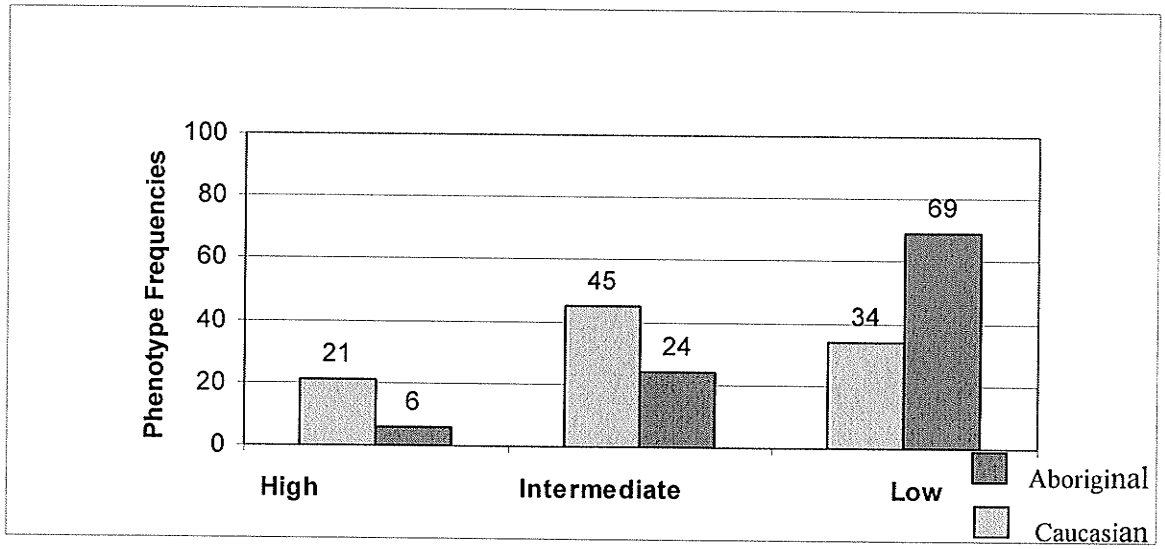


Figure 8. IFNγ phenotype frequencies for Aboriginal and Caucasian populations

In contrast to the Caucasian cohort, individuals of Aboriginal descent had a high frequency of the IL-6 (-174) allele that is associated with a higher production of this cytokine (Figure 9).

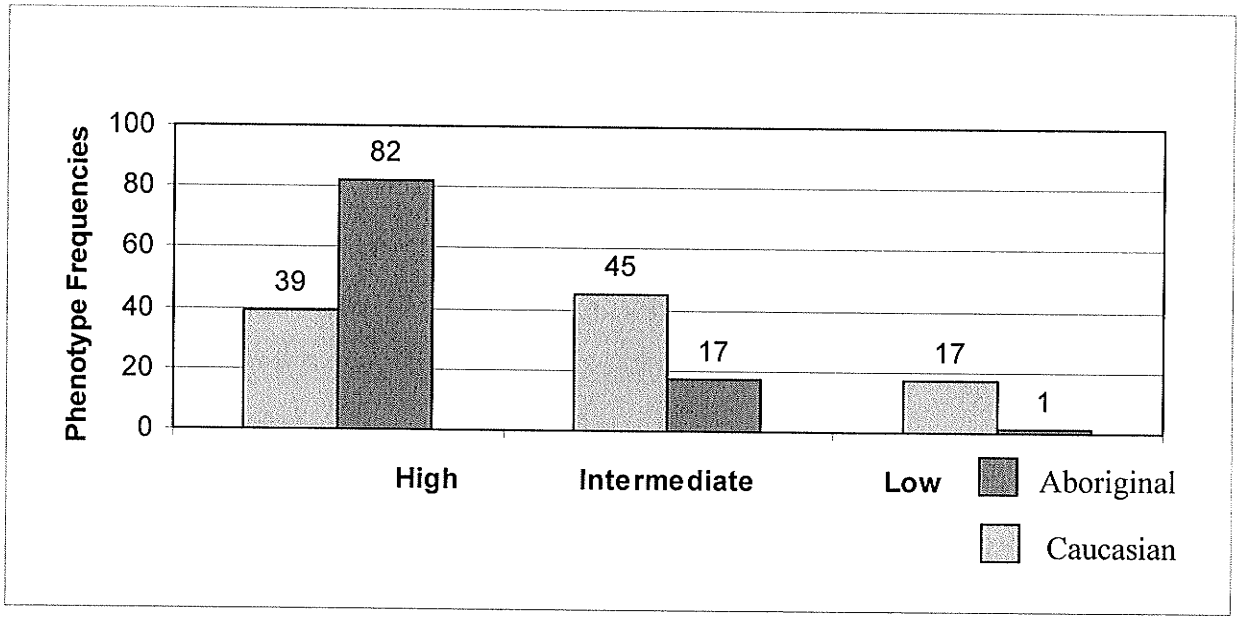


Figure 9. IL-6 (-174) phenotype frequencies for Aboriginal and Caucasian populations

The Aboriginal patients maintained a statistically higher frequency of the "G" allele at the TNFα (-308) loci and would therefore tend to be low producers of this cytokine as compared to the Caucasian cohort. Similarly, the Aboriginal patients maintained a statistically higher frequency of the "A" allele at the IFNγ loci, which would be associated with low production of IFNγ. In Aboriginals, all IL-10 loci phenotypes were skewed towards a lower production level of IL-10 as compared to the Caucasian cohort (Figure 10). In contrast, the Aboriginal group as compared to the Caucasian cohort,

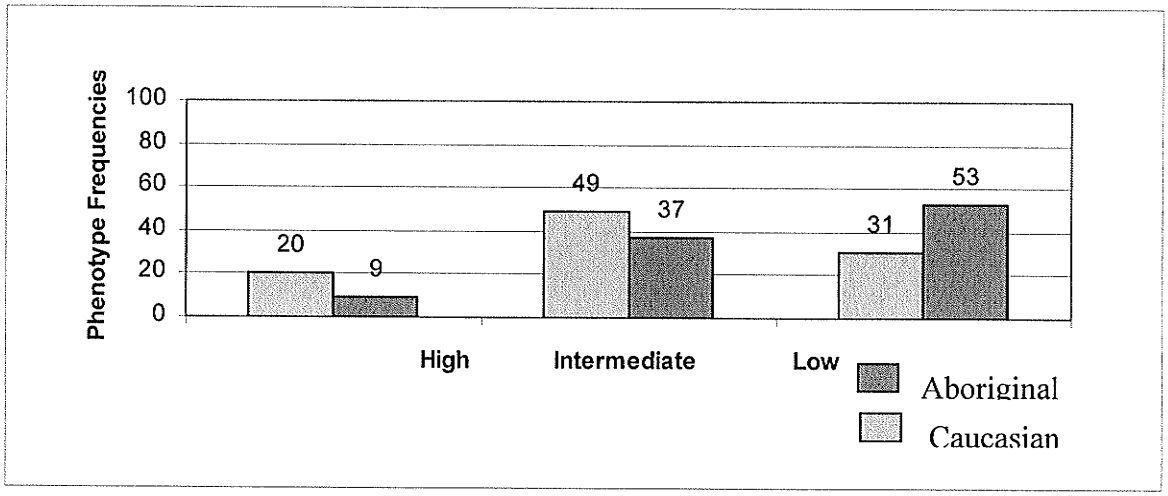


Figure 10. IL-10 (-1082) phenotype frequencies for Aboriginal and Caucasian populations

had alleles associated with a higher expression level for IL-6 (G allele at –174) and $TGF\beta_1$ (G allele codon 25) (Figure 11). There was no statistical difference between allele frequencies of the Aboriginal and Caucasian groups for $TGF\beta_1$ (codon 10) (Figure 12).

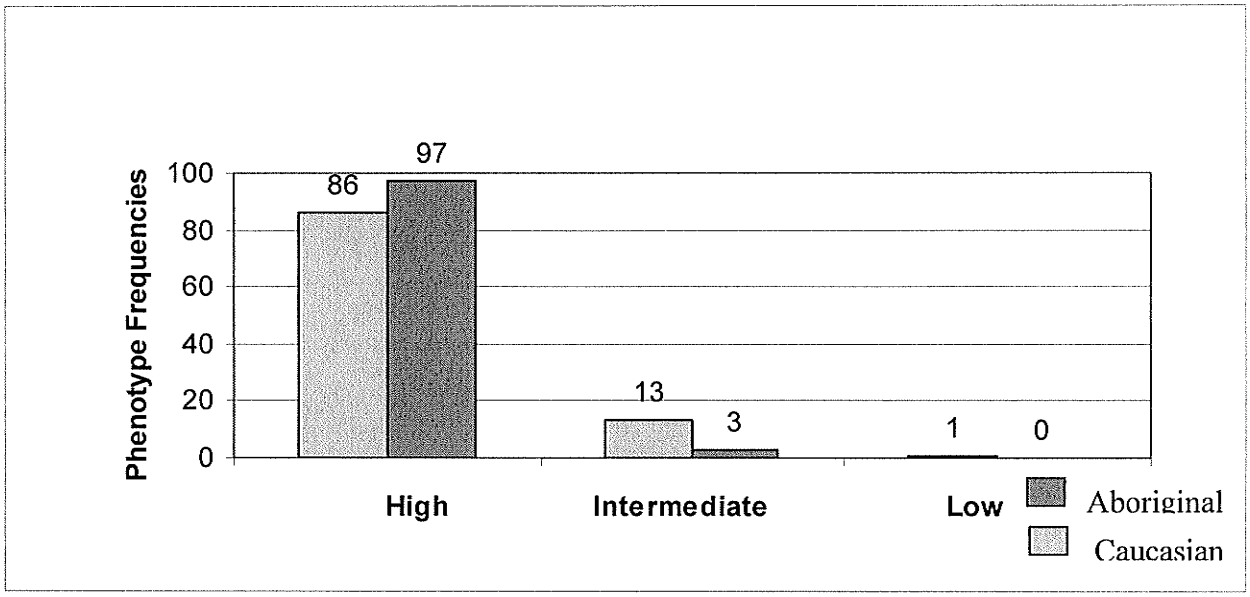


Figure 11. TGFβ Codon 25 phenotype frequencies for Aboriginal and Caucasian populations

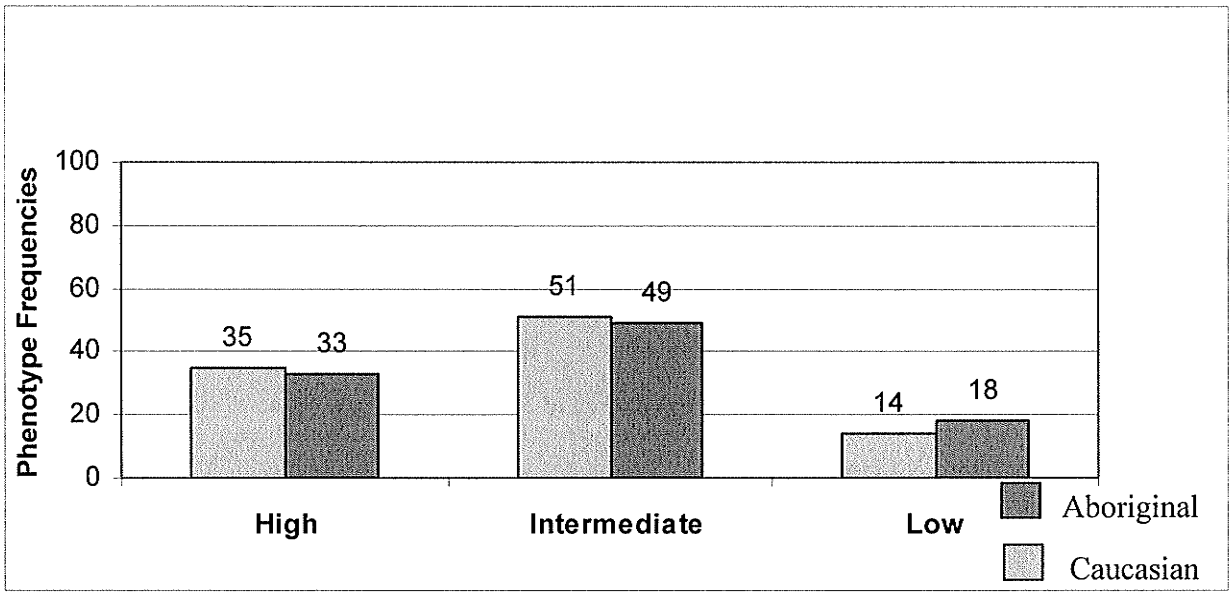


Figure 12. TGBβ Codon 10 phenotype frequencies for Aboriginal and Caucasian populations.

4.4 Population differences in allelic frequencies

The expected allele frequencies differed from the observed allele frequencies of the TNFα (-308) polymorphism in the North American Aboriginal with ESRD when

compared to the Caucasians with ESRD using the Hardy-Weinberg theorem (Table 3). If the Aboriginal study population is in H/W equilibrium, that is, no change in the allelic frequency was occurring, then we would expect to see an allele frequency similar to that of the Caucasian population. The expected values are based on the number of observations one would expect to see if the variable was independent (i.e. the allele frequency was not correlated to ethnicity). Instead, what we find is a statistically significant difference between the observed and expected phenotypes indicating that the frequency of certain alleles has changed in the Aboriginal population as compared to the Caucasians. These findings suggest that selective pressures exist as indicated by the differences in the expected and observed values. The Aboriginal patients with ESRD have a higher frequency of the "A" allele at the TNFα (-308) locus than would be expected if no selection was taking place and would therefore tend to be low producers of this cytokine as compared to the Caucasians patients. Alleles associated with the low production of IL-10 (-1082) and IFN-γ (+174) were found in higher frequencies among Aboriginal patients. Within an ethnic group the allele frequencies of IL-10 (-819) and IL-10 (-592) were identical in both the Aboriginal and Caucasian groups which would suggest that these loci are in linkage disequilibrium[6]. However, differences in allele frequencies were found between the two cohorts. The Aboriginal cohort had higher frequencies of the alleles that would result in the low to intermediate production of this cytokine as compared to the Caucasian group.

The alleles associated with the high production of IL-6 (-174) (G allele) and $TGF\beta_1$ (codon 25) (G allele) were found to occur in higher than expected frequency in the Aboriginal group. There was little difference between the expected and observed allele frequencies of $TGF\beta_1$ (codon 10).

[6] Linkage disequilibrium occurs when two or more loci do not association independently in a study population.

Table 3. Aboriginal expected and observed phenotype frequencies (n=78)

Cytokine Polymorphisms	Expected Frequency H, I, L	Observed Frequency H, I, L
TNFα (-308)	2, 18, 57	1, 8, 69*
IFNγ (+874)	12, 34, 31	5, 19, 54*
IL-6 (-174)	36, 30, 10	64, 13, 1*
IL–10 (-1082)	14, 35, 28	7, 29, 42*
IL-10 (-819)	41, 27, 9	29, 33, 16*
IL-10 (-592)	41, 27, 9	29, 33, 16*
TGFβ1 (codon 10)	28, 37, 12	26, 38, 14
TGFβ1 (codon 25)	70, 7, 1	76, 2, 0

(*P<0.05 when compared to the Caucasian ESRD study population).

4.5 Summary

Cytokine production is an immunological response to the presence of infection in the body. The level of cellular secretion of cytokine proteins that are responding is in part, determined by the promoter region of genes. Polymorphisms in the regulatory regions of TNFα, IL-6, IL-10, IFNγ, and TGFβ affect an individual's ability to express those cytokines. Alleles at certain SNP sites, influence the outward expression (phenotype) of these cytokines, so individuals in this study, are classified as high, intermediate or low producers. Studies have shown, for example, that a "G/G" genotype at the IL-6 (-174) site is associated with the high production of this cytokine, and that high levels of this cytokine are important for mounting an immune response against parasitic and fungal infections (T_h2 type of pathogens) (Marquet et al. 1999). In contrast, an individual who is "C/C" at IL-6 (-174) is a low producer of this cytokine, which means that when faced with a T_h2 type of pathogen, this individual's immune response may not be as strong as an individual's who is a high producer of IL-6 (Linker-Israeli et al. 1999; Pascual et al. 2000).

As compared to the Aboriginal population, the Caucasians in this study maintain a higher frequency of the TNFα (-308) and IFNγ allele SNPs whose phenotypic expression is associated with enhanced production of these cytokines. In contrast to the Caucasian cohort, individuals of Aboriginal descent had a high frequency of the IL-6 (-174) allele,

associated with a higher production of this cytokine. The Aboriginal individuals maintained a statistically higher frequency of the "G" allele at the TNFα (-308) loci and would therefore tend to be low producers of this cytokine as compared to the Caucasian cohort. Similarly, the Aboriginals maintained a statistically higher frequency of the "A" allele at the IFNγ loci, which would be associated with low production of IFNγ. The allele frequencies of IL-10 (-819) and IL-10 (-592) were similarly distributed in a given population, which would suggest that these loci are in linkage disequilibrium. However, the Aboriginals had IL-10 loci phenotypes skewed towards a lower production level of IL-10 as compared to the Caucasian cohort. In contrast, the Aboriginal group as compared to the Caucasian cohort, had alleles associated with a higher expression level for IL-6 ("G" allele at –174) and $TGF\beta_1$ ("G" allele codon 25). There was no statistical difference between allele frequencies of the Aboriginal and Caucasian groups for $TGF\beta_1$ (codon 10).

The Aboriginals in this study, as a group, maintain a cytokine SNP profile that may make them less able to mount an efficient T_h1 immune response to clear bacterial or viral infections and may therefore be at a greater risk of mortality or morbidity when challenged with pathogens requiring such a response. The observed distribution of cytokine genotypes among the North American Aboriginals with ESRD suggests that this population is skewed to favour a T_h2 immune response. This was also found to be true of a normal Aboriginal population (Larcombe et al. 2005).

It is not unexpected that the contemporary Aboriginal immunogenetic profile should differ from the Caucasians given that the two populations have adapted to vastly different social and ecological environments. The observed immunogenetic profile may be an accurate representation of the genetic differences that has changed little from that of that of the ancestral Aboriginal population. The contemporary Aboriginal study population maintains a distinctive immunogenetic profile despite genetic admixture with Caucasian populations. Szathmary et al. (1974) estimated that European admixture in three Ojibwa communities ranged from 3% to 30% based on analysis of nuclear genes. Analysis of the effect that admixture and other selective pressures may have had on the Aboriginal immune response can only be accomplished through the analysis of the cytokine genotypes of ancient individuals.

Chapter 5 Molecular analysis of human remains from Manitoba

5.1 Introduction

In the 1980's, researchers were excited by the discovery that DNA could be extracted from an extinct horse that had long been a museum specimen (Brown and Brown 1992; Higuchi et al. 1984). It was later reported that DNA had been extracted from a mummified Egyptian child who radiocarbon dated to 2430± 120 BP (Pääbo 1985). Indeed, Egyptian skin specimens dating to 13000 years ago have yielded DNA (Pääbo 1989). In 1986, Pääbo retrieved DNA from brain tissue from the Windover Pond site. The tissue yielded less than 1% of the amount of DNA that fresh tissue would yield however, human mtDNA was identified from this sample that dated to almost 8,000 B.P. (Pääbo 1986). Hagelberg (1989), described the amplification of mitochondrial DNA sequences from human bone that was several hundred years old. The acquisition of DNA first from tissue sources, and later from bone and tooth remains, had considerable impact on the archaeological and anthropological community. Bone typically preserves under a wider range of environmental conditions than does soft tissues. As such, the ability to recover genetic material from bones has broader research potential.

The use of new techniques in the field of molecular anthropology such as polymerase chain reaction (PCR) has vastly improved the potential for ancient DNA (aDNA) analysis (Hagelberg et al. 1991b). Samples of ancient organic material may contain low or sometimes single copies of DNA, and the PCR process effectively replicates the DNA thereby providing sufficient quantities for analysis. The environmental condition in which DNA is preserved in organic material is a source of some debate in the literature (Gilbert et al. 2003a; O'Rourke et al. 2000; Wandeler et al. 2003). Also issues concerning the ability to control for contamination have been intensively examined in conjunction with applications of aDNA technology (Pusch and Bachmann 2004; Schmerer et al. 1999; Yang et al. 2003). Dedicated laboratory facilities and equipment, methodological procedures that control contamination, and the

incorporation of validation tests into the research design have vastly reduced the risks of contamination in ancient DNA testing. However, research continues to focus on strategies for optimizing the recovery of valid ancient DNA template and for minimizing affects of PCR inhibitors and contamination (Gilbert et al. 2003b; Yang et al. 2003).

This chapter will examine the current methods and applications of ancient DNA technology and establish how this analytical technique can be used to explore the genetic relationship between past and present populations.

5.2 DNA preservation and recovery in human remains

Ancient DNA can potentially be extracted from any organic substance however, in the case of human remains, the archaeological context may affect the potential for DNA preservation and recovery. Understanding the conditions in which the skeletal remains were found is important because the environmental conditions will influence the protocols used for DNA extraction, and indeed, whether or not it is feasible to recover the DNA. Archaeologically recovered materials such as teeth, tissue, hair and coprolites can all potentially hold preserved DNA, although the best source of aDNA comes from hard tissues (molar teeth and bone) (O'Rourke et al. 2000). The degradation process of DNA after the death of the organism involves the breakdown of the bond between the sugar and base of the DNA strand in the presence of water (O'Rourke et al. 2000:218). This processes, called hydrolytic damage, causes both depurination (breakdown of the bond between the purines, guanine/adenine and depyrimidination breakdown of the bonds between the pyrimidines, cytosine/thymine (Burger et al. 1999; Gilbert et al. 2003a; Gilbert et al. 2003b). Oxidative damage caused by the direct interaction of ionizing radiation with DNA will also result in the modification of the bases (Hoss et al. 1996). These processes constitute the major part of DNA degradation and occur within a few hours or days after the death of an organism (Burger et al. 1999; Gilbert et al. 2003a; Gilbert et al. 2003b). The result is that DNA strands from ancient organisms are characteristically fragmented soon after death and the degree of fragmentation of DNA may not be related to the age of the specimen (Brown and Brown 1992:13). Dried skin of a specimen that was four years old and one that was 13,000-years-old, for example, has similar sizes of molecular fragments (Pääbo 1989).

It has been suggested that preservation of both bone and soft tissue from permafrost is more likely to yield ancient DNA than specimens recovered from hot climates (Kumar et al. 1999). Kumar et al. (1999) found that specimens from open-air sites in India did not yield sufficient DNA template for analysis. The researchers attributed rapid DNA degradation to the hot, moist climatic conditions. Krings et al. (1999) considered the microenvironment of the limestone cavern to be at least in part responsible for the successful amplified DNA from a Neanderthal specimen that was approximately 29000-years-old. Similarly, Burger et al. (1999) considered that the low temperatures at the Lichtenstein Cave site had an advantageous affect on DNA preservation in the skeletal remains. In addition, alkaline or neutral pH in the sample or in the soil can contribute to DNA preservation. In fact, these environmental conditions may be more significant than the age of the remains (Hagelberg et al. 1991b). Humic and fulvic acids found in the soils can also have inhibitory effects on the enzymatic reaction that takes place during PCR (Burger et al. 1999; Hagelberg and Clegg 1991), therefore samples that have greater amounts of these acids will have a lower rate of amplification success (Burger et al. 1999; Hagelberg and Clegg 1991).

No data has been systematically collected on the environmental conditions from which the samples from Manitoba were collected. The central and northern regions from which all of the samples were recovered are generally within the Boreal Forest ecological region or the transition region between the Aspen Parkland and Boreal Forest regions (Scott 1996). The soils in these regions are in general very acidic (4.0-6.5 pH) as a result of the organic matter that overlays the alluvial clays. Typically, archaeological occupations in Manitoba are embedded in the organic layers, which may overlie the clay, or they may be sandwiched between layers of clay and organic material. Burials, however, tend to be placed below this organic layer, either within or on top of the clay soils. Clay soils (especially those associated with limestone deposits) in contrast to the organic layer are alkaline, having a pH between 7.0 and 8.5. The high pH of the clay soils may have a significant affect on the preservation of DNA in skeletal materials found in these contexts (Hagelberg et al. 1991b; Matheson and Brian 2003). Data on the pH of the soils surrounding burials would contribute significantly towards understanding the conditions that enhance the preservation of DNA.

The tissue sample chosen for DNA testing is usually a function of availability resulting from differential preservation. Usually, hard tissues are better preserved and are therefore more likely to be available for DNA testing. In addition, hard tissues have a greater probability of yielding DNA than do soft tissues (Kaestle and Horsburgh 2002). In a test where DNA was extracted from a soft tissue sample and a bone sample from the same 3350 year-old animal, the soft tissue yielded only 150 base pairs (bp) in length whereas researchers were able to amplify a DNA fragment from the bone that was 438 bp in length (Richards et al. 1995:292). Rib bones are considered to be suitable specimens for aDNA extraction because of their spongy bone composition, they are numerous per individual, and they are usually less significant morphologically or palaeopathologically (O'Rourke et al. 2000:222). Hagelberg et al. (1991b) examined the histology of long bones from several sites of varying age and demonstrated that even in a poorly preserved bone there might be regions of the bone that have unchanged micro-structures that may make DNA recovery possible. Histological screening of samples might result in the identification of some well preserved hard tissue and allow for optimization of DNA recovery (Hagelberg et al. 1991b). Long bones are often well represented in the archaeological record because of their density and size; however, when intact and well preserved, these bones can be difficult to cut and sample.

The amplification of DNA fragments (measured in base-pairs), and in particular, nuclear short tandem repeats, was shown to be significantly higher with DNA from molar teeth rather than bone (Gilbert et al. 2003a; Zierdt et al. 1996). Zierdt et al. (1996) extracted DNA from both bone and tooth samples of 200 individuals and amplified a short tandem repeat locus. The success rate for recovering DNA from tooth was 14% higher than recovering DNA template from bone (Zierdt et al. 1996). Accessing DNA from the teeth is also advantageous because using multiple teeth from a single individual has the potential for providing sufficient sample for replicating the experiments. The potential for recovery of aDNA from any one of these sources is dependent upon the preservation conditions and the extraction methods. Ideally, multiple tissue samples from any one individual would be collected for DNA because preservation may differ even within an individual skeleton. Having several samples also ensures that sufficient tissue is available so that multiple extractions can be preformed to authenticate the test results.

5.3 Extraction and amplification of ancient DNA

To recover DNA from archaeologically recovered tissue samples, the DNA molecules must be separated from the bone, tooth or tissue matrix. This occurs through a process of mechanical and chemical reduction and separation (Kaestle and Horsburgh 2002). To minimize contamination, the exterior surface of the sample is sometimes removed by simply cutting off, scraping, or sanding the exterior surface. Stone et al. (1993b) described a process of removing the outer layer of bone by scraping it away with a sterilized razor blade. Richards et al. (1995) tested both chemical and mechanical methods of removing surface contaminants on pig bones that had been archaeologically recovered. Richards et al (1995) handled the bones in a way that intentionally contaminated the pig specimens and the bone was then treated using three different methods. One of the specimens was untreated, another was soaked in 0.5% sodium hypochlorite for one hour, and the other was thoroughly shot blasted. Each of the specimens was tested for the presence of both pig and human DNA sequences. It was found that the untreated pig sample yielded only human DNA. The human DNA introduced through handling had thoroughly out-competed the endogenous pig DNA during the amplification process. The two treated bone specimens both yielded equal amount of pig DNA. Richards et al. (1995) suggested that either bleaching or shot blasting the bone could serve to remove surface contaminates. Bleaching may also be useful in removing contamination from less well-preserved bone that is porous (Richards et al. 1995). While contaminates can be reduced by bleaching the tissue specimens, they cannot be eliminated, so other methods must be used additionally.

Irradiation by ultraviolet light (254nm) of surfaces of the sample as well as workbenches and equipment is considered a good method for removing DNA contaminates since the UV light fragments double-stranded DNA (Zierdt et al. 1996). However, UV light only affects DNA when it is in aqueous solution, and, in addition, the fragmentation of surface DNA may result in the amplification of surface contaminants and result in their identification as ancient DNA because of their artificially shortened strand length. The *inability* to amplify sequences longer than 300bp (presumably modern exogenous DNA) in a positive control cannot therefore be considered reliable proof that the DNA is ancient. Depending on the condition and the type of sample being used for

aDNA extraction, a combination of decontamination methods is usually necessary to reliably remove surface contaminants because no one method in itself is considered sufficient (Hummel 2003).

Accessing DNA from teeth or the pulp cavity either by powdering the entire tooth or by sectioning it are acceptable procedures (Gilbert et al. 2003b; O'Rourke et al. 2000:222). Matheson (per. comm.) used a technique of drilling a small hole into the molar tooth pulp cavity, powdering the pulp with a Dremmel® tool, and removing the powder through the drill hole. This technique leaves the tooth largely intact and is a viable procedure in situations where the tooth cannot be destroyed. Another advantage to this technique is that contamination of endogenous DNA is further reduced only the powdered pulp, which has not been previously exposed, is used in the sample. The primary disadvantage of this method rests in the fact that the quantity of sample is greatly reduced compared to the amount of sample obtained from powdering the entire tooth. Yet another method used only the roots of the molar teeth, thereby preserving the crown of the tooth (Burger et al. 1999).

After the outer surface has been treated (either chemically, mechanically or both) a tissue sample is then removed by drilling or scraping from the interior of the specimen and then powdered. Once in powdered form, the bone or tooth is chemically decalcified and rinsed. The resulting mixture contains not only DNA but also endogenous protein and possibly PCR inhibitors from the soil, such as proteins or other factors related to the burial conditions. Soils contain humic acids, tannins and fulvic acids derived from plant material that inhibit the action of the Taq during PCR (Hummel 2003). Hoss et al. (1993) experimented with the silica-based purification method and found that they could significantly reduce the amount of contaminates from both PCR inhibitors and modern DNA. The silica method uses guanidinium thiocyanate (GuSCN) to extract DNA and bind it to silica particles (Hoss and Pääbo 1993). This method reduces the amount of PCR inhibitors that might be co-extracted because GuSCN has the ability to lyse proteins and at the same time facilitates the binding of DNA to the silica particles. O'Rourke et al. (2000) cautioned that GuSCN can easily be contaminated because of its strong affinity to nucleic acids, both ancient and modern. Silica is a strong PCR inhibitor, so it is necessary that all of the silica is removed during the succeeding purification stage (Yang et al.

1998). While other methods have been demonstrated to be effective for ancient DNA extraction (i.e. phenol-chloroform) (Hummel 2003) the use of GuSCN and silica beads is effective and may lessen the possibility of co-extracting potential PCR inhibitors (Yang et al. 1998).

5.3.1 Techniques for amplifying ancient DNA

The degraded and fragmentary nature of DNA recovered from ancient specimens presents a challenge for researches to compare with modern reference collections. Before 1983, research in recombinant DNA relied on cloning to obtain isolated copies of DNA. This procedure was time consuming since it involved cloning as well as methods to detect target DNA sequences (Watson et al. 1997). In 1983, researchers began to use Polymerase Chain Reaction (PCR), which is a process that mimics the natural DNA replication process. PCR uses single strands of DNA as templates along with oligonucleotide primers and enzymes that affect the binding of the primers to the DNA template (Hummel 2003). The entire PCR cycle is a three-step process involving denaturing the DNA strand, annealing primers to the exposed strand and polymerization of the new strands. With the use of PCR, the short fragmented sections of aDNA can be amplified (replicated) in a relatively short period (Hummel 2003). Heating the molecule in a mixture of polymerase enzyme and oligonucleotide primers denatures a double-stranded DNA molecule. A double-stranded DNA molecule can be converted into a single strand by heating the DNA above its melting temperature (Tm) at 94°-95°C. The second stage of PCR involves cooling the mixture allows the primers to bind or anneal to the target region. Primers are usually 20 to 30 nucleotides in length and are specifically designed to flank and amplify a target region of DNA. The third PCR stage occurs as the DNA polymerase uses the primers to begin synthesis of the new strand of DNA. The reaction mixture can then be heated again and the originals and the newly synthesized DNA stands can serve as templates for further amplification (Watson et al. 1997). By varying the annealing time and temperature, the specificity of the amplification process can be enhanced so that mis-priming or mistakes in the annealing process do not occur.

Kolman et al. (2000) make the point that despite taking precautions in the field and in the laboratory, contamination of ancient DNA is "a given" for archaeological

samples. Given the highly degraded and damaged nature of aDNA, even minute quantities of modern DNA can out-compete the ancient template during the PCR amplification process. Because DNA from ancient material is typically present in low copy numbers increased numbers of PCR cycles (usually 40-45 cycles) are required to obtain sufficient DNA for analysis. Yang et al. (2003) demonstrated that higher numbers of PCR cycles resulted in the amplification of contaminates in the negative controls even though contaminate DNA might be present at extremely low levels. Using multiple positive controls in the PCR reaction and monitoring the sensitivity of the PCR amplification in these controls might be a strategy for optimizing the PCR and assessing the level of contamination (Yang et al. 2003).

Initially, the PCR process was slow because DNA polymerase E. coli was used. This enzyme is heat sensitive, and, after each denaturing cycle, fresh enzyme had to be added. An enzyme from a bacteria (*Thermus aquaticus*) that lives in hot springs works best at high temperatures and is therefore the preferred by many researchers (Watson et al. 1997). *Taq* polymerase, as it is called, is stable at 94°C so that this polymerase is added to the PCR reaction mix only once. The PCR product is visualized on an agarose or polyacrylamide gel using electrophoresis and is either directly sequenced, or cloned and sequenced.

5.3.2 Multiplex PCR

Multiplex amplification has been used for a long time by forensic sciences for autosomal Short Tandem Repeat (STR) analysis using commercially available kits (Hummel et al. 1999). STRs are short sequences of DNA, usually 2-5 base pairs in length, that are repeated numerous times, and the number of these repeats is highly polymorphic between individuals (see below). If the STR loci are amplified and the length analyzed, the variable number of repeats that exists between individuals (except for monozygotic twins) is visible. Because each STR is a single-locus event, the amplification of only short lengths of DNA from degraded template is not incompatible. The key to successful amplification of multiple loci using this method is dependent on good primer design.

Multiplex PCR amplifies several of these STR loci simultaneously. Fluorescently labelled primers allows the different STR PCR products to be visualized and identified using electrophoresis (Hummel and Schultes 2000). Hummel et al. (1999) simultaneously amplified nine STRs and the amelogenin locus from nine individuals. Although not all regions amplified reliably, the tests demonstrated that multiplex typing cannot only provide genetic profiles of the individuals, but it can also address questions of authenticity of the results (Hummel et al. 1999). In addition, this method uses a minimal amount of bone or tooth sample for the analysis of multiple genetic loci.

5.4 Applications of ancient DNA analysis

In each cell in the human body, there are two different sources of DNA. The DNA in the nucleus (nuclear DNA or nDNA) of each cell directs the biological functioning of the cell. Each person has a total of 46 chromosomes, twenty-three of which are inherited from their mother and twenty-three of which are inherited from their father. Forty-four of the chromosomes code for all of the biological functions of the human body and the other two chromosomes, (one from each parent) codes for the sex of the individual. The presence of two X chromosomes means that the individual is female; the presence of an X and Y chromosome indicates that the individual is male. It is the nuclear DNA therefore, that holds information about the sex of an individual and genetically related conditions or diseases.

In each cell, there are thousands of structures called mitochondria, which contain their own compliment of DNA specific to their structure. Each of the millions of cells in the human body contains hundreds of mitochondria organelles. In contrast, each cell contains only one nucleus and one complement of genomic DNA. Because mitochondria are so numerous, retrieval of DNA sequences from these structures is much more reliable than retrieval of the low copy numbers of nuclear DNA. The mitochondrial DNA consists of 16,570 base pairs coding for 37 genes, and within this genome are several non-coding regions that have a comparatively high rate of polymorphism as a result of earlier mutation events (Brown et al. 1998; Brown and Brown 1992). Table 4 lists the characteristics of nuclear verses mitochondrial DNA and emphasizes the fact that, while

mtDNA might be more available and more easily characterized, it limits the types research of questions that can be addressed.

The osteological analysis of human remains that have been recovered from archaeological contexts can provide information about specific individuals in terms of sex, age and cause of death as well as some sense of the individual's health before death. The traditional methods of analysis rely on observations and measurements of specific sites on bones to derive from the individuals as much information as possible. In many cases, human remains that have been archaeologically recovered have been subjected to erosion and weathering, resulting in differential preservation of the remains. These factors may result in a situation where information about the individual cannot be determined through traditional analytical techniques.

Table 4. Nuclear verses mitochondrial DNA

Nuclear DNA	Mitochondrial DNA
Linear structure	Circular structure
30-40,000 base pairs in length	16,500 base pairs in length
Inherited both paternally and maternally	Maternally inherited
Recombination of genetic material occurs	Recombination of genetic material does not occur
Low mutation rate	High mutation rate (5 times that of nuclear DNA)
Each cell contains one nucleus –single copy of nuclear genome	Each human cell contains 100's of mitochondria
Sex chromosomes are present	No genetic material for sex identification

If the pelvis is missing, for example, it is difficult to determine the sex of the individual. If the cranium is missing or if the grave offerings have been eroded away, it might not be possible to assess the ethnicity of the individual. In Manitoba, DNA analysis of ancient samples had been attempted but it was not until recently that the techniques and methods were refined sufficiently to obtain DNA from ancient human remains (Molto, per. comm.). In addition, First Nations communities have only recently become interested in the potential results of this type of analysis.

5.4.1 Determination of the sex of the individual

Genetic sexing of individuals is particularly important in the case of the recovery of juvenile or infants who cannot be sexed based on bone conformation. Confirmation of the sex of an adult based on genetic sexing can be used in circumstances where sex identification from bone morphological traits is uncertain. Knowing the sex of an individual might assist in matching particular bone elements to an individual, in a case where elements from a number of skeletons are co-mingled. The amelogenin gene, which codes for a protein of tooth enamel, is frequently analyzed for sex identification because the male and female variants differ by six base pairs. The X chromosome has a 6-bp deletion in an intron region of this gene (Mannucci et al. 1994). The single-copy gene is located on the Y chromosome at Yp11.2 and on the X chromosome at Xp22.31-p.22.1. Amplification of a single band (106 base pairs) on a gel indicates female and two bands (106 bp and 112 bp) indicate a male (Hummel 2003).

5.4.2 Familial Relationship

Methods for looking at familial relationship can use mitochondrial or nuclear DNA genetic markers. Each of the millions of cells in the human body contains hundreds of mitochondria organelles. Because mitochondria are so numerous, it is much easier to retrieve the DNA sequence from these structures in contrast to the relatively low number of nuclear DNA. The DNA contained in the mitochondria is identical in each of the mitochondrial cells and it is identical to the mtDNA of an individual's mother. The mitochondrial genome is maternally inherited and accumulates mutations, or substitutions at a much higher rate than the nuclear genome. The individuals in the kinship diagram in Figure 13, denoted in black, would share the same mitochondrial DNA. A mother and child should have nearly identical mtDNA, however, the mitochondrial genome accumulates mutations, or substitutions, at a much higher rate than the nuclear genome so that over many generations genetic distance will accumulate.

Hyper Variable regions I and II (HVR I and II) of the d-loop control region consists of about 600 base pairs, which have a high number of polymorphic sites (Hummel 2003) (Figure 14). A comparison of the HVR's in two randomly selected unrelated individuals would reveal differences in 3 out of every 100 nucleotide positions

(Stoneking 2000). If two individuals are closely related (mother and child for example) one would expect that a comparison of a 100 base pair region would show no differences.

Polymorphisms in the HVR II and I are used as markers to evaluate maternal relationship and phylogenetic relationship. A comparison of the HVR's in two randomly selected unrelated individuals would reveal differences in 3 out of 100 nucleotide positions (Stoneking 2000). This region is used in studies at the population level to investigate migration patterns and population affinities (Hagelberg and Clegg 1993; Torroni et al. 1992).

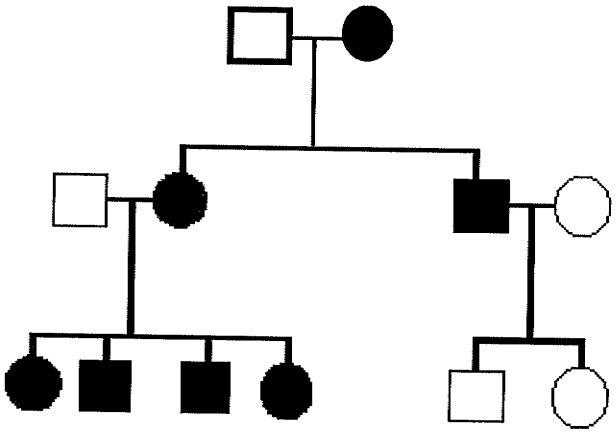


Figure 13. Maternal inheritance of the mitochondrial genome

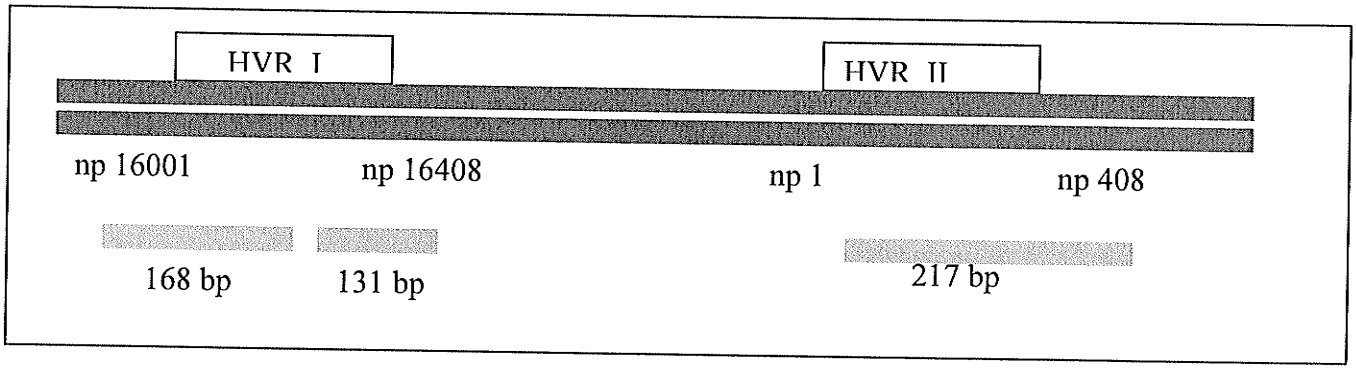


Figure. 14. Mitochondrial DNA Hyper Variable Regions I and II

(adapted from Handt et al. 1998)

5.4.3 *Haplogroup analysis of Aboriginal North American groups*

The analysis of genetic markers in contemporary North American Aboriginal populations has resulted in the identification of five haplogroups, or groups of individuals that share similar mitochondrial genetic markers. To-date, a total of five groups based on mitochondrial markers have been identified among North American Aboriginal populations and they are referred to as haplogroups A, B, C, D and X (Torroni et al. 1992; Torroni et al. 1993b). These haplogroups are a subset of the maternal haplogroups found in Eurasia which suggests to researchers that North American Aboriginals share genetic affinities to Eurasian populations (Schurr et al. 1990; Torroni et al. 1993a). Analysis of the mtDNA of ancient North American individuals has confirmed the presence of these haplogroups in the ancient populations (Kaestle and Smith 2001; Schurr 2000; Smith et al. 2000; Stone and Stoneking 1993b) (Figure 15). Recognition of each of the five different haplogroups is accomplished using Restriction Fragment Length Polymorphism (RFLP) and by direct sequencing of the Hyper Variable regions. Sequencing of the Hyper Variable I and II regions of human mtDNA allows for the identification of the genetic markers representing each of the haplogroups (Table 5).

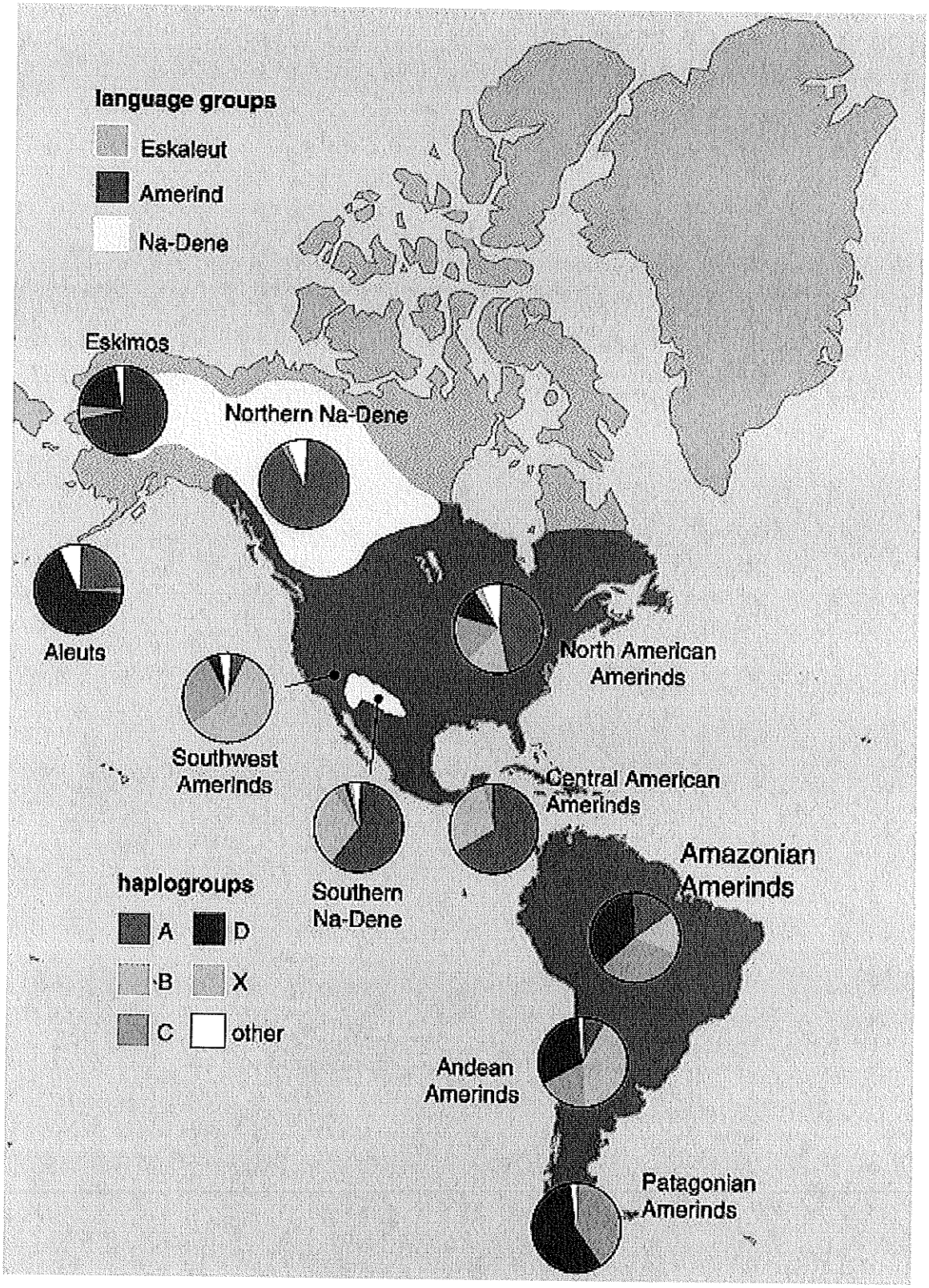


Figure 15. Native North American haplogroups (Schurr 2000:250)

Table 5. Genetic markers representing Native North American haplogroups.

Haplogroup A is identified by the presence of at least two of the following mutations

C – T transition at np 16111 (Malhi et al. 2001)

C - T transition at np16290 (Malhi et al. 2003; Schurr et al. 1990; Torroni et al. 1993a)

C - T transition at np 16223 (Malhi et al. 2003)

G - A transition at np 16319 (Malhi et al. 2003; Schurr et al. 1990; Torroni et al. 1993a)

T – C transition at np 16362 (Malhi et al. 2003; Schurr et al. 1990; Torroni et al. 1993a)

Haplogroup B is identified by the presence of the following two mutations:

A – C transversion at np 16183 (Malhi et al. 2003)

T – C transition at np 16189 (Malhi et al. 2003)

T – C transition at np 16217 (Malhi et al. 2003; Torroni et al. 1993a)

T - C transition at np 16519 (Malhi et al. 2003)

T – C transition at np 16819 (Torroni et al. 1993a)

Haplogroup C is identified by the presence of at least two of the following mutations:

T – C transition at np 16298 (Malhi et al. 2003; Torroni et al. 1993a)

C - T transition at np 16223 (Malhi et al. 2003)

C – T transition at np 16327 (Malhi et al. 2003; Torroni et al. 1993a)

T – C transition at np 16325 (Malhi et al. 2003; Torroni et al. 1993a)

Haplogroup D is identified by the presence of the following mutation:

C – T transition at np 16223

Haplogroup X is identified by the presence of the following mutations:

C – T transition at np 16223 and 16278 (Smith et al. 1999)

A – C transversion at np 16183 (Smith et al. 1999)

T – C transition at np 16189 (Smith et al. 1999)

Bolded mutations indicate those that are specific to that haplogroup (Torroni et al. 1993a)

Haplogroup X is the least common of the five haplogroups with a continent-wide frequency of about 3% but like the other haplogroups, it has a non-random distribution (Smith et al. 1999). The modern Chippewa (Ojibwa), Micmac and Kiowa are characterized by very high frequencies of haplogroup X (Smith et al. 1999). Among Manitoban Aboriginal populations haplogroup X is associated with Algonkian speaking groups (Malhi et al. 2002). Haplogroup D occurs less frequently in contemporary Manitoba populations (Torroni et al. 1993b) but it lacks a distinguishing polymorphism like those found in haplogroups A, B, and C. The polymorphisms for haplogroups A, B,

C and D are found in contemporary Ojibwa populations (Lorenz and Smith 1997; Torroni et al. 1993a).

These haplogroup genetic markers are all found on the mitochondrial DNA and therefore indicate only the maternal heritage of the individual. By tracing the occurrence and frequency of these genetic markers in individuals and populations, anthropologists have examined the migration and movement of Aboriginal people in the past.

5.4.4 Ancient DNA and the identification of pathogens

The use of molecular analytical techniques has substantially broadened the research design regarding the relationship between diseases and humans in the New World. The detection of *Yersinia pestis* (Drancourt et al. 1998), *Mycobacterium leprae* (Rafi et al. 1994), *Mycobacterium tuberculosis* (Salo et al. 1994; Spigelman and Lemma 1993) and the virus that caused the 1918 flu pandemic (Taubenberger et al. 1997) in ancient samples demonstrates that the historic or prehistoric characteristics of this pathogens are knowable. It may be possible, using molecular techniques, to gain a better understanding of the evolution of pathogens and parasites that have affected humans. The ability to investigate the disease pathogens through the analysis of ancient DNA is just beginning to shed light on their evolution and will likely contribute to a better understanding of the diseases that existed in the New World. The diagnosis of *Mycobacterium tuberculosis* among ancient populations, for example, has been greatly enhanced by using DNA probes for the direct detection of mycobacterial sequences (Braun et al. 1998; Eisenach et al. 1990).

The first studies of tuberculosis relied on the identification of tubercular lesions on bone or preserved soft tissue. Salo et al. (1994) first identified *Mycobacterium tuberculosis* from a pre-Columbian Peruvian mummy by taking a sample of lung tissue and a lymph node with lesions that were considered to be "tuberculosis-like". The individual had been ^{14}C radiocarbon dated to 1040±44 years. Salo et al. (1994) compared the target sequence of IS6110 of the ancient sample to that of contemporary *M. tuberculosis* and found them to be identical. The identification of *M. tuberculosis* from soft tissue led researchers to explore the potential for applying these methods to skeletal remains. Braun (1998) noted that infections with *Blastomycosis* spp. and healed

compression fractures can be similar in appearance to vertebral lesions involved with tuberculosis and that only10% of cases have skeletal involvement as a result of tuberculosis infection (Taylor et al. 1996). In cases where lesions are present, the interpretation of pathology is dependent upon bone preservation and the extent of diagenic damage. Because the presence of tuberculosis cannot be reliably diagnosed simply through visual inspection of bone, reliable identification of the disease through DNA analysis can help to confirm its presence.

Baron et al. (1996) detected *M. tuberculosis* in bone specimens without any tuberculosis lesions. Using autopsy specimens from a historical pathological collection, the researchers were able to amplify the IS6110 sequence from the bone samples and therefore confirm the presence of tuberculosis from visually uninvolved bone. The insertion element IS6110 is common to all members of the *Mycobacterium tuberculosis* complex, which includes *M. bovis, M. microti* and *M. africanum* so that it is impossible at this point to distinguish between the complexes using the IS6110 sequence.

Recent characterization of the genomic deletion events of Mycobacteria suggested that it is *M. bovis* rather than *M. tuberculosis* that has undergone extensive adaptation and has called into question the hypothesis that *M. tuberculosis* evolved from *M. bovis* (Mays et al. 2001). The fact that the *M. bovis* genome, because of genomic deletions, is much smaller than that of *M. tuberculosis* suggests the human form of the pathogen is older (Brosch et al. 2002). Brosch et al. (2002) identified a "modern" strain of *M. tuberculosis* that is recognized by the deletion of "TbD1". In order to assess whether or not the TbD1 strain was present prior to the human migration into the New World, Brosch et al. (2002) noted that it would be helpful to know whether the Egyptian and South American mummies (Arriaza et al. 1995) carried the TbD1 deleted gene. The resurgence of tuberculosis as an epidemic disease among Aboriginal populations might be explained by the introduction of a "modern" *M. tuberculosis* strain with the TbD1 deletion into a population who had survived with an older endemic form of the disease.

Infectious diseases that kill a human host quickly are usually not identifiable in the osteological record and, historic records provide only limited amounts of information about epidemics in the past. The 1918 influenza pandemic, for example, occurred long

before scientists were capable of isolating the virus, but the recovery of RNA and DNA from frozen tissues of flu victims, means that this virulent strain is now available for study. Researchers determined that the 1918 flu virus was most genetically similar to the human and swine influenza strains that existed in the 1930's, although it also had some avian characteristics (Reid et al. 1999). It is thought that in the past, wild birds were a major reservoir for influenza viruses, so it is logical to assume that historic or ancient forms human influenza might be genetically similar to the avian strains (Kilbourne 1997). The investigation of the genetic similarities and differences between the past and present flu strains can potentially shed light on how new viruses might emerge and adapt.

The development of a method for amplifying and identifying *Yersinia pestis* in ancient human remains is an important methodological advancement and has far reaching implications for the analysis of other blood-borne pathogens (Drancourt et al. 1998). The amplification of *Y. pestis* in DNA samples from the dental pulp cavity of historically documented plague victims demonstrates that the presence of systemic infectious diseases can be determined even when the soft tissues are absent. The methods developed by Drancourt (1998) hold considerable potential for analyzing infectious diseases such as typhus, leprosy and treponema that primarily affect soft tissues.

5.5 Polymorphisms and ancient nuclear DNA analysis

Genetic polymorphisms also arise from mutations and result from the insertion or deletion of a section of DNA. The most common types of polymorphisms are short tandem repeats (STRs) or microsatellites that are tandemly repeated arrays of 2-6 base-pairs. The following DNA sequence from Genbank D7S280 (Medicine 2004), a public DNA database, illustrates a four base-pair repeat sequence – "gata" (Figure 16).

```
121 ctaacgatag atagatagat agatagatag atagatagat agatagatag atagacagat
181 tgatagtttt tttttatctc actaaatagt ctatagtaaa catttaatta ccaatatttg
```

Figure 16. A 120 base pair long DNA sequence beginning at position 121 with four repeated "gata" sequences.

The number of times this tetrameric repeat occurs may vary between individuals from as few as six repeats, to as many as 15 tandem repeats. STRs are therefore useful markers for population genetic studies and individual kinship determination since the probability that two individuals will have the same number of repeats is low (Chambers and MacAvoy 2000; Hummel 2003; Hummel and Schultes 2000). The effective and statistically valid use of STR's for the analysis of kinship requires that multiple loci be analyzed. The amplification of STRs from degraded DNA extracted from bone was successfully demonstrated in 1991 (Hagelberg and Clegg 1991; Hagelberg et al. 1991a; Hummel et al. 1999), and later in 1995 the technique was used to determine kinship for prehistoric individuals (Zierdt et al. 1996).

The simplest type of polymorphisms result from a single base mutation where one nucleotide is substituted for another. The locus of such a change has recently been termed a 'single nucleotide polymorphism' (SNP) (Kwok and Chen 2003; Schork et al. 2000). A comparison of any two individuals' genomes will reveal that they are 99.9% identical (Cooper et al. 1985); however, within the 3.2 billion base-pair genome, each person will have approximately 3.2 million differences in his or her diploid genome. SNPs are found throughout the genome in both coding and non-coding regions, including promoter regions, and are therefore more likely to have a functional or physiological relevancy than other sorts of polymorphisms (Lazarus et al. 2002; Schork et al. 2000). A SNP in the coding region may impact a relevant protein, and, in the promoter region, a SNP can influence gene expression (Bayley et al. 2004; Fishman et al. 1998). Single nucleotide polymorphisms in the mtDNA hypervariable regions are currently used to characterize population haplogroups and are routinely identified in the analysis ancient DNA (Stone and Stoneking 1993a; Torroni et al. 1993a). The analysis of how nuclear DNA SNPs' may influence phenotypes such as disease risk and drug response differences is on-going (Schork et al. 2000), but these SNPs have, to-date, not been identified or examined in a prehistoric context.

5.6 Summary

Anthropologists have cautiously stepped into the new research arena of ancient DNA analysis. With each innovation, the arena has become bigger and the cautious steps

more confident, despite the occasional setbacks. With the benefit of new procedures such as PCR, the analysis of fragmented and damaged segments of DNA from ancient materials can be cloned effectively, facilitating the comparison of ancient biomolecules with modern reference collections.

It is the most fundamental and most often repeated conclusion in discussions about ancient DNA, that there is no single method that will effectively and reliably extract and amplify DNA from ancient material. The conditions in which the material was treated at the time of burial, the preservation conditions, recovery and conservation protocols will all influence the viability of the DNA. The resultant possibility of recovering and analyzing any preserved DNA will depend on extraction and amplification procedures that are tailored to the behavioural, taphonomic and conservation to which the remains were subjected. It is evident from the literature that each sample, from which an attempt will be made to extract, amplify and analyze ancient DNA, will vary considerably; indeed, samples from a single individual may also not yield consistent results.

The uniqueness of each DNA sample will affect the reproducibility of the experiments that are required to authenticate the results. Richards et al. (1995) stated that the reproducibility of test results is an essential criterion for assessing the validity of aDNA results. However, small sample sizes, differential preservation of bone elements and soil conditions may all cause test results to vary. While this may seem to be a daunting situation, it may in fact have positive implications. The failure of extracting DNA from a bone element from an ancient individual may not necessarily mean that the DNA is irretrievable. It may require that burial conditions be re-evaluated, soils be tested for inhibitory acids, extraction and amplification protocols be modified, and/or a different tissue type be selected for amplification.

The use of ancient DNA technology holds considerable potential for the analysis of past populations and issues related to health and disease. This technology allows for the comparison of genetic information of ancient populations and pathogens. We know that pathogens have shaped the course of human history. The use of molecular techniques can make a significant contribution to revealing the biological and genetic factors that

influenced these changes. The re-evaluation of the evolution of the mycobacterium genome, for example and the detection of *M. tuberculosis* in human skeletal remains has now made it possible to explore the genetic evolutionary relationship between mycobacterium, humans and animals. Ancient DNA technology will increasingly play a central role in addressing the relationship between host and pathogen by looking directly at the genetic evolution of the pathogen and by examining the genetics of the host's immune response to infection.

Ancient DNA technology has enabled researchers to make comparisons between ancient and modern populations to establish genetic relationships. The identification of Native North American haplogroup genetic markers in ancient human remains can contribute to the a broader understanding of the genetic relationship between past populations and their contemporary descendants. More recently, significant growth has occurred in the area of research regarding the identification of single nucleotide polymorphisms in contemporary populations. The identification of SNPs that influence susceptibility and/or resistance to diseases, and their differential distribution among contemporary Aboriginal and Caucasian populations, as demonstrated in the previous chapter, has raised questions regarding the selective pressures that influenced the population frequency of these genetic markers (Larcombe et al. 2005). The examination of SNPs in ancient individuals that influence the immune response could provide significant insight into the understanding the cytokine SNP frequencies that exist in the contemporary Aboriginal population.

Chapter 6 Genetic analysis and the detection of single nucleotide polymorphisms (SNPs) in ancient DNA

6.1 Approaches for the analysis of SNPs

The methods for detecting and identifying single nucleotide polymorphisms (SNPs) were developed using the labour intensive and expensive technique of comparing DNA fragment lengths that were digested with restriction enzymes (Kwok and Chen 2003). The bacterium *Hemophilus aegypticus,* for example, produces an enzyme called HaeIII that will cut a DNA strand wherever the sequence 5'GGCC3' and 3'CCGG5' is encountered. The human mitochondrial DNA sequence has close to 75 such GGCC sequences that can be cut using the HaeIII enzyme. The detection of human mtDNA haplogroup markers is an example of the use of restriction enzymes to digest a piece of DNA and subsequently, identify specific alleles or their variants (Wallace and Lott 2004). The following mitochondrial sequence (120bp in length) has one 'GGCC' grouping and it is at this location that the HaeIII enzyme would cut the strand (Figure 17). A cut strand would be identified on a gel after electrophoresis by the presence of two bands of almost equal size. A strand that did not have the GGCC at 663 would remain one long 120bp strand. The presence of a 'G' at np 663 is a polymorphism that occurs in high frequency among Asian and North American Aboriginal people and occurs in low frequency among Caucasians (Wallace and Lott 2004). Single nucleotide polymorphisms that were identified by evaluating the DNA fragment lengths were referred to by the method of analysis - restriction fragment length polymorphisms or RFLPs (Kwok and Chen 2003). The analysis of RFLPs is therefore a method for identifying the presence of polymorphisms if they occur at a location that can be cut with an enzyme.

SNPs that do not create or destroy a restriction site were not detectable using this method, and even the advent of polymerase chain reaction (PCR) did not improve the

situation because DNA sequence data were required to allow for the design of loci-specific PCR primers.

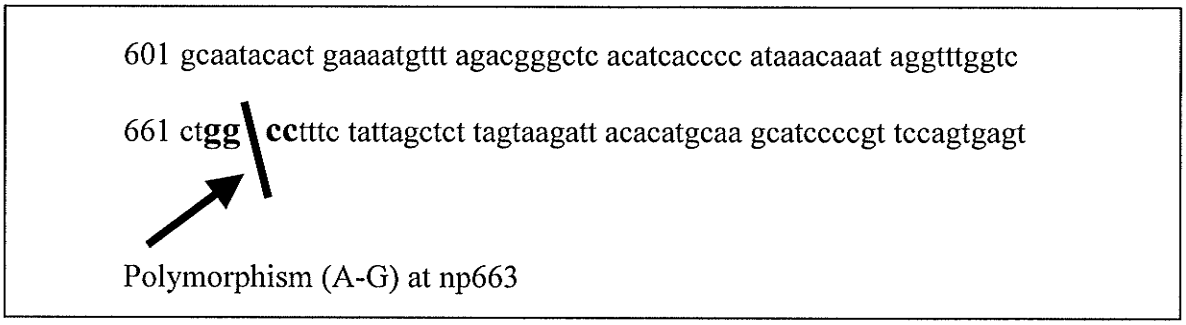


Figure 17. Polymorphic site at np663 and the site of cleavage by the enzyme HaeIII

Autoradiographs that allowed researchers to manually sequence SNP fragments were produced using radioactive phosphorus labelled nucleotides and gel electrophoresis, An X-ray film overlay, enabled radioactive DNA to strike the film and produce a high-resolution image (Russell 2002) (Figure 18). The "manual" part of the process referred to reading the sequence of nucleotide bases from the autoradiograph. Automated sequencing developed by Sanger et al. (1977), labels or "tags" each nucleotide base, which fluoresces as a different colour, which can be read by an automated sequencing device. Direct sequencing and oligonucleotide probing are new technologies that have improved the ease with which SNPs can be detected, and they are much more powerful than RFLPs in detecting the frequency of SNPs over the entire genome (Schork et al. 2000).

The analysis of SNPs in degraded and ancient DNA material has been done by amplifying the DNA using PCR and then analyzing fragment lengths using either gel electrophoresis alone or in combination with an automated fragment length analysis system. The assessment of fragment lengths using either agarose or polyacrylamide gels can resolve fragments that range in size from 50 to 500 bp, but the automated techniques such as capillary electrophoresis can assess small fragment lengths (Hummel 2003). Capillary electrophoresis along with appropriate software uses the Sanger et al. (1977) dideoxy method of DNA sequencing that incorporates dye labelled nucleotides into a PCR reaction that directly labels DNA nucleotides and inhibits further extension. Capillary electrophoresis analyzes the DNA by fragment length, a function of its

molecular weight, and scans for the fluorescently labelled dideoxynucleotide triphosphates (ddNTPs). A laser beam illuminates the tagged (ddNTPs) and an automatic scanner provides an image, called and electropherograms, of the sequence (Russell 2002) (Figure 16). In addition to providing increased accuracy for DNA sequencing, capillary electrophoresis requires smaller quantities of samples for analysis than does the gel electrophoresis method and requires significantly less handling.

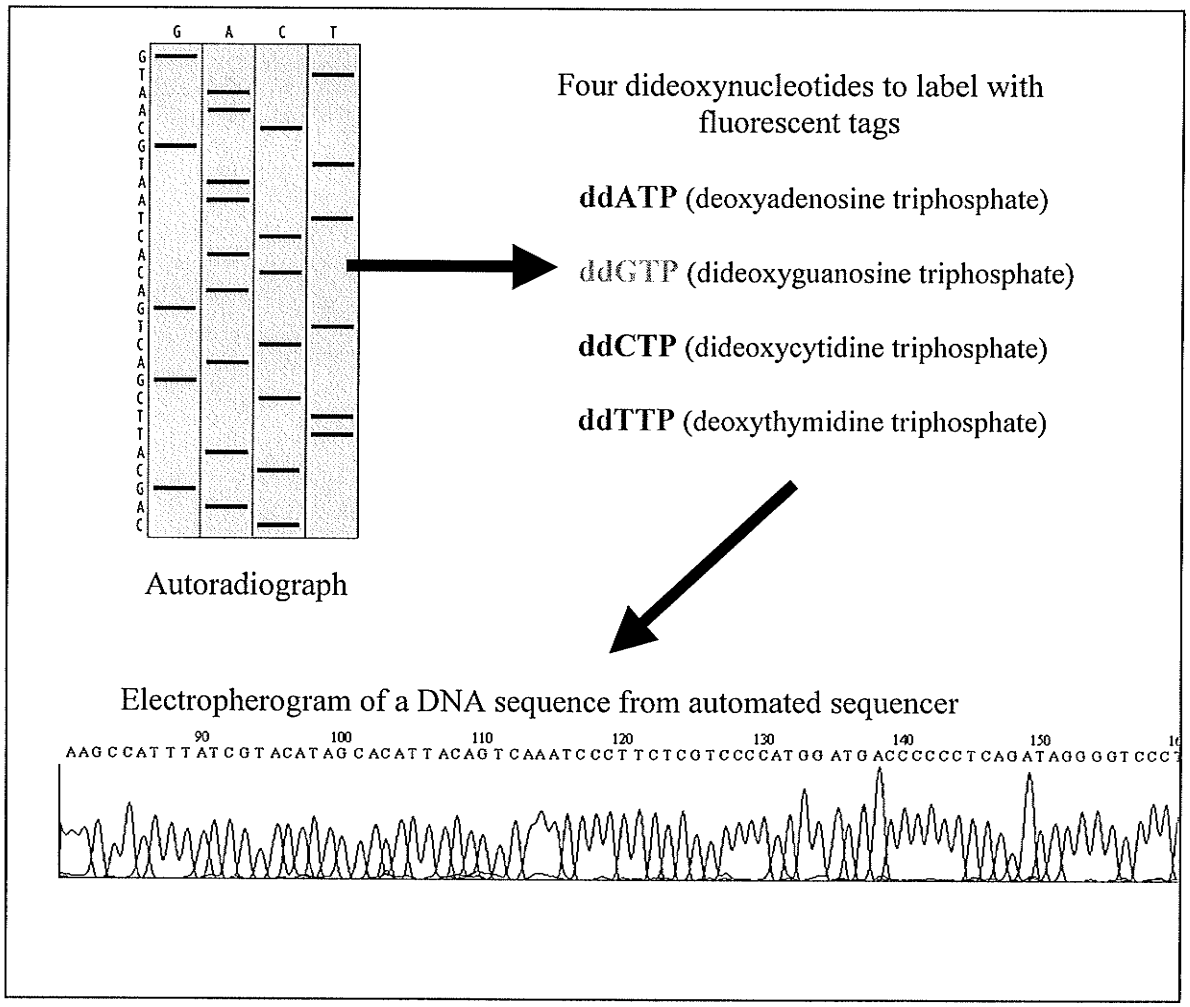


Figure 18. Automated sequence analysis of DNA

The use of polymerase chain reaction – sequence specific primer (PCR-SSP) kits (as described in chapter 4) is a well established method for amplifying and typing cytokine SNPS in modern tissue samples (usually white blood cells) that have high copy numbers of well-preserved nuclear DNA. However, nuclear DNA from ancient tissues is

typically highly degraded and fragmented, and, when present in archaeologically recovered remains, is maintained only in exceedingly low copy numbers (Burger et al. 1999; Hummel 2003; Kaestle and Horsburgh 2002). The study of cytokine SNPs in ancient material therefore requires an alternate approach that uses small sample sizes and accommodates DNA template shorter than 300 bp.

While much of SNP detection is turning to high throughput micro-array technology (Russell 2002), kits are currently available for labeling and detecting SNPs using a multiplex system that is considered moderate throughput level (Turner et al. 2002). SNaPshot (Applied Biosystems, Foster City, CA, USA) is a commercial minisequencing kit that uses extension primers and fluorescently labelled ddNTPs to prime and tag SNPs. Generally, the process is as follows

- an initial amplification of a target DNA sequence,
- purification of the PCR product and an additional to remove unincorporated ddNTPs
- SNaPshot amplification, which inserts a fluorescently labelled nucleotide at the polymorphic site.
- purification of the SNaPshot PCR product to remove unincorporated ddNTPs
- analysis of fragment length and scanning for fluorescent nucleotides using an automated sequencer and the appropriate software

The initial PCR uses primers designed to amplify a region surrounding the SNP. The PCR product is purified and a second amplification uses an extension primer, which binds immediately adjacent to the SNP site and has fluorescently labelled ddNTP that tag the SNP site at the 3' end (Figure 19). Turner et al. (2002) evaluated this method for a multiplex reaction which amplified nine different cytokine promoter loci were targeted simultaneously using nine primer sets. However in order to optimize the procedure for ancient DNA only a single set of initial primers and an extension primer was used in each reaction.

This chapter describes the materials and methods used to assess SNPs in the cytokine promoter regions of nuclear DNA from pre-contact and historic human remains from Manitoba. The general method described above, served as guidelines for

approaching the amplification of SNPs from ancient tissue but modifications were made to account for the fragmented DNA template.

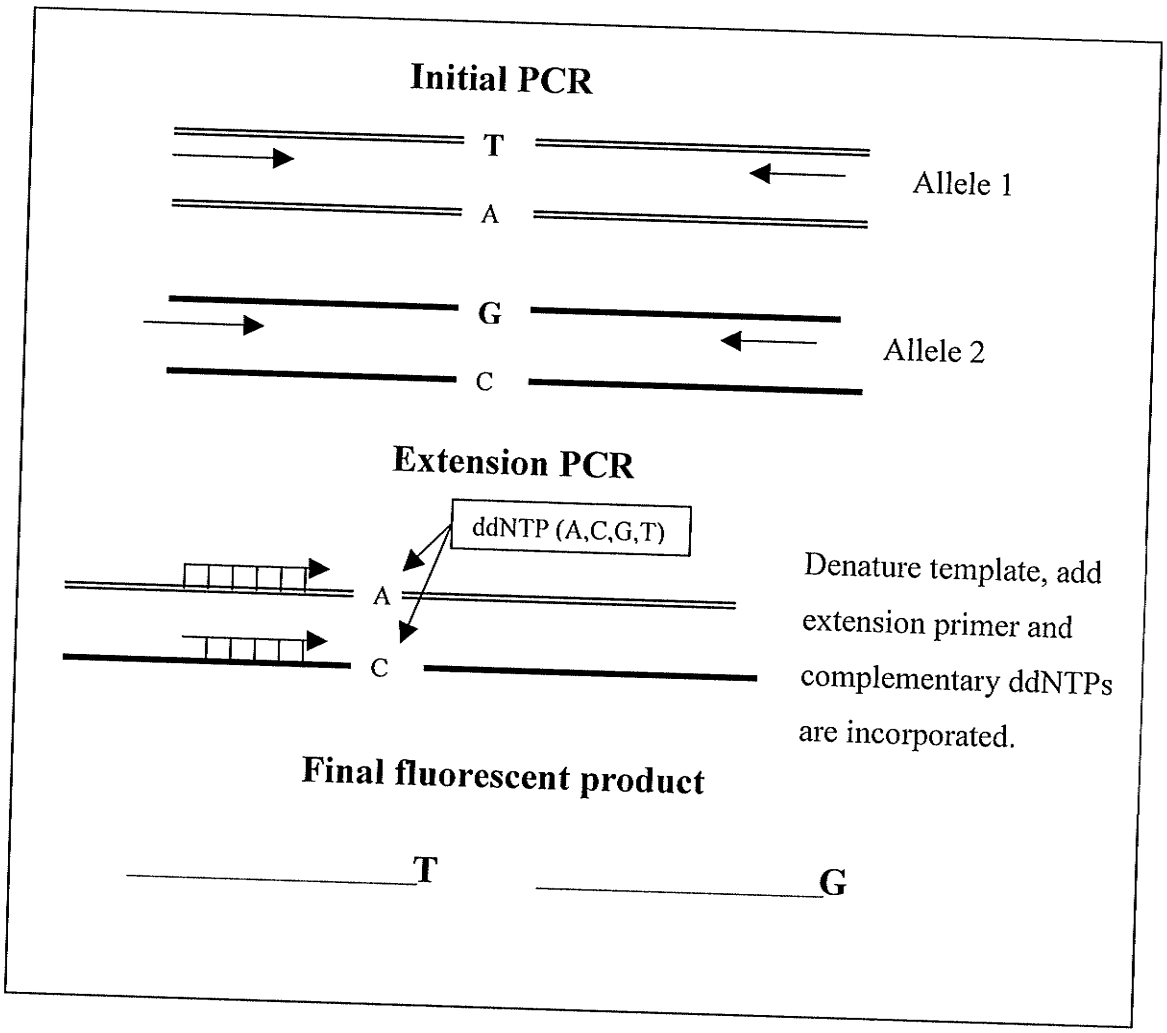


Figure 19. SNaPshot method

6.2 Materials and methods

There are numerous methods for extracting; purifying and amplifying degraded DNA from archaeologically recovered material (Hummel 2003). The methods vary between researchers, and the protocols are sometimes modified depending on the condition of the archaeological sample. For example, additional purification steps may be necessary if the sample contains an excessive amount of humic acid or, depending on the

type of tissue sample, changes in the volume of reagents used for extraction may be required. Although a general set of steps is followed, the individual condition of each sample was assessed and methods adapted accordingly.

The ancient DNA extractions, PCR reactions, and analyses, were conducted at the PaleoDNA Laboratory at Lakehead University in Thunder Bay, Ontario. The laboratory facilities are dedicated to ancient DNA analysis and research. The extraction, purification and amplification processes for this research were conducted in separate rooms in order to protect the reagents from contaminating DNA. Protective clothing including a full Tyvek suit, surgical mask, head covering, eyeglasses and a double layer of gloves that were changed frequently were worn throughout the pre-PCR procedures. Strict protocols for the sterilization of dedicated equipment are followed in the lab and regulated airflow all serve to eliminate contamination from exogenous DNA.

6.3 Archaeological samples

Skeletal samples were made available for this research from the University of Manitoba, University of Winnipeg, Manitoba Museum of Man and Nature and the Manitoba Historic Resources Branch. The skeletal remains were archaeologically recovered as part of the heritage protection policy that is currently in place in Manitoba. Permission and permits for the analysis of the samples were secured through the Manitoba Historic Resources Branch. Tissue samples from the skeletal remains of 18 individuals from 11 archaeological sites in Manitoba were examined to assess the extent of preservation, and one or two intact skeletal elements were chosen for DNA analysis. The amplification of short tandem repeats was shown to be significantly more successful from teeth rather than bone tissue (Zierdt et al. 1996); therefore, molar teeth were collected for analysis when available. Molar teeth without cracks or caries ensure that the dentine chamber is intact, thereby lessening the chance of contamination from exogenous DNA.

The bioarchaeological information available for each of the individuals in this study was for the most part, consistent in terms of the types of data collected (Mieklejohn 2001; Mieklejohn 2002a; 2002b). In the reports, it is evident that every effort was made to confirm that the individuals were of Aboriginal ancestry. Morphological assessment,

metric data, absolute dating, archaeological context and association with grave goods were all factors that were considered in assessing the ethnicity of the individuals. Dates for each of the individuals' come either directly from carbon[14] dates of the skeletal elements themselves, or indirectly from the archaeological context and/or associated grave goods. The morphological assessments and morphometric data that were collected further substantiates the ancestry of the individuals. It is likely safe to assume that the clearly dated pre-contact individuals had no genetic admixture with Caucasian people, but such admixture cannot be ruled out for the individuals who date to the historic period.

Each sample was assigned an "MSC" number, which is an accession number for the PaleoDNA laboratory. In addition, the archaeological site name and Borden number and the University of Winnipeg accession number ("ZW") are included as designators to further identify the sample.

6.3.1 Southeastern Manitoba

The human remains from the southeastern region in Manitoba come from three different archaeological sites - the Rivermouth Site (EcKx-37), the Two Eagles Site (EcKw-14) and the Whaley Cairn Site (EbKx-10). Figure 20 illustrates the general locations from which the individuals were recovered.

6.3.1.1 River Mouth Site (EcKx-37)

The two individuals (ZW-44 and ZW-45) from the Rivermouth Site (EcKx-37) were both C^{14} dated to the Late Woodland period (270 ± 60 yrs B.P. and 330 ± 40 yrs B.P.) (Ens 1999; Morlan 1999). It is not clear from the CARD database which date belongs to which individual, but the dates are consistent and demonstrate that the individuals could be contemporaneous. Both individuals have morphological features that are indicative of Aboriginal descent (Ens 1999). Rivermouth ZW-44 (MSC0004-03 & 07-03[7]) was determined through morphological assessment to be female while Rivermouth ZW-45 (MSC0008-03) was assessed as male (Ens 1999).

[7] MSC0004-03 was a left calcaneous and MSC0007-03 was a right lower 2nd molar from River Mouth ZW-44.

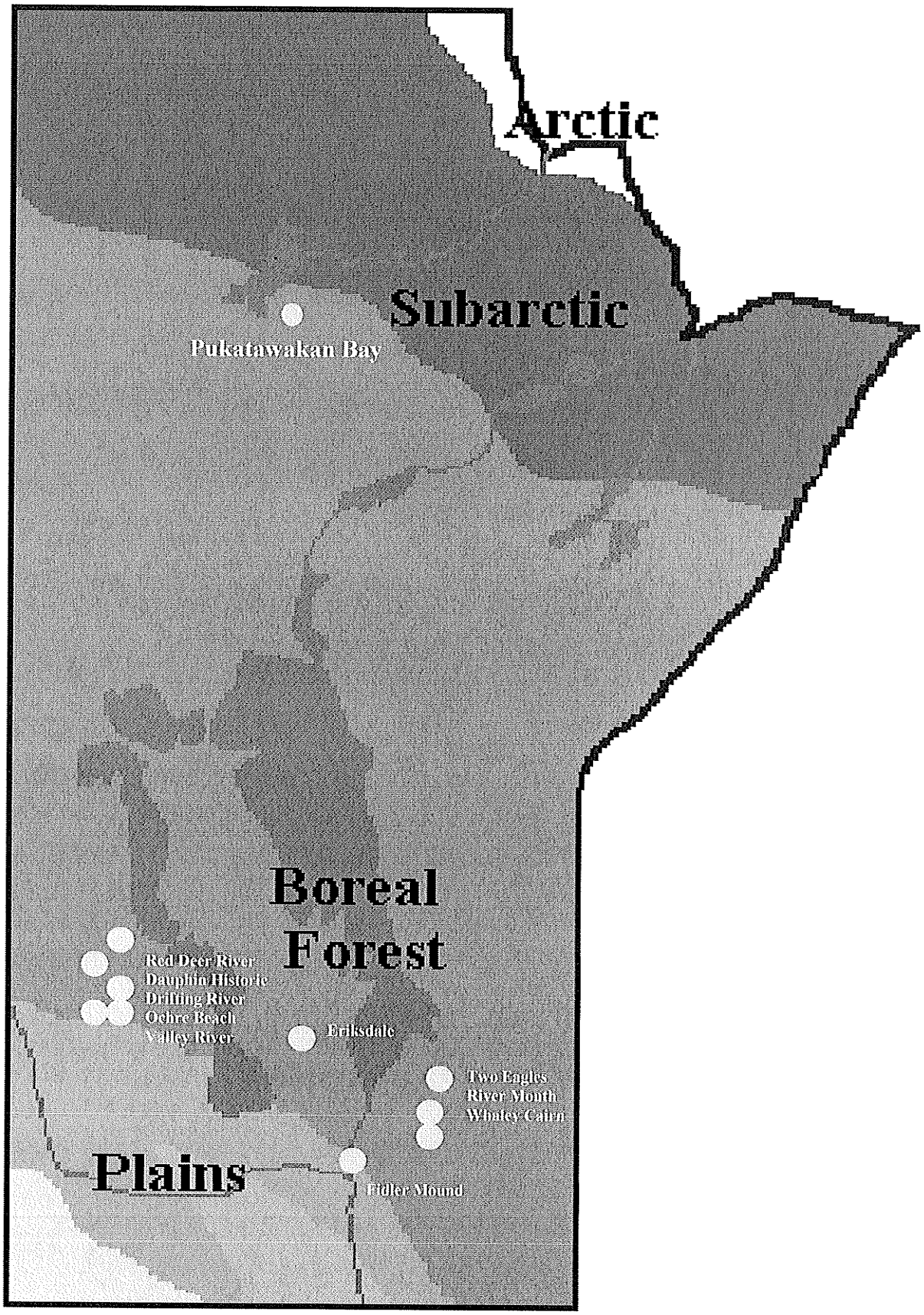


Figure 20. Location of archaeological sites

6.3.1.2 Two Eagles Site (EcKw-14)

MSC00039-03 Two Eagles (EcKw-14) was recovered from an eroding bank of Lac du Bonnet near the Lee River (Ens 1999). The individual was recovered in association with five projectile points, two harpoon points, a rolled copper bead and a perforated bone pendant (Ens 1999:5)). Although the individual has not been carbon dated, the artifact styles suggest that the burial is 2-3,000 yrs B.P. In addition, the Two Eagles individual has shovel shaped incisors and cranial measurements that are consistent with individuals of Aboriginal descent (Ens 1999). Based on the morphometric data, this individual was identified as male (Ens 1999).

6.3.1.3 Whaley Cairn Site (EbKx-10)

Eleven individuals were recovered from the two features within the Whaley Cairn burial; three of which are included in this study (ZW-61; ZW-65; ZW-68). Two individuals (Whaley Cairn ZW-68 and Whaley Cairn ZW-62) from the two different features from the site (EbKx-10), in southeastern Manitoba, had identical C^{14} dates of 1760± 60 yrs B.P. (Morlan 1999). The only directly dated individual from this burial feature that was included in this study was Whaley Cairn ZW-68. This individual was a 40-50 year old female who had considerable evidence of periosteal reactive bone on the anterior surfaces of both tibiae. Both fibulae showed considerable bone remodeling (Garlie 2001). This individual had bilateral periostitis on the ulnae and radii in addition to slight porosity on the frontal and parietal bones. This is the only individual in the Whaley Site population that might have some naso-palatal degeneration, a diagnostic feature of tremponema. These pathologies and their bilateral distribution on this individual are indicative of treponema.

MSC00016-03 (Whaley Cairn ZW-65) was recovered from the same burial feature as MSC00015-03 (Whaley Cairn ZW-68) and therefore these individuals are considered to be contemporaneous (Hill 1998b). The MSC00016-03 (Whaley Cairn ZW-65) individual was sub-adult at the time of death and had some bilaterally distributed pathologies that are suggestive of treponema (Mieklejohn et al. nd). The reactive bone on the left and right tibiae, radii and ulnae may be related to a generalized disease condition. A diagnosis of treponema is supported by occurrence of the porous bone on the supraobital tori. A complicating factor for diagnosing treponema is that the reactive bone

may in fact be remodeling due to bone growth particularly at the diaphyses of the long bones. However, the patches of reactive bone are located consistently on the midshaft areas of the long bones and on the anterior surfaces of the tibiae where the patches are extensive. This distribution of reactive bone is suggestive of a pathology rather than normal bone growth.

The third individual (MSC00018-03) from this site comes from a separate excavation unit and feature that was dated using a sample from another individual. Whaley Cairn ZW- 61(MSC00018-03)'s association with the dated individual and the conditions of the skeletal remains suggest that all of the burials from the site were relatively contemporaneous (Hill 1998b; Mieklejohn et al. nd). Whaley Cairn Site (MSC00018-03) was a 40-50 year old female that has some of the classic characteristics of treponemal infection including the bilateral sabre shin, thickened clavicles and bowed fibula, radii and ulna (Garlie 2001). The change in conformation of both the right and left tibiae is indicative of treponema. The right tibia is missing from the collection at present, but the slide taken of the element clearly shows that it is bowed in the manner referred to as sabre shin. The left fibula is bowed and has only very slight periosteal remodelling. Additionally, the bilateral distribution of periostitic reactive bone on the tibiae, fibula and the clavicles supports a diagnosis of treponema. Both the left and right radii are twisted. The involvement of both the hands and feet in degeneration may be indicative of treponemal infection particularly because it is bilaterally distributed.

The style of burial, associated grave goods (rolled copper bead and pendant), early C^{14} dates and the morphological analysis of the individuals (Garlie 2001) support the contention that these individuals are Aboriginal.

6.3.1.4 Fidler Mound (EaLf-3)

Three isolated molar teeth (MSC00029-04, MSC00030-04 and MSC00031-04) were taken from the collection of human remains from the Fidler Mound. The teeth do not have an exact provenience or date within the mound feature and are simply described as recoveries from the fill. Dates from eight individuals from the mound suggest that the site was used from as early as 1570 BP ± 40 BP to 540 ± 40 BP (Hewitt 2004).

6.3.2 Interlake region of Manitoba

6.3.2.1 Eriksdale 110-1(EfLl-1)

Other individuals (110-1 and 110-2) included in this study that were directly carbon dated are those from the Eriksdale Site (EfLl-1) in the Interlake region. The human remains from Eriksdale (EfLl-1) were recovered from a location where gravel was being extracted for road construction. The C^{14} date from Eriksdale 110-1 (MSC0003-03) was 3,470 ± 40 years BP with a calibrated age of BP 3,840 to BP 3,640 (Allingham et al. nd). Eriksdale 110-2 (MSC0005-03), however, returned a conventional age of 3,570 ± 60 years BP with a calibrated age of BP 4,230 to BP 3,870 (Allingham et al. nd). Syms (1983) noted that these C^{14} dates are somewhat older than expected because Eriksdale 110-1 has a small Pelican Lake point embedded in the femur. This "barbed" style of point which has been associated with more recent dates, can have some antiquity and has been found to have a wider more northern distribution than previously thought (Epp and Dyck 1997; Larcombe 2000). Although the site was disturbed, and number of artifacts were recovered and are believed to be grave goods belonging to the individuals. MSC0003-03 Eriksdale 110-1 had green staining on the skeletal elements and it is likely therefore that the individual was buried with copper artifacts that have since eroded away. Ten modified bone tubes were recovered that may have been part of a breast plate and over 300 shell beads were found at the site (Allingham et al. nd).

The early precontact dates clearly indicate that these individuals are of Aboriginal ancestry. Both were considered to be male based on morphological assessment (Allingham et al. nd).

6.3.3 Northern Manitoba region

6.3.3.1 Pukatawakan Bay (Hglr-3)

The Pukatawakan Bay Site (HgLr-3) is located on Southern Indian Lake on a small island in Pukatawakan Bay (Brownlee 2001:8). The Pukatawakan Bay (HgLr-3) burial was dated using dendrochronological analysis of the coffin boards of one of the two individuals recovered from the site (Brownlee 2001). Obviously, the presence of coffins boards indicates that the burials were historic. However, a more accurate date was

obtained by comparing the growth rings in the wood of the coffin boards to tree cores from the area. A colder than normal year in 1817 resulted in a distinctive set of growth rings that were present in the coffin board (Brownlee 2001). Based on a comparative analysis, it was determined that the tree used for the coffin boards was cut in 1878 and it is likely the individual in the coffin was buried that same year (Brownlee 2001:29)). Pukatawakan Bay ZW-124 (MSC00017-03) was found next to this dated individual and it is believed that they were roughly contemporary (Mieklejohn 2002b).

The skeletal remains are largely complete and have a uniform dark chocolate brown colour. This individual had pathologies on the ribs and spine that are consistent with tuberculosis. Resorptive lesions on the anterior surfaces of the vertebral bodies, pitting on the vertebral articular facets, and new bone formation on the internal surfaces of the right ribs are indicative of chronic transpleural inflammation - quite possibly tuberculosis (Mieklejohn 2002b). Mieklejohn's (2002b) morphological analysis of the remains indicate that Pukatawakan Bay ZW-124 (MSC00017-03) was likely of Aboriginal descent and was male.

6.3.4 *West central region of Manitoba*

The four individuals from the west central region of Manitoba include MSC00013-03 Ochre Beach (EgLw-5), MSC00014-03 Drifting River (EhMc 18), MSC0006-03 Red Deer River (FfMj-1) and MSC00011-03 Dauphin Historic Site (Hunter's Brother Site) (EhLx-8). All of these individuals are believed to be roughly contemporaneous, dating to the late 18th or early 19th centuries (Brownlee et al. 2003). Aboriginal ancestry was assigned to each of theses individuals by the archaeologists, based on the evaluation of the cultural material remains, which were indicative of Ojibwa-Saulteaux affiliation (Brownlee et al. 2003). In addition, the double shoveling of the incisors in the individuals and/or other morphometric data (i.e. cranial metrics), support an Aboriginal designation (Mieklejohn 2001; Mieklejohn 2002a). The marked shoveling of the upper central incisors is an effective diagnostic marker of Aboriginal ancestry. The three historic individuals (apart from Ochre Beach - a sub-adult individual) were determined to be male from an assessment of the skeletal morphology (Brownlee et al. 2003). The Red Deer River MSC00006-03 remains were recovered in a partially

mummified and partially skeletonized condition. The skeletal remains had some copper staining that might affect the recovery of DNA.

6.3.4.1 Ochre Beach (EgLw-5)

The skeletal remains of MSC00013-03 Ochre Beach (EgLw-5) were largely complete except for the terminal portions of both hands and feet, and one vertebra, which were missing. The skeleton is a dark brown to ochre brown colour and this colour, has penetrated the porous bone. This individual and MSC00014-03 Drifting River (EhMc-18) (see below) have skeletal pathologies that may have been genetic in origin. The fusion of the C2 and C3 vertebrae at the tip of the spinous process may be indicative of the Kippel-Feil syndrome (Mieklejohn 2001). The non-symmetrial shape of the manubrium and the other developmental defects on the spine of this individual may be related to the syndrome although there is no clear evidence for this. The morphologic features of the pelvis, humerus and dental eruption, indicated the age of this individual to be between 10 and 11 years of age at the time of death (Mieklejohn 2001). Because of the young age of this individual, biological sex assessment could not be determined from morphological features. The lower, left first molar tooth and the right calcaneous were removed from the skeletal material for DNA analysis. The complete pulverization of the molar tooth yielded 1.78g of powdered material

6.3.4.2 Drifting River (EhMc-18)

Drifting River ZW-118 (EhMc-18) had a uniform light ochre brown colour throughout that likely reflects the burial environment. This individual was mostly complete. The morphological features and metrics of the pelvis indicate that this individual was male. Using multiple markers on the pelvis, ribs, cranium, dental and the general skeleton, Meiklejohn determined that the individual was ± 30 years of age (2002a). The left upper M2 was taken from the skeletal remains for DNA analysis (MSC00014-03). A sample of 2.3g of powder was recovered from the molar tooth and used for mitochrondrial and nuclear DNA analysis.

MSC00014-03 Drifting River (EhMc-18) was noted to be "highly pathological" primarily in the postcranial skeleton (Mieklejohn 2002a). The spine shows obvious curvature in the cervical, thoracic and lumbar regions and abnormal fusion in the sacrum.

The feet have abnormalities that may have a genetic basis. The left proximal phalanges of the hand are shorter than those on the right, which is a pattern also found on the feet. The left humerus is also clearly shorter than the right but the diameter of the shaft is broader which might indicate heavier use of the right side. The cause of death of this individual could not be determined, although it is clear that he had major pathological features, some genetic in origin that may have contributed to poor health status. The presence of cribra orbitalia, which is diagnostic of anaemia and slight enamel hypoplasia are also indicative of non-specific stresses throughout this individual's life.

6.3.4.3 Dauphin Historic Site (Hunter's Brother Site)(EhLx-8)

MSC00011-03 Dauphin Historic Site (Hunter's Brother Site) (EhLx-8) was recovered in a partially mummified, partially skeletonized condition. The skeletal remains had some copper staining and because the copper might interfere with the recovery of DNA, only elements without staining were considered for DNA analysis; namely, the left lower first molar. The weight of the pulverized molar tooth was approximately 2g.

6.3.4.4 Red Deer River (FfMj-1)

This individual from Red Deer River (FfMj-1)(MSC0006-03) was recovered in a partially mummified, partially skeletonized condition. The skeletal remains also had some copper staining, and again, only elements without copper-staining were considered for DNA analysis. Indication on the ribs and spine of this individual are suggestive of tuberculosis. The left 4^{th} rib and samples of hair and mummified tissue were collected for DNA analysis. The rib was chosen so that pathogen analysis could be conducted, in addition to mitochondrial and nuclear DNA analysis.

6.3.4.5 Valley River (EiMg-3)

The two individuals (ZW-205 and ZW-206) from Valley River (EiMg-3) were recovered from a single grave feature in a gravel pit. No diagnostic grave goods were found, but the MSC00011-03 Valley River ZW-206 had three partial moose hyoid bones in close association to the skeleton. The bones show considerable polish that is consistent with considerable handling (Mieklejohn 2004). MSC0009-03 Valley River ZW-205 was morphologically determined to have been a female of Aboriginal ancestry. The highly

worn teeth and the possible presence of red ochre on the remains suggest a pre-contact date. Pathology of the spine is similar in pattern to that found in the historic individuals from Drifting River (EhMc-18 ZW-118), Ochre Beach (EgLw-5 ZW-134) and in Pukatawakan Bay (HgLr-3 ZW-124). The second individual MSC00011-03 Valley River ZW-206, is a child approximately 7 to 8 years old. The sex could not be determined. The morphological analysis of the child and adult skeletal remains, the presence of moose bones and the location of the burial all indicate that the individuals were Aboriginal ethnicity.

Table 6 summarizes the samples collected, their known or approximate dates, the element collected from each individual, and the morphologically determined sex, if available.

6.4 Sample preparation

A number of techniques were used to retrieve DNA from the available bone or tooth elements. The trabecular bone was collected in a bone preparation hood. The rib of MSC0006-03 Red Deer River (FfMj-1) was broken to expose the spongy interior bone. The trabecular bone was scraped or chipped, out from the central portion of the rib and placed in an autoclaved and cross-linked mortar. The small erosions on the talus of MSC0009-03 - Valley River ZW-205 (EgMi-3) and calcaneous of MSC0004-03 - River Mouth ZW-44 (EcKx-37) were probed to remove trabecular material from beneath the cortex of the bone. The trabecular bone was then scrapped or chipped out into a sterilized mortar. Once sufficient material (approximately 0.1g – 0.3g from each extraction) was collected, it was ground to a fine powder using a pestle. The powder was placed in a sterilized Eppendorf® collection tube that was sealed and labelled.

Table 6. Samples collected for analysis

	Accession # PaleoDNA lab	Sample	Date	Morphologic Sex	Tissue Type
1	MSC0003-03	Eriksdale 110-1 (EfLl-1)	3470± 40 yrs BP	Male	Right lower 1st molar
2	MSC0003-03	Eriksdale 110-1 (EfLl-1)	3470± 40 yrs BP		Left lower 3rd molar
3	MSC0004-03	River Mouth ZW-44 (EcKx-37)	Ca. 500 yrs BP	Female	Left calcaneous
4	MSC0005-03	Eriksdale 110-2 (EfLl-1)	3570± 60 yrs BP	Male	Tooth
5	MSC0006-03	Red Deer River (FfMj-1)	ca 200 BP	Male	Left 4th rib
6	MSC0007-03	River Mouth ZW-44 (EcKx-37)	300 yrs BP		Right lower 2nd molar
7	MSC0008-03	River Mouth ZW-45 (EcKx-37)	300 yrs BP	Male	Right talus
8	MSC0009-03	Valley River ZW-205 (EgMi-3)	Pre-contact	Female	Right lower 2nd molar
9	MSC0009-03	Valley River ZW-205 (EgMi-3)	Pre-contact		Left talus
10	MSC00011-03	Dauphin Historic (EhLx-8) (Hunter's Brother Site)	Ca. 200 yrs BP	Male	Left lower 1st molar
11	MSC00012-03	Valley River ZW-206 (EgMi-3)	Pre-contact	undetermined	Right lower 2nd molar
12	MSC00012-03	Valley River ZW-206 (EgMi-3)	Pre-contact		Left lower 2nd molar
13	MSC00013-03	Ochre Beach (ZW-134) (EgLw-5)	Ca. 200 yrs BP	undetermined	Left lower 2nd molar
14	MSC00014-03	Drifting River (ZW-118) (EhMc-18)	Ca. 200 yrs BP	Male	Left upper 2nd molar
15	MSC00015-03	Whaley Cairn ZW-68 (EbKx-10)	1760± 60 yrs BP	undetermined	Right lower 2nd molar
16	MSC00016-03	Whaley Cairn ZW-65 (EbKx-10)	1760± 60 yrs BP	Female	Right lower 1st molar
17	MSC00017-03	Pukatawakan Bay (HgLr-3) (ZW-124)	1878 A.D.	Male	Molar
18	MSC00018-03	Whaley Cairn ZW-61 (EbKx-10)	1840 yrs BP	Female	Right clavicle
19	MSC00039-03	Two Eagles (EcKw-14)	ca 2-3000 yrs BP	Male	Left lower 3rd Molar
20	MSC00029-04	Fidler Mound (EaLf-3)	Late Precontact	Undetermined	Isolated molar
21	MSC00030-04	Fidler Mound (EaLf-3)	Late Precontact	Undetermined	Isolated molar
22	MSC00031-04	Fidler Mound (EaLf-3)	Late Precontact	Undetermined	Isolated molar

The molar teeth were prepared by cleaning the exterior surfaces with 5% sodium hypochlorite then by UV irradiating the surfaces for 12 hours to degrade any surface contaminating modern DNA. During the irradiation process, the teeth were cleaned with sodium hypochlorite several times to ensure that the tooth surfaces were damp because UV irradiation is not effective on dry surfaces (Hummel 2003). Each molar tooth was then powdered in its own autoclaved and cross-linked mixer mill (25 ml stainless steel mortar and 20 mm ball) that crushed the entire tooth into a fine powder in a sterile environment. The entire tooth was used in order to obtain a maximum amount of sample. The powder was carefully divided into 0.1g -0.3g aliquots and placed into sterile Eppendorf® collection tubes.

Another technique for the collection of sample from molar teeth effectively removed the dentine from within the tooth cavity but left the enamel of the tooth intact. A small hole was drilled into the cementum of the molar root of MSC0007-03 River Mouth ZW-44 using a Dremmel Tool®, and the enclosed dentine was powdered, removed and placed in a sealed sterilized Eppendorf tube. The advantage of this method was that the tooth remained largely intact, except for a small hole. The element remains available for other types of analysis or for repatriation. However, this process resulted in a smaller amount of processed sample and thereby severally limited the number of tests that could be conducted on this individual.

The powdered bone and tooth aliquots were stored in their sealed Eppendorf tubes at room temperature in the extraction area of the laboratory.

6.5 Extraction and purification procedures

DNA was extracted and purified using a modified guanadinium thiocyanate (GuSCN) and silica-based DNA binding method as described by Boom et al. (1990) and Höss and Pääbo (1990; Höss and Pääbo 1994; Pääbo 1985). A 0.1-0.3g sample of either tooth or bone was incubated at 56°C with 500 – 700 ul of GuSCN for at least 8 hours. The mixture was boiled at 94°C for ten minutes and centrifuged at 14000 rpms for 1 minute to pellet the tissue powder. The supernatant was removed and pipetted into a clean Eppendorf® tube. The residual bone powder was stored in a –80C° freezer. The supernatant was transferred to a purification hood where 10μ of silica beads were pipetted

into the tube. The mixture was carefully stirred and placed on ice for one hour. The silica particles were washed with wash buffer and then with ethanol. The DNA/silica bead suspension was air dried and then re-suspended using 150-µl ddH_20 and incubating the silica bead and ddH_20 at 56°C for 1 hour. Two aliquots of 75-µl of the suspension were placed onto BioRad® P-30 spin columns and collected into a sterile 1000ml Eppendorf tubes. A negative control containing all reagents but no sample was subjected to every extraction and purification process including PCR amplification and the final fragment analysis.

6.5.1 Amplification procedures

All PCR reactions were set-up in a clean lab, designed specifically for ancient DNA, and is separate from the extraction and purification rooms. Each PCR reaction was set up in a 2.0ml thin-walled reaction tube in a dedicated positive airflow hood and then transferred to another positive airflow hood where the DNA template was added. The tubes were moved through a UV irradiated "pass-thru" to the post-PCR area and the thermocycler room.

6.5.2 Mitochondrial DNA amplification

To validate the ancient DNA extraction method, a 191 base-pair (bp) region of HVR I and II (16210 – 16401), of the mtDNA in each of the samples was amplified. A forward primer of 5'- CCC ATG CTT ACA AGC AAG TA –3' and a reverse primer of 5 –TGA TTT CAC GGA GGA TGG TG-3' were used for the amplification of this mtDNA region (Kolman and Tuross 2000; Vigilant et al. 1989). Each reaction contained 20mM Tris-HCl (pH 8.0), 50mM KCl (PCR Buffer supplied), 0.2 mM dNTP mix, 1.5mM $MgCl_2$, 5mM of each of the forward and reverse primers and 1-U Platinum Taq. In each reaction, 10.0µl of DNA template was added and ddH_20 was used to bring the reaction volume to 50 µl. The following cycling conditions were used for the initial PCRs: 94°C for 60 seconds; 60°C for 30 seconds and 72°C for 60 seconds for 45 cycles. A PCR negative control which contained all reagents but no DNA template was carried through to the final fragment analysis. The amplified target mtDNA regions were electrophoresed on polyacrylamide gels to visualize the PCR product and the successfully amplified product was sequenced using the Big Dye Terminator Sequencing Kit®.

6.5.3 Cytokine promoter region amplification (pre-SNaPshot amplification)

The use of the SNaPshot method requires the amplification of a target region surrounding the SNP of interest. Turner et al. (2002) used primers described previously for the amplification of target cytokine SNPs, although some were designed based on observation of the published sequences (Perrey et al. 1998). The primers used in Turner et al.'s (2002) research typically amplified a region of approximately 1000 bp in length. Due to the fragmented nature of aDNA, the successful amplification requires primers that are designed to amplify a region of not more than 300 bp (Hummel 2003). Two different methods were used to amplify the cytokine promoter target regions in order to test various methods – booster PCR and nested primers.

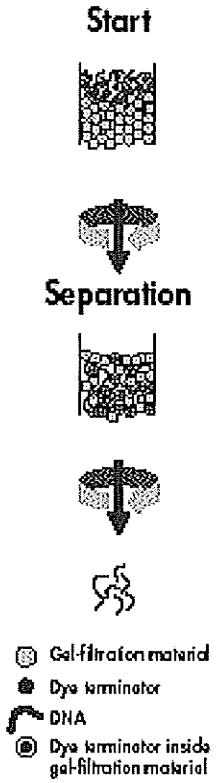
Nested primers were designed to target a 200 bp region around the (–308) region of interest in order to increase the specificity of the product. An initial reaction used a forward primer 5'-GCC CCT CCC AGT TCT AG -3' and a reverse primer 5'-CAC ACT CCC CAT CCT CC-3' to amplify a 200bp region of the TNFα (-308) promoter region. Each reaction contained, 20mM Tris-HCl (pH 8.0), 50mM KCl (PCR Buffer supplied), 0.2 mM dNTP mix, 1.5mM $MgCl_2$, 5mM of each of the forward and reverse primers and 1-U Platinum Taq. In each reaction 10.0µl of DNA template was added and ddH_20 was used to bring the reaction volume to 50 µl. The following cycling conditions were used for the initial PCRs: 45 cycles of 94°C for 60 seconds; 60°C for 30 seconds and 72°C for 60 seconds. A second reaction targeting a 100 bp region of the TNFα (-308) promoter region used the PCR product of the initial reaction in place of DNA template and a forward primer of 5'-CCA CAG ACC TGG TCC C -3' and a reverse primer of 5'-CAC TGA CTG ATT TGT GTG T-3'. All other reagents and cycling parameters remained the same. The primers were tested on the modern DNA control samples in a separate laboratory, and sequences were obtained to demonstrate the specificity of the primers.

The "AT' rich region which is located just down from the Interleukin-6 (-174) promoter region, made it difficult to design nesting primers that would be effective in amplifying the short fragments of DNA material. Therefore, a single initial PCR reaction used primers designed to amplify a 235 bp region of IL-6 (-174) on chromosome 7 using polymerase chain reactions (Forward 5'-CTT CGT GCA TGA CTT CAG C-3'; Reverse

5'-GTT GGA GGG TGA GGG TG-3'). Likewise, the GC rich Interleukin-10 (-819) promoter region seriously limited the use of nested primers. A single initial PCR reaction was therefore designed to amplify a 179 bp region on chromosome 1 surrounding the IL-10 (-819) polymorphic site (Forward 5' – GGA GAT GGT GTA CAG TAG GG –3'; Reverse 5'- GAG TAG TCT GCA CTT GCT G –3'). After the primers were validated on modern control samples, PCR reactions using DNA extracted from tooth and bone were conducted using the same PCR reagents and cycling parameters as described for TNFα above. A PCR negative control that contained all reagents but no DNA template was carried through to the final fragment analysis. Instead of a second PCR using nested primers, fresh Taq was added to each PCR product of the IL-6 reactions and an additional round of cycling occurred. As described by others (Ruano et al. 1989), a booster PCR was preformed by adding 0.2-µl Platinum Taq to the initial PCR product and performing an additional 20-cycle regime of 94°C for 60 seconds; 60°C for 30 seconds and 72°C for 60 seconds.

6.5.4 *PreSNaPshot purification*

After the booster or nested amplification, 6.0 µl of PCR product was incubated at 37°C for 60 minutes with 5-U shrimp alkaline phosphatase (SAP) and 2-U Exonuclease I (Exo1). This was followed by a 15-minute incubation at 75°C to inactivate the enzymes. To remove the excess primer and unincorporated dNTPs, the PCR product was further purified using a DyeEx® spin column (Figure 21). This column uses a gel filtration system that separates molecules based on molecular weight which traps the dye terminators used in the PCR reaction but lets the DNA pass through the filters.



DyeEx separation principle.

Figure 21. DyeEx 2.0 Spin Kit Product Insert

6.5.5 SNapShot reaction

Following purification, 4.0 µl of digested PCR product was mixed with 5µl SNaPshot (Applied Biosystems, CA) ready reaction premix, 0.4 µl of 5.0 mM the appropriate extension primer (see Table 7) as previously tested and 0.6µl ddH20. Two extension primers were designed for IL-6, but, after both primers were tested, the shorter 20 bp primer was used. The reaction preparation was done keeping all primers, PCR product, and ready reaction mix on ice. The standard SNaPshot method was modified for

Table 7. Extension primers

Tumor Necrosis Factor-α (-308) (29 bp)
5' - AAT GGA GGC AAT AGG TTT TGA GGG GCA TG –3'
Interleukin-6 (-174)
5'- TTC CCC CTA GTT GTG TCT TGC-3' (21 bp)
5' – AGC TGC ACT TTT CCC CCT AGT TGT GTC TTG C – 3' (30 bp)
Interleukin-10 (-819) (20 bp)
5' – GCA AAC TGA GGC ACA GAG AT –3'

amplifying ancient samples by increasing the volume of PCR product to take into account the potentially low copy number of DNA in the samples.

The SNaPshot reaction was amplified using 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 30 seconds. Upon completion, of the amplification, 0.5-U SAP was added and the reaction mixture was again incubated for 60 minutes at 35°C and 15 minutes at 75°C. The SNaPshot PCR product was put through a DyeEx® spin column to again remove any unincorporated ddNTPs. Before loading onto the ABI Prism 3100, 9.5µl formamide was added to 0.5µl of reaction mixture, and samples were heated to 95°C for 5 minutes. The fragments were analysed using ABI Prism Genescan® version 3.7 software.

Chapter 7 Results of SNP detection in ancient human remains

7.1 Mitochondrial DNA analysis

The Hyper variable Region I (HVRI) (16210-16401) of the D-loop of the mitochondrial DNA was amplified in fourteen samples. The purpose of sequencing this region was threefold. The mtDNA HVRI was amplified to ensure that the sample was not inhibited and would yield DNA. A comparison of the ancient sequences against both the reference sequence and the researcher's sequence could verify that they were unique and therefore could be considered authentic. The identification of polymorphisms that are markers for the Native North American haplogroups could also verify that the samples are not contaminated by the researcher and that they are Aboriginal. Chart 1 in the Appendix lists all of the sequences for each of the samples. All of the sequence information is provided for the purposes of documenting the information and to demonstrate some of the inherent problems with mtDNA sequence results. The variations (polymorphisms) from the Cambridge reference sequence (Anderson et al. 1981) are highlighted. Chart 2 in the Appendix summarizes the polymorphisms that were identified in each of the fourteen successfully amplified samples. The Native North American haplogroup with which the polymorphisms are associated are also shown. Figure 22 illustrates the relative frequencies of each of the identified haplogroups.

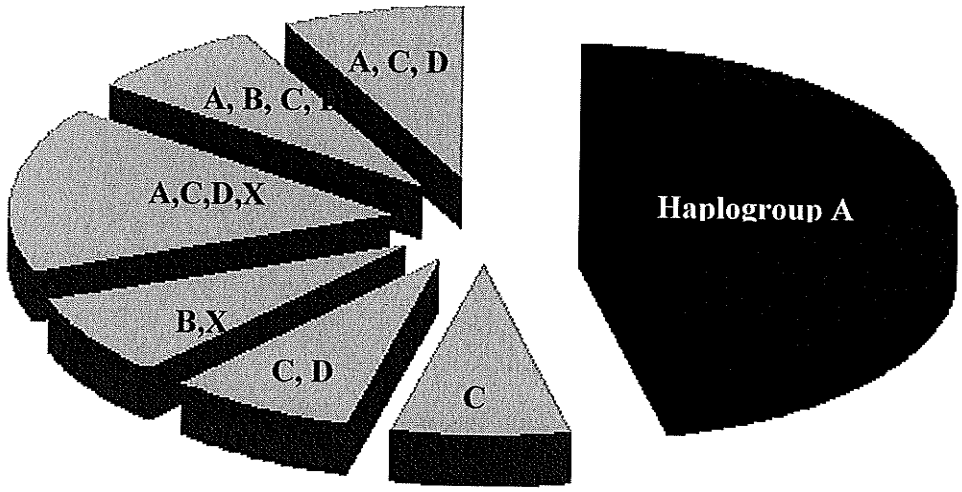


Figure 22. Frequency of Native North American haplogroups in Manitoba

It is an important first point to that note that Larcombe, the primary researcher, has four polymorphisms (np16224, np16270, np16297, and np16311). None of these polymorphisms were found in any of the ancient individual's sequences. A fifth polymorphism at np16189 however, was found in one of the ancient individuals' sequences (MSC0008-03 River Mouth (ZW-45)). This polymorphism is associated with haplogroup B and X. While haplogroup B is uniquely Aboriginal, the markers for haplogroup X have been found in both European and Aboriginal populations (Brown et al. 1998; Malhi et al. 2004). It is possible therefore that both the researcher and the ancient sample possessed this marker. Because MSC0009-03 River Mouth (ZW-45) has three other polymorphisms that are not present in my sequence, it is unlikely that the presence of the polymorphism at np16189 in the ancient individual was due to contamination.

The mitochondrial sequences of the two individuals from the Eriksdale site were analyzed to detect haplogroup markers and to assess whether or not they might be related. The C-T transition at np16223 is a polymorphism found in haplogroups A, C, D and X

and is marker for Aboriginal decent therefore, a specific haplogroup cannot be assigned to MSC0003-03 (Eriksdale 110-1). MSC0005-03 (Eriksdale 110-2) has one additional C-T transition; at np16290 which is a polymorphism found only in haplogroup A. Two more polymorphisms found in the HVRI region of MSC0003-03 (Eriksdale 110-1) at np1630 and np16391 indicate that although these two individuals were recovered from the same site they were not maternally related. The presence of three differences in the genetic code in a relatively small mitochondrial region suggests that there would likely be many more differences if one sequenced and compared a longer segment or the entire mitochondrial D-loop of these individuals. If the two had been related maternally, it would be expected that the genetic sequences of the sequenced regions would have been identical.

The four historic individuals from the Interlake region (Red Deer River MSC0006-03, Dauphin Historic MSC00011-03, Ochre Beach MSC00013-03 and Drifting River MSC00014-03) have some interesting similarities in their mtDNA sequences. Drifting River and Ochre Beach have the same three polymorphisms at np16290, np16362 and np16319. The first two markers are found only in haplogroup A, while the latter is a polymorphism found in haplogroups A and B. Based on the similarities in this short segment of mtDNA of Drifting River and Ochre Beach, further testing may reveal that these individuals were maternally related. Red Deer River, Dauphin Historic and Ochre Beach have a C-T transition at np16362. A sequence was not amplified at this location for Drifting River. Three of the four historic individuals (Red Deer River, Ochre Beach and Drifting River) can be assigned to haplogroup A, while the fourth individual (Dauphin Historic), can only more generally be assigned to haplogroup A, C, D or X in the absence a more conclusive polymorphic marker. Further testing of the mtDNA HVRI would likely result in a more specific haplogroup assignment.

The HVRI region of Valley River (ZW-205) MSC0009-03 was sequenced six times throughout the course of analysis. The polymorphic markers for haplogroup C (np16298 and 16327) were present in three of four amplifications of that region. In addition, a C-T transition was present at np16223 that is found in haplogroups A, C, D and X. All but one sample, Valley River (ZW-206) MSC00012-03, have polymorphisms associated with one of the haplogroups A, B, C, D or X. Only a short 80 bp sequence was

obtained for this sample, although multiple attempts were made to amplify a longer sequence.

Discrepancies between sequences became evident when multiple extractions and sequences were obtained for a single individual. Table 8 illustrates three sequences, 20 bp in length that were amplified from the Valley River (ZW-205) MSC0009-03 sample. One sequence amplified a 'T' at np16292, while the other two sequences amplified a 'C". The double underlined 'T' at np16292 (C-T transition) is not an identified haplogroup marker but is a polymorphism that was also observed in the sequence from Dauphin Historic MSC00011-03. It is not likely that one sample contaminated the other because they were extracted and amplified separately. The 'C''s in two of the sequences at np16298 are haplogroup markers, but they were not reliably amplified in all three sequences.

Table 8. Three sequences from Valley River (ZW-205) MSC0009-03

Sequence 1)	16291	CCACCCTCAA	16321	CATTTACCGT
Sequence 2)		CTACCCTTAA		CATTTACCGT
Sequence 3)		CCACCCTCAA		CATTTATCGT

The Valley River (ZW-205) MSC0009-03 sample contrasts somewhat with the sequences obtained for Ochre Beach (ZW-134) MSC00013-03 The Ochre Beach sample was sequenced five times and nearly identical sequences were obtained in every test except for the occurrence of a A-C transversion at np16300 in one sequence (Table 9). This polymorphism was unique to this individual. Further, along the fragment at np16362 a A-G transition was detected in all five sequences including the reverse sequence.

Table 9. Four sequences from Ochre Beach (ZW-134) MSC00013-03

Sequence 1)	16281	ACAAACCTAT	CCACCCTTAA	CAGTACATAG	TACATAAAAC
Sequence 2)		ACAAACCTAT	CCACCCTTAA	CAGTACATAG	TACATAAAAC
Sequence 3)		ACAAACCTAT	CCACCCTTAA	CAGTACATAG	TACATAAAAC
Sequence 4)		ACAAACCTAT	CCACCCTTAC	CAGTACATAG	TACATAAAAC

It is possible that the Valley River (ZW-205) MSC0009-03 individual was heterozygous at the nucleotide positions that display differences. Obtaining reverse sequences for this same region might be useful to sort out these discrepancies.

The four individuals from the southeastern part of the province have potentially the most diversity in mtDNA haplogroup types. Three different individuals have markers from haplogroup B. Mitochondrial sequences were successfully amplified for only two of the three individuals from the Whaley Cairn Site. One unsuccessful attempt was made to amplify Whaley Cairn (ZW-61) MSC00018-03. No further attempts were made to amplify the small sample that remains from this individual. Whaley Cairn (ZW-65) MSC00016-03 was successfully amplified and has five different polymorphisms at np16223, np16294, np16298, np16325 and np16327. This individual also has an unusual C-G transversion at np16290. There is strong evidence that the individual belongs to either haplogroup C or D. Whaley Cairn (ZW-68) MSC00015-03 shares the polymorphism at np 16325 with Whaley Cairn (ZW-65) MSC00016-03 which is found in both haplogroups C and D (Malhi et al. 2003; Torroni et al. 1993a). Whaley Cairn (ZW-68) MSC00015-03 also has a C-T transition at np16319 and the C-T transition at np 16223. The former polymorphism is found in haplogroups A and B while the later is found in A, C, D and X. The number of differences in the short DNA segments that were amplified for these two individuals from the Whaley Cairn site indicates that they were probably not maternally related. Additional testing would be required to confirm this finding and to refine the haplogroup assignment.

The Rivermouth Site individuals were probably not maternally related given the fact that Rivermouth (ZW-45) MSC0008-03 has four polymorphims (at np16152, np16182, np16183 and 16189) that were not found in Rivermouth (ZW-44) MSC0007-03. The polymorphic markers at np16183 and np 16189 have been identified in haplogroups B and X (Malhi et al. 2004; Smith et al. 1999). Rivermouth (ZW-44) has a C-T transition at np16111 and a C-T transition at 16223. The former polymorphic site is identified with haplogroup B or X and the later with A, C, D or X (Malhi et al. 2004). Also from this same general geographic region is Two Eagles MSC00039-03. Native North American polymorphic markers were identified at np16290 (C-T transition) and

np16319 (G-A transition) and np16362 (T-C transition) (Malhi et al. 2004). It is likely therefore, that Two Eagles belongs to haplogroup A.

The single individual from northern Manitoba, Pukatawakan Bay (ZW-124) MSC00017-03, has the same three polymorphisms as Two Eagles MSC00039-03. The C-T transition at np16290, a G-A transition at 16319 and a T-C transition at np 16362 are all identified in haplogroup A. There are a number of polymorphisms that have not previously been identified in North American Aboriginal populations. The C-T transition at np16292 in Dauphin Historic and Valley River (ZW-205) is particularly interesting because it was detected in two individuals.

7.1.1 Sex Determination

The determination of sex through the amplification of target regions on the amelogenin gene was undertaken primarily to assess the recovery of nuclear DNA. In five out of the seven individuals tested, the genetic sex determination corroborated the findings made from the morphological analysis (Table 10). In two of the samples, the results of the two methods for sexing the individuals did not agree. Eriksdale (110-2) MSC0005-03 and River Mouth (ZW-45) MSC0008-03 were morphologically assessed as males but genetically determined to be females. Testing of four target regions on the Y chromosome ensures that the determination of an individual as male is reliable. A determination of female rests on the fact that females do not have a Y chromosome. It is possible therefore, that an individual could be incorrectly considered female (a false positive result) if the target regions of a Y chromosome were to amplify. The amelogenin gene, for example, is found on both the X and Y chromosome. Amplification of this gene would result in a fragment of 106 bp on the X chromosome and 112 bp on the Y chromosome (Hummel 2003). Two fragments would therefore be expected in a sample from a male individual (one fragment from each of the X and Y chromosomes). If the Y chromosome failed to amplify, the detection of only a single X chromosome would falsely indicate a female. The assignment of female to an individual is therefore less reliable than the assignment of male for these individuals.

Table 10. Comparison of the morphologically and genetically determined sex identification for seven individuals.

Sample	Morphologically determined sex	Genetically determined sex
MSC0003-03 (Eriksdale 110-1).	Male	Male
MSC0005-03 (Eriksdale 110-2)	Male	Female*
MSC0006-03 Red Deer River	Male	Male
MSC0004-03 Rivermouth ZW-44	Female	Female
MSC0007-03 Rivermouth ZW-44	Female	Female
MSC0008-03 Rivermouth ZW-45	Male	Female*
MSC0009-03 Valley River ZW-205	Female	Female
MSC0012-03 Valley River ZW-206	Undetermined (subadult)	Male
MSC0030-04 Fidler Mound	Undetermined	Male
MSC0031-04 Fidler Mound	Undetermined	Male

The fact that in five out of seven samples the nuclear amelogenin gene was successfully amplified indicated that the nuclear genome was accessible in these samples. Because the sample sizes from the individuals are small, additional testing was not conducted on the samples that were questionably determined to be female.

7.2 SNaPshot analysis

An important factor in the development of a new method for detecting SNPs in ancient material was the ability to reproduce the results. It is the standard protocol for authenticating results to replicate the findings from two separate extractions (temporally separate) and two PCR amplifications from each extraction (Hummel 2003). A careful strategy therefore had to be adopted so that the development and refinement of this novel method did not deplete the entire sample of tooth powder from any one individual. The quantity of sample from each individual was compromised by the frustrating fact that amplification of the DNA or the SNP did not always occur. Even minute changes in the quality of the multiple sample aliquots from one individual, or small changes in the extraction and purification procedures, or PCR set-up could result in the failure of the DNA or SNP to amplify. Consequently, every effort was made to replicate the method for SNP amplification and detection using multiple samples. A second SNP PCR was attempted to replicate these initial results if sufficient sample was available. A second extraction and SNP amplification was attempted only if the sample from that individual

was not depleted. Although all of the samples were donated specifically for the development of this technique, it was considered prudent to keep some sample in reserve until the technique is refined for more cytokine SNP loci or for the collection of other specific genetic data.

Chart 1 (Appendix A) lists the results of the detection of cytokines SNPs in fourteen samples. The extraction number includes, the number assigned to each extraction, the year and the aliquot number. The aliquot number indicates the number of tissue samples that were accessioned for each individual and then the number of samples that were retrieved from each tissue sample.

Eriksdale 110-1

MSC0003-03

Extraction 65-03 (2:5)

The label indicates the PaleoDNA Laboratory accession number (MSC0003) and year of accession (-03). For the Eriksdale 110-1 individual, two molar teeth were accessioned for this study (samples 1 and 2), and each of those teeth produced 4 or 5 aliquots of tooth powder. The aliquots of tooth powder were numbered 1:1, 1:2, 1:3 etc and 2:1, 2:2, 2:3 etc. This numbering system makes it easy to see in the Chart the replication of results from multiple aliquots from each individual. Eriksdale 110-1 MSC0003-03 was replicated five times with four different extractions, and, for one extraction, the results were replicated with two PCRs.

7.2.1 Interleukin-6 (-174)

Modern control samples that were homozygous and heterozygous for the IL-6 (-174) SNP were typed using the modified SNaPshot method as described above. The fluorescent peaks in the modern control samples represent "G/G", "G/C" or "C/C" alleles of IL-6 (–174) SNP. Figure 23 illustrates the electropherograms of the homozygous and heterozygous IL-6 (-174) SNPs. A size ladder was used with the modern control samples to accurately measure the fragment length, which, for IL-6 (-174) is 21 bp (Figure 24). A size standard was also run with a multiplex control positive

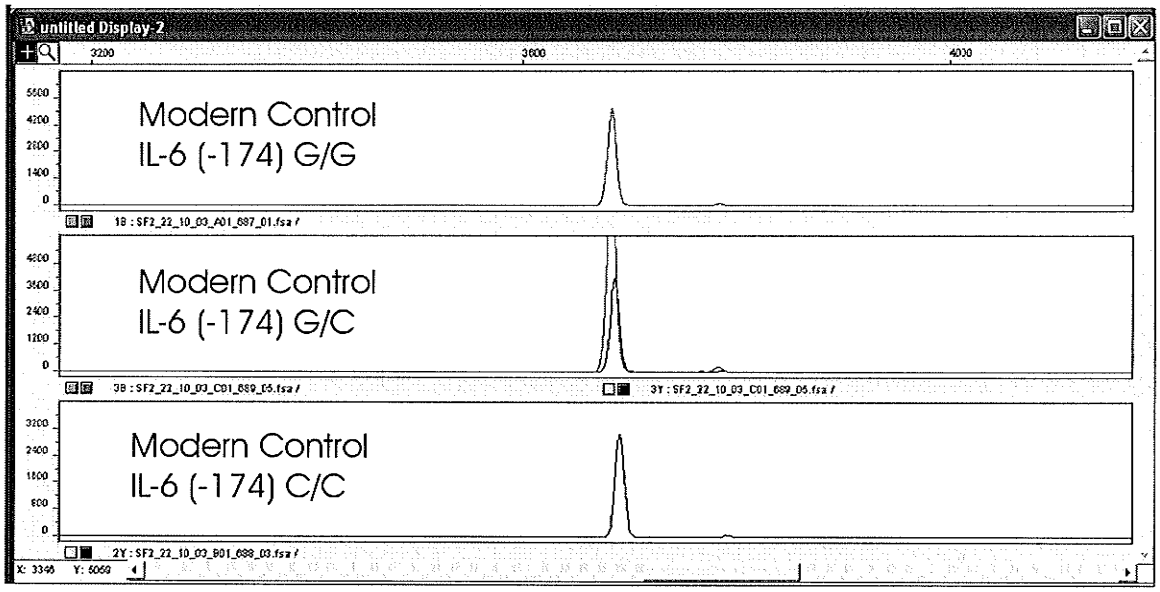


Figure 23. Homozygous and heterozygous interleukin-6 (-174) SNPS in modern control samples.

along with three ancient samples to compare fragment mobility and peak height (Figure 25). The multiplex positive control uses the SNaPshot reaction mix, control primers and control DNA, which result in pre-determined fragment lengths as shown in Figure 24. In the control multiplex reaction, the "A" amplifies at 24 bp, the "A/G" at 29 bp, the second "G" at 36 bp, the "T" at 46 bp, the "C/T" at 53 bp and the final "C" at 60 bp. Each of the control fragment lengths are detected as a slightly longer fragment than the actual length of the primer due to the influence of the dyes. Fragment shift or mobility is especially true for the shorter fragment lengths because there is a greater contribution of dye. In each of the ancient samples (Valley River (ZW-205) MSC0009-03, Dauphin Historic MSC00011-0-3 and Ochre Beach MSC00013-03), a "G" peak was detected between 24 and 29 bp. In subsequent tests, the "G" allele was consistently detected within this size range.

The SNaPshot PCR products that are illustrated in Figure 24 were run a second time with the GeneScan™ 120 Liz™ size standard (Figure 25). The size standard incorporates a molecular ladder that provides a standard against which the fragment lengths of the ancient and multiplex control samples can be aligned. In the negative

control in Figure 25 the only peaks are those of the 120 Liz™ size standard. These orange peaks correspond to different fragment lengths (15, 20, 25, 35, 50 62, 80, 110 and 120) as indicated below each peak in Figure 23. The peaks in the multiplex control positive are positioned appropriately in relation to the size standard thereby giving an accurate measure of the IL-6 (-174) extension primer product. The size standard was not used regularly with the ancient DNA samples because in many cases, the size standard dye obscured the ancient results with "pull ups" and "bleed through" result in multiple extraneous peaks as seen in the Ochre Beach sample in Figure 24.

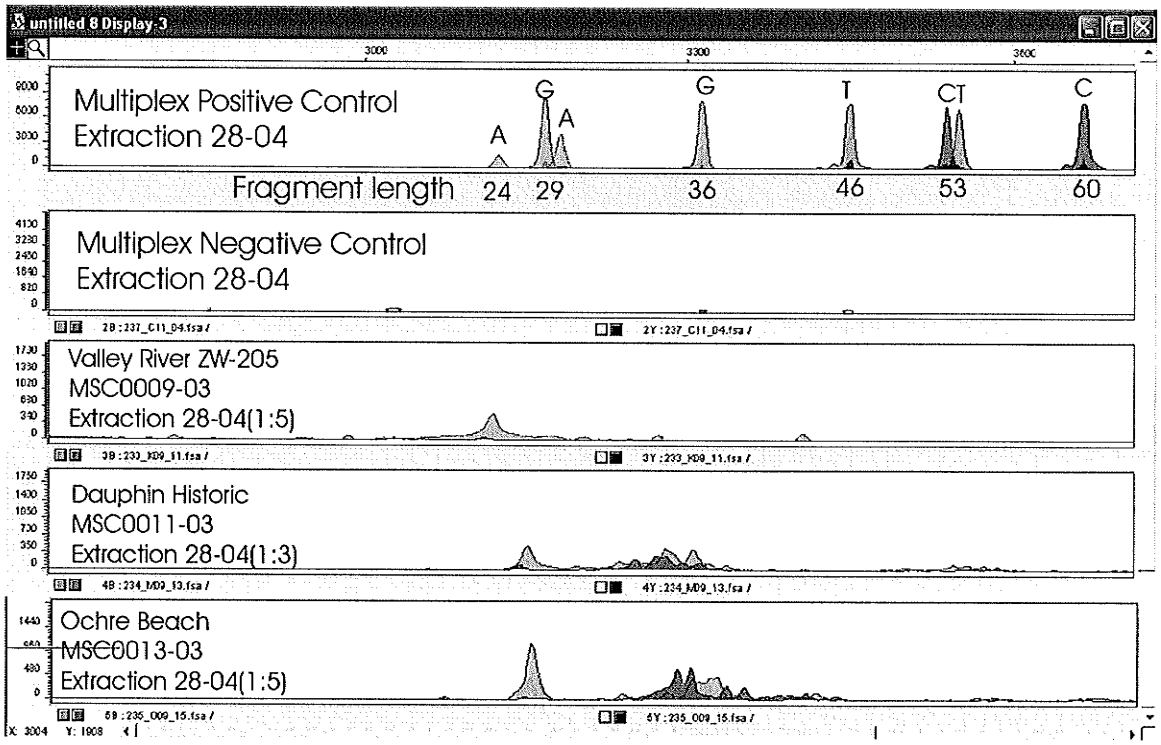


Figure 24. Multiplex positive control and three ancient samples.

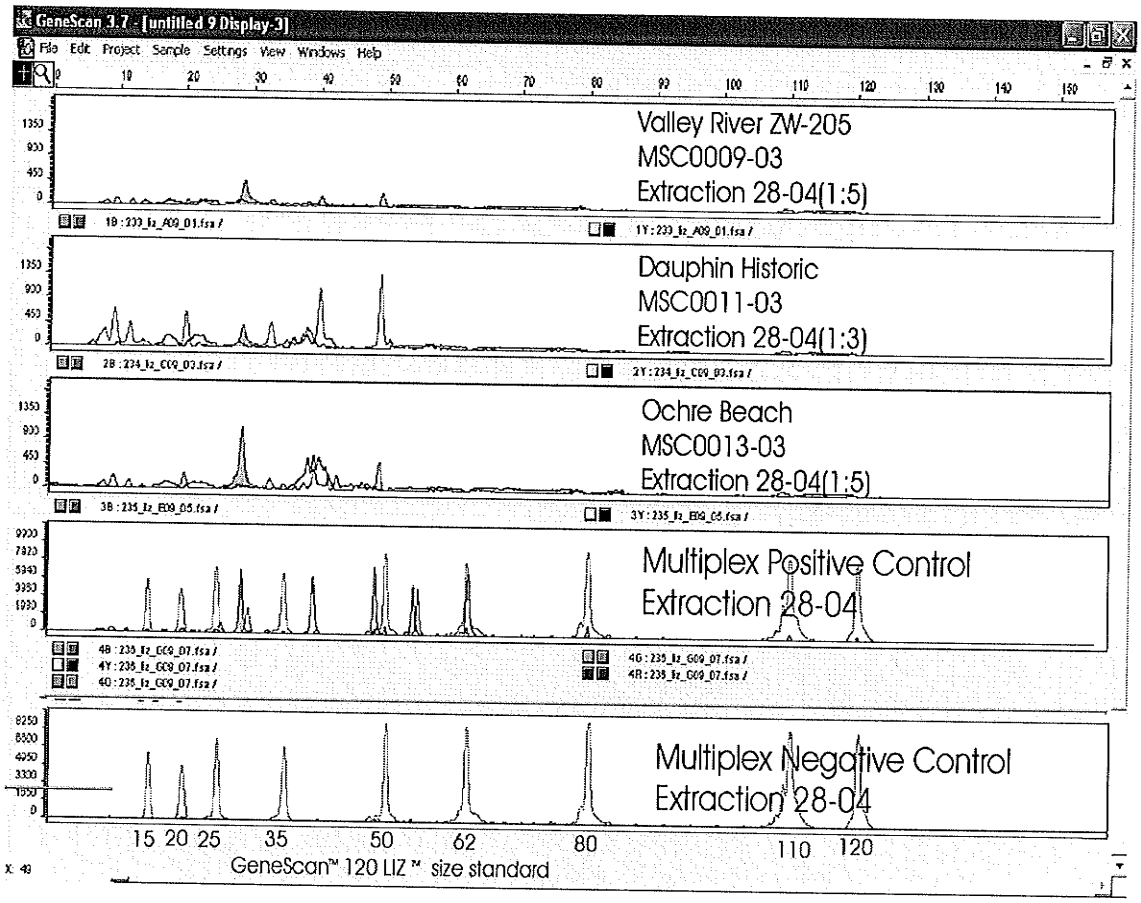


Figure 25. Multiplex positive control and three ancient samples analyzed with the Genescan™ 120 Liz™ size standard.

7.2.2 Typing homozygotes

Figure 26 illustrates the results of the amplification of the IL-6 (-174) SNP in four ancient samples (Eriksdale MSC0003-03 110-1; Whaley Cairn (ZW-68) MSC00015-03; Whaley Cairn (ZW-65) MSC00016-03 and Pukatawakan Bay (ZW-124) MSC00017-03). The three individuals (Eriksdale 110-1 MSC0003-03, Whaley Cairn (ZW-68) MSC00015-03 and Pukatawakan Bay (ZW-124) MSC00017-03), were typed as "G/G" because a single peak occurred at the expected fragment length position. A "G/G" genotype was consistently detected for Eriksdale 110-1 MSC0003-03. Three extractions yielded the same genotype, but the results could not be replicated beyond one PCR reaction

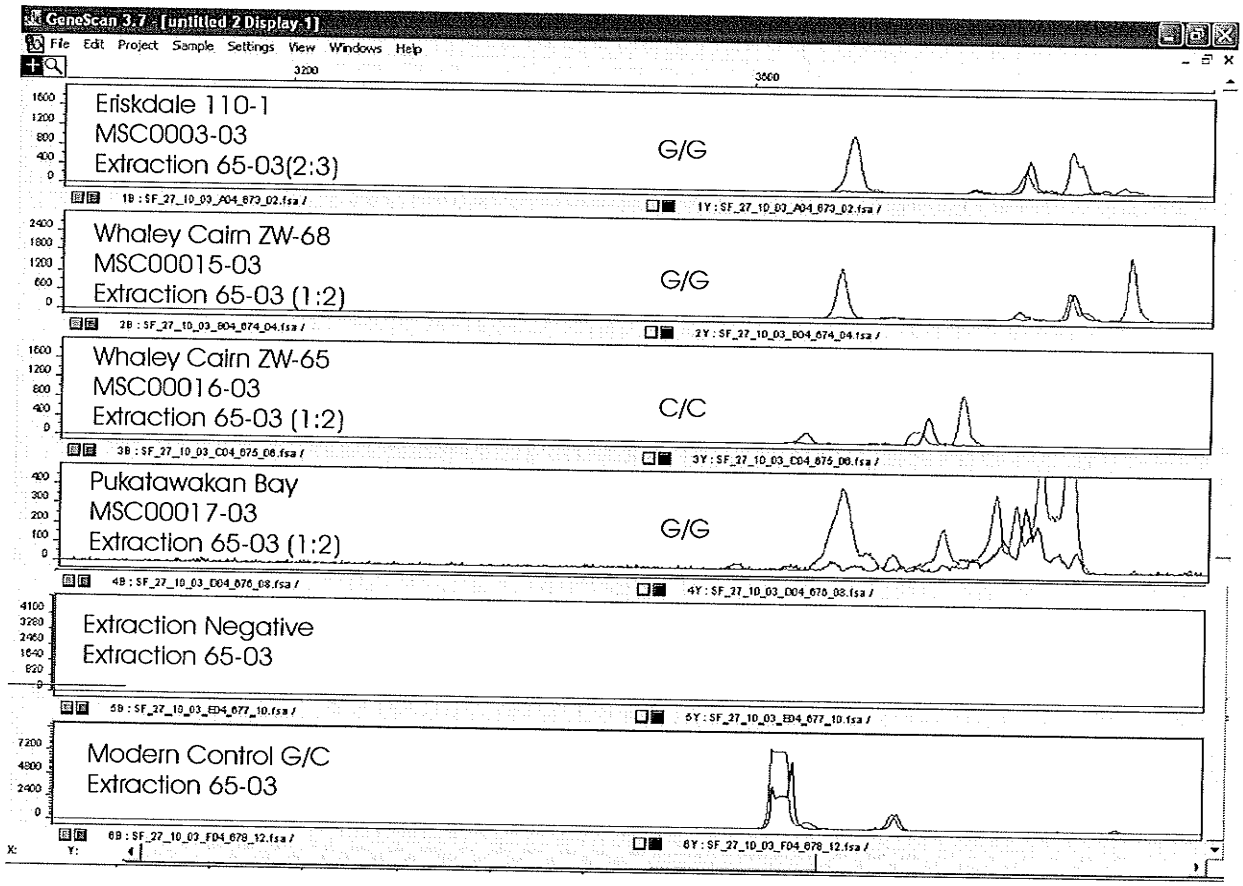


Figure 26. Interleukin-6 (-174) SNP's detected in ancient samples (Extraction 00065-03) and modern control.

for each extraction. Pukatawakan Bay (ZW-124) MSC00017-03 appears to be a "G/G" genotype as well. Whaley Cairn (ZW-65) MSC00016-03 was called a "C/C" even though the peak height for the "C/C" amplification was smaller than the other ancients in that run. However, the "C" peak for Whaley Cairn (ZW-65) MSC00016-03 measured 225, which makes the "C/C" a valid call. In a subsequent test, Whaley Cairn (ZW-65) MSC00016-03 amplified as a "G/G" (see Figure 26). Unfortunately, no additional amplifications were successful. It is reasonable to suspect that allelic dropout occurred in each of the two amplifications, one where the cytosine dropped out and one where the guanine dropped out. Allelic dropout arises when one nucleotide fails to amplify and is a phenomena that has been investigated in both ancient DNA analysis and the context of single gene research (Piyamongkol et al. 2003). In the case of ancient DNA, allelic dropout is likely due to degradation or fragmentation of the DNA (Hummel 2003; Miller

et al. 2002; Piyamongkol et al. 2003). If a homozygous genotype is detected, it is impossible to know whether or not the results are authentic or if they represent a false positive. Allelic dropout should always be considered with the detection of a homozygote and additional replications are necessary to resolve the genotype (Hummel and Schultes 2000). Additional amplifications would be necessary to securely type Whaley Cairn (ZW-65), but it is likely that this individual is a heterozygote.

The problem of detecting a heterozygote is complicated given allelic dropout and because the height of the two peaks usually varies so that one peak might obscure the other (this may well be what allelic dropout is). The peak height varies even in the electropherograms of modern DNA sequences of the IL-6 (-174) SNP (Figure 23). In a modern control sample the amplification of the "G/C" SNP shows a significant difference in the height of the nucleotide peaks. In Figure 27, the "C" nucleotide is considerably larger than the "G" nucleotide and the BioEdit® software program only called the "C" nucleotide. The authenticity of the genotypes of the modern controls was verified in a separate laboratory using the PCR-SSP method. Allelic dropout is a problem not only in

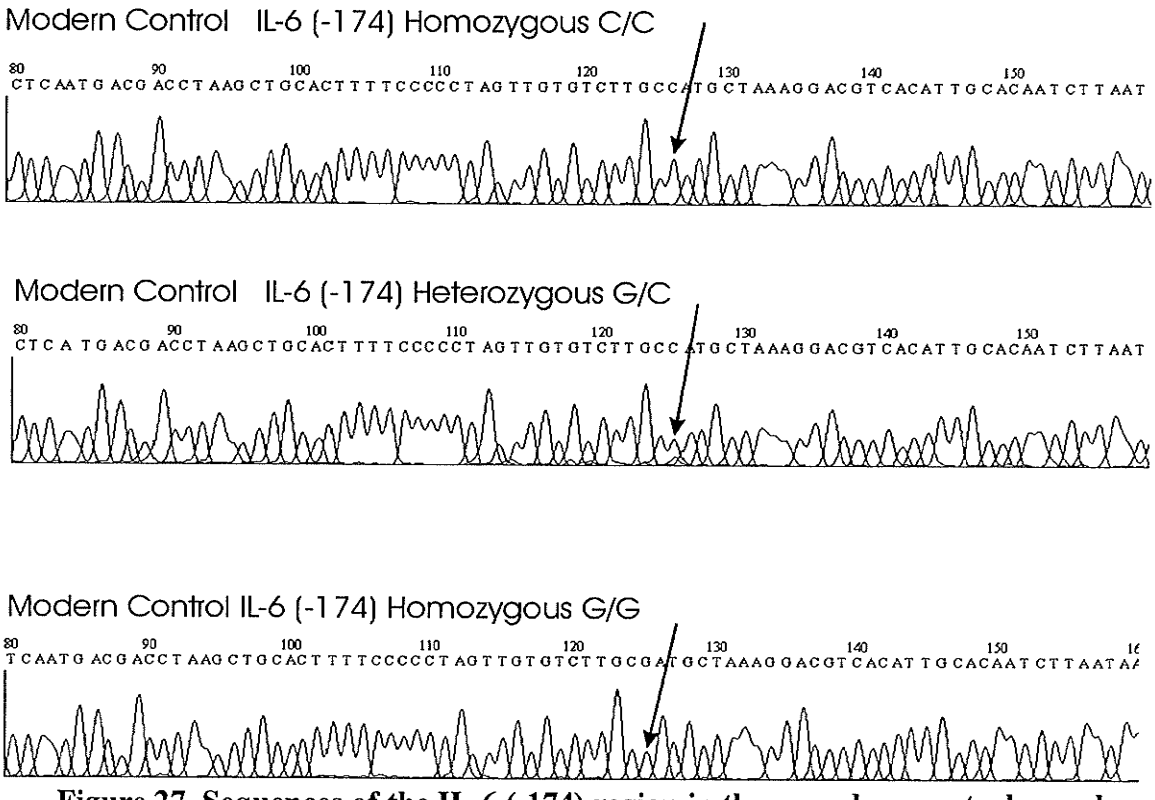


Figure 27. Sequences of the IL-6 (-174) region in three modern control samples.

ancient DNA but also in the detection of SNPs in modern DNA samples (Miller et al. 2002). The problem of variation in peak size and the ability to accurately identify a heterozygote is not unique to SNaPshot.

The other Whaley Cairn individual, MSC00015-03, presents an even more complicated problem. From one extraction (65-03), this individual amplified twice as homozygous "G/G". In a second extraction (11-04), a "G/C" polymorphism was detected.

Although the "C" peak was considerably smaller than the "G", it was large enough to identify. This individual cannot be reliably typed without further replications. The detection of the "G/C" from the second amplification suggests that the amplifications from the first extraction may have allelic dropout.

No replicated examples of homozygotes for cytosine were detected in any other of the ancient individuals. This outcome is not unexpected because the expected frequency of the "C" allele is only 1% in the contemporary Aboriginal population.

Figure 28 shows extraction 72 in which the results from extraction 65-03 were replicated for Eriskdale 110-1 MSC0003-03, Pukatawakan Bay ZW-124 MSC00017-03 and Whaley Cairn ZW-65 MSC00016-03. Drifting River (ZW-118) MSC00014-03 amplified as a "G/G" genotype in two extractions (see Figure 29). Peak heights with a vertical amplification of less than 100 at the expected fragment length position were not considered definitive and so, along with Valley River (ZW-206) MSC00012-03 were not assigned a genotype.

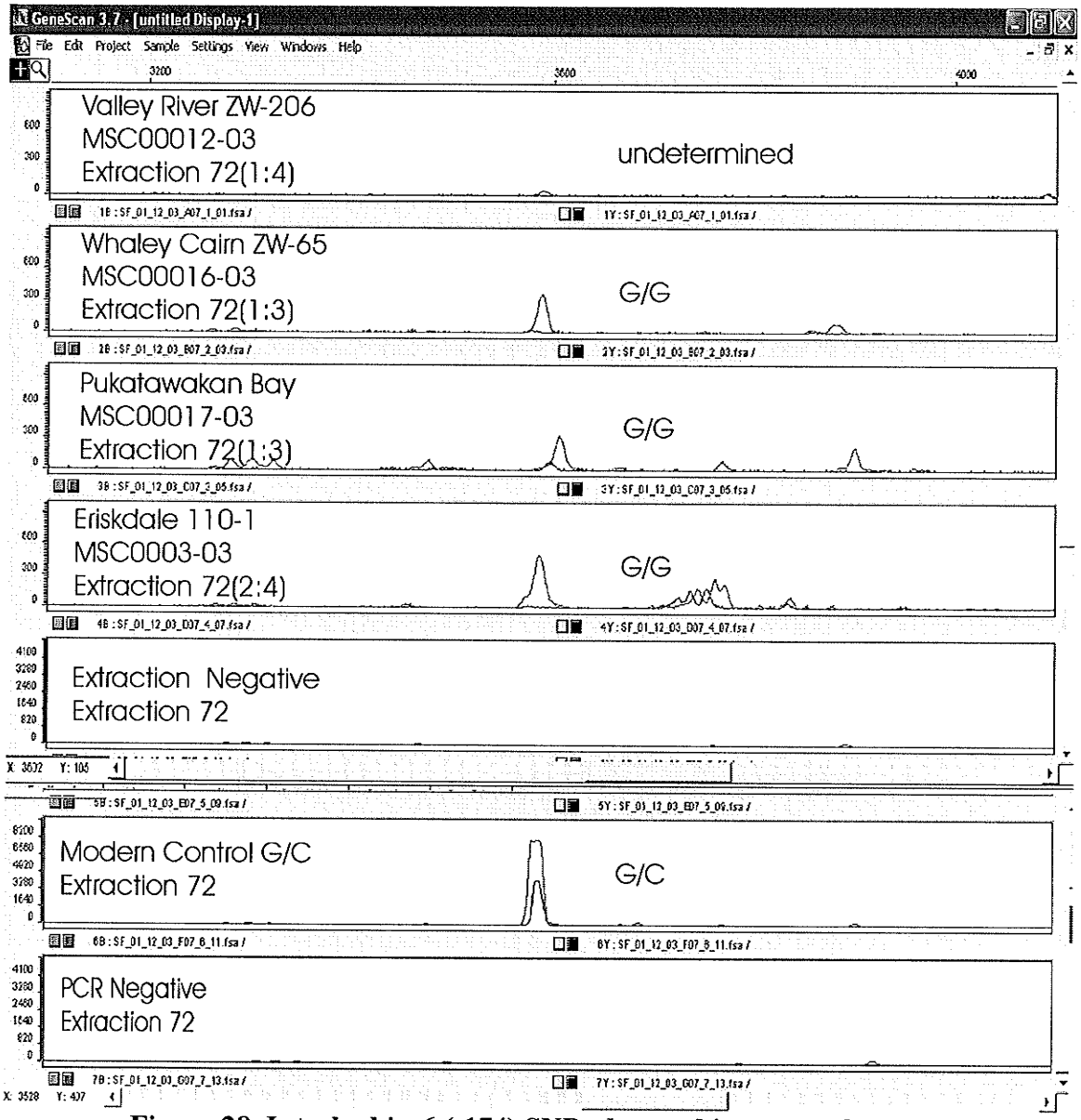


Figure 28. Interleukin-6 (-174) SNPs detected in a second extraction (Extraction 00072-03) in ancient samples.

7.2.2.1 Typing heterozygotes

Four samples, Valley River (ZW-205) MSC0009-03, Valley River (ZW-206) MSC00012-03, Ochre Beach (ZW-134) MSC00013-03 and Fidler Mound MSC00030-04 are considered heterozygotes. Each of these samples was extracted at least two times and amplified at least three times. In every case, one amplification resulted in a call of a

"G/G" genotype. For the Valley River ZW-206 MSC0012-03 and Fidler Mound MSC00030-04 individuals, two of three amplifications were "G/C".

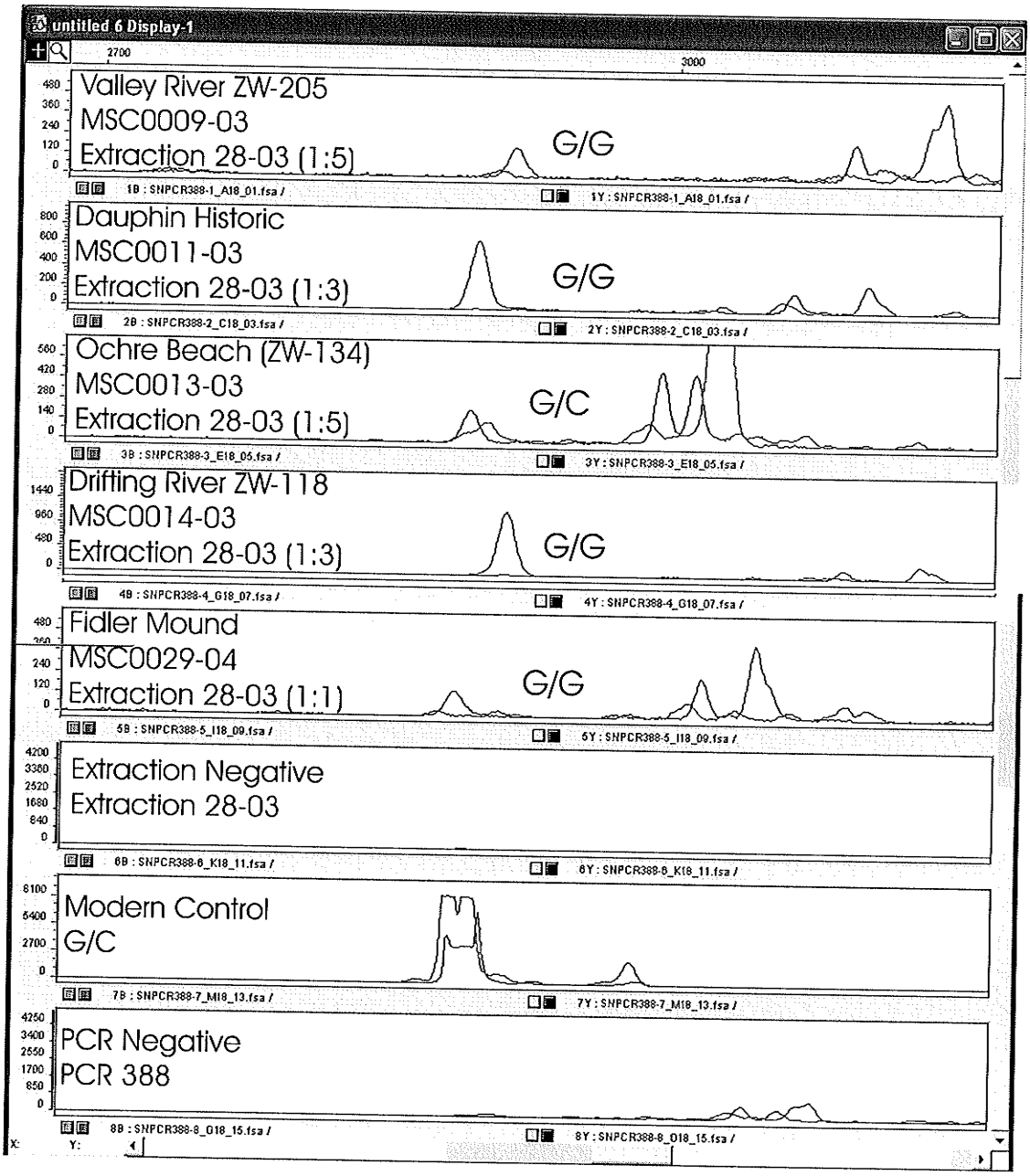


Figure 29. Heterozygote G/C IL-6 (-174) SNP in Ochre Beach (ZW-134) MSC00013-03.

For the five amplifications of the Fidler Mound sample, in five amplifications "G/C" was detected in three and two detected "G/G". Ochre Beach (ZW-134) MSC00013-03 was extracted twice and had two PCRs from each extraction. In three of the electropherograms, either the cytosine or guanine, peak heights were considerably smaller than its counterpart. However, in every case where the cytosine was successfully amplified, the peak was larger than 100 in amplitude therefore securing a "G/C" call. Figure 30 shows three ancient samples from the same extraction, PCR and SNaPshot PCR that were typed as "G/C". All of the "C" peaks have amplitudes close to 200.

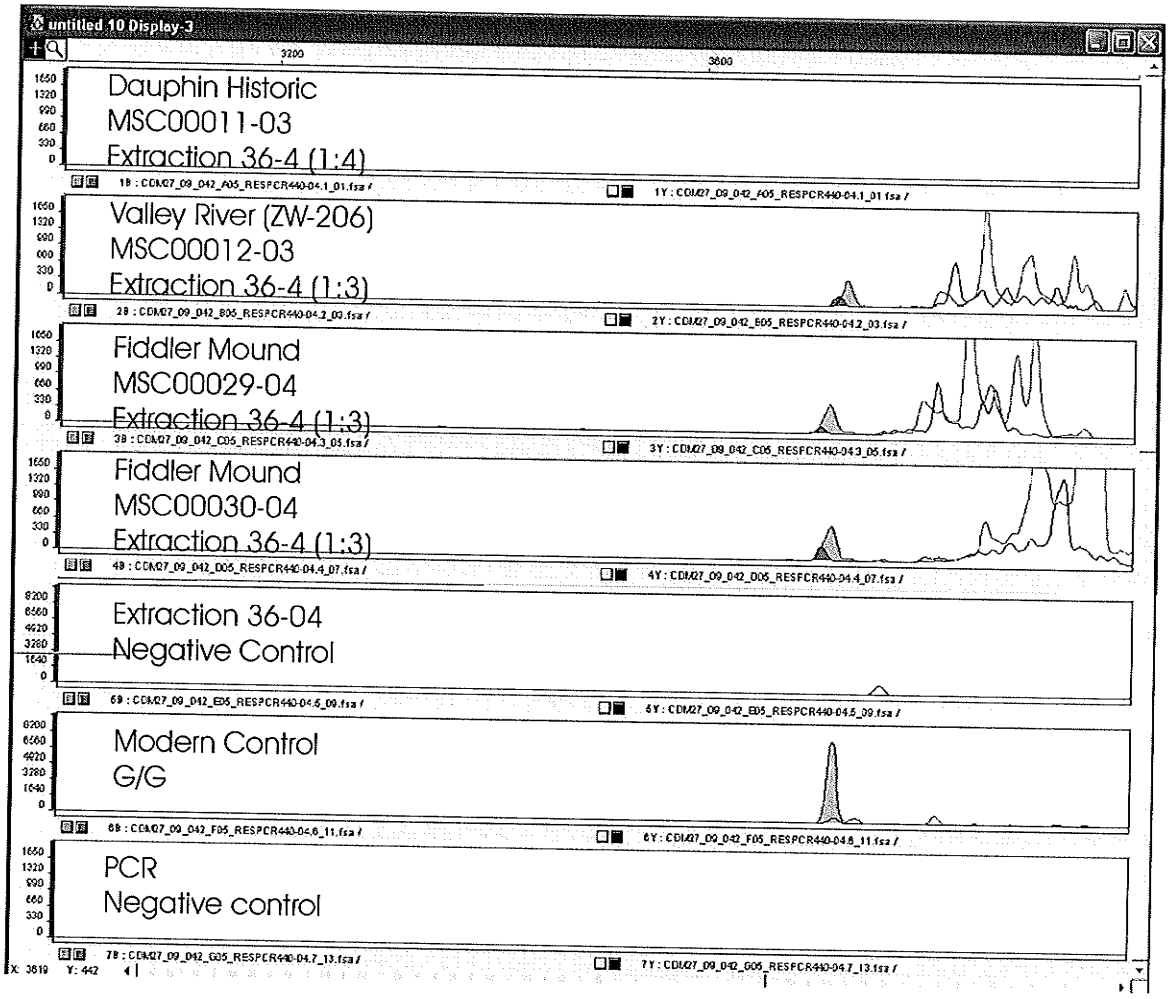


Figure 30. Detection of "G/C" IL-6 (-174) SNP in three ancient samples.

The failure to replicate the initial detection of the homozygote "G/G" genotype in these samples suggests that allelic dropout likely occurred in these runs. The only

recourse for authenticating these results is retesting the sample with additional extractions and amplifications. Indeed, the initial detection of a "G/G" genotype for IL-6 (-174) SNP in all of the individuals in this study raised a red flag that allelic dropout might be occurring. Although the results could make phylogenetic sense, subsequent amplifications have resulted in a much more balanced gene frequency that includes both homozygotes and heterozygotes.

Fidler Mound MSC00031-04 was amplified three times from one extraction (35-04) that was chemically separated by molecular weight into two aqueous solutions. The homozygous genotype in the first amplification was called into question with the heterozygous detection in the second and in the amplification of the B phase of the solution. It is likely therefore that this individual it is heterozygote. Two Eagles MSC00039-03, Red Deer River MSC0006-03 and Dauphin Historic MSC00011-03 were typed only once, and a homozygote "G/G" genotype was detected for each. Given the occurrence of allelic dropout these results need to be replicated before they can be considered conclusive.

7.2.2.2 Extraneous peaks

The electropherograms of the IL-6 (-174) SNPs almost uniformly have additional peaks that appear to represent larger fragment lengths in addition to the expected fragment. Extraneous peaks can be observed in the analysis of the IL-6 (-174) SNP in the ancient samples which is a phenomenon more likely to occur in ancient rather than modern material for a number of reasons. The production of unextended product during the initial and booster PCRs would occur at a significantly higher rate compared to modern DNA. The limited amount of template and fragmentation of ancient DNA may also generate unextended or incompletely extended product. Because of the low template and high cycle numbers used to amplify ancient DNA, the activity of the enzyme is reduced causing an increase in incomplete amplification. Altering the PCR parameters, the volume of primer and/or the purification protocols, may result in less unincorporated ddNTP's and fewer peaks. Extended digestion time of the SAP and/or the use of fresh SAP could eliminate the extraneous peaks if they are interfering with the interpretation. Additional testing and the use of nested primers for the initial amplification may result in

the reduction of the number of extraneous peaks in all samples. This may also enhance the peak of interest in future tests of IL-6 in Sample 3.

7.2.3 Interleukin-10 (-819)

The extension primer for IL-10 (-819) was designed to amplify the reverse strand, therefore the expected peaks are "G/G", "A/G" and "A/A" rather than the "C/C", "C/T" and "T/T" that would be expected if the forward strand had been amplified. Figure 31 illustrates the results of the amplification of IL-10 (-819) in three ancient samples (Whaley Cairn (ZW-68) MSC00015-03; Pukatawakan Bay (ZW-124) MSC00017-03 and Ochre Beach (ZW-134) MSC00013-03).

The modern control amplified with these ancient samples illustrates the expected peaks associated with the heterozygote genotype. The amplification of all three genotypes of IL-10 (-819) is exciting, and the lack of extraneous peaks and "clean"

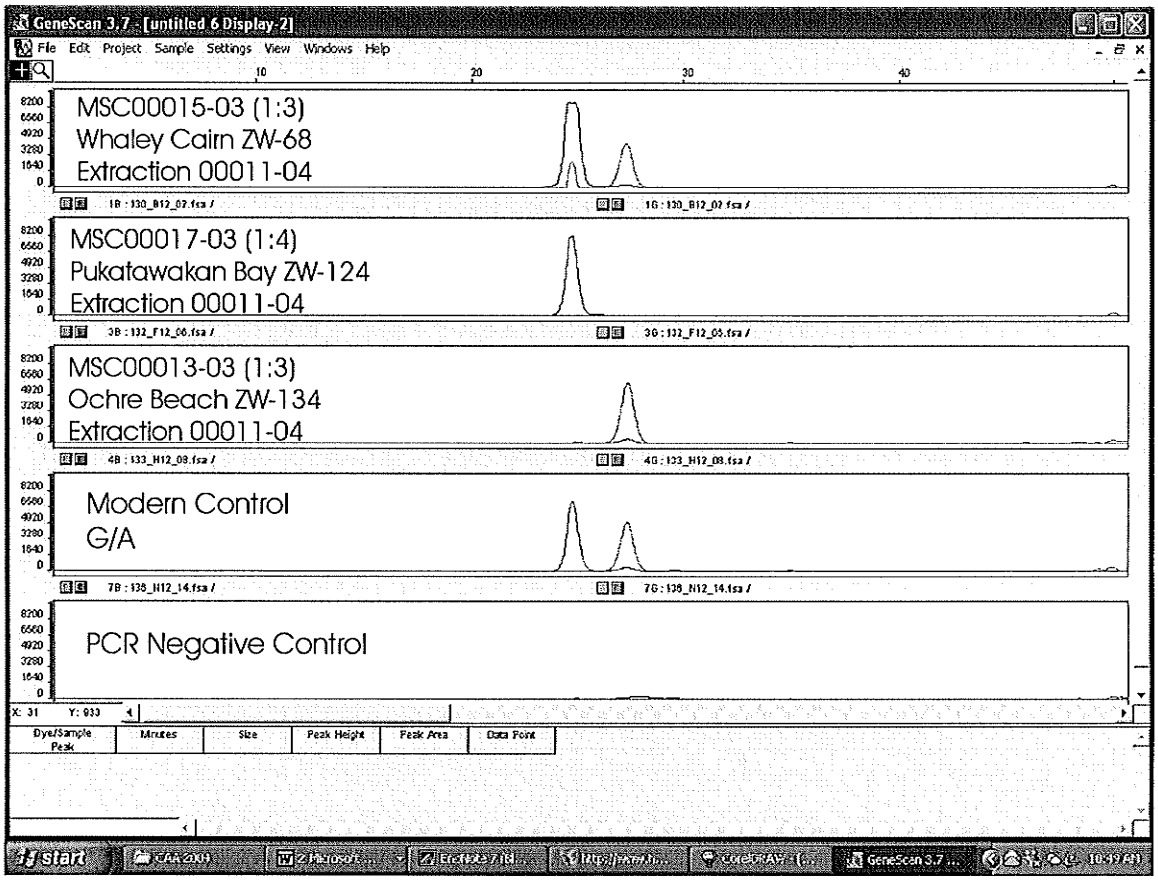


Figure 31. Interleukin-10 (-819) SNP's detected three ancient samples.

electropherograms contrasts significantly with those obtained for IL-6 (-174). To date four other attempts have been made to replicate these results; however, the SNP failed to amplify. These results show some interesting peak characteristics of the "G" and "A" nucleotides. The "A" nucleotide is consistently smaller than the "G" even in the modern control sample and it is always to the left of the "G" peak. This arrangement is consistent with the expected fragment product for the heterozygote "A/G" (Biosystems n.d.). The inability to replicate the initial results raises some concerns about the detection of this SNP. It is evident that contamination was not an issue because three different genotypes were detected. It is not unusual for a significant number of failures to occur particularly with nuclear DNA. Continued analysis of IL-10 (-819) using fresh primers and reagents or slightly altered cycling parameters may sort out some of the problems.

7.2.4 *Tumor Necrosis Factorα (-308)*

The first experiments that attempted to amplify a cytokine SNP focused on TNFα (-308) and initially, the results seemed good. Subsequently, the detected genotypes were found to be incongruent with the genotypes of the contemporary Aboriginal population. The first three individuals tested were Eriksdale 110-1 MSC0003-03, Pukatawakan Bay (ZW-124) MSC00017-03 and Whaley Cairn (ZW-65) MSC00015-03 for the A/G SNP in the TNFα(-308) promoter region. The test included samples from two temporally separate extractions (65-03 and 68-03), and in the case of Eriksdale 110-1 MSC0003-03, two molar teeth were tested. Each of the three ancient samples amplified a strong green "A" peak and a smaller but significant blue "G" peak (Figure 32). A second PCR was run using the individuals from the first test, and Whaley Cairn (ZW-68) MSC00016-03 was included. Figure 33 illustrates the results of the second PCR and on first examination, the amplifications looked excellent.

Compared with the IL-6 (-174) SNP amplifications from later tests, these first electropherograms of a cytokine SNP were very clean (very few extraneous peaks) and showed good peak height. It is possible that the results are as they indicate – that all four ancient samples are heterozygous A/G for TNFα (-308). However, several factors place this interpretation in doubt. The difference in size between the "A" peak and the "G" peak is unexpected. In the modern control samples, some variation exists in peak height

but not usually to the extent seen in the ancient samples. The smaller "G" peaks have a vertical height larger than 100, so they are therefore large enough to be called. The "A" peaks however range in vertical height from 800 to 1600, which is a considerable discrepancy when compared with the "G" peaks.

The peaks are arranged in the expected position relative to each other - that is the "G" peak is to the right of the "A" peak and with very little background "noise" or extraneous peaks likely because the nested primers and the purification steps increased the specificity of the amplification.

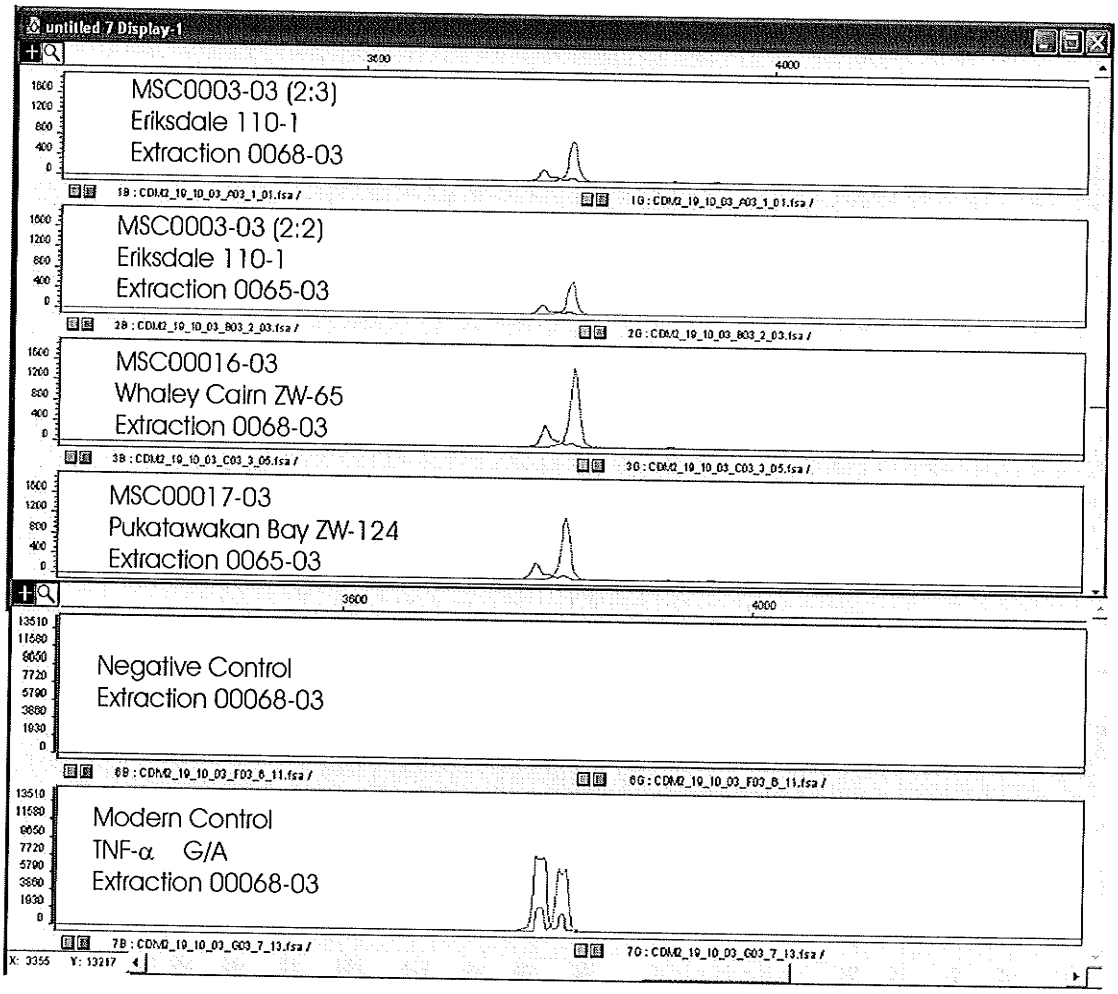


Figure 32. Initial TNFα (-308) amplification in four ancient samples from three individuals.

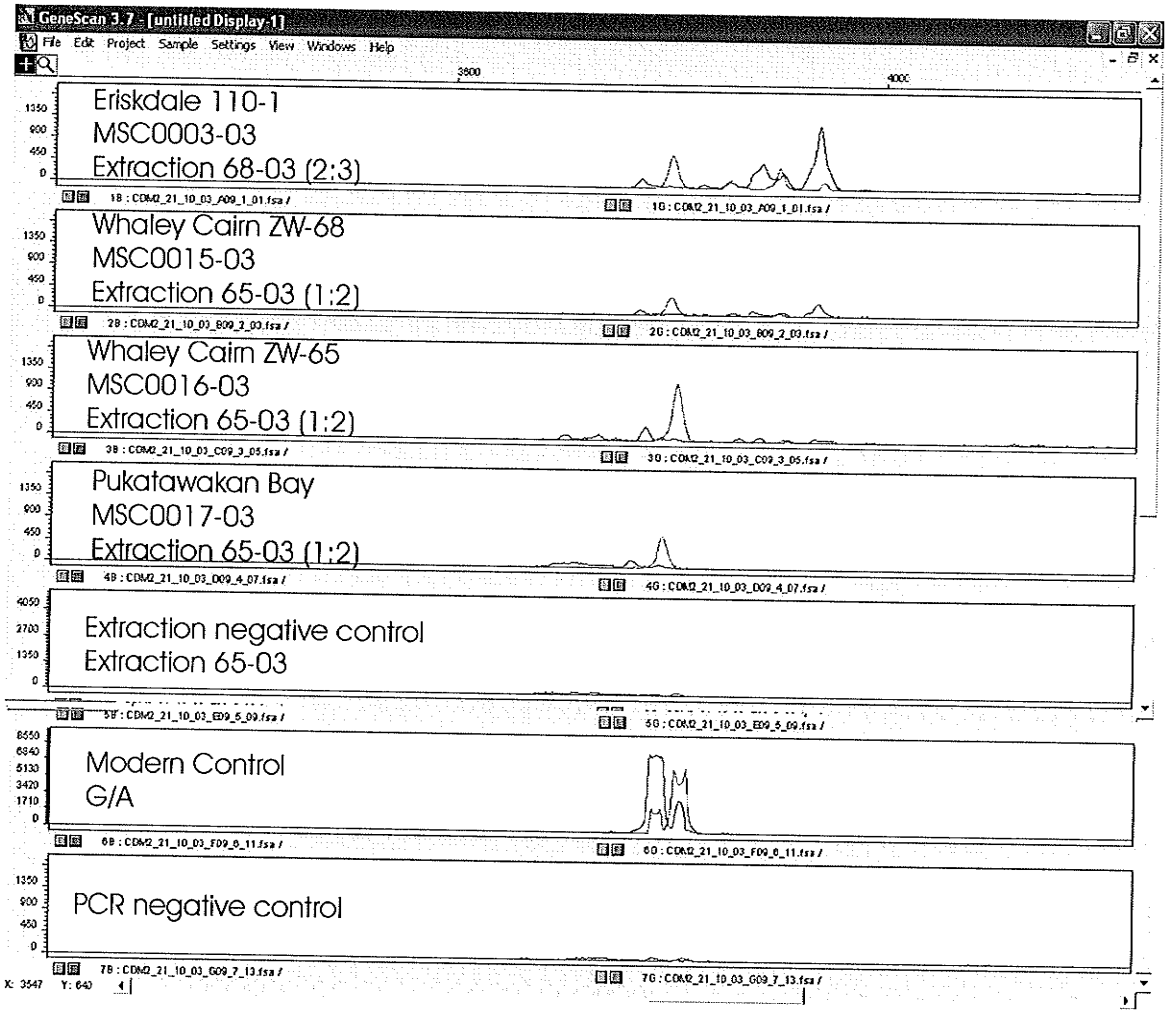


Figure 33. TNFα (-308) amplification in four ancient samples

7.3 Summary

The methods used to extract and amplify the cytokine promoter region of interleukin-6 (-174), interleukin-10 (-819) and TNFα (-308) modified the existing technologies and applied them to the analysis of degraded DNA from ancient human remains. Figure 34 illustrates the procedure that was developed and tested including the steps for mtDNA and sex determination. Given the relatively clean electropherograms of the IL-10 (-819) and TNFα (-308) it is possible that the purification steps could be altered or reduced in number. However, additional purification steps are likely required for the IL-6 (-174).

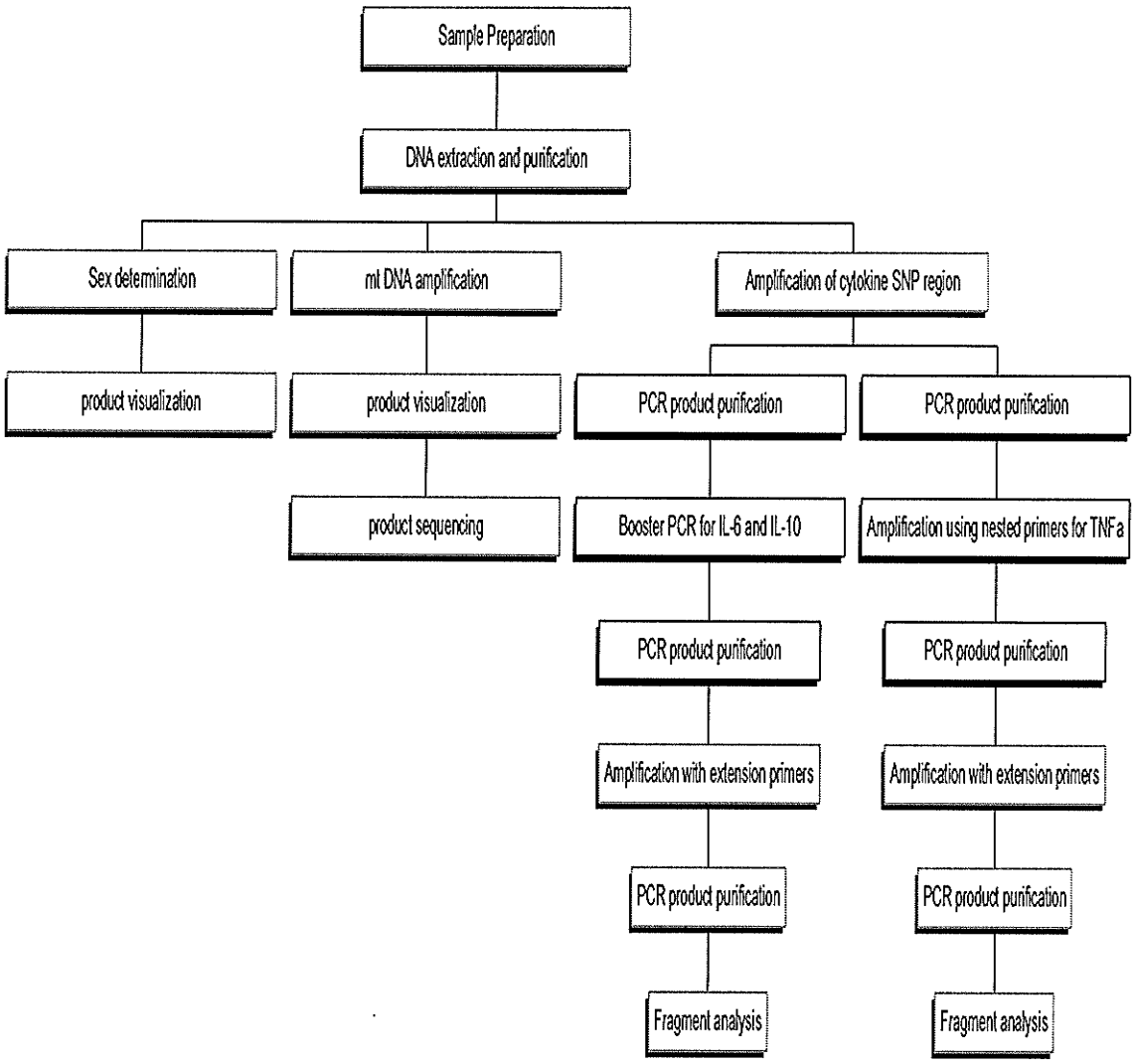


Figure 34. Method for detecting cytokine SNPs from ancient human remains.

Table 11 summarizes the genotypes and phenotypes that were detected in the ancient human remains. The IL-6 (-174) SNP was successfully detected and the results replicated for four ancient individuals (Eriksdale 110-1 MSC0003-03, Dauphin Historic MSC00011-03, Drifting River (ZW-118) MSC00014-03 and Pukatawakan Bay (ZW-124) MSC00017-03). To-date the IL-6 (-174) SNP has been analyzed for fourteen of the eighteen individuals included in this study. In the process of development of the detection method, the tissue sample from three individuals was entirely depleted (MSC00003-03 Eriksdale 110-1, MSC00005-03 Eriksdale 110-2 and MSC0007-03 Rivermouth ZW-44).

Table 11. Summary of detected cytokine SNPs for IL-6 (-174) and IL-10 (-819) in ancient human remains

	PaleoDNA lab Accession #	Sample Name	Genotype for IL-6 (-174)	Phenotype for IL-6 (-174)	Genotype for IL-10 (-819)	Phenotype for IL-10 (-819)	Genotype for TNF-a (-308)	Phenotype for TNF-a (-308)
1	MSC0003-03	Eriksdale 110-1	G/G	H			A/G	I
2	MSC0005-03	Eriksdale 110-2						
3	MSC0006-03	Red Deer River	G/G*	H				
4	MSC0007-03	River Mouth (ZW-44)						
5	MSC0008-03	River Mouth (ZW-45)						
6	MSC0009-03	Valley River (ZW-205)	G/C*	I				
7	MSC0011-03	Dauphin Historic	G/G	H				
8	MSC0012-03	Valley River (ZW-206)	G/C	I				
9	MSC0013-03	Ochre Beach (ZW-134)	G/C	I	T/T*	L		
10	MSC0014-03	Drifting River (ZW-118)	G/G	H				
11	MSC0015-03	Whaley Cairn (ZW-68)	G/C*	I	C/T*	I	A/G	I
12	MSC0016-03	Whaley Cairn (ZW-65)	G/C*	I			A/G	I
13	MSC0017-03	Pukatawakan Bay (ZW-124)	G/G	H	C/C*	H	A/G	I
14	MSC0039-03	Two Eagles	G/G*	H				
15	MSC00029-04	Fidler Mound	G/C*	I				
16	MSC00030-04	Fidler Mound	G/C	I				
17	MSC00031-04	Fidler Mound	G/C*	I				
	Researcher	L.L.	G/C	I	C/T	I	A/G	I
	Technician	K.V.	G/G	H	C/T	I		

(* indicates that the genotype was not replicated. An empty cell indicates that no amplification was attempted).

The SNP in the promoter region of IL-10 (-819) was detected in four of the six ancient samples tested, but attempts to replicate these results have not yet been undertaken. The results for detecting the TNF-a (-308) SNP are inconclusive given that

all of the ancient samples were determined to be heterozygotes. Additional testing and modifications in the procedures are necessary to amplify this target site.

A critical limiting factor in the development of this novel method was the small size of the tissue sample and DNA extract. Ideally, each successful result should be followed by a second PCR, an additional extraction, and two amplifications. Each molar tooth produced enough powder for four or five, but mtDNA testing, sexing of the individuals and testing for three cytokine loci, essentially used the entire amount of DNA extract. Ideally, there should be enough sample and DNA extract to obtain at least a three loci genotype. In reality, the process of developing the method for cytokine SNP amplification and detection required multiple PCR's and extractions to optimize the procedure. Additionally, the mtDNA testing and sex determination depleted the stock of sample in cases where the DNA was inhibited are the amplification failed and the tests were repeated.

It was the goal of this component of research to develop a method for typing multiple cytokine promoter SNP loci of ancient human remains. The successful amplification and detection of IL-6 has demonstrated that cytokine SNP detection is possible in degraded DNA material. It is evident from the results presented here that SNPs can be detected in human remains as old as 3500 BP. It is also apparent that each cytokine SNP requires individualized amplification and detection protocols that have not yet been refined. It is clear from this preliminary work that the detection of cytokine SNPs in ancient human remains is possible. Future work on development and refinement of cytokine SNP detection should continue to focus on ancient human DNA, but other types of ancient mammalian tissue from archaeological sites might also be used. Experiments for optimizing nuclear DNA recovery, SNP amplification, thermocycling parameters and SNP detection could all use non-human ancient tissues. This type of experimentation, in conjunction with continued analysis of ancient human cytokine SNPs, could significantly improve the quality of SNP detection.

Chapter 8 An evolutionary perspective of cytokine SNP frequencies in Aboriginal populations

8.1 Introduction

New molecular technologies are forcing the re-evaluation of long held theories regarding the spread of disease in the New World at the time of contact with Europeans. The virgin-soil hypothesis is commonly invoked to explain the spread of disease in the New World, but this model does not take into account the recent genetic information about New World pathogens and their evolution. Additionally, this concept describes the condition of immunologic naiveté among the affected population without exploring the processes that may have led to this condition. The description of the dramatic demographic decline in the New World at the time of European contact as a virgin-soil epidemic, does not necessarily consider that morbidity and mortality was geographically and temporally unequal, and it disregards individual human immunogenetic variation. Complex historic and evolutionary events led to the immunogenetic profile of the North American indigenous population, and it is only within an evolutionary context that we can make sense of the differential rates of morbidity and mortality that occurred and continue to occur between ethnic groups.

Humans have a long established relationship with disease-causing pathogens, and the complexity of the human immune system attests to the evolution of a diverse and flexible biological immune response. The genes that are active in the human immune response to infection were identified as a result of tissue transplantation research and it is the genes that are responsible for identifying "self" from "non-self"; the HLA molecules that are the most polymorphic in the human genome. The extensive polymorphic nature of the HLA molecules is a mammalian evolutionary response to the equally extensive array of foreign bodies or pathogens that can compromise the functioning of the mammalian body (Bontrop 2000). Livingstone (1983), published the classic anthropological example of the genetic balance at the HLA –A locus that occurred within

a population as a consequence of adaptation to a pathogen. The identification of population variation based on HLA frequencies of contemporary populations has been instrumental for anthropologists for reconstructing historic relationships between human groups and the genetic ancestry. The genetic distance between two populations has been measured based on the number of differences in HLA polymorphisms and allele frequencies. The term 'haplotype' was in fact based on the multilocus combination of alleles on a chromosome and was coined as a result of research on the major histocompatibility complex (Cavalli-Sforza and Feldman 2003). This method of assessing genetic closeness is based on the assumption that at some point in the past humans were similarly adapted to a specific environment and maintained an ancestral human HLA profile. As groups dispersed from the parent population they became isolated and adapted to new pathogen environments or experienced genetic drift (or both) and these selective pressures resulted in changes in HLA allele frequencies (Bias 1981; Bontrop 2000; Lechler and Warrens 2000). With the introduction of electrophoresis, RFLP and sequencing methods, it became evident that genetic variation was not a rare event but rather almost every protein had genetic variation and that the identification of these variations were useful for population studies (Cavalli-Sforza and Feldman 2003). Certain HLA alleles and specific markers are known to have ethnic affiliation, and, not surprisingly, the occurrence of some markers is related to the unequal risk of acquiring certain diseases or to an increased resistance to certain pathogens. Studies have shown that certain HLA alleles are associated with specific diseases, although it may not necessarily be clear what the functional relationship between the allele(s) and the disease(s) might be. However, it is the function of the alleles, and the unequal frequencies of allelic variants in certain populations, that may hold the key to understanding their evolutionary importance.

8.2 Ethnic differences observed in cytokine genotype frequencies in contemporary populations

It was observed in our study of contemporary Aboriginal and Caucasian individuals with ESRD that differences exist in the frequency of SNPs in the cytokine promoter regions (Larcombe et al. 2005). The contemporary Caucasian cohort in this

study maintained a significantly higher frequency of cytokine SNPs that would enable them to produce a strong T_h1 type of immune response against an infection. The SNP frequencies in the Aboriginal cytokine promoter regions would tend to favour a T_h2 immune response. In order to account for these genetic differences one should consider the historical context in which these populations evolved in relationship to their microbial environments.

The Old World Caucasian populations, during the past 11,000-year period, traded a hunting and gathering lifestyle for one that included the domestication of plants and animals, sedentism and subsequently urbanization. These changes in subsistence and settlement patterns resulted in profound changes in the microbial environment in the Old World (Cohen 1989; McNeill 1998). With the advent of agriculture and urbanization the so-called "crowd infections" became prevalent and epidemics of smallpox, measles, plague and tuberculosis occurred throughout Europe (Cohen 1989; Newport et al. 1996). The high mortality rates associated with these pathogens likely exerted intensive selective pressures on the population (Lipsitch and Sousa 2002). For example, given the high prevalence of tuberculosis infection leading to premature death in Europe (ca. 18^{th} century) survival would have in part favoured those individuals who could effectively mount a T_h1 type of immune response. This type of response is characterized at a cytokine level by IFNγ and TNFα expression (Newport et al. 1996). Indeed, when anti-TNFα was used to treat patients with inflammatory bowel disease it was associated with the occurrence, or the reactivation of tuberculosis, thereby confirming the important role of TNFα as an immune modulator or neutralizer for tuberculosis (Lim et al. 2002; Mira et al. 1999). Similarly, IFNγ has an important role in a host's resistance to *Mycobacterium tuberculosis,* although IFNγ production cannot adequately control the infection without the presence of other critical cytokines (Flynn and Chan 2000). In terms of other cytokines, evidence from animal models recently found that IL-6 expression by macrophages can prevent the effective containment of *Mycobacterium tuberculosis* infection (Nagabhushanam et al. 2003). Coming back then to the Caucasian population in our study, it is interesting to note that they maintain a relatively higher frequency of cytokine SNPs that would favour a T_h1 type immune response (i.e. IFNγ, TNFα) and a lower IL-6 expression level compared to the Aboriginal cohort. Given the

aforementioned high prevalence of pathogens requiring a T_h1 type immune response in Europe, since ca. 1700 to the early 1900's when effective anti-microbial therapy was developed, the selection for individuals with a cytokine SNP profile observed today in the Caucasian population likely occurred.

In contrast, Aboriginal North American populations experienced a unique set of evolutionary pressures because of their migration across the Bering Land Bridge (ca. 15-20,000 years ago) and their relative isolation in North America from ca.10,000 B.P. to at least 1,000 B.P. with increasingly greater contact after 500 B.P. (Dobyns 1983; Ramenofsky 1987; Schweger 1990; Turner 1986). The North American Aboriginal populations maintained an ancient mobile hunting and gathering lifestyle, one that had existed in the Old World and was conducive to the maintenance of diffuse settlements with low population densities (McGhee 1994; Merbs 1992; Ramenofsky 1987; Trimble 1985; Wright 1995). The analysis of pathologies from human skeletal remains of ancient Aboriginal individuals indicate that parasitic and fungal infections and malnutrition were present among the early populations (Buikstra 1976; Buikstra 1981; Cybulski 1977; Pfeiffer 1984; Zimmerman et al. 2000). Indeed, the osteological, archaeological and demographic data demonstrate that pre-contact Aboriginal populations lived with, and died from a range of disease-causing pathogens including helminths, fungi and bacterial infections causing tuberculosis and treponema (Arriaza et al. 1995; Buikstra 1976; Cook 1984; Cybulski 1977; Powell 1988; Powell 1992; Saunders et al. 1992; Waddell 1994).

The bio-archaeological analysis of human remains from hunter-gatherer and early horticultural populations support the contention that Aboriginal populations were adapted for thousands of years to pathogens that were specific to the New World. The consumption of raw or undercooked meat, living conditions that were confined during the winter months, and the pattern of collecting wild plants brought these people into close contact with fungi and parasites that left them vulnerable to seasonal nutritional deficiency (Buikstra 1976; Cook 1984; McGhee 1994; Zimmerman et al. 2000). The New World microbial environment would tend to favour positive selection for a T_h2 type adaptive immune response in order to survive. Indeed, studies of South American Aboriginal families with *Shistosoma mansoni* infection have confirmed the protective role of T_h2 type cytokines IL-4, IL-6 and IL-13, in protection against parasitic infection

(Marquet et al. 1999). In the context of the current study, among Canadian Aboriginals, the cytokine SNP profile is skewed if favour of high levels of IL-6 and relatively, low levels of TNFα and IFNγ production. These observations support the concept that this population is generally adapted to an environment where parasitic and fungal pathogens were once prevalent.

Recent studies of South American Aboriginal populations indicate that the high susceptibility to tuberculosis among the relatively isolated Aché and Yanomami may, in part, be a function of immunogenetics (Hurtado et al. 2003; Sousa et al. 1997). Despite the suggestion among some studies that socio-economic status and nutrition may be implicated in disease susceptibility among the Aché, individuals with high nutritional and socio-economic status they are equally at risk as those with low nutritional and socio-economic status (Hurtado et al. 2003). It has been suggested that the high rates of antibody production and T_h2 mediated activation by South American Aboriginal individuals competes with the T_h1 mediated defenses required to effectively fight against infectious diseases such as tuberculosis (Hurtado et al. 2003; Sousa et al. 1997). Similarly, North American Aboriginal populations in Manitoba have the highest incidence of tuberculosis of any local population Canada (Canada 1999; FitzGerald et al. 2000; Smeja and Brassard 2000).

While the unequal risk of disease between ethnic populations maybe partly explained by environmental and socio-economic differences, cytokine SNPs may play a role in this variability. It was hypothesized that in the contemporary Manitoba Aboriginal study population the observed cytokine SNP profile was developed as a result of selective pressures in a specific pathogenic environment. One would expect therefore, that in the ancient population the immunogenetic profile would be skewed even more heavily to favour a T_h2 immune response. Figure 35 depicts this hypothesis where the pre-contact Aboriginal immunogenetic genotype is currently unknown but was influential in the observed immunogenetic genotype of the contemporary population.

The hundreds of years since contact with individuals of European-descent may have caused changes in the allele frequencies as a result of genetic drift in response to selective pressures, such as from novel diseases (crowd infections), or through admixture

with European-descent populations. The pre-contact Manitoba population may have been subjected to regular or continuous helminthic and/or fungal pathogens that constantly primed the T_h2 immune response; indeed, survival necessitated the selection for individuals with the genetic code for producing the cytokine proteins to illicit a T_h2 type

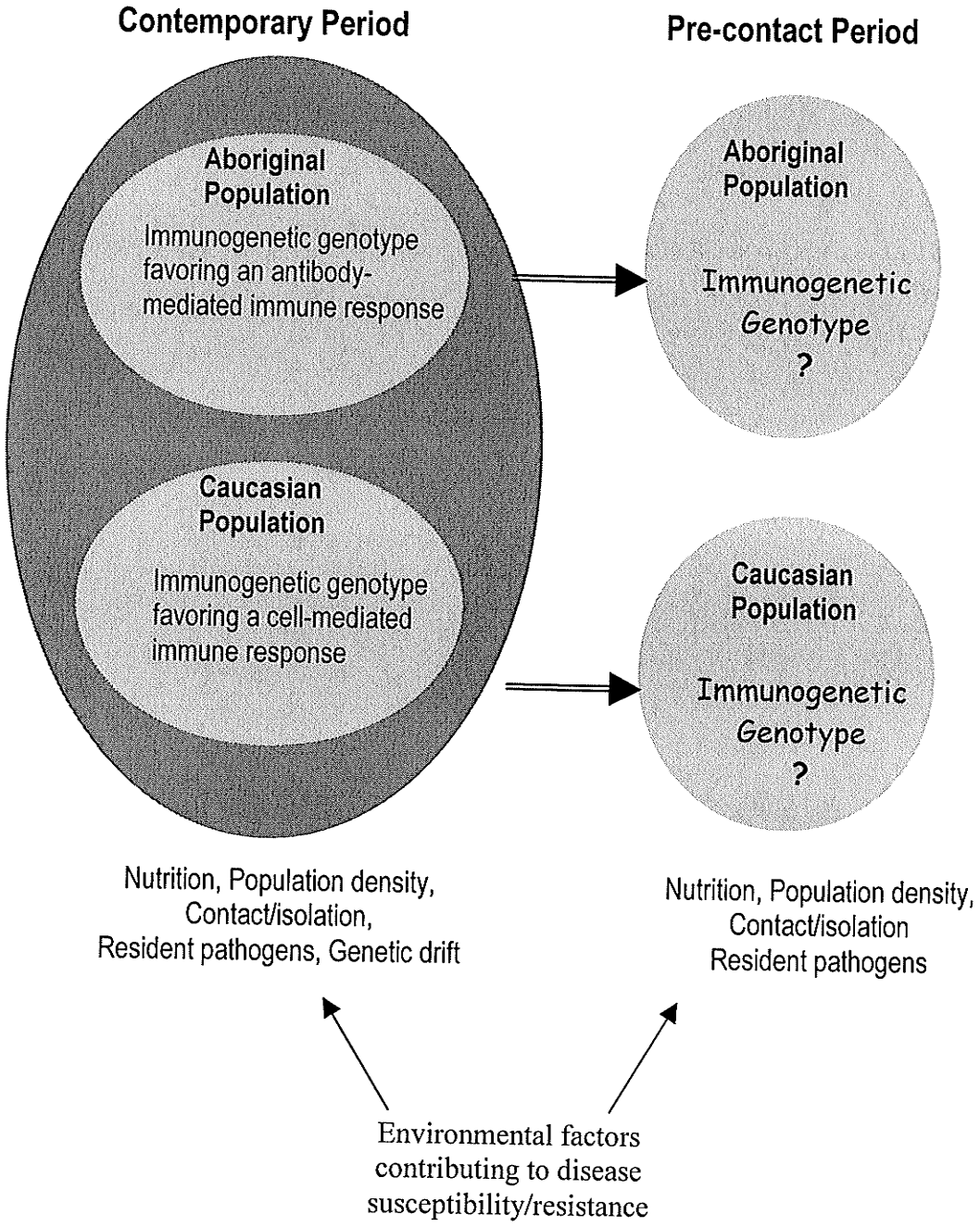


Figure 35. The relationship between the contemporary and precontact immunogenetic genotypes.

of response. This genetic advantage may, however have had disastrous implications when measles, small pox and novel strains of influenza, requiring a T_h1 immune response, were introduced at the time of contact with European-descent populations.

8.2.1 Archaeological context of the skeletal remains

The archaeologically recovered skeletal remains ranged in age from 3,500 B.P. to the historic period and were from geographically diverse regions of Manitoba. Mitochondrial DNA sequences were obtained from all individuals sampled[8] and neither the age of the skeletal remains, nor their geographic provenience, inhibited amplification. The tissue type that amplified the most reliably was from the molar teeth.

The tooth powder, from all samples, was uniformly white in colour. This would suggest that humic acids, which can inhibit DNA amplification, were not present. The fact that the teeth were free from cracks and caries facilitated the amplification of authentic DNA from each tooth. The intact condition of each tooth ensured that surface contaminates could be successfully removed. An mtDNA sequence was not obtained from the mummified skin tissue from the Red Deer River (MSC0006-03) individual although the IL-6 (-174) SNP was detected. The recovery of nuclear DNA but not mitochondrial DNA might indicate that the mtDNA was too fragmented to be recovered, although the investigation of a nuclear SNP was still possible. Further analysis of the mtDNA from this specimen should be undertaken to assess use of other tissues sources such as skin or bone for the detection of nuclear SNPs.

The success with which DNA was extracted and amplified from skeletal remains that were archaeologically recovered in Manitoba was extraordinary, and is likely a reflection of the soil conditions in which these remains were buried. Clay soils in general maintain a neutral or basic pH and if the parent material is limestone (as is the case for the Interlake region), the soil will be even more highly basic. It is likely that the pH of the soils contribute to the preservation of the skeletal remains and the DNA in the teeth. Additional work would be required to compare the relative quality of preservation of the DNA in bone and soft tissues compared to the teeth. A systematic evaluation of the pH

[8] Mitochondrial DNA analysis was not conducted on the three individuals from Fidler Mound (EaLf-3). A detailed analysis of the genetic relationship of these individuals is required and it was deemed necessary to conserve these samples.

environment of the burials would enable a detailed analysis of the preservation conditions. All of the human remains were subject to dramatic freezing, thawing and warming environments in their respective burial environments and it is apparent that these conditions did not prevent the amplification of DNA from the molar teeth.

8.3 Native North American Mitochondrial Haplogroup analysis

Polymphisms that occur frequently in contemporary Native North American populations were identified in the mtDNA sequences in each of the fifteen ancient individuals from Manitoba. Haplogroup A markers occurred the most frequently and were identified in both historic (Ochre Beach (ZW-134), Drifting River (ZW-118) and Pukatawakan Bay (ZW-124), and pre-contact individuals, (Eriksdale (110-2), River Mouth (ZW-44) and the individual known as Two Eagles. The genetic markers for Haplogroup B and X occurred in only one individual (River Mouth (ZW-45)). Although considerably more analysis is required to verify the precise Haplogroup association for the ancient individuals, it has been demonstrated that mitochondrial sequences can be obtained from human remains from archaeologically recovered skeletal remains in Manitoba. The identification of the Native North American haplogroup markers in historic and pre-contact individuals indicate Aboriginal genetic heritage; however, it would be hasty and inaccurate to rely only on this data to establish ethnicity. The mtDNA markers cannot address the patrilineal ancestry nor can it indicate, in the case of the historic individuals, the extent of potential genetic admixture with non-Aboriginals.

8.4 SNPs in the cytokine promoter regions detected in ancient human remains

SNPs in the cytokine promoter regions of nuclear DNA have never before been explored in ancient human remains. Indeed, the analysis of nuclear DNA in ancient tissues has only been feasible within the past five years (Hummel 2003). The experiments undertaken in this research resulted in the first-ever detection of SNPs in ancient human remains that would have influenced cytokine production. The success of this research is largely the result of new advances in molecular technologies that have focused on genome wide sequencing and detection of nuclear allelic variants. The technological progress was and continues to be driven by research that seeks to identify associations

between SNPs and the occurrence of diseases and detection of SNPs that may be related to resistance and/or susceptibility to diseases.

The methods used for this analysis combined the extraction methods designed for ancient DNA, and a modified micro-sequencing kit, to extract, amplify and detect SNPs in the cytokine promoter regions of IL-6 (-174), IL-10 (-819) and TNFα (-308). In total, eighteen ancient individuals, from eleven different archaeological sites were included in the molecular analysis and SNPs in the cytokine promoter regions were detected in fourteen.

The two slightly different methods that were used to isolate a target region of approximately 200 bp need to be more closely evaluated. Nested primers were used for the amplification of TNFα (-308) and this method appeared to increase the specificity of the target region and eliminate spurious amplification of non-target regions. The relatively clean electropherograms that resulted from the detection of the TNFα (-308) SNP may be a product of this increased specificity. However, the IL-10 (-819) electropherograms were also extremely clean, yet nested primers were not used; rather, a booster PCR was performed instead. This alternative method, the booster PCR, was used for IL-10 and IL-6 because it was not possible to design a set of nested primers for the target regions. The extraneous peaks that appear in the electropherograms for the IL-6 (-174) SNP may be simply a function of the chemistry of that target region.

The most problematic step in the SNP amplification protocol were the multiple purifications that are required to remove the extraneous ddNTPs. This was particularly problematic for IL-6 (-174). Each time the PCR product is subjected to a purification step, the risk of contamination increases, as does the risk of loosing the DNA through pipetting errors. Each time the PCR product is purified there is a chance that the DNA may not be properly transferred. As the method developed for this research becomes more reliable in terms of reproducible results, alternative methods for purifying the PCR product or removing purification steps should be considered and tested. The electropherograms produced using the Genescan software for IL-10 (-819) and TNFα (–308) were relatively clean and it may not be necessary to include all of the steps to purify the PCR products for these SNPs.

The other problem that is more difficult to solve is the occurrence of allelic dropout. It is a phenomenon that occurs with some regularity in the analysis of genetic material from a single or a small numbers of cells (Piyamongkol et al. 2003). With ancient DNA, the stochastic amplification of only a single nucleotide base of heterozygote alleles, is that much more of an issue since damage to the DNA sequence is expected. The occurrence of allelic dropout was usually identified, in the samples used for this research, in the second extraction and amplification of a tissue sample. In some cases, a single extraction resulted in the identification of a homozygous genotype in two PCR products, but subsequently, in a second extraction and amplification, the second allele was amplified. This was illustrated in the Fidler Mound MSC00030-04A samples where a homozygous genotype was detected in one PCR from the first extraction and the first PCR from the second extraction. Minimally, two extractions and two amplifications from each are essential to verify a homozygous genotype. A subsequent PCR from the second extraction and two PCRs from a third extraction resulted in the detection of the second allele. Cloning of the PCR product and sequencing the cloned product is a widely accepted method for replicating the results. However, in order to use this method to ensure the correct typing of a heterozygote individual, several independent clones of each PCR product would have to be sampled to pick up a poorly or under represented allele (Zhang and Hewitt 2003). Cloning is costly, labour intensive, and can still potentially produce false positives depending on the quality of the original DNA template and the success of the amplification process.

8.4.1 Interleukin-6 (-174) cytokine SNP in ancient human remains

The amplification and detection of the IL-6 (-174) SNP was successful in fourteen of the ancient samples. Table 12 lists the frequency of the IL- 6 (-174) genotypes that were detected in the ancient samples and compares the genotype frequencies of these ancient individuals, against that of the contemporary study population. If just the replicated results are considered, the ancient study population has a lower frequency of high expressers and a higher frequency of intermediate expressers of IL-6 (-174) than the contemporary Aboriginal study population.

Table 12. Frequency of IL- 6 (-174) genotypes for the ancient individual and percent of phenotypes for the study populations (( ) denotes number of individuals for which the results have not been replicated).

IL-6 (-174) phenotype	IL-6 (-174) genotype	Frequency of genotypes of ancient study population	Percent of genotypes for the ancient study population	Percent of genotypes for the contemporary Aboriginal study population	Percent of genotypes for the contemporary Caucasian study population
High	G/G	6 (2)	42	82	39
Intermediate	G/C	8 (3)	56	17	45
Low	C/C	0	0	1	17

It is impossible at this point to make any definitive statements regarding the frequency of IL-6 (-174) genotypes in the larger Manitoba ancient population given the small number of individuals that have been typed. However, in general, this small ancient population tended towards being intermediate or high producers of IL-6. In fact, the frequency of the "G" allele is 42% and the "C" allele 57% in the ancient population from which results have been obtained. The absence of any individuals that were homozygous "C/C" conforms to the genotype pattern observed in the contemporary Aboriginal cohort. Both the ancient and contemporary Aboriginal groups have significantly fewer low expressers of IL-6 than does the Caucasian study group. It is interesting that the ancient study group has a higher frequency of the "C" allele than do their contemporary counterparts. Two possibilities exist that may explain the observed allele frequencies. The observed frequency of the ancient populations may represent a sampling biases or it may be that the observed frequencies are truly representative of the larger ancient population.

It may well be that the pattern of maintaining a higher frequency of the "C" allele that is demonstrated in this ancient study population was true of the larger population in the past, although it does seem unlikely for a number of reasons. If we assume a low frequency of the "G" allele in the ancient population as indicated in these results, the only mechanism through which an increased frequency of the "G" allele in the contemporary Aboriginal population would occur would be through selective pressure. The contemporary Caucasian population maintains a frequency of the "G" allele of only 60%

and while admixture might boost the "G" allele frequency somewhat, it could not account for occurrence of an 82% frequency in the modern Aboriginal population. If selective pressure was a factor in an increase in the frequency of the "G" allele we would expect to see, the oldest of the ancient samples maintain a higher frequency of the "G" allele and the more recent, historic samples include a higher frequency of the "C" allele. In fact, the opposite is true. Four of the five historic individuals are "G/G", while the individuals that date to the pre-contact period are predominately "G/C" (Table 13).

Table 13. IL-6 (-174) genotypes of 14 individuals

Accession #	Sample Name	IL-6 (-174) Genotype	IL-6 (-174) Phenotype	Sample Age
MSC0006-03	Red Deer River	G/G*	High	Historic
MSC0011-03	Dauphin Historic	G/G	High	Historic
MSC0013-03	Ochre Beach (ZW-134)	G/C	Intermediate	Historic
MSC0014-03	Drifting River (ZW-118)	G/G	High	Historic
MSC0017-03	Pukatawakan Bay (ZW-124)	G/G	High	Historic
MSC0016-03	Whaley Cairn (ZW-65)	G/C*	Intermediate	Middle Pre-contact
MSC0003-03	Eriksdale 110-1	G/G	High	Middle Pre-contact
MSC0015-03	Whaley Cairn (ZW-68)	G/C*	Intermediate	Middle Pre-contact
MSC0039-03	Two Eagles	G/G*	High	Middle Pre-contact
MSC00029-04	Fidler Mound	G/C*	Intermediate	Late Pre-contact
MSC00030-04	Fidler Mound	G/C	Intermediate	Late Pre-contact
MSC00031-04	Fidler Mound	G/C*	Intermediate	Late Pre-contact
MSC0009-03	Valley River (ZW-205)	G/C*	Intermediate	Pre-contact
MSC0012-03	Valley River (ZW-206)	G/C	Intermediate	Pre-contact

It is more likely that sampling bias might have affected the allele frequency in the study population. Given that the sample is small and may represent even smaller pockets of potentially related individuals, the cytokine genotype detected for these individuals may not accurately represent the larger ancient population. The two Valley River individuals for example, may have been related given their archaeological context and the closeness of their mtDNA sequences. Even if maternal relationship is not evident from

the mtDNA analysis as with the Whaley Cairn individual's, a paternal relationship between these two is a possibility. The fact that the ancient study population includes clusters of individuals, who may be related, may have caused a significant bias in the results.

The three Fidler Mound individuals were typed (two provisionally) as "G/C". The samples were isolated teeth recovered from a large mound feature (Hewitt 2004) and likely represent three distinct individuals, although it is possible the teeth were scattered from one or two individuals. In a broader context, the horticulturalists in the Fidler Mound area may have been immigrants from the Missouri and Mississippi drainage system horticultural villages (Flynn 2002). The early horticultural villages in the Missouri River drainage area were more densely inhabited and the people were more sedentary than the Manitoba Aboriginal hunter and gather peoples. It is likely that the horticultural groups experienced diseases that may not have been as prevalent among hunter-gatherer groups. The immunogenetic profile of the small population that may have migrated north to population the Fidler Mound area may be indicative of a group that survived diseases that were endemic (i.e. treponema, tuberculosis) in the southern horticultural villages (Buikstra 1981; Cook and Buikstra 1979). Individuals who produced a poor protective cell-mediated immunity as required for defense against treponema and tuberculosis, would have been selected against. Only individuals who could effectively produce a T_h1 immune response would ultimately become the predominate phenotype of the population.

Alternatively, it is possible that our ability to interpret the results depicted in the electropherograms is hampered by the fact that these results are the first of their kind. Although nuclear DNA has been amplified and detected using the Genescan® software for the X and Y-chromosomes, each gene loci amplifies differently and the height of the peaks and the amount of background "noise" on the electropherograms varies considerably. It is possible that the small peaks that were identified as amplification of a "C" are not necessarily large enough to be reliably called. Continued work to detect the "C" allele, perhaps using other ethnic populations that have a higher frequency of that allele or working with highly diluted modern DNA, might clarify what we would expect to see in terms of peak height for each base.

The intermediate or high expression of the cytokine IL-6 (-174) is important for an effective T_h2 immune response in a host's defense against parasitic and fungal infection. The IL-6 genotypes detected in this ancient sample suggest that these individuals were able to mount an effective immune response against these types of pathogens. A foraging pattern of subsistence would have likely exposed hunter-gatherer populations to a constant load of parasitic and fungal pathogens resulting in selection for intermediate or high producers of IL-6. An individual who was genetically capable of producing high levels of IL-6 and was coping with an antibody mediated parasitic infection, might not been able to resist the devastating effects of pathogens requiring a cell-mediated immune response.

8.4.2 Interleukin-10 (-819) cytokine SNP in ancient human remains

Table 14 lists the frequency of the IL-10 (-819) genotypes that were detected in the ancient samples and compares the phenotype frequencies of these against that of the contemporary study population. The results obtained for IL-10 (-819) are particularly interesting because all three genotypes were detected in the small sample providing an excellent illustration of what we might expect to see for the different peak intensities on the electropherograms. The fact that the results that were obtained have not yet been replicated despite numerous attempts is a concern however; many options exist for modifying the method so that results might be reliably detected. A comparison of the genotype frequencies of the contemporary Aboriginal population to that obtained from the ancient samples shows that the frequencies are very similar.

Table 14. Frequency of IL-10 (-819) genotypes for the ancient individual and percent of phenotypes for the study populations

IL-10 (-819) phenotype	IL-10 (-819) genotype	Frequency of genotypes of ancient individuals	Percent of genotypes for the ancient study population	Percent of genotypes for the contemporary Aboriginal population	Percent of genotypes for the contemporary Caucasian population
High	C/C	1	25	37	59
Intermediate	C/T	2	50	42	32
Low	T/T	1	25	20	9

IL-10 has a key role in a T_h2 immune response and also functions to down-regulate a T_h1 response by inhibiting T_h1 cells and macrophage function (Benjamini et al. 2000). High and intermediate producers of IL-10 would have a selective advantage in an environment where parasitic and fungal infections predominated. Much more work is required to determine if the pattern observed in this ancient, study population is indicative of the larger population. However, the results suggest that the detection of this cytokine SNP in ancient individuals could contribute significantly to understanding the immunogenetic adaptation of Aboriginal populations.

8.4.3 *Tumor Necrosis Factorα (-308) cytokine SNP in ancient human remains*

The amplification and detection of the TNFα (-308) cytokine SNP followed a different method than was used for IL-6 (-174) or IL-10 (-819). The use of nested primers should improve the specificity of the amplification and is a preferable method to using booster PCRs. Boostering the PCR product through the addition of more Taq enzyme and PCR cycles, is a proven method for producing sufficient DNA for analysis from a small number of initial copies. However, the risk of contamination increases significantly. Using a series of primers to amplify a target region and then re-amplify a nested segment of the target region not only increases the specificity of the amplification but also is far less risky in terms of contamination. Designing nested primers around a specific nucleotide site is not always possible given the restrictions of working with fragmented ancient DNA. Despite the ability to design nested primers for TNF-α (-308), the results of detecting this SNP in ancient human remains was not as successful as hoped.

The primary reason why the results for TNFα (-308) were considered problematic was not the amplification and detection of the SNP, but that the frequency of the observed genotype in the ancient samples is quite different from that observed in the modern Aboriginal study population. Table 15 lists the frequency of the TNFα (-308) genotypes that were detected in the ancient samples and compares the phenotype frequencies of these against that of the contemporary study population.

Table 15. Frequency of TNFα (-308) genotypes for the ancient individual and percent of phenotypes for the study populations

TNFα (-308) phenotype	TNFα (-308) genotype	Frequency of genotypes of ancient individuals	Percent of genotypes for the ancient population	Percent of genotypes for the contemporary Aboriginal population	Percent of genotypes for the contemporary Caucasian population
High	A/A	0	0	1	2
Intermediate	A/G	4	100	10	29
Low	G/G	0	0	88	69

If the four ancient individuals are indeed all heterozygous at the TNFα (-308) SNP, then their genotypes differ from the expected frequency based on the genotypes of the modern Aboriginal population. Only 10% of contemporary Aboriginal individuals are heterozygous "A/G" for TNFα (-308). It would seem unlikely therefore, that 100% of an ancient population would have this genotype. It is unlikely that the ancient SNPs were contaminated by the researcher (L.L.) who is "A/G" for the TNFα (-308) SNP since the negative controls were not contaminated. In addition, a small section of the mtDNA HVRI region of each ancient sample was sequenced and the individual sequences were determined to be authentic. In light of the initial results obtained for TNFα (-308) the primers, ddNTPs, reagents and enzymes were subjected to scrutiny to assess the quantity and function of each chemical in the DNA amplification and SNP detection and to test for contamination. While no contamination was found it was discovered that the polymerase used in the SnaPshot kit (Amplitaq Gold®) has a "proof-reading" capability, which means that it adds a nucleotide, generally adenine, to the ends of the amplified DNA fragments. Terminal nucleotide additions do not occur with all types of polymerase and it is recommended that the polymerase that are used with primer extension assays should not have the proofreading activity (Syvanen 1999). Because the amount of sample was limited, the subsequent tests turned to amplifying cytokine loci that did not include adenine at the polymorphic site. The validity of the TNFα (-308) results therefore remains uncertain. The SNaPshot method needs to be tested using other primers that are designed to amplify cytokine loci that include adenine (i.e. IL-10 (-1082)) or alternatively to reassess the reaction.

The experiments with TNFα demonstrate how a modern control population can be used to identify inconsistencies in the ancient DNA work. While some change in the genotype frequency of the ancient and modern population might be expected, a dramatic shift would not. However, the results of the TNFα experiments cannot be entirely dismissed since the ancient sample size was small and there is a chance that the individuals were indeed heterozygotes. Two of the individuals for which the TNFα (-308) SNP was detected are from the same burial cairn and could well be paternally related. This potential relatedness could have biased the sample in which case, the results may in fact be accurate.

8.5 Ethnic differences observed in contemporary cytokine genotype frequencies in an evolutionary framework

The virgin-soil hypothesis is the most fundamental model that continues to guide how anthropologists think about pathogens and disease transmission in the New World (Black 1972; Cockburn 1971; Crosby 1976). However, archaeological, osteological, historical documentation, and now molecular evidence, have demonstrated a more complex interaction of North American Aboriginal populations to novel pathogens at the time of European contact (Decker 1989; Herring 1992; Ramenofsky 1987; Trimble 1985). The archaeological, osteological and historic records all indicate that the North American Aboriginal populations did not live in a pathogen-free environment (Larsen 1994; Merbs 1992; Powell 1992; Saunders et al. 1992). Certainly, new pathogens were brought to the New World by the early explorers; however, the archaeological, osteological and historic records all support the contention that the North American Aboriginal population did not live in a pathogen-free environment. Generally, it is hypothesized that some pathogens may have been filtered out during the human migration from Eurasia to the New World some 15,000 years ago (Merbs 1992). Some pathogens that had a long association with human hosts and that were tolerant of less temperate climates likely survived the trip into the New World. The predominate pathogens that affect hunter-gatherer's are those that are environmentally specific but do not necessarily differentiate between an animal or human host. Consequently, when the foragers made their home in the New World, some pathogens (*Rikettsia* for example),

which are comfortable in both animals and humans, likely quickly became a factor in Aboriginal health.

The archaeological and osteological record supports the contention that a number of diseases were present among the pre-contact populations of the New World. Infectious diseases that affect the growth or development of the skeleton are primarily those caused by bacteria. However, the frequency of occurrence of these pathogens is not always accurately represented in the archaeological record. The diagnosis from skeletal lesions in pre-contact human remains of tuberculosis, metabolic disorders, conditions of arthritis, treponema, and fungal and bacterial infections clearly demonstrate that the New World people were forced to adapt to pathogens and conditions that may have compromised their health. The range of diseases that may have affected the New World populations may not have been limited to those that are still present today. Additionally, diseases such as tuberculosis and influenza were likely present in both hemispheres. However, the molecular evidence suggests that the subspecies or strains of these disease-causing pathogens were likely different from those that exist more recently (Ramenofsky 2003). Indeed, the speed at which pathogens are known to mutate makes it very likely that diseases not present today might have affected past populations. In addition, many infectious diseases that cause death do not leave evidence on hard tissue. Viruses, plague, cholera, meningitis and dysentery all can cause acute infection and death, yet leave no evidence on skeletal remains. The molecular analysis of viral pathogens such as influenza has shown that the New World could have experienced episodes of influenza depending on whether or not a group had contact with the pathogens primary reservoir. In addition to the archaeological and osteological evidence, which rather conclusively demonstrates the occurrence of disease in New World pre-contact populations, the historic records at the time of contact demonstrate that the disease load changed. At the time of contact, the Aboriginal people still faced the immune challenges of their own pathogen environment; in addition, they encountered diseases and/or disease strains that were new.

The dramatic demographic collapse that occurred at the time of contact between Aboriginal and European descent explorers in the New World supports the virgin-soil hypothesis - that Aboriginal people had little or no immunity to the novel diseases such as measles, smallpox or influenza (Dobyns 1983). However, the occurrence of the virgin-

soil epidemics tends to overshadow the bio-archaeological and historic record that demonstrates that the Aboriginal individuals and populations experienced diseases caused by an array of native pathogens. The virgin-soil hypothesis also obscures the fact that the North American Aboriginals were likely genetically well adapted to resident pathogens, thereby ensuring the survival of the Aboriginal people as foragers living in dispersed and in some cases highly mobile and isolated groups.

Disease-causing pathogens (crowd –diseases such as measles, smallpox and plague) that are associated with factors such as an increase in population size, and the development of large settlements and urbanization in the Old World were notably absent in the New World among hunting and gathering populations. In the Old World, Cohen (1989) identified changes in the health and disease status of populations after the advent of agriculture during the Neolithic period. The types of infectious disease and the amount of stress placed on certain populations by disease changed because of modifications to subsistence and settlement patterns. Interestingly, a similar pattern is hypothesized for New World populations during the Middle Pre-contact period when a shift to sedentism, and the intensification of horticulture resulted in archaeologically detectable changes in the health of New World populations (Buikstra 1981; Cook 1984; Goodman et al. 1984). Neither the 12 th to 14th century Mississippians, nor the Iroquois villages ever reached the population size, density or persistence of the Old World early civilizations, however the osteological evidence indicates that the health of these populations changed significantly as they adopted a more settled life (Cook 1984; Goodman et al. 1984; Pfeiffer 1984; Saunders et al. 1992).

Indeed, the archaeological and osteological investigation of the early horticultural village populations in North America indicate that they were afflicted with diseases that were thought not to be present in the New World until the time of contact. Specifically, tuberculosis has been identified in these populations and has been diagnosed from skeletal remains that date to an even earlier period (Allison et al. 1981; Saunders et al. 1992). A comparative molecular analysis of *Mycobacterium tuberculosis* and *Mycobacterium bovis* surprised researchers when it was discovered that *bovis,* and not *tuberculosis* was found to be the more recent strain of *Mycobacterium* (Brosch et al. 2002). Molecular analysis of the species of *Mycobacterium* has laid open the possibility

that tuberculosis may be much older than previously thought and that stains of tuberculosis existed in antiquity that are no longer present today (Brosch et al. 2002; Donoghue et al. 2004; Ramenofsky 2003). It is also evident from the molecular analysis that diseases, that were once though to be absent from the New World, may have had an historic presence albeit in a modified form. Shigella, for example, was once thought to have developed with agriculture, but a comparison of some of the genetic code of the various strains of this bacteria suggests that it is far older than the Neolithic period and may have survived in small hunter gather bands (Pupo et al. 2000).

Molecular analysis of other disease-causing pathogens continues to complicate our understanding of the New World environment that was once thought to be disease free. For example, domesticated, as opposed to wild animals were thought to be integral to the occurrence and spread of influenza viruses. Although domesticated pigs and fowl were traditionally considered the reservoirs for influenza, gene sequencing of the pandemic influenza virus (Influenza A) has implicated wild migratory fowl as the primary reservoir of all influenza viruses and that whales, seals and horses can act as reservoirs for influenza (Cox and Subbarao 2000). It is not unrealistic to consider that strains of influenza may have been present in the New World long before the arrival of European-descent explorers.

It was observed that in a contemporary population the Aboriginal cohort maintains a high frequency of cytokine SNPs that enhance a T_h2 type immune response as compared to the SNPs maintained in the Caucasian cohort. The study population was composed of individuals who were enrolled in a study that examined the cytokine genotype of patients with end-stage renal disease at a Winnipeg health care center. The distinction between the Aboriginal and Caucasian cohorts (ethnicity) is based on loosely defined cultural constructs, but self-identification with either group serves as an important proxy indicator for genetic ancestry (Collins 2004). The contemporary Aboriginal study population, therefore, represents a relatively small regional cohort in relation to the larger North American Aboriginal population. However, the same pattern that was observed in this study population was validated in a separate, independent study of a larger population of normal Aboriginal and Caucasian individuals from a reserve in

Manitoba (Larcombe et al. 2005). If the observed pattern is indeed representative of the larger North American Aboriginal population, the results from the analysis of the ancient populations suggest that one might expect some regional or familial variation in cytokine genotype frequency given differential admixture and exposure to pathogens. More regional and temporal variation might exist in cytokine phenotypes than previously thought given the range of factors that have yet to be considered regarding Aboriginal disease history and biological adaptation. The fact that the frequency of the IL-6 (-174) SNP in the ancient study population deviated from the expected profile may support the contention that regional and temporal variations may have existed between and among pre-contact Aboriginal populations.

The contemporary Aboriginal population in our study maintained a high frequency of T_h2 cytokine SNPs than did the Caucasian even though conventional wisdom would suggest that individuals with a T_h2 genetic profile might have been at an immunologic disadvantage when faced with epidemics of measles, smallpox, and influenza epidemics at the time of contact. The important role of cytokine expression in disease outcome, and the differential distribution of SNPs in the cytokine promoter regions of ethnically diverse populations, makes it likely that selective forces influenced the contemporary genetic profile. However, a number of factors have likely influenced the maintenance of the high frequency of T_h2 cytokines in the Aboriginal population.

Early contact between Aboriginal people, missionaries, merchants and explorers was not uniform nor where the outbreaks of whooping cough, smallpox, influenza and tuberculosis. Indeed, economic and social factors played a major role in isolating certain populations while bringing others into constant or persistent contact with diseased people or communities. Social behaviors in response to the occurrence of disease in a community, settlement pattern, a community's ability to address nutritional needs and more recently, access to medical assistance, are factors that would have resulted in differential responses disease. In addition, the archaeological and osteological records indicate that infectious diseases such as treponema, certain strains of tuberculosis and parasitic and fungal infections that may have been specific to certain local environments differentially affected the Aboriginal populations. This research included only a relatively

small sample of the entire North American Native population but a closer look at the cytokine SNP frequencies in distinct groups of Aboriginal people that are culturally and geographically separated, might reveal differences in their immunogenetic profiles.

The differential spread of early disease outbreaks was likely influenced by socio-economic factors however, the interaction of the host's genetics with the novel pathogens and the existing disease load, likely influenced the intensity of the outbreak, the pattern of morbidity and mortality and the disease progression. It is likely that susceptibility and/or resistance to pathogens is influenced by a large number of polymorphic genes and there is not a single immunity gene. On-going research of the host's genetics support the contention that a number of gene polymorphisms that may contribute to disease susceptibility and/or resistance. In addition, a greater understanding of the highly complex host-pathogen interaction, their evolutionary relationship, and the environmental conditions that determine disease progression are vital for explaining differential susceptibility and/or resistance to pathogens. It is evident therefore that a simple Darwinian explanation of natural selection or the survival of the immunologically fittest, cannot explain the contemporary Aboriginal cytokine SNP frequency. Indeed, it is not possible to fully understand the contemporary Aboriginal susceptibility to certain infectious disease without exploring the genetic, biological and cultural factors that work together to influence an individuals experience with disease.

8.6 Future directions for SNP research

The analysis of human resistance and susceptibility to infectious disease must consider that the response to infectious diseases is a biological, social and evolutionary process. As such, the integration of research from archaeology, molecular anthropology, and immunogenetics can provide the longitudinal perspective required for exploring a populations' adaptation to their environment. This research explored a functional and evolutionary interpretation of the observed differences between the cytokine SNP frequencies maintained by the Aboriginal and Caucasian populations. As a result of this investigation, several avenues of research in both the contemporary and ancient contexts have been identified.

8.6.1 Ancient DNA research

In Manitoba, archaeologists have cultivated a working relationship with numerous First Nations communities over the last 5 to 10 years. Archaeologists from the Historic Resources Branch, the Manitoba Museum and the Universities of Winnipeg and Manitoba, have involved First Nations communities and individuals in archaeological research, field and laboratory work. Some First Nations communities, in collaboration with the Province and the University of Winnipeg, have a well-established recovery, bio-archaeological analysis, and reburial policy for human remains that are found within Aboriginal traditional land-use areas. This established procedure, and the working relationships that archaeologists have developed with certain First Nations communities, was critical in developing a strategy for assessing the immunogenetic markers in ancient Manitoba Aboriginal individuals.

The development of a method for detecting cytokine SNPs in ancient human remains from Manitoba required a careful strategy of processing samples from multiple individuals in a way that allowed for both development of a new procedure, and also for the collection of genetic data that would be useful for the First Nations. As such, while the identification of Native North American Haplogroup markers was key to validating the ethnicity of the individuals, the information was also used to assess potential maternal relatedness of certain individuals and to provide a more complete understanding of the individuals who were recovered in single burials or in related burial features. Similarly, the determination of sex using molecular techniques, served not only validate the recovery of nuclear genomic material, but also to provide specific biological information about the ancient individuals. This information was of value to the First Nations communities. At this stage of developing molecular techniques for investigating the immunogenetic profile of people in the past, it will be important to keep the First Nations communities involved in the goals, successes, and limitations of this type of research.

Continued collaboration with First Nations communities will be key to the future of molecular analysis of archaeologically recovered human remains in Manitoba.

The evaluation of the mitochondrial DNA haplogroup markers in the the ancient study population yielded partial profiles for fourteen individuals from Manitoba. This is the first time that haplogroups markers have been detected in the ancient Manitoba population. In order to contribute to the larger anthropological investigation of human migration patterns in the New World, a strategy for assessing mtDNA markers in ancient individuals should be established to facilitate the development of a regional database.

The analysis of DNA from ancient human remains from Manitoba, and more specifically the development of a method for the detection of SNPs in the cytokine promoter region of nuclear DNA, has broken new ground in ancient DNA research. The development of an alternative method to the modified SNaPshot procedure developed here, should be pursued so that a broader panel of cytokine SNPs can be detected. The experimentation with the TNFα (-308) SNP suggested that the proof-reading Taq in the SNaPshot kit was causing false results. Breaking apart the kit into it's constiuent parts - fluoresecently labelled ddNTPs, taq and reaction buffer - would allow the use of a more appropriate Taq (without the proof-reading capability) and more flexibility for amplifying SNPs. The development of an alterantive method for SNP detection to compliment the modified SNaPshot protocol, will allow for a more extensive investigation of the ancient cytokine SNP profile.

The ability to compare a broad panel of ancient cytokine SNPs in Manitoba Aboriginals, to that of their contemporary descent populations, may contribute to explaining the observed differences between the contemporary Aboriginal and Caucasian populations. In addition, the paleopathological and archaeological records indicate that some ancient North American Aboriginal populations were differentially exposed to

infectious diseases such as treponema, tuberculosis and rheumatoid arthritus. The analysis of the cytokine SNP profiles of affected individuals from these populations, and the consideration of the cultural distinctiveness of these groups, may shed considerable light on the relationship between these selective pressures and the maintainance of certain cytokine SNPs.

The host immunogenetics is adapted to a specific pathogen environment and is responsive to changes in that environment and likely to changes in the pathogen itself. Since cytokine expression is a key regulator of the immune response, it is suggested that the genes that regulate cytokine expression may be responsive to the selective pressures of a pathogen that itself is evolving. The amplification and typing of the molecular structure of pathogen causing diseases in ancient individuals has already proven to be important in exploring the host/pathogen relationship and has implications for understanding contemporary host/pathogen interaction (Brosch et al. 2002). The identification of the *Mycobacterium tuberculosis* in an ancient South American mummy has changed the longheld position that *Mycobacterium bovis* was the more ancient of the two types of tuberculosis (Brosch et al. 2002). Continued analysis of the ancient strains of tuberculosis, treponema and other infectious diseases, may play an important role in understanding the evolution of these diseases, their spread in human populations, and their evolution in relation to human populations.

Clearly, molecular anthropology has and will continue to make significant contributions towards understanding the complex nature of the host/pathogen relationship. Using the molecular techniques, not only to verify the presence of a pathogen, but to compare the structure of that pathogen to others in the past and present, will provide an important body of data for understanding a populations resistance and/or susceptibility to infectious diseases.

8.6.2 *Genetic analysis in contemporary Aboriginal populations*

This research identified that Aboriginal and Caucasian populations maintain differences in the frequency of SNPs in their cytokine promoter regions and it is suggested that this may be an adaptive response to differing environments. The interaction between host and pathogen is influenced by social and economic factors, however the genetic structure of both the host and the pathogen and their evolutionary relationship, may be key to understanding the current differential rates of disease prevalence. In Manitoba, DNA finger-printing of pathogens such as *Mycobacteria tuberculosis* revealed that multiple strains exist (Blackwood et al. 2003). This suggests a complex evolutionary history between mycobacterium strains and human hosts. Coupled with potential differences in the cytokine SNP profiles in Manitoba Aboriginal populations this might, in part, explain the occurrence of isolated outbreaks of tuberculosis among Aborignal people.

Complementary to this work, research involving the molecular characterization of the genome of mycobacteria, would provide a means of investigating the other half of the host/pathogen relationship. Ancient DNA work has changed our understanding regarding the relationship between an *Mycobacterium bovis* and *Mycobacteria tuberculosis.* The on-going research which has identified that multiple strains of *Mycobacteria tuberculosis* exist and that they differ in their virulence suggests that mycobacteria, and likely other pathogens have been and are responsive to selective pressures.

It is apparent from the archaeological and historic records that NewWorld Aboriginal populations experienced differences with respect to their exposure and experiences to disease both pre- and post-contact. As such, we might expect to see diffences in the cytokine SNP profiles of geographically and culturally distinct Aboriginal groups within Manitoba. A more detailed analysis of the cytokine SNP profiles of geographically and culturally distinct populations may reveal differences. Of

particular interest would be a comparison of individuals within the Aboriginal communities who are currently experiencing an infectious disease (i.e. tuberculosis) in contrast to those that are exposed but have not become diseased. With respect to tuberculosis, SNPs in the cytokine promoter regions of Interleukin-12 and Interleukin-4 have been implicated in disease susceptibility and therefore should be investigated along with the previously investigated panel of SNPs (Akahoshi et al. 2003; Bai et al. 2004; Flynn 2004; Muller-Berghaus et al. 2004). Expanding the research to a broader panel of cytokine SNPs and the inclusion of other resistance and/or susceptibility genes, that may exist in both ancient and contemporary populations in Manitoba should be explored.

8.7 Conclusions

This research has demonstrated that the frequencies of cytokine genotypes of TNFα (-308), IFNγ (+874), IL-10 (-1082), IL-10 (-819), IL-10 (-592), IL-6 (-174) and TGFβ codon 25 of the modern Aboriginal study population are statistically different from those of the Caucasian study population. This difference may be explained within an evolutionary and functional framework, which highlights the fact that environmental pressures may have selected for a strong T_h2 type of immune response in New World pre-contact Aboriginal populations. While this overall pattern may be true, a more complex situation likely existed where regionally and temporally dispersed North American Aboriginal groups may have been exposed to different pathogens.

It is possible, therefore, that pre-contact Aboriginal groups experienced differential selective pressures because of local and temporal variation in the pathogen environment. In some geographic regions, the pre-contact populations may have experienced episodes of selection for individuals who could produce enhanced levels of T_h1 immunity if they were exposed to viral or bacterial infections such as tuberculosis, treponema or influenza. On the other hand, some Native North American populations may have remained relatively isolated and may not have experienced pathogens that required a T_h1 immune response. Therefore, little or no selective pressure for cytokine SNPs that favour a T_h1 immune response would have occurred, or perhaps occurred only

recently. Despite the fact that some Aboriginal communities encountered pathogens that required strong T_h1 immune responses at the time of contact, it cannot be assumed that the disease load associated with the need for a high frequency of T_h2 cytokine SNPs, disappeared. There may have been a selective advantage therefore for maintaining a cytokine SNP profile that favoured the T_h2 immune response. Localized differential selective pressures, variations in the disease history of past populations, and more recently, differential access to medical care and treatment, may all have contributed significantly to the observed cytokine profiles of the ancient population.

The analysis of the cytokine profile in ancient populations may contribute to a better understanding of the relationship between disease and cytokine expression. A comparative examination of diseased and disease-free, pre-contact skeletal remains could contribute to understanding the selective pressures that existed. A more directed population approach to compare the cytokine profiles of different regional groups may shed light on the degree of genetic drift that may have occurred as a result of local disease events. A comparison between an incipient horticultural group and an established horticultural group that had experienced diseases and selection for T_h1 type immune response could begin to unwind the selective processes that served to differentiate the Aboriginal and Caucasian cytokine profiles. The North American Aboriginal populations are ideally suited for the study of the evolution of the cytokine profile because these groups maintained variability of subsistence and settlement patterns and they experienced different disease histories within a relatively recent timeframe.

The outcome of this research is that a method was developed that enables researchers to access the immunogenetic program of ancient individuals. This method for cytokine SNP amplification and detection still needs refinement and experimentation to replicate the cytokine SNPs for IL-10 and TNF-α. However, the preliminary results presented here suggest that this avenue of research will provide an exceptional way of assessing the evolutionary relationship between pathogens and the human immunogenetic program, and health in past populations.

Clearly, the characterization and functional analysis of the human immunogenetic profile in contemporary and past populations will contribute significantly towards understanding human resistance and susceptibility to infectious diseases. Although the ancient immunogenetic research is still in its infancy, methods for more complete and efficient detection of the genome of ancient individuals will provide a more detailed profile of these ancestral populations. In conjunction to this, a more detailed analysis of the immunogenetic profiles of geographically and culturally distinct groups within the contemporary Aboriginal population needs to be addressed. This research explored how the virgin-soil epidemic hypothesis is inadequate as an explanatory model for the post-contact Aboriginal demographic collapse and population recovery. The analysis of the cytokine SNP frequencies maintained by the contemporary Aboriginal population, within a functional, adaptive context, suggests a substantially complex pattern of evolution of the immune response. The virgin-soil epidemic hypothesis only describes the affect of novel pathogens on Native North Americans without exploring the specific, underlying causes. The combined analytical techniques of immunogenetics and ancient DNA have made a significant contribution to the development of an explanatory model for host/pathogen interaction. The research described in this thesis engaged the past and present to develop a functional and adaptive explanation for the observed immunogenetic profile observed in the contemporary Manitoba Aboriginal populations.

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Appendix A

Mitochondrial DNA sequences of Ancient Aboriginal Study Population

Accession # Paleo lab	Sample Name	Sequence Number	16021	16031	16041	16051	16061	16071	16081	16091	16101
	Cambridge Ref. Seq.		CTGTTCTTTC	ATGGGGAAGC	AGATTTGGGT	ACCACCCAAG	TATTGACTCA	CCCATCAACA	ACCGCTATGT	ATTTCGTACA	TTACTGCCAG
	Larcombe		**********	**********	**********	**********	**********	**********	**********	**********	**********
MSC0003-03	Eriksdale 110-1	117									
MSC0003-03	Eriksdale 110-1	304									
MSC0003-03	Eriksdale 110-1	79									
MSC0003-03	Eriksdale 110-1	88									
MSC0005-03	Eriksdale 110-2	80									
MSC0006-03	Red Deer River	307									
MSC0006-03	Red Deer River	329				**********	**********	**********	**********	**********	**********
MSC0007-03	River Mouth (ZW-44)	90	**********	**********	**********	**********	**********	**********	**********	**********	**********
MSC0008-03	River Mouth (ZW-45)	93		**********	**********	**********	**********	**********	**********	**********	**********
MSC0009-03	Valley River (ZW-205)	94	**********	**********	**********	**********	**********	**********	**********	**********	**********
MSC0009-03	Valley River (ZW-205)	305									
MSC0009-03	Valley River (ZW-205)	306									
MSC0009-03	Valley River (ZW-205)	238									
MSC0011-03	Hunters Brother	415									
MSC0011-03	Hunters Brother	430									
MSC0012-03	Valley River (ZW-206)										
MSC0013-03	Ochre Beach (ZW-134)	119									
MSC0013-03	Ochre Beach (ZW-134)	302									
MSC0013-03	Ochre Beach (ZW-134)	416									
MSC0013-03	Ochre Beach (ZW-134)	R141									
MSC0013-03	Ochre Beach (ZW-134)	239									
MSC00014	Drifting River (ZW-118)	303									
MSC00014	Drifting River (ZW-118)	417									
MSC00014	Drifting River (ZW-118)	240									
MSC0015-03	Whaley Cairn (ZW-68)	418									
MSC0015-03	Whaley Cairn (ZW-68)	R138									
MSC0016-03	Whaley Cairn (ZW-65)	419									
MSC0016-03	Whaley Cairn (ZW-65)	730									
MSC0016-03	Whaley Cairn (ZW-65)	258	**********	**********	**********	**********	**********	**********	**********	**********	**********
MSC0017-03	Pukatakwakan Bay (ZW-124)	420									
MSC0017-03	Pukatakwakan Bay (ZW-124)	733									
MSC0017-03	Pukatakwakan Bay (ZW-124)	118									
MSC0039-03	Two Eagles	731									

Mitochondrial DNA sequences of Ancient Aboriginal Study Population

Accession # Paleo lab	Sample Name	Sequence Number	16111	16121	16131	16141	16151	16161	16171	16181	16191
	Cambridge Ref. Seq.		CCACCATGAA	TATTGTACGG	TACCATAAAT	ACTTGACCAC	CTGTAGTACA	TAAAAACCCA	ATCCACATCA	AAACCCCCTC	CCCATGCTTA
	Larcombe		**********	**********	**********	**********	**********	**********	**********	********C*	******C***
MSC0003-03	Eriksdale 110-1	117									
MSC0003-03	Eriksdale 110-1	304									
MSC0003-03	Eriksdale 110-1	79									
MSC0003-03	Eriksdale 110-1	88									
MSC0005-03	Eriksdale 110-2	80									
MSC0006-03	Red Deer River	306									
MSC0006-03	Red Deer River	329	**********	**********	**********	**********	**********	**********	**********	**********	**********
MSC0007-03	River Mouth (ZW-44)	90	T*********	**********	**********	**********	**********	**********	**********	**********	**********
MSC0008-03	River Mouth (ZW-45)	93	**********	**********	**********	**********	*C********	**********	**********	*CC*****C*	
MSC0009-03	Valley River (ZW-205)	94	**********	**********	**********	**********	**********	**********	**********	**********	**********
MSC0009-03	Valley River (ZW-205)	305									
MSC0009-03	Valley River (ZW-205)	306									
MSC0009-03	Valley River (ZW-205)	238									
MSC0011-03	Hunters Brother	415									
MSC0011-03	Hunters Brother	430								**********	**********
MSC0012-03	Valley River (ZW-206)										
MSC0013-03	Ochre Beach (ZW-134)	119									
MSC0013-03	Ochre Beach (ZW-134)	302									
MSC0013-03	Ochre Beach (ZW-134)	416									
MSC0013-03	Ochre Beach (ZW-134)	R141									******C***
MSC0013-03	Ochre Beach (ZW-134)	239									
MSC0014-03	Drifting River (ZW-118)	303									
MSC0014-03	Drifting River (ZW-118)	417									
MSC0014-03	Drifting River (ZW-118)	240									
MSC0015-03	Whaley Cairn (ZW-68)	418									
MSC0015-03	Whaley Cairn (ZW-68)	R138									
MSC0016-03	Whaley Cairn (ZW-65)	419									
MSC0016-03	Whaley Cairn (ZW-65)	730									
MSC0016-03	Whaley Cairn (ZW-65)	258	**********	**********	**********	**********	**********	**********	**********	**********	**********
MSC0017-03	Pukatakwakan Bay (ZW-124)	420									
MSC0017-03	Pukatakwakan Bay (ZW-124)	733									
MSC0017-03	Pukatakwakan Bay (ZW-124)	118									
MSC0039-03	Two Eagles	131									

Mitochondrial DNA sequences of Ancient Aboriginal Study Population

Accession # Paleo lab	Sample Name	Sequence Number	16201	16211	16221	16231	16241	16251	16261	16271	16281
	Cambridge Ref. Seq.		CAAGCAAGTA	CAGCAATCAA	CCCTCAACTA	TCACACATCA	ACTGCAACTC	CAAAGCCACC	CCTCACCCAC	TAGGATACCA	ACAAACCTAC
	Larcombe		**********	**********	***C******	**********	**********	**********	*********T	**********	**********
MSC0003-03	Eriksdale 110-1	117							**********	**********	**********
MSC0003-03	Eriksdale 110-1	304					**********	**********	**********	**********	**********
MSC0003-03	Eriksdale 110-1	79			**T*******	**********	**********	**********	**********	**********	**********
MSC0003-03	Eriksdale 110-1	88			**T*******	**********	**********	**********	**********	**********	**********
MSC0005-03	Eriksdale 110-2	80			**T*******	**********	**********	**********	**********	**********	*********T
MSC0006-03	Red Deer River	306				**********	**********	**********	**********	**********	**********
MSC0006-03	Red Deer River	329	**********	**********	**T*******	**********	**********	**********	**********	**********	**********
MSC0007-03	River Mouth (ZW-44)	90	**********	**********	**T*******	**********	**********				
MSC0008-03	River Mouth (ZW-45)	93									
MSC0009-03	Valley River (ZW-205)	94	**********	**********	**T*******	**********					
MSC0009-03	Valley River (ZW-205)	305					**********	**********	**********	**********	**********
MSC0009-03	Valley River (ZW-205)	306						**********	**********	**********	**********
MSC0009-03	Valley River (ZW-205)	238					**********	**********	**********	**********	**********
MSC0011-03	Hunters Brother	415							**********	**********	**********
MSC0011-03	Hunters Brother	430	**********	**********	**T*******	**********	**********	**********			
MSC0012-03	Valley River (ZW-206)										
MSC0013-03	Ochre Beach (ZW-134)	119	**********	**********				**********	**********	**********	*********T
MSC0013-03	Ochre Beach (ZW-134)	302							**********	**********	*********T
MSC0013-03	Ochre Beach (ZW-134)	416					**********	**********	**********	**********	*********T
MSC0013-03	Ochre Beach (ZW-134)	R141	**********	**********	**T*******	**********	**********	**********	**********	**********	*********T
MSC0013-03	Ochre Beach (ZW-134)	239					**********	**********	**********	**********	*********T
MSC0014-03	Drifting River (ZW-118)	303									
MSC0014-03	Drifting River (ZW-118)	417						**********	**********	**********	*********T
MSC0014-03	Drifting River (ZW-118)	240						**********	**********	**********	*********T
MSC0015-03	Whaley Cairn (ZW-68)	418						**********	**********	**********	*********T
MSC0015-03	Whaley Cairn (ZW-68)	R138	**********	**********	**T*******	**********	**********	**********	**********	**********	*********T
MSC0016-03	Whaley Cairn (ZW-65)	419							**********	**********	*********G
MSC0016-03	Whaley Cairn (ZW-65)	730									
MSC0016-03	Whaley Cairn (ZW-65)	258	**********	**********	**T*******	**********	**********	**********			
MSC0017-03	Pukatakwakan Bay (ZW-124)	420						**********	**********	**********	*********T
MSC0017-03	Pukatakwakan Bay (ZW-124)	733									
MSC0017-03	Pukatakwakan Bay (ZW-124)	118						**********	**********	**********	*********T
MSC0039-03	Two Eagles	731						**********	**********	**********	*********T

Mitochondrial DNA sequences of Ancient Aboriginal Study Population

Accession # Paleo lab	Sample Name	Sequence Number	16291	16301	16311	16321	16331	16341	16351	16361	16371	16381
	Cambridge Ref. Seq.		CCACCCTTAA	CAGTACATAG	TACATAAAGC	CATTTACCGT	ACATAGCACA	TTACAGTCAA	ATCCCTTCTC	GTCCCCATGG	ATGACCCCCC	TCAGATAGGG
	Larcombe		**********	**********	C********* *	**********	**********	**********	**********	**********	**********	**********
MSC0003-03	Eriksdale 110-1	117	**********	**********	C*********	**********	**********	**********	**** *****	**********	**********	**********
MSC0003-03	Eriksdale 110-1	304	**********	**********	**********	**********	**********	**********	**********	**********	**********	
MSC0003-03	Eriksdale 110-1	79	**********	***C******	**********	**********	**********	**********	**********	**********	**********	**********
MSC0003-03	Eriksdale 110-1	88	**********	***C******	**********	**********	**********	**********	**********	**********	**********	**********
MSC0005-03	Eriksdale 110-2	80	**********	**********	**********	**********	**********	**********	**********	**********	**********	**********
MSC0006-03	Red Deer River	306	**********	**********	**********	**********	**********	**********	**********	*C********	**********	
MSC0006-03	Red Deer River	329	**********	**********	**********	**********	**********	**********				
MSC0007-03	River Mouth (ZW-44)	90										
MSC0008-03	River Mouth (ZW-45)	93										
MSC0009-03	Valley River (ZW-205)	94										
MSC0009-03	Valley River (ZW-205)	305	*******C**	**********	**********	**********	**********	**********	**********	**********	**********	**********
MSC0009-03	Valley River (ZW-205)	306	*T********	**********	**********	**********	**********	**********	**********	**********	**********	
MSC0009-03	Valley River (ZW-205)	238	*******C**	**********	**********	******T***	**********	**********	**********	**********	**********	**********
MSC0011-03	Hunters Brother	415	*T********	**********	**********	**********	**********	**********	**********	**********	**********	**********
MSC0011-03	Hunters Brother	430										
MSC0012-03	Valley River (ZW-206)					**********	**********	**********	**********	**********	**********	**********
MSC0013-03	Ochre Beach (ZW-134)	119	**********	**********	********A*	**********	**********	**********	**********	*C********	**********	**********
MSC0013-03	Ochre Beach (ZW-134)	302	**********	**********	********A*	**********	**********	**********	**********	*C********	**********	**********
MSC0013-03	Ochre Beach (ZW-134)	416	**********	**********	********A*	**********	**********	**********	**********	*C********	**********	**********
MSC0013-03	Ochre Beach (ZW-134)	R141	**********	**********	********A*	**********	**********	**********	**********	*C********	**********	**********
MSC0013-03	Ochre Beach (ZW-134)	239	*********C	**********	********A*	**********	**********	**********	**********	*C********	**********	**********
MSC0014-03	Drifting River (ZW-118)	303				**********	**********	**********	****TC****	*******C**	**********	*********A
MSC0014-03	Drifting River (ZW-118)	417	**********	**********	********A*	**********	**********	**********	**********	*C********	**********	**********
MSC0014-03	Drifting River (ZW-118)	240	**********	**********	********A*	**********	**********	**********	**********	*C********	**********	**********
MSC0015-03	Whaley Cairn (ZW-68)	418	**********	**********	**********	**********	**********	**********	**********	**********	**********	**********
MSC0015-03	Whaley Cairn (ZW-68)	R138	**********	**********	********A*	****C*****	**********	**********	**********	**********	**********	**********
MSC0016-03	Whaley Cairn (ZW-65)	419	***T***C**	**********	**********	****C*T***	**********	**********	**********	**********	**********	**********
MSC0016-03	Whaley Cairn (ZW-65)	730				****C*T***	**********	**********	**********	**********	**********	**********
MSC0016-03	Whaley Cairn (ZW-65)	258										
MSC0017-03	Pukatakwakan Bay (ZW-124)	420	**********	**********	********A*	**********	**********	**********	**********	*C********	**********	**********
MSC0017-03	Pukatakwakan Bay (ZW-124)	733							**********	*C********	**********	**********
MSC0017-03	Pukatakwakan Bay (ZW-124)	118	**********	**********	********A*	**********	**********	**********	**********	*C********	**********	**********
MSC0039-03	Two Eagles	731	**********	**********	********A*	**********	**********	**********	**********	*C********	**********	**********

Chart 1

Mitochondrial DNA sequences of Ancient Aboriginal Study Population

Accession # Paleo lab	Sample Name	Sequence Number	16391	16401	16411	16421	16431
	Cambridge Ref. Seq.		GTCCCTTGAC	CACCATCCTC	CGTGAAATCA	ATATCCCGCA	CAAGAGTGCT
	Larcombe		**********	**********	**********		
MSC0003-03	Eriksdale 110-1	117	**********	**********	**********		
MSC0003-03	Eriksdale 110-1	304					
MSC0003-03	Eriksdale 110-1	79	A*********	**********	**********		
MSC0003-03	Eriksdale 110-1	88	A*********	**********	**********		
MSC0005-03	Eriksdale 110-2	80	**********	**********	**********		
MSC0006-03	Red Deer River	306					
MSC0006-03	Red Deer River	329					
MSC0007-03	River Mouth (ZW-44)	90					
MSC0008-03	River Mouth (ZW-45)	93					
MSC0009-03	Valley River (ZW-205)	94					
MSC0009-03	Valley River (ZW-205)	305	**********	**********	**********		
MSC0009-03	Valley River (ZW-205)	306					
MSC0009-03	Valley River (ZW-205)	238	**********	**********	**********		
MSC0011-03	Hunters Brother	415	**********	**********	**********		
MSC0011-03	Hunters Brother	430					
MSC0012-03	Valley River (ZW-206)		**********	**********	**********		
MSC0013-03	Ochre Beach (ZW-134)	119	**********	**********	**********		
MSC0013-03	Ochre Beach (ZW-134)	302					
MSC0013-03	Ochre Beach (ZW-134)	416	**********	**********	**********		
MSC0013-03	Ochre Beach (ZW-134)	R141	**********	**********	**********		
MSC0013-03	Ochre Beach (ZW-134)	239	**********	**********	**********		
MSC0014-03	Drifting River (ZW-118)	303	********G*	**********	**********		
MSC0014-03	Drifting River (ZW-118)	417	**********	**********	**********		
MSC0014-03	Drifting River (ZW-118)	240	**********	**********	**********		
MSC0015-03	Whaley Cairn (ZW-68)	418	**********	**********	**********		
		R138					
MSC0016-03	Whaley Cairn (ZW-65)	419	**********	**********	**********		
MSC0016-03	Whaley Cairn (ZW-65)	730	**********	**********	**********		
MSC0016-03	Whaley Cairn (ZW-65)	258					
MSC0017-03	Pukatakwakan Bay (ZW-124)	420	**********	**********	**********		
MSC0017-03	Pukatakwakan Bay (ZW-124)	733	**********	**********	**********		
MSC0017-03	Pukatakwakan Bay (ZW-124)	118	**********	**********	**********		
MSC0039-03	Two Eagles	731	**********	**********	**********	**********	

Chart 2

Polymorphisms identified in Ancient Aboriginal Study Population

	Identified polymorphisms	16111	16152	16182	16183	16189	16197	16223	16224	16270	16290	16292	16294	16298	16304	16311	16319	16325	16327	16355	16356	16362
Accession # Paleo lab	**Sample Name**																					
	Cambridge Ref. Seq.																					
	Larcombe					B,X	None		None	None						None						
MSC0003-03	Eriksdale 110-1							A,C,D,X							None	None						
MSC0005-03	Eriksdale 110-2							A,C,D,X			A											
MSC0006-03	Red Deer River							A,C,D,X														A
MSC0007-03	River Mouth (ZW-44)	A						A,C,D,X														
MSC0008-03	River Mouth (ZW-45)		None	None	B,X	B,X		n/a														
MSC0009-03	Valley River (ZW-205)							A,C,D,X				None		C					C			
MSC0011-03	Hunters Brother							A,C,D,X				None										
MSC0012-03	Valley River (ZW-206)							n/a														
MSC0013-03	Ochre Beach (ZW-134)							A,C,D,X			A						A,B					A
MSC0014-03	Drifting River (ZW-118)							n/a			A						A,B			None	None	A
MSC0015-03	Whaley Cairn (ZW-68)							A,C,D,X			A						A,B	C,D				
MSC0016-03	Whaley Cairn (ZW-65)							A,C,D,X			C-G		D	C				C,D	C			
MSC0017-03	Pukatawakan Bay (ZW-124)							n/a			A						A,B					A
MSC0039-03	Two Eagles							n/a			A						A,B					A

Chart 2

Polymorphisms identified in Ancient Aboriginal Study Population

	Identified polymorphisms	16368	16390	16391	16398	Haplogroup Assignment
Accession # Paleo lab	Sample Name					
	Cambridge Ref. Seq.					None
	Larcombe					
MSC0003-03	Eriksdale 110-1			None		A,C,D,X
MSC0005-03	Eriksdale 110-2					A
MSC0006-03	Red Deer River					A
MSC0007-03	River Mouth (ZW-44)					A
MSC0008-03	River Mouth (ZW-45)					B,X
MSC0009-03	Valley River (ZW-205)					C
MSC0011-03	Hunters Brother					A,C,D,X
MSC0012-03	Valley River (ZW-206)					None
MSC0013-03	Ochre Beach (ZW-134)					A
MSC0014-03	Drifting River (ZW-118)	None	None		None	A
MSC0015-03	Whaley Cairn (ZW-68)					A,B,C,D
MSC0016-03	Whaley Cairn (ZW-65)					C,D
MSC0017-03	Pukatawakan Bay (ZW-124)					A
MSC0039-03	Two Eagles					A

Chart 3

Interleukin-6 (-174) Genotypes from Ancient Human Remains

	Interleukin-6 (-174)	Eriskdale 110-1 MSC0003-03	Valley River ZW-205 MSC0009-03	Valley River ZW-206 MSC00012-03 A	Ochre Beach MSC00013-03	Dauphin Historic MSC00011-03	Drifting River ZW-118 MSC00014-03	Whaley Cairn ZW-68 MSC00015-03	Whaley Cairn ZW-65 MSC00016-03	Pukatawakan Bay ZW-124 MSC00017-03	Fidler Mound MSC00029-04	Fidler Mound MSC00030-04 A
Replicate 1	Extraction #	65-03 (2:2)	28-04(1:5)	35-04	28-04(1:5)	28-04 (1:3)	28-04(1:3)	65-03(1:2)	68-03(1:2)	65-03(1:2)	28-04(1:1)	31-04 (1:1)
A	RESPCR#	396-2	367-1	430-04.1	367-2	367-3	380-4	396-3	396-4	396-5	380-5	383-4
	SNPCR#	400-2	369-1	430-04.1	369-2	369-3	388-4	400-3	400-4	400-5	388-5	390-4
	Genotype	G/G	G/G	G/G	G/G	G/G	G/G	G/G	no result	no result	G/G?	G/G
B	RESPCR#		380-1	432-04.1	380-3	380-2		396-1	396-2	396-3		
	SNPCR#		388-1	432-04.1	388-3	388-2		412-1	412-2	412-3		
	Genotype		G/G	G/C*	G/C	G/G		G/G	C/C	G/G		
Replicate 2	Extraction #	68-03 (2:3)	11-04(1:3)	36-04 (1:3)	11-04 (1:3)		37-04(1:4)	11- 04(1:3)	72-03 (1:3)	72-03 (1:3)	35-04	35-04
C	RESPCR#	396-1	170-5	440-04.2	170-4		443-04.3	170-1	431-2	431-3	430-04.2	430-04.3
	SNPCR#	400-1	179-5	440-04.2	179-4		443-04.3	179-1	436-2	436-3	430-04.2	430-04.3
	Genotype	G/G	no results	G/C*	G/C		G/G	G/C	G/G	G/G	G/G	G/G
D	RESPCR#	96-1	188-5		188-4			188-1				432-04.3
	SNPCR#	412-1	190-5		190-4			190-1				432-04.3
	Genotype	G/G	G/C		G/C			No results				G/C*
Replicate 3	Extraction #	72-03 (2:4)								11-04(1:4)	36-04	36-04
E	RESPCR#	431-4								170-3	440-04.3	440-04.4
	SNPCR#	436-4								179-3	440-04.3	440-04.4
	Genotype	G/G								G/G?	G/C *	G/C*
F	RESPCR#									188-3		
	SNPCR#									190-3		
	Genotype									No results		
Replicate 4	Extraction #	11-04(2:5)										
G	RESPCR#	170-2										
	SNPCR#	179-2										
	Genotype	G/G										
H	RESPCR#	188-2										
	SNPCR#	190-2										
	Genotype	No results										

*- indicates unequal peak heights ?- indicates a result that is borderline acceptable

Interleukin-6 (-174) Genotypes from Ancient Human Remains

	Interleukin-6 (-174)	Fidler Mound MSC00030-04B	Fidler Mound MSC000031-04 B	Fidler Mound MSC000031-04 A	MSC00039-03 Two Eagles	Red Deer River MSC006-03 B
Replicate 1	Extraction #		35-04	35-04	37-04(1:3)	37-04 (2:2)
A	RESPCR#		432.4.4	430-04.4	443-04.5	443-04.2
	SNPCR#		432.4.4	430-04.4	443-04.5	443-04.2
	Genotype		G/C*	G/G	G/G	G/G
B	RESPCR#			432-04.4		
	SNPCR#			432-04.4		
	Genotype			G/C*		
Replicate 2	Extraction #	35-04				
C	RESPCR#	432.4.3				
	SNPCR#	432.4.3				
	Genotype	G/C*				
D	RESPCR#					
	SNPCR#					
	Genotype					
Replicate 3	Extraction #					
E	RESPCR#					
	SNPCR#					
	Genotype					
F	RESPCR#					
	SNPCR#					
	Genotype					
Replicate 4	Extraction #					
G	RESPCR#					
	SNPCR#					
	Genotype					
H	RESPCR#					
	SNPCR#					
	Genotype					

Interleukin-10 (-819) Genotypes from Ancient Human Remains

	Interleukin-10 (-819)	Eriskdale 110-1 MSC0003-03	Valley River ZW-205 MSC0009-03	Valley River ZW-206 MSC00012-03 A	Ochre Beach MSC00013-03	Dauphin Historic MSC00011-03	Drifting River ZW-118 MSC00014-03	Whaley Cairn ZW-68 MSC00015-03	Whaley Cairn ZW-65 MSC00016-03	Pukatawakan Bay ZW-124 MSC00017-03	Fidler Mound MSC00029-04	Fidler Mound MSC00030-04 A
Replicate 1	**Extraction #**	11-04(2:5)	Not Tested	11-04(1:3)	11-04(1:3)	Not Tested	37-04	11-04(1:3)	Not Tested	11-04(1:4)	Not Tested	Not Tested
A	**RESPCR#**	171-2		171-5	171-4		444-04.4	171-1		171-3		
	SNPCR#	180-2		180-5	180-4		444-04.4	180-1		180-3		
	Genotype	no results		no results	T/T		C/T	C/T		C/C		
B	**RESPCR#**	171-2		171-5	171-4			171-1		171-3		
	SNPCR#	191-2		191-5	191-4			191-1		191-3		
	Genotype	no results		no results	no results			no results		no results		
Replicate 2	**Extraction #**											
C	**RESPCR#**											
	SNPCR#											
	Genotype											
D	**RESPCR#**											
	SNPCR#											
	Genotype											

Chart 4

Interleukin-10 Genotypes from Ancient Human Remains

	Interleukin-10 (-819)	Fidler Mound MSC00030-04B	Fidler Mound MSC000031-04 B	Fidler Mound MSC000031-04 A	Two Eagles	Red Deer River MSC006-03 B
Replicate 1	**Extraction #**	Not Tested	Not Tested	Not Tested	Not Tested	Not Tested
A	**RESPCR#**					
	SNPCR#					
	Genotype					
B	**RESPCR#**					
	SNPCR#					
	Genotype					
Replicate 2	**Extraction #**					
C	**RESPCR#**					
	SNPCR#					
	Genotype					
D	**RESPCR#**					
	SNPCR#					
	Genotype					

Chart 5

TNF-α (-308) Genotypes in Ancient Human Remains

		Tumour Necrosis Factor-a (-308)	Eriskdale 110-1 MSC0003-03	Valley River ZW-205 MSC0009-03	Valley River ZW-206 MSC00012-03 A	Ochre Beach MSC00013-03	Dauphin Historic MSC00011-03	Drifting River ZW-118 MSC00014-03	Whaley Cairn ZW-68 MSC00015-03	Whaley Cairn ZW-65 MSC00016-03	Pukatawakan Bay ZW-124 MSC00017-03	Fidler Mound MSC00029-04	Fidler Mound MSC00030-04 A
Replicate 1		Extraction #	65-03 (2:2)	Not Tested	Not Tested	Not Tested	Not Tested	Not Tested	65-03(1:2)	68-03(1:2)	65-03(1:2)	Not Tested	Not Tested
	A	RESPCR#	398-2						398-3	398-4	398-5		
		SNPCR#	399-2						399-3	399-4	399-5		
		Genotype	G/A						G/A	G/A	G/A		
	B	RESPCR#							402-3	402-2	402-4		
		SNPCR#							406-3	406-2	406-4		
		Genotype							G/A	G/A	G/A		
Replicate 2		Extraction #	68-03 (2:3)										
	C	RESPCR#	398-1										
		SNPCR#	399-2										
		Genotype	G/A										
	D	RESPCR#	402-1										
		SNPCR#	406-1										
		Genotype	G/A										

Chart 5

TNF-α (-308) Genotypes in Ancient Human Remains

	Tumour Necrosis Factor-a (-308)	Fidler Mound MSC00030-04B	Fidler Mound MSC000031-04 B	Fidler Mound MSC000031-04 A	Two Eagles	Red Deer River MSC006-03 B
Replicate 1	**Extraction #**	Not Tested	Not Tested	Not Tested	Not Tested	Not Tested
A	**RESPCR#**					
	SNPCR#					
	Genotype					
B	**RESPCR#**					
	SNPCR#					
	Genotype					
Replicate 2	**Extraction #**					
C	**RESPCR#**					
	SNPCR#					
	Genotype					
D	**RESPCR#**					
	SNPCR#					
	Genotype					