Pacific People, Metabolic Disease and Evolutionary Processes

A MITOCHONDRIAL DNA STUDY

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Abstract

Data clearly indicate that there is a high burden of metabolic disease including gout, type 2 diabetes, and cardiovascular heart disease, among Polynesians. Many of the metabolic diseases have a shared aetiology and often present as comorbidities. To date, a number of studies have been undertaken, with varying success, attempting to disentangle the genetic and environmental components of these complex diseases.

The high disease burden has led some to propose that Pacific populations possess a 'Thrifty Genotype'. Here I argue that not only do the data not support this hypothesis, but that this is not in line with what is known of the evolutionary history of the region. Instead, a model for selection by a mechanism of infectious disease resistance is proposed. All of these metabolic conditions have a significant immunological component, namely the involvement of the NLRP3 inflammasome, an important component of innate immunity.

A number of recent studies have indicated that mitochondria play an integral role in the activation of the NLRP3 inflammasome. Thus, a study of mitochondrial genetic variation was undertaken, exploring how genetic variation in mitochondrial DNA (mtDNA) could contribute to differences in immune response and altered metabolic efficiency. Gout was used as a proxy for altered immune response and obesity as an indicator for metabolic efficiency. Whole mitochondrial genome sequencing was undertaken on 442 Māori and Polynesian men, selected from a cohort of individuals participating in a study of gout genetics.

Few genetic associations were detected between either of these proxies, however there was a statistically significant association detected between variation in the region of the poly-cytosine tracts (16179-16189) in hypervariable region 1 (HVR1) and gout status. There was some indication that sequence length heteroplasmy in this region (which coincided with individuals possessing mitochondrial genomes belonging to the B macrohaplogroup) was associated with gout.

Mitochondrial DNA copy number was also examined in the context of gout. Quantitative PCR was undertaken to measure the relative amount of mtDNA in 484 Polynesians. There appeared to be a general reduction in the amount of mtDNA in participants with gout compared to healthy controls. Given the involvement of the mitochondria in the co-localisation of components of the

NLRP3 inflammasome, this novel finding may explain some of what is occurring in the inflammatory processes underlying gouty disease, and possibly other metabolic diseases more generally.

From this preliminary study, it appears that mitochondrial genetics may provide insights into susceptibility to metabolic conditions. This has wider implications when it comes to considering genetic ancestry and disease susceptibility, and may explain some of the high prevalence of metabolic disease among Polynesian and other Pacific populations.

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I know I will have missed people out, as there are so many people who have come into my life and been kind and helpful in the last three years, so what follows is by no means an exhaustive list of those to whom I am grateful. Please don't feel offended if I have missed you out. It is not a deliberate snub, and more likely down to my thesis-frazzled brain. Listed here are just those who would fit within my self-inflicted one page limit. (Sorry mum and dad – no space for you!)

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ABCG2: Gene encoding the protein ABCG2 (ATP-binding cassette sub-family G member 2), a gene that has been found to associate with gout

ASC: Apoptosis-associated speck-like protein containing a CARD (a component of the NLRP3 inflammasome)

ATP: Adenosine triphosphate

BMI: Body mass index (kg/m^2)

BP: years before present

bp: base pairs (DNA)

BWA: Burrows-Wheeler Alignment

CI: Confidence interval

C_t: Cycling threshold

Cyclic AMP: Cyclic adenosine monophosphate

DAMPs: Damage-associated molecular pattern

ΔC_t: relative amount of DNA ascertained by calculating the difference in C_t between a sequence of interest and a reference sequence

DISH: Diffuse idiopathic skeletal hyperostosis

DNA: Deoxyribonucleic acid

EHH: Extended haplotype homozygosity

FTO: A gene found to correlate with obesity

G6PD: Glucose-6-phosphate dehydrogenase

GATK: Genome Analysis Tool Kit

HDL: High density lipoprotein

HLA: Human leukocyte antigen

HVR1: Hyper-variable region I

IAPP: Islet amyloid polypeptide

IGV: Integrated Genomics Viewer

iHS: Integrated haplotype score

IL-1β: Interleukin 1β

ISEA: Island South-east Asia

kB: kilo-bases (DNA)

kya: thousand years ago

LDL: Low density lipoprotein

LEPR: Gene for the LEPR (leptin receptor) protein

MAF: Minor allele frequency

MAVS: Mitochondrial antiviral-signalling protein

MSU: Monosodium urate

mtDNA: mitochondrial DNA

NGS: Next generation sequencing

NET: Neutrophil extracellular trap

NLRP3: NACHT, LRR and PYD domains-containing protein 3; component of NLRP3 inflammasome

NUMT: Nuclear mitochondrial DNA

OECD: Organization for Economic Cooperation and Development

OR: Odds ratio

OXPHOS: Oxidative phosphorylation

PAMPs: Pathogen-associated molecular pattern

PCR: Polymerase chain reaction

PGC1α: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha, encoded by the gene *PPARGC1A*

Poly-C: Poly-cytosine

PPARGC1A: Gene encoding the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α)

PPARGC1B: Gene encoding the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1β)

qPCR: Quantitative polymerase chain reaction

rCRS: Revised Cambridge reference sequence (mtDNA)

RNA: Ribonucleic acid

rRNA: Ribosomal RNA

ROS: Radical oxygen species

SKAT: Sequence kernel association test

SLC2A9: Gene encoding the urate transport SLC2A9 *solute carrier family 2, facilitated glucose transporter member 9)

SNP: Single nucleotide polymorphism

SU: Serum urate

TERT: Telomerase reverse transcriptase

TFAM: Mitochondria transcription factor A

TLR2/4: Toll-like receptor 2/Toll-like receptor 4

tRNA: Transfer RNA

TXNIP: Thioredoxin interacting protein

VCF: Variant call file

1 Introduction

1.1 Opening statements

This thesis is founded on two undeniable facts: 1) Metabolic disease is a problem among Māori and Pacific peoples; 2) These populations share a common ancestry that may be contributing to the development of these diseases. As such, exploring ancestry, the process of colonisation and the evolutionary history of the people living in this geographic region is pertinent to understanding their health. This thesis explores the genetic predisposition to metabolic disease, its prevalence and epidemiology in the Pacific, and aims to propose and test some hypotheses regarding the geographic patterning of disease in the region.

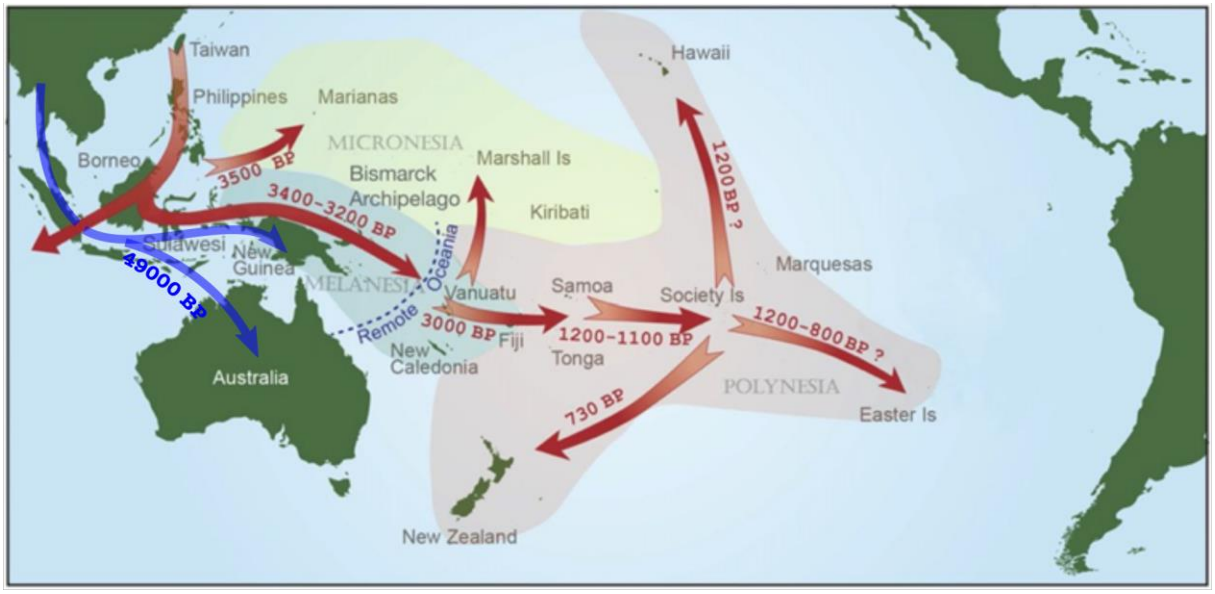


Figure 1.1: Dates for the Pacific settlement. Blue lines indicate the timing and expansion of the first groups of people into what was then Sahul (prior to sea level rise), while the red lines indicate the movement of Austronesian-speaking colonists (Modified from Matisoo-Smith, 2015). Base map courtesy of Ceridwen Fraser.

1.2 Background

The Pacific region spans a huge geographical area and is the home to a large number of culturally diverse people. Here I focus mainly on Pacific Islands, in particular Polynesians. When considering human variation in the Pacific it is important to consider how the region was settled (Figure 1.1). There have been multiple movements of people into the Pacific region throughout the 50,000 year history of human occupation. People first arrived on the continent of Sahul, which became New Guinea and Australia with subsequent sea level changes, at least 49,000 years ago (Summerhayes et al., 2010b). Genetic analyses have shown that there were probably

multiple populations involved in these Late Pleistocene and Early Holocene migrations out into New Guinea and the Solomon Islands, a region known as Near Oceania (Rasmussen et al., 2011). Subsequently, there have been several influxes of people from the Island Southeast Asia region into the islands of the Bismarck Archipelago and beyond as early as 5,000 years ago but certainly by some 3,350 years ago with the appearance of the Lapita cultural complex (Summerhayes et al., 2010a, Kirch et al., 1989). These people mixed with resident coastal populations prior to expanding out into the wider Pacific where they were the initial colonists on the previously unoccupied islands of Remote Oceania. The archaeological sites associated with these early Lapita colonists have distinctive trademarks, including highly decorated dentate-stamped pottery, which has been identified in sites from the Bismarck Archipelago, the Reef Santa Cruz (Southeast Solomon Islands), Vanuatu, Fiji, New Caledonia (Bedford et al., 2006) and as far east as Tonga (Burley et al., 2012) and Samoa (Spriggs, 2011). This settlement occurred very rapidly – with the earliest Lapita sites in New Ireland dating to ~3,350 years ago, and the latest settlements in Tonga only 2830-2846 years ago (Burley et al., 2012). There was a delay before the settlement of East Polynesia, where all the main island groups were settled within a three hundred year window between AD 1025-1290 (Wilmshurst et al., 2011).

While there is likely to have been ongoing small-scale movements of people between archipelagoes with continuous contact and trade networks, the next major movement of people into the region were the 18th and 19th century movements of Europeans. This has had a more profound effect in some populations than others. For instance, in New Zealand and Hawai'i there was largescale European settlement compared to Tonga which maintained its own sovereignty, which has likely resulted in differing amounts of acculturation and admixture.

The recent nature of the initial settlement of Remote Oceania has significant implications surrounding the cultural and genetic similarities and differences between populations in the region. Polynesians and Micronesians have been found to be more genetically similar to certain Asian populations than to many Near Oceanic populations which are highly heterogeneous (Friedlaender et al., 2008). Phenotypic and genotypic differences within Polynesia are more subtle but evident (Phipps-Green et al., 2010). Because of these differences in ancestry, there are both genetic and phenotypic differences between Pacific populations, even those within a reasonably close geographical distance to one another and with shared Lapita ancestry.

Overlying this shared ancestral background of Polynesians and other Pacific people, there is an overrepresentation of metabolic disease burden. This is particularly evident among Māori and Pacific people in New Zealand compared to New Zealanders of other ancestral backgrounds

(Figure 1.2). Included in this bundle of metabolic diseases are gout, type 2 diabetes, obesity and cardiovascular disease, conditions which often present as comorbidities (Winnard et al., 2013).

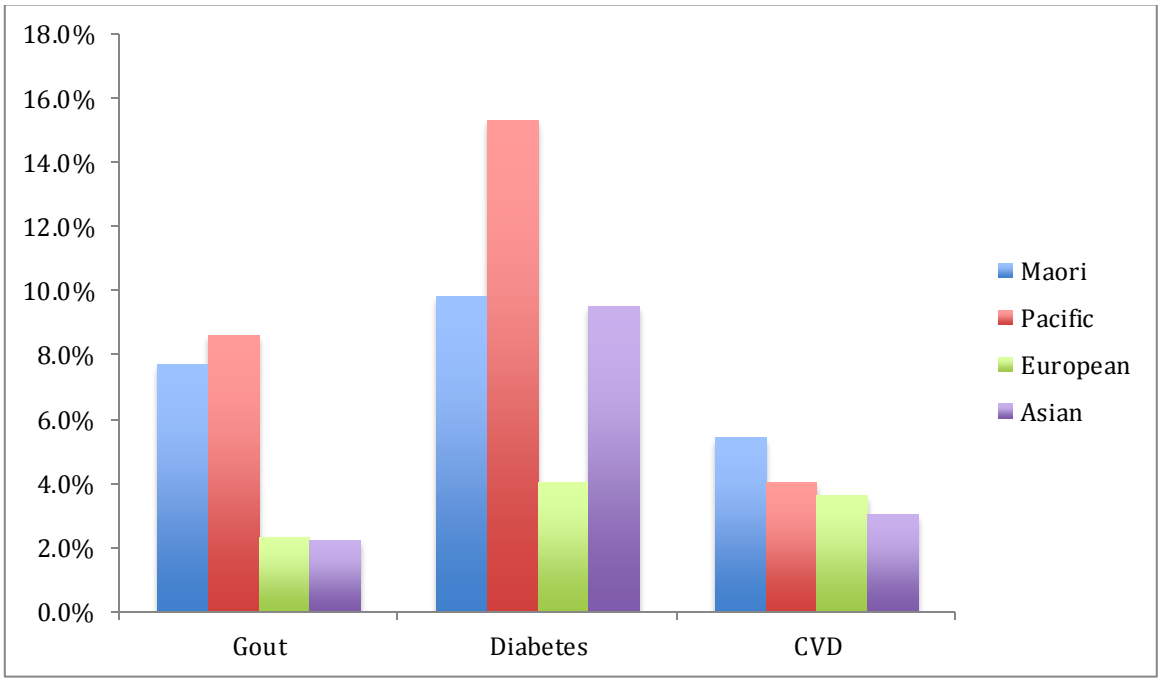


Figure 1.2: Age-standardised prevalence of metabolic diseases among New Zealanders of different ancestry (Winnard et al., 2013)

The Merriman Laboratory and their collaborators have collected DNA samples from a large cohort of Māori and Pacific people for the purposes of gout research. As such, gout will be primarily focussed on in this thesis, however, the other prevalent metabolic diseases will be discussed at points because of the likelihood of shared aetiological factors between them.

Gout is a chronic metabolic disease defined by inflammatory arthritis, with a prerequisite state of hyperuricaemia (serum urate levels exceeding 0.42 $mmolL^{-1}$). High serum urate (SU) levels can result in nucleation and formation of monosodium urate (MSU) crystals, which accumulate in synovial joints. The subsequent inflammatory response to these crystals results in the onset of gouty symptoms, including inflamed joints and extreme pain. Usually gout presents as a series of acute attacks, which appear swiftly and are reasonably rapidly resolved. However, when left untreated, chronic gout may develop where bouts are more prolonged and damage to bony tissue may result (Dalbeth et al., 2009).

The aetiology of gout is not well understood; MSU crystals have been isolated from hyperuricaemic patients not suffering gout, so clearly there are other unknown factors contributing, however its development is reliant on elevated SU levels in the patient. The development of hyperuricaemia is better understood and is known to have a multifactorial causation. There is a genetic component, with ~30 loci implicated in the control of SU levels

(Köttgen et al., 2012). Variants in the genes *SLC2A9* and *ABCG2*, in particular, have been found to have strong effects, accounting for ~4% of variation in serum urate levels in European Caucasians (Köttgen et al., 2012), and are a significant cause of gout in Māori and Pacific Island New Zealanders (Hollis-Moffatt et al., 2009, Phipps-Green et al., 2010).

In New Zealand, gout is of particular interest because of its previously mentioned high prevalence among Māori and Pacific people, particularly Polynesians (Winnard et al., 2012). Recently, it was found that not only do Māori and Pacific people in New Zealand have a higher gout prevalence, but they also have an earlier age of onset of gout (on average earlier by 9 years), a higher flare frequency and more features of joint inflammation than non-Māori/Pacific (Dalbeth et al., 2013a).

Given the shared ancestral background of Māori and Polynesians, it is reasonable to suggest that there is a shared innate genetic predisposition contributing to the higher prevalence of gout. While this concept has been widely accepted, it has often been assumed that gout in the Pacific is a phenomenon related to the changes away from traditional diets and lifestyles impacting upon a genetic predisposition, with some commentators (Lenane et al., 1960, Rose, 1975, Johnson & Rideout, 2004, Schlesinger, 2005, Álvarez-Lario & Macarrón-Vicente, 2010, Merriman & Dalbeth, 2011) suggesting that gout prior to the twentieth century was uncommon. Contact with Europeans has changed traditional life particularly in terms of diet and activity patterns (Waqanivalu, 2010). This is most evident in places like New Zealand and Hawai'i, where the indigenous peoples have become a minority within the entire population, and significant urbanisation and industrialisation have been introduced. Most Pacific Islands have similarly been influenced by western cultures, becoming more dependent on imported foods, as the populations have risen beyond what the traditional agricultural practices can support. The high prevalence of a range of metabolic diseases including type 2 diabetes, obesity and cardiovascular disease can be attributed to the high energy foods and increasingly sedentary lifestyles in modern Pacific nations (Sundborn et al., 2008). Gout is part of this spectrum of diseases.

There have been a number of studies identifying genetic variants that appear to be contributing to the elevated urate levels that underlie the high gout prevalence. These are also increasingly focusing on population-specific effects (Phipps-Green et al., 2010, Hollis-Moffatt et al., 2012, Flynn et al., 2013, Phipps-Green et al., 2016). However, when, how and why this probable genetic predisposition to hyperuricaemia and gout developed is something that has not been explored extensively. Further consideration of the population histories of Pacific people may help elucidate potential reasons for this phenomenon.

Not all possibilities of genetic inheritance in relation to gout have undergone systematic analysis. Most of the loci which have previously been found to be contribute to gout or hyperuricaemia susceptibility in Polynesians as yet, have previously been identified in larger cohorts elsewhere (e.g. (Dehghan et al., 2008, Woodward et al., 2009, Köttgen et al., 2012), etc.). This is consistent with the common disease, common variant hypothesis, wherein common disease-causing alleles will be found in all human populations, though may exist at varying frequencies (Pritchard & Cox, 2002).

Mitochondrial DNA (mtDNA), a small extra-nuclear genome within the mitochondria of each cell, has not undergone systematic testing in relation to gout, despite its adaptive potential. Mitochondria are organelles that play an essential role in energy production and immune processes. mtDNA encodes genes that are essential for mitochondrial function and population-specific variation is known to exist within mitochondrial genomes worldwide. Genetic variation within this DNA may influence immune response, something that is salient in auto-inflammatory conditions like gout and other metabolic diseases.

1.3 Motivation for the study

Ethnographic studies carried out in Māori gout sufferers suggest that there is a great sense of embarrassment, self-blame and guilt associated with gouty disease (Lindsay et al., 2011, Te Karu et al., 2013). This, in some, leads to a reluctance to seek medical help to manage the disease, which would improve the quality of life of the gout sufferers. Urate-lowering therapies can significantly reduce the likelihood of gout flares. This reluctance to seek help is not unique to gout, and crosses over to other metabolic conditions such as type 2 diabetes where there is a three- and four-fold higher prevalence of undiagnosed disease among Māori and Pacific people compared to New Zealand Europeans respectively (Joshy & Simmons, 2006). Through this work of examining the evolutionary context in which susceptibility to gout and other metabolic disease-contributing variants may have developed, it is hoped that it may lessen some of this stigmatisation.

One aim is to investigate a historic precedent for gout among Māori and other Pacific people, something that has been hinted at in the palaeopathological literature (Buckley, 2011), but which has not yet been fully embraced by the medical community. Establishing and understanding the history of this disease would imply that it is not only changes in the dietary environment and lifestyle habits of gout sufferers that is contributing to disease, but there is likely a pre-existing genetic predisposition which pre-dates Polynesian settlement. Raising awareness of this history of gout may take some of the onus off the individuals themselves and encourage them to seek treatment.

Also, by studying gouty disease in Polynesians, it may be possible to identify fundamental metabolic and immunological processes that contribute to the development of disease. Understanding these underlying immunological processes may allow for alternative drug therapies to be applied. It may also allow for the identification of other at-risk Pacific populations, allowing targeted education and awareness before the levels of disease increase to a level at which they will be recognised.

1.4 Purpose of the study

Seldom in academia does something fall firmly within a single field of research. With the increasing amounts of knowledge accumulating as academic pursuits continue, there is a growing need for interdisciplinary research. Genetics, immunology, archaeology, rheumatology; these all have facets which are shared and feed into the other, embroidering each other to make the complex picture that exists in the world around us. It is naive to work on the genetics of metabolic diseases without considering the people who are suffering these diseases, the populations to which they belong, the cultural and physical environments they inhabit, and their evolutionary histories. It is also naive to study the genetics of metabolic disease without considering the biology of the diseases themselves, the interactions between innate immunity and the metabolic system, the immune pathways, the role of organelles like mitochondria, and the complex and bemusing web of cytokine interactions.

While interdisciplinary research has increased in recent times, there are still distinct divisions between fields of research. Reading widely in multiple disciplines, in the case of this thesis in the fields of archaeology, biological anthropology, medical genetics and immunology, assists in the synthesis of diverse ideas and the development of new hypotheses that may contribute to our understanding of disease. This effectively is the purpose of this thesis.

The evolutionary and biocultural perspectives of biological anthropological research have the potential to enhance medical genetics studies. There has been increasing recognition of the need to consider inter-population differences in medical genetics studies because of differences in population histories contributing to variation in allele frequencies of risk variants and even novel risk loci (Rosenberg et al., 2010). While *Homo sapiens* as a species arose in Africa some 200,000 years ago, there have been multiple migrations out of Africa, with populations both within and outside of Africa being exposed to different environments, pathogens (Fumagalli et al., 2011, Karlsson et al., 2014), subsistence patterns (Hancock et al., 2010), with different timings of migrations and interactions with extinct hominin species (Reich et al., 2011, Huerta-Sanchez et al., 2014) – all things that are likely to be contributing to genotypic and ultimately phenotypic variation in modern populations. Combined with other factors like recent admixture

and changing lifestyles following increasing industrialisation, this variation contributes to metabolic disease susceptibility. These are all things that require acknowledgment when dealing with human variation, whether looking at human variation from an anthropological viewpoint or from a medical one.

The study aims to examine the population history and the patterning of disease prevalence across the Pacific region to see whether there are trends that make sense in an evolutionary context. The prehistoric settlement of the Pacific is a process which is increasingly being disentangled with ongoing archaeological, linguistic and genetic studies, and incorporating this knowledge into studies of medical genetics may help elucidate reasons for the high prevalence of metabolic conditions in these peoples.

An understanding of the population histories in the region allows for the generation of evolutionary explanations for the high burden of metabolic disease. There are a number of pre-existing hypotheses, such as the 'Thrifty Genotype' hypothesis, which have been put forth which will be assessed in light of the evolutionary history of the region. A novel hypothesis is suggested which poses that selection by infectious disease exposure may have contributed to susceptibility to metabolic disease, particularly via selection of genetic variants contributing to the NLRP3 inflammasome, an important innate immune response which is inherently involved in the inflammation characteristic of metabolic diseases such as gout and type 2 diabetes.

1.4.1 Specific aims

- To establish a historical precedent for the high rates of gout through examination of the historic literature – is gout really a modern phenomenon and something just seen in a few remarkable archaeological sites?
- To explore potential evolutionary explanations for the elevated prevalence of gout and other metabolic diseases among Polynesian populations;
- To investigate how the mitochondrial genome may affect metabolic disease, including susceptibility to gout.

1.5 Significance of the research

The significance of the research is twofold. A new hypothesis regarding the susceptibility to hyperuricaemia and gout is proposed with consideration of the evolutionary history of the region. While this does not get tested using selection statistics such as the integrated haplotype score (iHS) (Voight et al., 2006) or extended haplogroup homozygosity (Sabeti et al., 2002), it represents an alternative to the current out-dated models such as the 'Thrifty Genotype' which are widely accepted but have remained virtually untested in the 53 years since it was first proposed (Neel, 1962).

Second, the mitochondrial DNA component of this thesis constitutes the single largest study of mitochondrial diversity in Polynesia thus far. There have been few complete mitochondrial genomes sequenced in Polynesians thus far (Pierson et al., 2006, Duggan et al., 2014), so the data produced may be able to give pertinent information not only on disease susceptibility, but the settlement of the Pacific region.

1.6 Thesis structure

This thesis is divided into seven chapters. A large portion of this thesis comprises a review of the literature. This is primarily because of the attempt to bridge multiple disciplines. The first three chapters (Chapters 2-4) comprise a thorough review of the literature, synthesising material from diverse fields, and sets the context for the laboratory work described in Chapters 5 and 6. Three academic publications (attached in Appendix 1) have arisen from the literature reviewing the Chapters 2 and 3.

In Chapter 2, a genetic predisposition to the high prevalence of gout among Pacific people is established by a review of the epidemiological literature. In particular, hyperuricaemia and elevated serum urate levels are explored, not only in the Pacific but also on a worldwide scale. While elevated urate levels alone are not sufficient to cause gout – it still requires an inflammatory immune response - urate levels are something more easily quantified and examined on a population level than immune function. Further, the chapter outlines the historical and prehistoric evidence for gout among Pacific populations. Establishing a historic genetic predisposition to gout in these early periods of pre- and post-European contact is important as it suggests that the genetic background is having a large contribution to phenotype in absence of the westernised diets that have been recently introduced to many contemporary Pacific populations.

In Chapter 3, current hypotheses surrounding the high prevalence of metabolic disease among Pacific people are explored and contextualised with reference to current knowledge surrounding the settlement processes and prehistory of the region. In particular, the oft-cited 'Thrifty Genotype' hypothesis is a particular focus. In this section, a novel hypothesis is also put forward to explain the high rates of hyperuricaemia, which contribute to the high burden of metabolic disease in the region.

Chapter 4 acts as a bridge between the literature reviewing and hypothesis building from Chapters 2 and 3, and the laboratory work later in the thesis. This chapter focuses on the innate immune responses that underlie the metabolic diseases that are discussed throughout this

thesis, and introduces the link to mitochondria and mitochondrial variation. The NLRP3 inflammasome is thought to be responsible for a number of auto-inflammatory conditions, including gout, and the role of mitochondria in this immune pathway is explored.

Mitochondrial genetic variation and its potential role in disease is focussed on in Chapter 5 and the results of sequencing 442 whole mitochondrial genomes from Polynesian gout cases and controls are discussed. Because variation in the mtDNA sequence is not the only form of genetic variation, Chapter 6 explores the potential effect of mitochondrial DNA copy number (as a proxy for mitochondrial mass) on susceptibility to gout.

Finally, Chapter 7 includes an overall discussion and conclusion for the thesis.

2 Gout and metabolic diseases among Pacific peoples through space and time

Foreword

This chapter comprises a synthesis of the following three publications, which are included in Appendix 1.

- Gosling, AL., Matisoo-Smith, E. and Merriman, TR. 2014. Hyperuricaemia in the Pacific: why the elevated serum urate levels? *Rheumatology International* 34(6): 743-757

- Gosling, AL., Matisoo-Smith, E. and Merriman, TR. 2014. Gout in Māori. *Rheumatology* 54(5): 773-774

- Gosling, AL., Buckley, HR., Matisoo-Smith, E. and Merriman, TR. (2015). Pacific populations, metabolic disease and 'Just-So Stories': a critique of the 'Thrifty Genotype' hypothesis in Oceania. *Annals of Human Genetics* 79(6): 470-480

2.1 Introduction

This chapter aims to contextualise metabolic disease in the Pacific, to establish the region-wide trends and to explore the history of this phenotype in the region. Establishing the existence of the phenotype prior to the influences of westernisation and the changes in diet and lifestyles which accompanied European contact is important in suggesting that selection is something that may have contributed to the development of the phenotype. It is imperative when carrying out any genetic study to take into consideration the context in which the disease is occurring.

2.2 Epidemiology of metabolic disease in Pacific populations

As previously mentioned in Chapter 1, it is well established in the literature that there is a high metabolic disease burden among Pacific peoples, including Māori. This is particularly evident when looking at the breakdown of disease prevalence in mixed populations such as in New Zealand where there is a diverse range of people of different ancestral backgrounds.

Additionally, the World Health Organization has raised concerns over the increasing rates of chronic diseases including type 2 diabetes and cardiovascular diseases. Here, I have broken these diseases down and looked at the prevalence of certain conditions across the Pacific, and in the context of other populations worldwide, where there are data available.

2.2.1 Gout and hyperuricaemia

As previously discussed, gout is an inflammatory arthritis characterised by the accumulation of MSU crystals in synovial joints which results in an inappropriate inflammatory response. As such, a state of hyperuricaemia (serum urate levels exceeding 0.36 $mmolL^{-1}$ or 6 mg/dL (Bardin, 2015)), is generally necessary for gout to develop. In this section, the prevalence of both of these are discussed in the context of the Pacific.

It was noted relatively early on that the Pacific people seemed to be "one large gouty family" (Kellgren, 1964, p. 116), and there were quite a number of studies carried out assessing gout prevelance by Isadore Rose, Ian Prior and others (e.g. Lenane et al., 1960, Rose & Prior, 1963, Prior & Rose, 1966, Prior et al., 1966, Rose, 1975). The findings of these early studies have stood the test of time and even today, a high prevalence of gout is seen among many Pacific populations, particularly those with high western influences, such as New Zealand, where Māori and Pacific Islanders have a much higher prevalence of disease compared to their New Zealand European and Asian counterparts (Winnard et al., 2012). The presently available prevalence data for populations throughout the Pacific region is presented in Table 2.1 (though the date of publication of the studies should be kept in mind, as the prevalence of gout is likely to have increased with increasing sedentism and dietary change (Klemp et al., 1997)).

Given the role of elevated serum urate levels in the aetiology of gout, it is perhaps not surprising that many Pacific populations have high urate levels compared to many other populations worldwide (Figure 2.1)(Gosling et al., 2014). To this end, a summary of the published data available on the mean serum urate levels worldwide is provided in Appendix 1. Only male data, unless otherwise stated, have been presented because of the confounding effect of hormones on urate levels in pre-menopausal women (Hak & Choi, 2008).

Examination of these data show that most Pacific Island populations (Polynesians and Micronesians) and their possible ancestral populations (Taiwanese aboriginals) in particular, have higher rates of hyperuricaemia, with few other populations showing high mean serum urate levels (in the 6.5 mg/dL + range). Most populations have average serum urate concentrations of between 4 and 6 mg/dL, below the hyperuricaemic threshold. While some of this variation could relate to differences in lifestyles, subsistence and environment, ancestry is also a likely contributing factor. Given the varied evolutionary experiences of populations

worldwide since the expansions out of Africa, it is feasible that higher serum urate concentrations may have been positively selected under certain environmental conditions, leading to the variability which we see in modern populations globally. This idea will be explored further in the Chapter 3.

Table 2.1: Prevalence of gout in Pacific and related populations

Locality	Population	Gout % (men only)	References
New Zealand	Māori	11.7%	(Winnard et al., 2012)
	Pacific Islanders	13.5%	(Winnard et al., 2012)
	European	3.7%	(Winnard et al., 2012)
	Asian	3.7%	(Winnard et al., 2012)
Cook Islanders	Rarotonga	2.4%	(Prior & Rose, 1966)
	Pukapuka	5.3%	(Prior & Rose, 1966)
Tonga	Tongans	2.8%	(Finau et al., 1983)
Tokelau	Migrants in NZ	5.3%	(Wigley et al., 1987b)
	Non-migrant	0.7%	(Wigley et al., 1987a)
Tuvalu	Funafuti	0.0%	(Zimmet & Whitehouse, 1981)
Samoa	Rural	6.2%	(Zimmet & Whitehouse, 1981)
	Urban	7.2%	(Zimmet & Whitehouse, 1981)
Fiji	Urban (Suva)	0.002%	(Tuomilehto et al., 1988)
Nauru	Nauruans	6.9%	(Zimmet et al., 1978)
Mariana Islands	Guam	1.5%	(Reed et al., 1972)
	Rota	4.1%	(Reed et al., 1972)
Java	"Malayo-Polynesians"	1.7%	(Darmawan & Lutalo, 1995)
Taiwan	Atayal Aborigines	11.7%	(Chou & Lai, 1998)

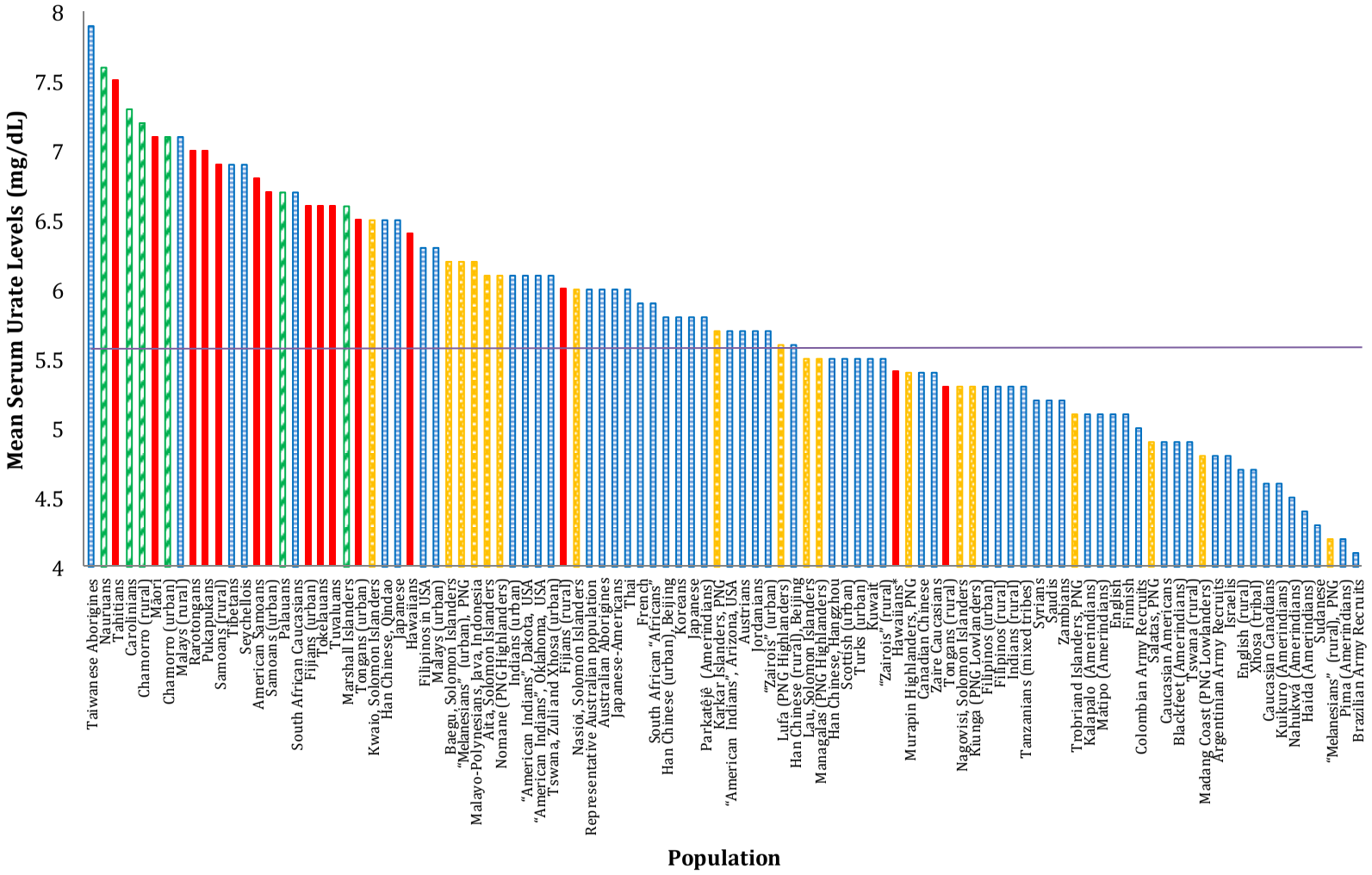


Figure 2.1: Mean serum urate concentrations (measured in mg/dL) world-wide, red bars indicate Polynesian populations, green bars indicate Micronesian populations, yellow bars indicate Melanesian populations, non-Pacific populations indicated by blue bars. The intersecting blue line indicates the mean urate levels worldwide, as calculated from the collated data in the appended material. The Hawaiian data indicated by the asterisk are unexpectedly low, and as discussed in text, probably anomalous.

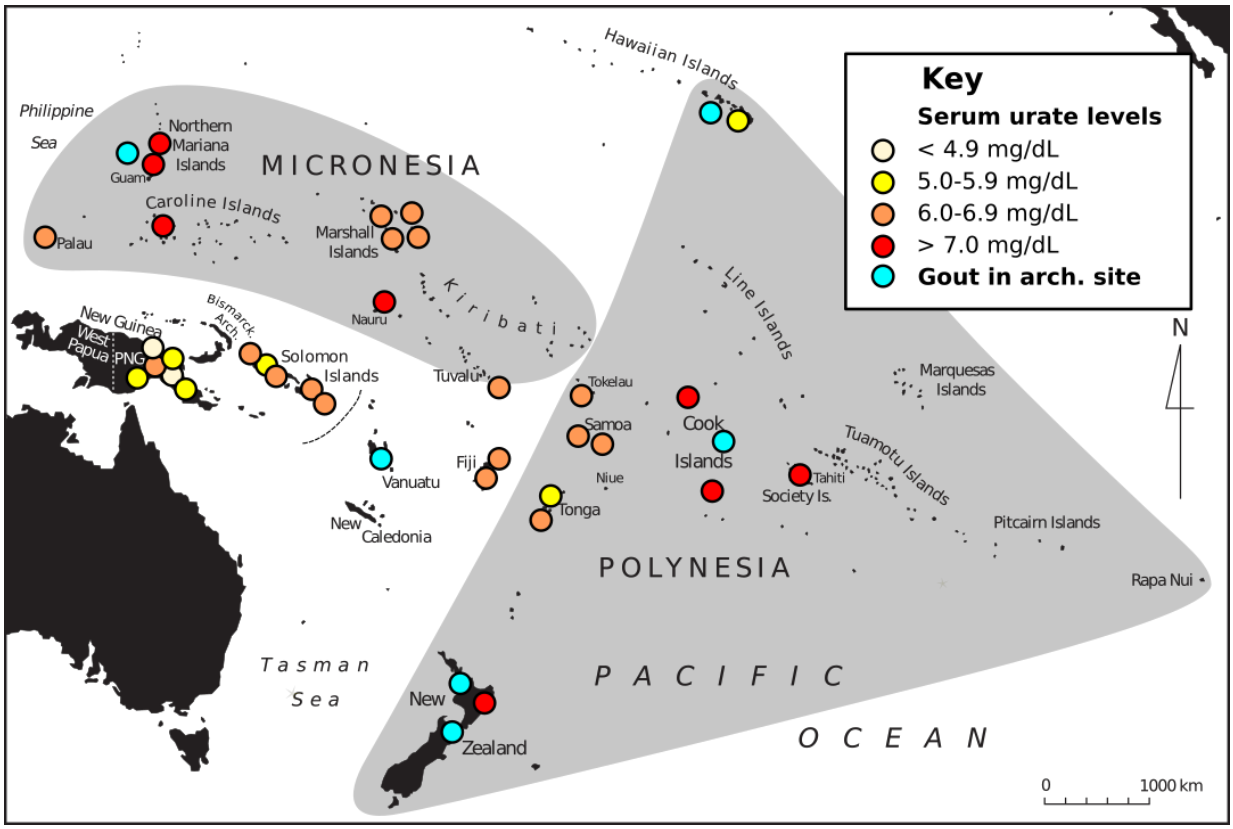


Figure 2.2: Summary of distribution of mean serum urate levels (in men) from throughout the Pacific. Modified from Gosling et al. (2014). Base map courtesy of Les O'Neill.

Though Polynesian populations tend to show high levels of serum urate, there is reported variation (Figure 2.2). For instance, the urate levels from a small sample of 49 Polynesians in Hawai'i (5.4 mg/dL), none of which showed any gouty disease, are unexpectedly low (Healey et al., 1966)– this has been used to argue that this population may be metabolically different than other Polynesian populations (Prior, 1981). These unusual results have not been revisited in the literature despite the study being carried out nearly 50 years ago. Currently there is a relatively high disease burden of gout in Indigenous Hawaiians (Pers. Comm.: K. Chong-Hanssen), and likely gout has been identified in skeletal remains from ancient Hawaiians (Suzuki, 1993). A high disease burden of gout can be taken as an indication of a high rate of hyperuricaemia in Indigenous Hawaiians.

Environment clearly also has an effect on serum urate levels; a number of studies have sampled from both urban and rural cohorts from within the same population, presumably to help understand the effects of urbanized living (Healey et al., 1967, Duff et al., 1968, Reed et al., 1972, Wyatt et al., 1980, Jackson et al., 1981, Finau et al., 1983, Tuomilehto et al., 1988, Prior et al., 1987). Higher urate levels were generally observed in those inhabiting an urban environment. For instance, Tongan men from urban areas had mean serum urate levels of 6.5 mg/dL

compared with 5.3 mg/dL in those living in rural areas (Finau et al., 1983). Similar trends were seen in Papua New Guinea, with an urban Melanesian population living in Port Moresby having mean serum urate levels of 6.2 ± 1.3 mg/dL, compared to 4.2 ± 1.3 mg/dL in a rural cohort (Wyatt et al., 1980), though this could also be related to genetic differences between the Port Moresby population and the rural population given the heterogeneity in Papua New Guinea. In general, living in an urban environment exacerbates the tendency toward elevated serum urate levels, concomitant with the increased consumption of foodstuffs (such as sugar-sweetened beverages and alcohol) that increase urate (Choi et al., 2004, Choi & Curhan, 2008, Batt et al., 2014). However, the fact that even those living rurally and with more traditional lifestyles in Polynesia have high rates of hyperuricaemia, compared to other populations worldwide, suggests a genetic predisposition.

2.2.2 Obesity

Defined by having a body mass index (BMI) exceeding 30 kg/m^2, obesity, particularly abdominal obesity, is considered a risk factor for most other metabolic syndromes, including type 2 diabetes and cardiovascular disease (Mokdad et al., 2003, Despres & Lemieux, 2006). However, BMI is certainly not the best measure of metabolic fitness, with studies suggesting that populations can have different lean mass: fat mass ratios (Swinburn et al., 1999). Rush et al. (2009) found that Pacific people have less adipose mass than other ethnicities when normalised for gender and BMI. They found that for a given body fat content, the BMI for the NZ Asian population was 25 kg/m^2, for Europeans 30 kg/m^2, and for Māori and Pacific Islanders 35 kg/m^2. Thus, the utility of BMI as a marker of adiposity has been contested. However, others have argued that the undeniable statistics surrounding the high type 2 diabetes and heart disease prevalence among Pacific people suggest that whether BMI varies in relation to body fat is immaterial (Taylor et al., 2010).

In the 2014 OECD obesity report, New Zealand had the third highest prevalence of obesity (below Mexico and the USA) (Ng et al., 2014). In general, obesity in New Zealand is on the rise, with the Ministry of Health reporting that 31% of adults are obese and a further 34% are overweight (http://www.health.govt.nz/nz-health-statistics/health-statistics-and-data-sets/obesity-data-and-stats). These statistics may be contributed to by the significant portion of New Zealanders being of Māori or Pacific ancestry (~22% according to 2013 census data; http://www.stats.govt.nz/Census/2013-census/profile-and-summary-reports/infographic-culture-identity.aspx). The New Zealand Ministry of Health reports that 48% of Māori and 68% of Pacific adults in New Zealand are obese. These trends of high rates of obesity translate out to the wider Pacific region.

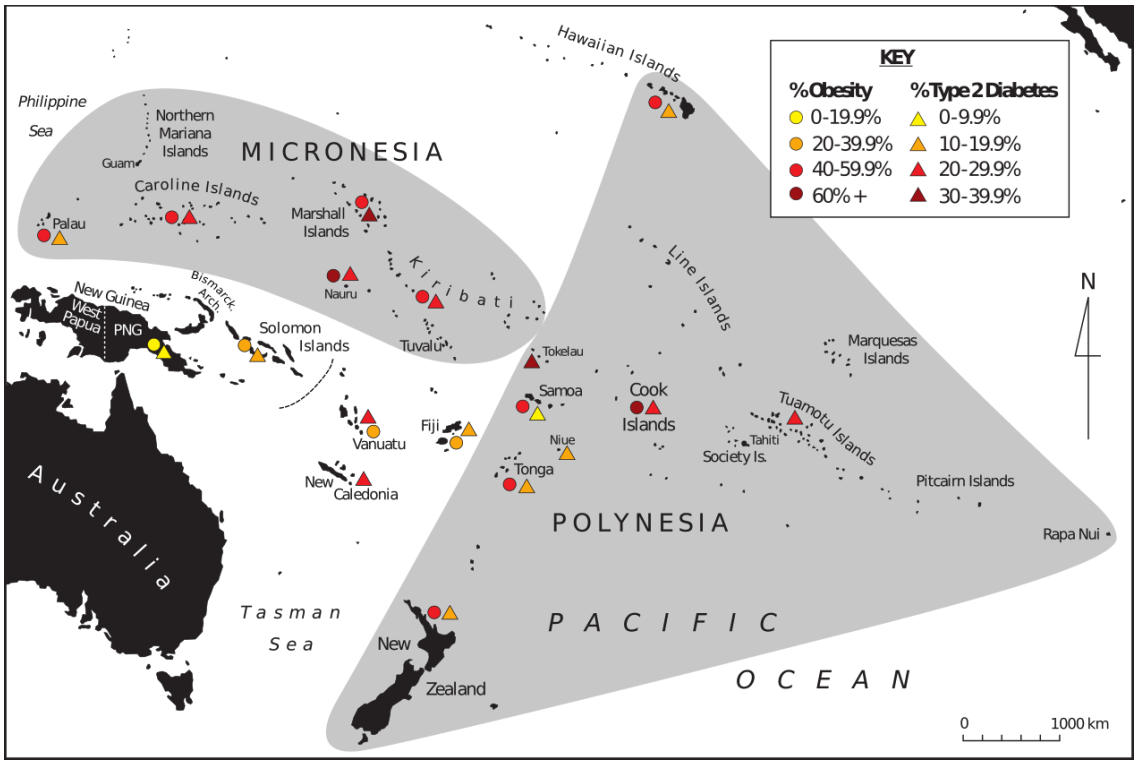


Figure 2.3: Prevalence of type 2 diabetes and obesity in the Pacific. The dashed line indicates the division between near and remote Oceania. (From Gosling et al., 2015).

The increasing rates of obesity among Pacific Island countries have come to the attention of the World Health Organization and have been highlighted as a concern (Waqanivalu, 2010). Figure 2.3 shows prevalence data for obesity throughout the Pacific region. Like the serum urate levels and gout discussed in the previous section, there are some large-scale region-wide trends, with the highest prevalence existing among Polynesian and Micronesian populations. As shown, nine of the populations for which data exist, possess a prevalence exceeding 50%. In Near Oceania, there are lower rates of obesity. This is likely due to a combination of less penetrance of westernised diets and lifestyles, and also a different genetic background. This will be more extensively explored in Chapter 3.

This epidemic of obesity is likely to be something that has arisen out of changes in lifestyle since contact with Europeans and with increasing amounts of foreign foods being imported into many localities (World Health Organization, 2012). A 1978 study of urban and rural populations in three areas in Samoa showed large differences in the prevalence of obesity, with 74% of women in Apia (urban) being obese, compared to 62% and 56% in Putasi and Tuasivi (rural) respectively. The data for the men was comparable with 57%, 44% and 36% obesity being measured in Apia, Poutasi and Tuasivi respectively (Dowse et al., 1995). Between the years of 1978 and 1991, there was a marked increase in obesity prevalence, particularly in rural areas. In

Poutasi (Samoa), the rate of obesity increased 297% in men and 115% in women (Hodge et al., 1994).

2.2.3 Type 2 diabetes

As previously mentioned in Chapter 1, another metabolic condition that disproportionately affects Māori and Pacific people in New Zealand is type 2 diabetes. The New Zealand Ministry of Health reports that belonging to this ancestral group is associated with a three times higher risk of developing disease compared to other New Zealanders (http://www.health.govt.nz/your-health/conditions-and-treatments/diseases-and-illnesses/diabetes). The International Diabetes Federation reported that in 2014, 138 million people in the Western Pacific region alone suffer diabetes. While their classification includes East and South East Asia in the Western Pacific region, the prevalence data for Pacific Island nations make up some of the highest in the world (Chan et al., 2014).

Studies of populations from the region report an increase in disease over the last 30 years or so. For instance, in Tonga the prevalence has increased from 7.5% in 1973 to 15.1% in 1998 (Colagiuri et al., 2002). Similar escalations in disease frequency have also been observed among three Samoan populations (Collins et al., 1994). In Apia (urban), prevalence increased from 8.1 to 9.4% in men and 8.2 to 13.4% in women between 1978 and 1991. In Poutasi (rural), prevalence increased from 0.1 to 5.3% in men and 5.4 to 5.6% in women, while in Tuasivi, men's prevalence increased from 2.3 to 7.0% in men, and 4.4 to 7.5% in women. This mimics the trend of increasing obesity in the same populations.

The geographic distribution of high prevalence of diabetes follows very similar patterns to those of obesity and serum urate levels (Figure 2.3), where high prevalences are seen almost universally throughout Polynesia and Micronesia (although in this case, the Samoans do have slightly lower prevalence), and lower rates of diabetes are seen in areas which were settled earlier, for instance the Solomon Islands and Papua New Guinea. This lends credence to the hypothesis that these may be interrelated and there may be a shared evolutionary origin for them.

2.2.4 Ischemic heart disease

As mentioned in Chapter 1, Māori and Pacific people in New Zealand have a higher incidence of ischemic heart disease than their New Zealand Asian and European counterparts (Winnard et al., 2013). A study from Wallis Island suggests that while the prevalence of hypertension and heart disease were low during the early 1980s, social and economic changes over the last 30 years have lead to a dramatic increase in the rate of cardiovascular disease (Linhart et al., 2015). Unfortunately there is not a lot of epidemiological data presently available on prevalence in

different Pacific populations, however the World Health Organization advise that alongside diabetes and hypertension, cardiovascular disease accounts for three quarters of all deaths throughout the Pacific (Waqanivalu, 2010).

2.2.5 Discussion

From where data are available, there do appear to be region-wide trends with these related metabolic conditions discussed here. The high serum urate levels and the prevalence of gout, obesity and type 2 diabetes are almost universal across the Pacific, with the notable exception of Near Oceanic regions, which broadly do not show this trend. This may partly be a result of differences in the penetrance of westernisation, but it may also be because of genetic differences in populations which arise from the settlement patterns in the region (discussed in Chapter 1).

Polynesian and Micronesian peoples speak languages belonging to the Austronesian language family. Austronesian languages are thought to have originated in Island South East Asia (ISEA), perhaps Taiwan, around 5000 years ago before being dispersed throughout ISEA, Oceania and beyond (Gray et al., 2009). Non-Pacific Austronesian-speaking populations also have elevated serum urate levels. Taiwanese aborigines have particularly high serum urate concentrations (Chang et al., 1997, Chou & Lai, 1998), while Filipinos also seem to have elevated serum urate levels (Healey et al., 1967). The Merina population in the Highlands of Madagascar, who have also been ancestrally linked to an Austronesian expansion out of ISEA (Cox et al., 2012), have likewise been observed to have high rates of gout and hyperuricaemia (Capdevielle et al., 1980). Similarly, the Seychellois, who may also have an ancestral genetic contribution from Austronesian-speaking colonists (Fitzpatrick & Callaghan, 2008) exhibit elevated serum urate concentrations (Conen et al., 2004).

Mitochondrial DNA analyses indicate that Polynesian and many other Austronesian-speaking populations have a significant genetic contribution from South East Asia (Kayser, 2010), where mean SU levels range from 5.5 mg/dL to 6.5 mg/dL (Appended data from hyperuricamia in Pacific paper). Y-chromosome STR analyses of Polynesians show that there is also a significant ancestral contribution from ancient Melanesian or Near Oceanic, non-Austronesian-speaking populations (Kayser, 2010). This is supported by genome-wide SNP data that indicate a genetic contribution from these ancient Near Oceanic lineages of around 13% in Polynesians (Wollstein et al., 2010), though it should be noted that there was minimal sampling from East Polynesia in this analysis. Figure 2.2 shows that the hyperuricaemic phenotype does not seem to be present in many Melanesian populations on the mainland of New Guinea and in the Solomon Islands, where sampled populations show mean SU concentrations ranging from 4.2 mg/dL to 6.2 mg/dL, compared with Polynesians whose mean SU range from 5.3 mg/dL to 7.1 mg/dL. Near

Oceanic populations are not genetically homogenous (Friedlaender et al., 2007), so these data should not be taken to be representative of all populations living in this area. However, the raised urate levels as seen in other Austronesian-speaking populations predominate over the lower serum urate concentrations in non-Austronesian speakers. Indeed, anecdotal data from personal communication with a physician working in the New Britain province of Papua New Guinea suggests that there are differences in gout and diabetes prevalence between populations of different ancestral backgrounds; he reported relatively high rates of diabetes among the Tolai (an Austronesian-speaking population) which did not occur in the Baining (a non-Austronesian-speaking population) (Pers. Comm.: Tim Fletcher). Further anecdotal reports have arisen through communication with physicians in the New Ireland province, where there are populations with a high Austronesian-influence and increasing prevalences of metabolic disease (Pers. Comm.: Lisa Matisoo-Smith). This indicates that this is an area that requires more research.

Together, the data suggests that there is a broad geographic trend of heightened susceptibility to metabolic disease, which may be underwritten by the almost uniformly high serum urate levels among the Austronesian-speaking populations in the region. Because of the wide distribution of the phenotype, it seems that the genetic variants contributing to this were likely to be increased in frequency prior to the settlement of the region. This may have resulted from selection to a more hyperuricaemic phenotype, or more broadly, a phenotype susceptible to metabolic disease, in Austronesian populations. This possibility will be elaborated on in Chapter 3. If this hypothesis is correct, it might be expected to see traces of metabolic disease in early skeletal material from the region. This will be explored in the coming section.

2.3 A history of gout?

It is widely accepted that Māori and Polynesians share an innate genetic predisposition contributing to the higher prevalence of gout. Despite this, much emphasis has often been placed on the changes away from traditional diets and lifestyles, with many commentators (Lenane et al., 1960, Rose, 1975, Johnson & Rideout, 2004, Schlesinger, 2005, Álvarez-Lario & Macarrón-Vicente, 2010, Merriman & Dalbeth, 2011) suggesting that gout prior to the twentieth century was uncommon. Contact with Europeans has changed traditional life particularly in terms of diet and activity patterns (Waqanivalu, 2010), which is evident in places like New Zealand and Hawai'i, where the indigenous peoples have become a minority, and there has been significant urbanisation and industrialisation. As previously noted in Chapter 1, most Pacific Islands have similarly been influenced, becoming reliant on imported foods. The high prevalence of a range of

metabolic diseases including type 2 diabetes, obesity and cardiovascular disease is influenced by over-consumption of high energy foods and the increasingly sedentary lifestyles in modern Pacific nations (Sundborn et al., 2008). Gout is part of this spectrum of metabolic diseases.

The assertion that modernity alone is responsible for the high rates of gout in Māori has been brought into question by recent bioarchaeological studies of prehistoric Māori which have identified bony lesions that are thought to be signs of gout (Buckley et al., 2010, Campbell & Hudson, 2011). The gouty immune response can result in osteological damage and the formation of sclerotic lesions (Figure 2.4) – it is these lesions which can be observed in skeletal analysis (Buckley, 2007). Typically, lesions are located on the margins of the first metatarso-phalangeal joint, as this joint seems to be the most common site of the development of gouty tophi. In dry bone, these lesions are distinguishable from other forms of arthritis because of the particular types of bony changes. Osteoarthritis, for instance, appears as polishing of the articular surfaces of a synovial joint, while the lesions attributed to gout by bioarchaeologists have a combination of osteoblastic (bone formation) and osteoclastic (bony destruction) changes associated with them.

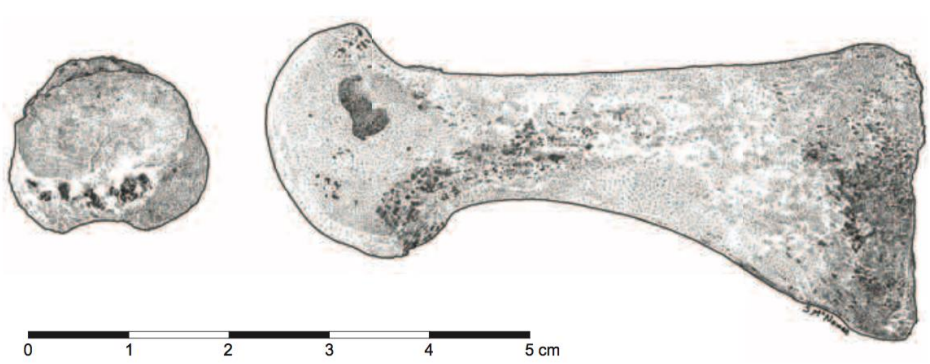


Figure 2.4: Examples of eroded holes that are characteristic of gout. The bones shown are those that make up the metatarso-phalangeal joint. From (Campbell & Hudson, 2011) (Used here with iwi permission)

Six of the forty-two (14%) individuals, all six of whom were male, recovered in archaeological excavations from Wairau Bar, the oldest known archaeological site in New Zealand, were found to possess these osteoclastic lesions (Buckley et al., 2010). Furthermore, eleven of the fifty-three (21%) adult individuals excavated from the Ihumatao site in Mangere, near Auckland, showed evidence of gout, including two of the fourteen identified females (14%) and four of the seventeen identified males (23.5%) (Campbell & Hudson, 2011). The tendency towards males possessing the lesions rather than the females, and the location of the lesions, which were predominantly located on the margins of the first metatarso-phalangeal joint, in both

assemblages, are consistent with the hypothesis that the lesions are a consequence of gout. It should also be noted that while the individuals from Wairau Bar were likely part of an immigrant population (dating to 1288 to 1300 AD) making them amongst the first New Zealanders (Buckley et al., 2010), the Ihumatao site dates to the 1620-1690 AD (Campbell & Hudson, 2011) by which time Māori populations were well established in New Zealand.

In light of this archaeological evidence for gout, it is clear that Māori suffered from gout prior to contact with Europeans. This challenges the view that the elevated prevalence of gout in contemporary NZ Māori is attributable primarily to Western influences on lifestyle. It is important that this paradox is resolved in order to better understand the causes of gout in Māori, both pre- and post-European contact. Examination of the historical literature sheds light on how the idea that gout is a disease of modernity has been perpetuated, so as to become the orthodox view.

2.3.1 The historical literature

The earliest reports of health in the Pacific by observers such as Sir Joseph Banks, who accompanied Captain James Cook in the Endeavour in 1769 (Hooker, 1896, p. 239-240), describe the excellent fitness of the Māori and other Polynesians. However, the episodic nature of gout (Prior, 1981), as well as the practice of moving chronically unwell individuals to special purpose-built dwellings away from the main settlement (Goldie & Best, 1904, Buck, 1910), would have reduced the likelihood of these earliest of spectators to witness gout if it were present. William Colenso (1868, p. 20), a missionary in the Hawke's Bay region, noted that Māori "rarely ever died in a good house; mostly in the open air, or under some wretched shed; this was done because the house in which anyone died would have to be forsaken to tapu".

Another confounding factor could be that during this early nineteenth century period, there were two co-existing schools of medical thought. Brought in by the colonising Europeans was Westernised medicine, which put the causation of disease as having a biological basis. Access to westernised medical practitioners, particularly for Māori, was limited as there were often long distances required to travel in order to see a doctor (with many Māori living rurally, while most doctors lived in cities or towns). Alongside this, there were also the pre-existing traditional medicines centred around more esoteric causes of illness (Buck, 1910). The underlying idea of the traditional Māori perception of medicine is that there was no natural disease: illness was a result of either transgression against an *atua* by the means of breaking some law of *tapu*, or else was the result of witchcraft or *makutu* (Buck, 1910). A *tohunga* or priest would be consulted in order to determine the cause of the complaint, and appropriate action was taken to either appease the *atua* or to remove the *makutu*. Thus, illnesses were often perceived as being either

mate Māori (traditional Māori conditions) or *mate Pakeha* (diseases brought in by the Europeans), and whether the disease was considered *mate Māori* or *mate Pakeha* had some influence over who Māori consulted in order to get rid of it (Best, 1904).

Given the archaeological evidence for gout in prehistoric Māori, the disease may have been considered *mate Māori,* especially given that gout did not have an obvious physical cause (i.e. not like a physical injury). This is supported by Sir Peter Buck's (1910, p. 43) recounting of how the Tohourangi *iwi* from the central North Island, had a god which they called Tatariki who they thought caused the swelling of the big toe joint and ankle. This, in itself, is compelling evidence that this particular *iwi* must have had enough of a history of gout for there to be an *atua* implicated in its causation to be passed on to Buck with the transmission of oral histories. What may also be surmised is that other *iwi* had attributed the disease to different *atua*, meaning that there was no universal term (or disease-*atua*) for the disease – this is supported by the late entry of a term for gout (*porohau*) in a Māori-English dictionary (Tregear, 1891). This word differs from the most frequently used Māori term in contemporary times (*kaute* – a transliteration of the word 'gout') (Ryan, 2008). Interestingly, other Polynesian languages (but not Māori) do have a shared cognate for rheumatic pain, swelling to the joints and gout-like symptoms (**ŋu-ŋu*), which indicates that the disease was likely ancient and not uncommon in the Austronesian world (Osmond, In Press).

Western medical care available to Māori during the earliest times post-European contact was via the missionaries. This was predominantly through providing pharmaceutical products. The missionaries themselves were not trained in medicine, and most made little mention of the state of health in Māori, let alone discuss the presence or absence of gout (e.g. Colenso, 1868, p. 7; Taylor, 1855, p. 253). The earliest official inquiry into the state of Māori health was a survey carried out by Dr Fairfowl in 1821 (Fairfowl, 1908). Being a physician, Dr Fairfowl presumably was better positioned to recognise the symptoms of gout; however it was not one of the common diseases that were mentioned in his report. It is interesting to note, however, that rheumatism was reported as being prevalent (Fairfowl, 1908). This apparent high frequency of rheumatism was also commented on almost two decades later by John Bright (1841), but again, no specific mention of gout was made.

The next accounts of health, and probably the most thorough account of Māori health dating back to this pre-twentieth century era, are the observations of Arthur S. Thomson, Surgeon of the 58th Regiment of Foot. Thomson wrote a detailed two-volume work on New Zealand, with much focus on the indigenous peoples (Thomson, 1859), in addition to a series of articles he wrote specifically on Māori health (Thomson, 1854, p. 361). It was in one of these articles that he

stated that "gout is unknown" among the Māori (p. 361). However, like Fairfowl before him, he noted a high frequency of "rheumatic affections". Thomson's observations of the absence of gout among native New Zealanders were rapidly picked up by others writing on the geographic distribution of various pathologies (Hirsch, 1885).

Thomson was not alone in describing this apparently high rate of rheumatism among Māori – Dr Alfred Newman (1883, p. 499) also noted this trend, while also indicating that Māori "show scant tendency to the gouty diathesis". "When gout does appear", Newman (1883, p. 499) states, "it is always in the person of an immigrant".

Belying some of these statements of apparently non-existent gout among the Māori are a smattering of reports of gout in Māori men in regional newspapers from around this period (Table 1). For instance, in 1885, the Grey River Argus (1885) reported the suicide of Ihaia Tainui, at the Māori School House, and a recent attack of gout was cited as a possible contributing factor to his "despondency and gloomy forebearings." Topai Turoa, a chief of high rank, was described as "the oldest and most infirm member of the embassy. He is afflicted with rheumatic gout, leans heavily on a stick, and moves about with some difficulty" (Wanganui Herald, 1884). The northern chief, Paora (Paul) Tuhaere of Orakei, was on multiple occasions reported to suffer from bouts of gout. In 1879, he was part of a greeting party for Sir George Grey, and was "mounted on a charger, as he suffer[ed] acutely from gout" (Thames Advertiser, 1879), and in 1884, a further attack of gout forced him to abandon the notion of visiting England (New Zealand Herald 1884). In this account, it was said that "the natives are not all satisfied on that head, and are under the belief that the Government in order to prevent Paul from going, have employed other Māoris to makutu him, and give him rheumatism and gout" (New Zealand Herald, 1884). This supports the idea that Māori may not have perceived gout as a disease in its own right, rather something that is caused by more spiritual means, whether it be disease *atua* or *makutu* and witch-craft. This possibility is supported by the previously mentioned Tohourangi iwi, who attributed gout to a god.

2.3.2 Disparities in the historic definitions of gout

Many of the historic documents discussing health in indigenous New Zealanders in the nineteenth century have been interpreted by more contemporary commentators (Lenane et al., 1960, Rose & Prior, 1963) to indicate that gout was not a health concern for Māori prior to the twentieth century. Given that the apparent absence of gout has been explicitly mentioned in many of these accounts (Thomson, 1854, Newman, 1883, Hirsch, 1885), this is not surprising. Rose (1975) argues that because gout was familiar to European colonists arriving in New Zealand they would have recognised its symptoms in the indigenous population. Therefore, he

argues, these historic accounts of people denying the presence of gouty symptoms among Māori can be taken seriously. However, it could also be argued that the definition of gout in the nineteenth century is quite different to what it is now, and that the reported high prevalence of rheumatism in Māori during this period may in fact reflect a misdiagnosis of gout as rheumatism.

Since the perception at the time was that gout was a disease that chiefly affected those living an affluent lifestyle (Hirsch, 1885), enjoying the "luxuries of civilised life" (Garrod, 1859), gouty pathologies observed in indigenous peoples in apparently "uncivilised" countries may not have been regarded as being the same disease, and thus were described as rheumatism instead. Garrod, who was the first to describe high uric acid levels in the blood of individuals suffering from gout (Garrod, 1848) and thus a contemporary authority and influential on the matter, stated (Garrod, 1859) "among nations in an uncivilised state, living chiefly on the produce of the chase obtained by personal exertion, or subsisting at least on the simplest fare, gout, according to the reports of eminent travellers, is entirely unknown even at the present day; but in our own country [England], and in many other parts of the civilised world, the case is far otherwise" (p. 2).

In their review of the history of gout, Porter and Rousseau (1998, p. 167) describe how in the mid-nineteenth century, gout was used as "a makeshift or umbrella diagnosis for all manner of aches, pains and complaints" in the wealthier echelons of society, with the prevailing perception being that while there was a hereditary element, luxurious living could also trigger the disease. Indeed, as early as 1816, Heberden bemoaned the fashionable nature of the diagnosis, reporting "I have seen several intoxicate themselves with strong liquors for two or three days together, upon presumption that they wanted a gouty fit, and that this was a proper way of procuring one", and further suggested that physicians allow "the criteria of [diagnosis] to be very obscure… I think hardly any which give us ground to suspect this disease, where there is no pain, nor redness, nor swelling in the first joint of the great toe, or in any other part of the foot…" (Heberden, 1816, p. 36-37). Even by 1914, Wohlmann (1914, p. 89) observed that "nowhere else in medicine is there such confusion of terminology. Thus the unqualified names 'gout', 'rheumatism', and 'rheumatoid arthritis' are almost without meaning, for they may bear absolutely different interpretations to different readers". Indeed, this perceived link between gouty pathology and social status is evident in the contemporary literature from New Zealand; in a description of a Court proceedings published in the New Zealand Herald in 1865, the link between gouty pathology and social status made in conjunction with a Māori chief, who apparently came to Court "with a long pole and his boot off, actually troubled by that peculiar

disease only claimed by English aristocracy as their hereditary right – the 'gout'." The reporter commented that the chief, Winiata, was probably "aware of this complaint being a sign of good blood" and an attending physician, Dr Nicholson was quoted saying that "it was the only case he ever met with among the natives, and is conclusive evidence that we can boast of the best blood among our Kaipara natives as well as amongst the Pakehas" (New Zealand Herald, 1865).

2.3.3 Gout and prehistoric diet

While the lifestyles of Māori had undoubtedly been influenced by European contact, on average, most Māori would not have been living the way of life that the physicians thought contributed to the development of gout. This is supported by a statement by Dr Newman (1879, p. 434) who considered that "not one of all the descendants of the canoes that came from Hawaiki ever suffered from gout... Imagine an old Māori Chief suffering from rich gout, when he had for food irregular and scanty allowances of fern root, dried eels……; his favourite tipple being a mild infusion of tutu berries, or perchance a little pure water, weakly flavoured with the juice of flax flowers. Of course no genuine Māori ever had gout". Clearly the state of living of the Māori did not align with the expectations of the British doctors in terms of what predisposes one to gout, thus they may not have diagnosed it on these grounds, lumping any gouty episodes into the broader term of rheumatism.

There is no way now to determine for sure whether or not the prevalence of gout in Māori has been understated due to a misdiagnosis of the disease during this period. However, given how little is written on the matter, the ambiguity in the definition of the terms in medical encyclopaedias from the time and the fact that there is archaeological skeletal evidence for gout in Māori populations well before the arrival of Europeans (Buckley et al., 2010, Campbell & Hudson, 2011), it is not intuitive to believe that there would have been a lack of gout directly after the arrival of Europeans.

Contemporary epidemiological data support the hypothesis that at least part of the cause of gout is the impact of diet (sugar-sweetened beverages, alcohol, purines) on an individual with a genetic predisposition to elevated serum urate (Dalbeth et al., 2013b) and to risk of gout in Māori (Batt et al., 2014). Presumably the fundamental cause of gout in non-Westernised Māori also held – impact of a (different) environment on individuals with a similar genetic predisposition.

Much time and energy was required for the production and preparation of food in prehistoric New Zealand. Horticultural production of kumara and taro were important in subsistence in

northern regions, however was marginal in the northern part of the South Island and completely absent to the south of Banks Peninsula due to climatic factors (Burtenshaw & Harris, 2007). Hunting of forest and sea birds, gathering of shellfish and seasonal fruit, and exploitation of both marine and freshwater fish contributed to the diets of prehistoric Māori (Buck, 1949). The absence of fermented beverages and other stimulants such as kava, was commented on in a number of the earliest reports on Māori (Colenso, 1868, Hooker, 1896), however, sugars were available for consumption. Studies have shown that the stems and rhizome of the *ti* or cabbage tree (*Cordyline australis*) are high in fructans, which when cooked in an *umu-ti* or earth-oven hydrolyse to fructose (Fankhauser, 1986). Fruit such as the drupes of the *karaka* tree (*Corynocarpus laevigata*) (Klinac et al., 2009) have also been found to be high in sucrose (a disaccharide consisting of both fructose and glucose). Both of these plants are thought to have been economically important and have a wide distribution throughout New Zealand (Leach & Stowe, 2005, Brooker et al., 1989). Since high sugar intake in modern populations, the intake of sweet sugary beverages for instance, has been found to contribute to higher serum urate levels (Choi & Curhan, 2008), it may be that the high sugar content of some of these traditional foods, such as the *ti* and the *karaka* berries, likewise contributed to higher urate levels in prehistoric Māori. There may well be other contributing factors that have been overlooked because of the reported apparent absence of gout in prehistory.

2.3.4 Conclusion

Palaeopathological evidence indicates that gout is a condition that has been present in New Zealand since the very first people arrived on the shores. Given the paucity of archaeological skeletal assemblages which have undergone systematic analysis, it is of note that the two best described skeletal assemblages both have a relatively high proportion of individuals with presumptive gouty lesions (14% at Wairau Bar and 21% at Ihumatao)(Buckley et al., 2010, Campbell & Hudson, 2011). The fact that these assemblages date to different time periods in prehistory, and are from quite different parts of the country, is also notable, as it shows a diversity in the conditions contributing to prehistoric gout. One can therefore assume that people who had recently arrived in a virgin land had different lifestyles and diets than those living in an established, horticultural society, and furthermore, that the lifestyles of both groups differed markedly from the lifestyle of excess and extravagance which typified gout in Europe at the time.

The under-representation of gout as a serious pathology in Māori as reported in historic documents, is likely related to a combination of factors: the episodic nature of the condition in its initial phases (Prior, 1981), a lack of epidemiological study of these populations prior to the

mid-twentieth century, as well as the perceived prerequisite lifestyle conditions for the development of gouty pathology, leading to a misdiagnosis of the condition in indigenous peoples.

2.4 Concluding remarks

I have presented here significant evidence that gout has a long history in Pacific populations, dating back at least 3000 years. Other metabolic complaints have a similarly long history in the Pacific. Analysis of the skeletal population at Teouma, Vanuatu shows evidence of diffuse idiopathic skeletal hyperostosis (DISH) (Pers. Comm.: Hallie Buckley), which is thought to be an indicator of underlying metabolic disease (Mader et al., 2013). While prevalence has been on the increase over the past 50 years since there have been systematic studies of the phenomenon, the occurrence of metabolic diseases has a deep history in the Pacific.

Modern epidemiology has demonstrated that raised serum urate levels have a multifactorial aetiology, with both environmental and genetic factors contributing. It must be recognised that lifestyle, diet in particular, influences not only the prevalence of obesity, but also the concentration of SU – more specifically, the consumption of purine and fructose-rich foods (Choi et al., 2004, Choi & Curhan, 2008, Batt et al., 2014). The last five decades in particular have seen radical changes in dietary habits in the Pacific. This is most evident in urban settings, but these changes are also occurring in many rural communities. There is now a reliance on the importation of foreign foodstuffs, because population sizes have reached points where the islands are now no longer self-sustainable (Hughes & Marks, 2009). Exacerbating this shift in subsistence practices is the decreasing popularity of traditional production practices and diets (Hanna et al., 1986, Coyne et al., 2000). Many of the imported foods are high in fat and sugar, leading to the coining of terms such as “dietary colonisation”, “Coca-colonisation” and “dietary genocide” (Hughes & Marks, 2009).

It is undeniable that these shifts in dietary and activity patterns are having an adverse effect on health, however these are not acting in isolation. The existence of prehistoric cases of disease, in particular gout which is more easily identified, suggests that there is a longstanding genetic predisposition to disease. Genes encoding proteins involved in renal and gut excretion of uric acid have a strong effect on serum urate levels, though genes involved in glycolysis are also associated (Köttgen et al., 2012). There have been several studies carried out on Māori and Pacific Island people living in New Zealand which have found that at least some of these genetic variants contribute to the risk of gout (Hollis-Moffatt et al., 2009, Phipps-Green et al., 2010, Hollis-Moffatt et al., 2012, Phipps-Green et al., 2016).

Collectively, the evidence suggests an inherent biological (genetic) predisposition to hyperuricaemia, and probably other metabolic conditions, in Polynesians. How might this have occurred? There are a number of processes by which genetic variants contributing to these phenotypes may have increased in frequency at the population level, including ancestry, random genetic drift and natural selection. The circumstances under which these may have operated will be discussed in the next chapter.

3 Evolutionary explanations for high metabolic disease burden

Foreword

This chapter comprises a synthesis of material from the following two publications, which are included in Appendix 1.

- Gosling, AL., Matisoo-Smith, E. and Merriman, TR. 2014. Hyperuricaemia in the Pacific: why the elevated serum urate levels? *Rheumatology International* 34(6): 743-757

- Gosling, AL., Buckley, HR., Matisoo-Smith, E. and Merriman, TR. (2015). Pacific populations, metabolic disease and 'Just-So Stories': a critique of the 'Thrifty Genotype' hypothesis in Oceania. *Annals of Human Genetics* 79(6): 470-480

3.1 Introduction

The antiquity of metabolic conditions in the Pacific, as evidenced by the identification of gouty lesions among prehistoric Pacific people and the historic evidence summarised in Chapter 2, suggests that there is likely to be a stronger genetic predisposition to gout and metabolic disease among the people living in this region. There are a number of different (not mutually exclusive) possibilities for how genetic variants contributing to this high metabolic disease burden may have reached appreciable frequency. This chapter assesses proposed hypotheses and puts forth a new one.

3.1.1 The settlement of the Pacific and possibility of founder effects and drift

One of the explanatory models put forward for the high rates of metabolic disease is that they are due to founder effects (Merriman & Dalbeth, 2011). As summarised in Chapter 1, Pacific settlement has been a long process, involving multiple movements of people out of continental Asia, starting very early in human history. The migration of people onto the ancient subcontinent of Sahul (which is now Australia and New Guinea) around 49 thousand years ago (Summerhayes et al., 2010b) represents one of the most significant migrations both in terms of distance travelled and technology. These peoples are genetically heterogeneous, and there are few mitochondrial and Y-chromosome haplogroups shared between the indigenous inhabitants

of Australia and New Guinea, despite their ancient shared ancestry (Duggan & Stoneking, 2014). As established in Chapter 2, high rates of metabolic disease have not been observed among these populations in Papua New Guinea.

Many Pacific populations, at least in part, are descended from peoples associated with the archaeologically defined Lapita cultural complex. The spread of the Lapita culture is thought to accompany the movement of Austronesian languages through the region (Gray et al., 2009) and represents the movement of a genetically distinct population from a more recent Asian source. There was some admixture and cultural exchange between the pre-existing people in the region and the incoming Austronesians, as evidenced by the abundance of ancient Near Oceanic derived Y-chromosomes in Polynesian populations (Kayser, 2010) and genome-wide SNP analysis showing a genetic contribution of around 13% (Wollstein et al., 2010). The Lapita culture first appeared in the Bismarck Archipelago approximately 3350 years ago and people carrying this culture rapidly sailed eastwards colonising the Reef Santa Cruz (Southeast Solomon Islands), Vanuatu and New Caledonia (Spriggs, 2011) within a few hundred years. Lapita expansion stopped at the edge of Polynesian Triangle, in Tonga, which was settled by 2830-2846 years before present (BP)(Burley et al., 2012), and Samoa, settled at a similar time (Petchey, 2001) (Spriggs, 2011). The most recent dates for settlement of East Polynesia suggest that around 1200 to 1500 years later, further migrations were undertaken from Samoa into Central and East Polynesia, the Marquesas and the Cook and Society Islands (Kirch & Kahn, 2007), and even later into the more remote islands, with settlement of Rapa Nui at 800 BP (Hunt & Lipo, 2006), Hawai'i between 800 - 1200 BP (McCoy, 2007), and New Zealand around 700 BP (Higham et al., 1999, Walter et al., 2010, Wilmshurst et al., 2011).

Hence modern Polynesians are the end product of a voyaging process that occurred over many thousands of years. Often founder effects are employed to explain an apparent lack of genetic variation in Polynesian populations and the apparent dissimilarity phenotypically between Polynesia and Melanesia (Pietrusewsky, 1996) – though continuous contact and voyaging would have reduced these effects. The basis of the underlying idea of limited genetic diversity in the Pacific resulting from founder effects lies in the observation that on average 94% of the mitochondrial variation in Polynesian populations are derived from East Asian haplogroups specifically those derived from the B4a1a1 haplotype (Kayser et al., 2006). However, to suggest that these founder effects have led to the chance emergence of a hyperuricaemic phenotype susceptible to a wide range of metabolic diseases in Polynesians is simplistic and unlikely. Also, when the data from the wider region is reviewed, the Polynesian serum urate levels and high rates of metabolic disease are not out of context (Chapter 2, Figures 2.2 and 2.3) because the

phenotype seems to be present in other populations in the homeland region, Taiwanese aboriginals for instance, who were unlikely to have had the same demographic founder alterations resulting from serial migration.

3.1.2 Natural Selection

Another mechanism by which genetic variants contributing to elevated serum urate may have come to prominence is natural selection. Natural selection describes the differential survival or reproductive success of individuals with phenotypic traits that contribute to fitness under specific environmental parameters. It may be expected that individuals who have greater fitness in a given environment will have a greater number of offspring and thereby be more successful in transmitting their genotype onto the next generation. Over time, this differential reproductive success would cause a shift in the allele frequencies of genetic variants underlying the phenotypic variation.

Natural selective forces arising from exposure to different environments worldwide likely contribute to the inter-population differences in genetic variation and disease-related phenotypic diversity (Barreiro et al., 2008). Evidence already exists that certain human phenotypes, such as resistance to malaria (Higgs & Weatherall, 2009) and lactase-persistence in adulthood (Sabeti et al., 2006, Tishkoff et al., 2007) have resulted from natural selection in response to certain environmental conditions. There has been growing interest in selection, and as a result, various algorithms have been formulated in an attempt to quantify the likelihood of a locus having been subject to selection (Sabeti et al., 2002, Voight et al., 2006, Karlsson et al., 2013).

In next section, a number of hypotheses based on the concept of selection are discussed, including the frequently applied 'Thrifty Genotype'. Later in the chapter, a novel hypothesis to explain the high serum urate levels so common in the Pacific region is proposed.

3.2 A critique of the 'Thrifty Genotype' Hypothesis

3.2.1 Background to the 'Thrifty Genotype" Hypothesis

In 1962, the American geneticist, James Neel, put forth a revolutionary hypothesis that the increasing prevalence of type 2 diabetes among many indigenous populations was a result of an evolutionary adaptation to periods of famine (Neel, 1962). Thus, the 'Thrifty Genotype' hypothesis, which also relates to other metabolic conditions, was born. The hypothesis was an important first step in employing evolutionary explanations for differences in disease prevalence between populations. Over the following fifty years, the hypothesis was modified in conjunction with advances in understanding of the complexity of metabolic disease and the

anthropology of hunter-gatherer and early Neolithic cultures and their subsistence patterns (Neel, 1982, Neel, 1999, Corbett et al., 2009).

The premise of the original hypothesis was that contemporary type 2 diabetes was caused by a disjunction between genes and environment. Specifically, variation in genes which facilitated survival during periods of famine in pre-industrialised societies, by allowing for the more efficient storage of energy during periods of plenty, were at odds with a modern, industrialised environment of plenty (Neel, 1962). Consequent to the replacement of traditional diets with a Westernised diet, these so-called 'thrifty genes' were proposed to contribute to the onset of diabetic symptoms. Following the publication of the hypothesis, other possible explanations relating to reasons for metabolic disease were discussed in the literature. These include the 'Thrifty Phenotype' hypothesis wherein inadequate nutrition early in life predisposes an individual to type 2 diabetes (Hales & Barker, 1992), and the 'Drifty Genotype' hypothesis which postulates that the removal of heavy predation pressure resulting from increased social behaviour, the use of fire and invention of weapons removed selective pressures permitting genetic drift which has allowed for genetic variants causal of obesity and type 2 diabetes to accumulate (Speakman, 2008).

Despite many critiques of the hypothesis (Speakman, 2006, Paradies et al., 2007, Beil, 2014, Sellayah et al., Benyshek & Watson, 2006), and a general lack of genetic evidence, for instance, the detection of selection signatures in metabolic genes (Helgason et al., 2007, Steinthorsdottir et al., 2014, Ayub et al., 2014), the 'Thrifty Genotype' hypothesis continues to be cited as a reason for disparities in metabolic health between populations. In their seminal paper, Gould and Lewontin warned against the acceptance of evolutionary narratives without due testing and critical thought (Gould & Lewontin, 1979), and this sentiment remains as true now as it did in 1979 (Nielsen, 2009).

The 'Thrifty Genotype' hypothesis is merely another 'Just-So Story' (to borrow the analogy used by Gould and Lewontin in their critique of the 'adaptationist programme' (Lewontin, 1991, Gould, 1997)), a possibility but one for which there is little or no supporting data. This is particularly true for Pacific Island populations, for whom the 'Thrifty Genotype' hypothesis has been a recurring theme in describing reasons for the high rates of type 2 diabetes, obesity and other metabolic disorders; Pacific Island populations, along with Pima Indians, have long been considered archetypal examples of a 'Thrifty Genotype' in action (Zimmet et al., 1990, Bindon & Baker, 1997, Diamond, 2003, Myles et al., 2007). Other populations, such as Australian aborigines (O'Dea, 1992), Asian Indians (Mohan et al., 2007), sub-Saharan Africans (Van der Sande, 2003), and Latin Americans (Filozof et al., 2001), have also been subject to the

hypothesis. It seems that the 'Thrifty Genotype' hypothesis has been used in a piecemeal, one-hypothesis-fits-all manner, where one of the few populations that seems exempt from these 'thrifty genes' are European-derived populations. As Allen and Cheer (1996) pointed out, it may be that the Europeans themselves were subject to some selection process which caused this discrepancy. But does the 'Thrifty Genotype' hypothesis fit the genetic and anthropological data?

As discussed in Chapter 2, there are indeed high rates of obesity, type 2 diabetes and hyperuricaemia (Gosling et al., 2014) among many populations throughout the Pacific – both among those still living in their ancestral homelands, and those who have migrated to urban centres in places such as New Zealand, the United States and Australia. While the prevalence of disease is higher among those living in more westernised contexts, the occurrence of metabolic disease in those living a more traditional lifestyle (pre-mid twentieth century and prehistoric) suggests a genetic contribution to disease. This is supported by skeletal indicators of metabolic disorders (gout and diffuse idiopathic skeletal hyperostosis (DISH) in particular) in bioarchaeological assemblages from the region (Buckley, 2007, Buckley et al., 2010, Buckley, 2011) and the epidemiological evidence of Ian Prior and colleagues from the 1950s through to the 1970s (Prior et al., 1966, Prior, 1981). Although it is reasonable to expect that indigenous Pacific populations will have diabetes and obesity risk alleles at a higher prevalence and penetrance (I note that there are currently no systematic genome-wide studies on genetics of diabetes and obesity in any Pacific population) the theory that this is due to selection as a result of food deprivation is not supported when one considers the population history, settlement process and environment of the Pacific in more depth.

3.2.2 The Migratory History of the Pacific

Understanding the evolutionary history of the region and the colonisation process is instrumental to place the 'Thrifty Genotype' hypothesis in a better context when applied to Oceanic populations. As previously discussed, there have been multiple movements of people into the Pacific region throughout the 50,000 year history of human occupation. Given this time depth and population diversity, it might be expected to see significant differences in the metabolic disease frequencies in the various populations – i.e. prevalences are likely to be influenced more by the genotype of ancestral populations rather than events occurring during the migratory history *per se*. People first arrived on the continent of Sahul, which became New Guinea and Australia with subsequent sea level changes, at least 49,000 years ago (Summerhayes et al., 2010b). Genetic analyses have shown that there were probably multiple populations involved in these Late Pleistocene and Early Holocene migrations out into New Guinea and the Solomon Islands, a region known as Near Oceania (Rasmussen et al., 2011). Subsequently, there have been several influxes of people from the Island Southeast Asia region

into the islands of the Bismarck Archipelago and beyond as early as 5,000 years ago but certainly by some 3,350 years ago (Summerhayes et al., 2010a, Kirch et al., 1989). These people mixed with resident coastal populations prior to expanding out into the wider Pacific where they were the initial colonists on the previously unoccupied islands of Remote Oceania. Thus, Polynesians and Micronesians have been found to be more genetically similar to certain Asian populations than to the general Near Oceanic populations which are highly heterogeneous (Friedlaender et al., 2008). Because of these differences in ancestry, there are both genetic and phenotypic differences between Pacific populations, even those within a reasonably close geographical distance to one another. The implication of this is that population history should be a major consideration when discussing differences in disease prevalence in the Pacific. For instance, while rates of type 2 diabetes and obesity are high among Polynesian and Micronesian populations, the rates are much lower in Papua New Guinea (Figure 2.3), whose general population has a larger proportion of their ancestry being derived from the earliest inhabitants of the region than the later Austronesian-speaking colonists. A proponent of the 'Thrifty Genotype' hypothesis might suggest other explanations for this phenomenon – namely the selection for 'thrifty genes' occurred after moving through this region, but these arguments are inconsistent when examined in context of the Pacific archaeology and anthropology.

3.2.3 'Thrifty Genotypes' and Oceanic Voyaging

One of the inherent weaknesses of the 'Thrifty Genotype' hypothesis in the context of the Pacific is the reliance on significant population loss mediated by restricted food supply. A number of mechanisms describing how the hypothesis might operate in the Pacific have been put forth. The scenario laid out by Bindon and Baker (1997) is that the selection for these 'thrifty' traits occurred during the voyaging and settlement of the Pacific. This is based on the assumption that the voyaging process was perilous and that there was a reasonably high mortality rate. Diamond (2003) takes this a step further by stating that 'in many attested examples of such lengthy voyages, many or most of the canoe occupants died of starvation, and only those who were originally the fattest survived'. No citations were given for this claim. However, given that colonisation voyages are not something that have happened for at least a couple of centuries (Irwin, 1989), it is likely that Diamond is referring to more recent examples of people who have not intentionally ventured out and are not necessarily prepared for long periods in a boat in the middle of the ocean (for instance, fishermen lost at sea). It is certainly not in line with studies which have found that the process of colonising the Pacific is likely to have involved safe, systematic and planned exploration prior to the colonisation (Hiroa, 1954, Irwin, 1994). Quite simply, the speed at which successful settlement of the wider Pacific region occurred could not be sustained if there was great loss of life. While there had been some doubt during the 1950s

and 1960s about the deliberate nature of Pacific voyaging and navigation, with theorists such as Sharp (1956) arguing for 'accidental voyaging', most scholars now agree that preliminary scouts were sent out on two-way exploratory voyages prior to the departure of a well-provisioned colonisation party who were travelling to a known destination with known resources and for a known period of time (Kirch, 2000). These colonisation events may have been assisted by weather patterns such as El Niño (Goodwin et al., 2014), which would have made the voyaging times faster.

Starving at sea is not the only voyaging-related proposal put forth to explain genetic diversity in the Pacific. Houghton (1990, 1996) and Bindon and Baker (1997) have suggested that the increased body mass among New Zealand Māori and other Polynesian populations is a result of selection due to the cold temperatures faced during open ocean voyaging, the premise being that higher body mass would insulate one against wind chill and ocean spray (an adaption of a hypothesis known as Bergmann's rule). Supporting this hypothesis, sophisticated calculations were devised to show the likelihood of survival for 10 days voyaging on open ocean at various latitudes (Houghton, 1996). These calculations assume naked bodies that have no cold protection, which does not fit with knowledge of Polynesian voyaging and evidence for protective clothing (Hiroa, 1924) or structures in the canoe allowing for some weather protection (van Dijk, 1991). Similarly, these simulations are undermined by the recent archaeological data supporting a general shortening of Polynesian prehistory (Wilmshurst et al., 2011). For rapid, successive population movements, a larger founding population would be expected, which in turn would require voyaging to have a much reduced mortality rate than this particular application of Bergmann's rule. This is supported by a recent study investigating the cold induced vasodilation response in a number of populations worldwide, which has indicated that the cold adaptation seen in Polynesian populations is likely an ancestral trait which evolved in Asia, prior to the start of the Pacific colonisation process (Wilberfoss, 2012).

There is also archaeological evidence to support extensive sailing between archipelagos (Collerson & Weisler, 2007), at least in the early periods of colonisation, though this interaction dropped off in later prehistory (Irwin, 1994). This reduction in inter-island contact may be due to the development of greater socio-political complexity in some of these island groups, possibly triggered by climatic change (Field & Lape, 2010). The focus of the island communities turned inwards, rather than maintaining strong links with distant islands. Thus, it seems improbable that the voyaging process would be a strong selective pressure. The mortality rates described to select for the postulated 'thrifty' traits indicate a lack of appreciation for the sophistication of sailing technology and expertise of early Pacific Islanders (Hiroa, 1954) and is inconsistent with the speed of settlement based on the archaeological record.

3.2.4 Fragile island environments

It has been argued that Pacific Island populations might be subject to more famine events because of their relative isolation and their susceptibility to cyclonic weather patterns and tsunami (Zimmet et al., 1990, McGarvey, 1994). This might result in the destruction of horticultural crops and impact staple foods such as shellfish and other marine foods, which were mainstays to many prehistoric Pacific Island populations. As evidenced by the devastation caused by these sorts of phenomena in modern times (for example, the Samoan tsunami in 2009), it is not unreasonable to assume that such occurrences have been persistent but intermittent and relatively localised problems for many populations since initial colonisation. Indeed, skeletons from archaeological sites in the region show skeletal pathology consistent with nutritional deficiencies (Snow, 1974, Buckley, 2000, Buckley et al., 2014). The palaeopathology record can indicate other skeletal and dental changes reflecting growth disruption for assessing periods of food insufficiency in the past (Goodman et al., 1984). However, these signs of growth disruption may also be a response to infection, especially in a tropical environment where pathogen loads are high (Buckley, 2006). Therefore these growth disturbances are generally considered to be a reflection of non-specific or more generalised stress during growth.

This permutation of the hypothesis does not appreciate the variation in subsistence patterns across the Pacific, partly as a result of the different geological origins of the inhabited islands. Pacific peoples have taken to inhabiting atolls, volcanic and continental islands (Neall & Trewick, 2008), and these different island-types offer different subsistence opportunities due to variation in ecological diversity (Kirch, 2000). Factors such as water availability and soil composition alter what horticultural domesticates are likely to grow, and any surpluses which might be produced. As such, different islands are likely to have had different levels of vulnerability when faced with natural disasters. The distribution of obesity-prone populations throughout the Pacific, under this model, would require multiple independent episodes of selection for a 'Thrifty Genotype', to which end we would expect to see different genetic contribution to phenotype and not the broad patterning to their distribution which present today.

The high prevalence of obesity throughout the Pacific seems indicative of increased body mass index (BMI) being an ancestral trait, which was already present among the colonising population rather than selection for 'thrifty' traits *in situ*. Finally it has remained largely unrecognised that in the context of Pacific prehistory, the scale of population loss needed to result in the selection of so-called 'thrifty genes' is simply not supported by demographic models of colonisation and pre-European population estimates.

3.2.5 Genetic studies of 'Thrifty Genes' in the Pacific

It seems telling that despite multiple genetic studies (Ohashi et al., 2007, Furusawa et al., 2010, Myles et al., 2011), there is no direct evidence for 'thrifty genes' among Pacific Islanders or other related populations, as manifest by signatures of selection at known diabetes and obesity loci. Furthermore, loci which have been strongly associated with obesity among European populations, for instance intronic variants in the *FTO* gene (Frayling et al., 2007, Dina et al., 2007), have been found to have allele frequencies consistent with what is seen among Asian populations, so have been argued to be unlikely 'thrifty' candidates (Ohashi et al., 2007).

The Gln223Arg variant of *LEPR* has also been suggested as a 'thrifty gene' on the basis of differences in allele frequency across Oceania (Furusawa et al., 2010). *LEPR* encodes a protein which is the receptor for the fat cell-specific hormone leptin which regulates satiety, and is associated with BMI (Park et al., 2006). An elevated allele frequency would be expected in specific populations if the locus had indeed been subject to selection. However, there are other explanations for differences in allele frequency, such as genetic drift. This variation in allele frequency could very well be related to serial founder effects – as indicated by other genetic markers, for instance, mitochondrial genome haplogroups which show a similar gradient across the Pacific (Kayser et al., 2006). This, indeed, seems more likely than selection when one considers the lack of evidence for a functional effect of alleles at this particular locus (Stratigopoulos et al., 2009).

Some variants have been associated with metabolic traits in certain populations and not others, for instance the Gly482Ser variant in *PPARGC1A,* a gene involved in energy metabolism, has been found to associate with high BMI among Tongans but not New Zealand Māori (Myles et al., 2011) although this is not necessarily indicative of this being a 'thrifty gene' *per se*. A recent study has demonstrated no evidence for a selection signature at the *PPARGC1A* locus, nor was the association with BMI in Tongans able to be replicated (Cadzow, Merriman & Wilcox, unpublished data). The higher allele frequency of the *PPARGC1A* variant (Myles et al., 2011) in islands colonised relatively late, such as New Zealand (670-720 BP (Wilmshurst et al., 2011)) compared with Tonga (2838 ± 68 BP (Burley et al., 2012)), one of the first Polynesian islands colonised, is consistent with the manifestation of serial founder populations, though inconsistent with a population model of return voyaging and sustained contact between populations which has been suggested through the archaeological evidence.

The higher allele frequency of the Gly482Ser variant in *PPARGC1A* has also been cited as a potential 'thrifty gene' on the basis of its higher allele frequency among Tongans compared with Han Chinese and Papua New Guinean Highlanders (Myles et al., 2007). This same study

suggested that this locus has been subject to selection based on the application of F_{ST} analyses on these populations – a rather high F_{ST} value was generated when comparing Highland New Guineans with Tongans (0.703). However, no recognition is given to the fact that these are populations with vastly different ancestral backgrounds. Polynesian populations have been found to have a higher degree of Asian ancestral contribution to their autosomal DNA than Near Oceanic (New Guinea) (Wollstein et al., 2010). Given their dissimilar histories, it would therefore be expected to observe genetic differences between these populations. Additionally, the small sample sizes used in this study are unlikely to capture the genetic variation in any of the populations - data from only 23 Polynesians (nine Cook Islanders, eight Samoans, four Tongans and two Niueans), 23 Highland New Guineans and 19 Han Chinese were used (Myles et al., 2007). A better approach for detecting more recent selection (i.e. selection consistent with the 'Thrifty Genotype' hypothesis in the Pacific) is probably the haplotype-based Extended Haplotype Homozygosity (EHH) concept (Sabeti et al., 2002).

There is evidence for founder effects in Remote Oceania, Polynesians in particular (Kayser, 2010). This has significant implications when it comes to impact on genome-wide variation and may complicate the detection of signatures of selection if relying on tools that detect changes in allele frequency. Focused, large-scale genetic studies of populations worldwide with known founder effects, for instance Ashkenazi Jews and Icelandic populations, have assisted in identification of important susceptibility loci associated with other complex phenotypes (Helgason et al., 2005, Steinthorsdottir et al., 2007, Guha et al., 2012). The only such study that has been carried out in the Pacific was a genome-wide association study of a cohort from Kosrae, an isolated island located in the Federated States of Micronesia (Lowe et al., 2009). This study found a relatively genetically homogenous population and showed that a majority of the common variants contributing predisposition to disease in Europeans have little effect on Kosraens. Novel disease associations were identified which further underlines the need for studies focused on populations with well-defined ancestry and population history.

3.2.6 The 'Thrifty Genotype' Hypothesis worldwide

The lack of support for the 'Thrifty Genotype' hypothesis is a pattern which has emerged from other studies of populations worldwide, including Europeans and Asians (Helgason et al., 2007, Southam et al., 2009, Steinthorsdottir et al., 2014, Ayub et al., 2014, Koh et al., 2014). Ayub et al. (2014) critically evaluated the hypothesis by testing for signatures of selection at type 2 diabetes loci ('thrifty genes'). They examined 65 loci and found a lack of consistent evidence for selection; when the loci were collectively tested, there were no signatures of selection. Although when scrutinized on a locus-by-locus basis, some nominal evidence for positive selection was detected. Furthermore, Koh et al. (2014) looked for signatures of selection at loci associated

with obesity and type 2 diabetes identified in a genome wide association study of an East Asian cohort and likewise did not find consistent support for the 'Thrifty Genotype' hypothesis. Together, the findings of these studies indicate that the 'Thrifty Genotype' hypothesis should be reassessed as a way of considering evolutionary explanations for higher rates of obesity and type 2 diabetes among various populations.

The 'Thrifty Genotype' hypothesis does not account for the fact that famines are likely to have been a periodic problem for all human populations even before the emergence of our species from other hominins (Prentice, 2005). Mathematical modelling suggests that if 'thrifty genes' did exist, given the number of famine-events faced by *Homo sapiens* since the divergence from our hominin ancestors these alleles would likely have reached fixation (where the advantageous allele becomes homozygous) (Speakman & Westerterp, 2013). This is in line with the recent suggestion that we should perhaps be looking deeper in human evolution for these 'thrifty genes'. Uricase has been highlighted as a putative 'thrifty gene' (Kratzer et al., 2014), but given that the inactivation of the gene occurred during the Miocene, prior to the divergence of humans from the ape lineage, uricase is also unlikely to account for differences in metabolic health between modern populations – nor is there any direct evidence that the loss of uricase function was driven by periods of famine. In other words, it 'assume[s] rather than demonstrate[s] the operation of natural selection' (Pigliucci & Kaplan, 2000). Given the multiple roles of urate (as an anti-oxidant, an adjuvant and in maintaining blood pressure (Gosling et al., 2014)), there may have been selective forces at work other than nutritional stress.

This is not to say that nutritional exposures have not led to the selection of certain genetic variants (Stover, 2006). Variation in allele frequencies for enzymes involved in the digestion of lactose (Bersaglieri et al., 2004), starch (Perry et al., 2007), and the metabolism of alcohol (Eng et al., 2007) and fructose (Ali et al., 1998) have been detected between populations. That some populations have locally adapted genetically to optimise their nutrient-uptake is unsurprising given the role of food in health and wellbeing, and indeed survival in general. The array of different adaptations is symptomatic of the diversity in subsistence patterns and nutritive resources available in different geographical regions. Some might argue that these variants themselves could be classed as 'thrifty genes', since the ability to digest lactose from cow's milk or to better break down starches from tubers and grains would undoubtedly be advantageous in situations of famine, though being advantageous is not the same as being 'thrifty'.

3.2.7 Concluding comments

The 'Thrifty Genotype' hypothesis overlooks other important factors that are likely to have had more significant impacts on populations and their genetic diversity, such as migration and

disease epidemics (O'Rourke, 2012). As discussed previously in relation to genetic differences between New Guinean and Tongan populations, genetic ancestry and the specific evolutionary history of a population has a huge impact on the particular variants present within a population and indeed the relative proportions of various alleles. Founder effects are clearly something that require consideration in Oceanic populations, as serial migrations have played a large part in the settlement of the area. Admixture, and the degree and nature of that admixture, is another factor which impacts the genetic variability of a population.

Infectious disease is one of the strongest drivers of genetic change (Karlsson et al., 2014): major human pathogens such as malaria (Kwiatkowski, 2005) and HIV (Schliekelman et al., 2001, Nkenfou et al., 2013) have shaped the genome in exposed populations. Given the links that are increasingly being made between metabolic diseases and innate immunity (Pickup & Crook, 1998, Lumeng & Saltiel, 2011, Robbins et al., 2014), genetic selection resulting from infectious disease exposure may contribute to an underlying susceptibility of certain populations to metabolic diseases. An example of the striking effect that infectious disease can have on a population is the introduction of Western diseases including measles, whooping cough and influenza to the Pacific during the 19th century; it is thought that these introductions caused up to 75% mortality in some East Polynesian populations (Harrison et al., 1988). Clearly this is likely to have had a large impact on the genetic diversity in the modern population.

For its time, the 'Thrifty Genotype' hypothesis was revolutionary. It reflected one of the first efforts to integrate our knowledge of modern epidemiology with human evolution. However, the hypothesis is also inherently reductionist and does not recognise other factors which are likely to have a greater impact on the development of genetic traits. In the 53 years since its introduction, contributions by archaeology and biological anthropology to our understanding of colonisation processes and prehistoric demography have remained overlooked in genetic studies and other reviews exploring the possibility of 'thrifty genes' among Pacific Islanders – and indeed, among other populations globally. When the evolutionary and cultural history of Pacific people is considered, the application of the 'Thrifty Genotype' hypothesis is no longer useful; the scenario certainly does not mesh with our current understandings. The continued reiteration of the 'Thrifty Genotype' hypothesis in general and in its application to Pacific populations in particular gets in the way of our formulating alternative, better supported and testable hypotheses based on our knowledge of the context and histories of the populations in question. Alternative hypotheses, such as selection by infectious disease exposures, need to be explored.

3.3 An alternative hypothesis

In this section, the obesity phenotype will largely be ignored. Instead, the uniformly high serum urate levels will be focussed on for the reason that the high rates of hyperuricaemia underpin the high prevalence of gout among many Pacific peoples. However, given that elevated urate levels have been suggested to be a predictor of weight gain (Masuo et al., 2003), the high rates of obesity in the Pacific may in part be contributed to by this genetic predisposition to hyperuricaemia. Elevated urate levels have also been detected in those who suffer other metabolic conditions such as type 2 diabetes (Hayden & Tyagi, 2004).

In order to assess the possibility of selection for heightened serum urate concentrations, it is necessary to consider the different biological roles of urate in humans. What follows is a brief description of these roles and how these may have provided a selective advantage to those with hyperuricaemia.

3.3.1 Antioxidant effect of urate

Urate can function as an antioxidant (Ames et al., 1981, Scott & Hooper, 2001). Antioxidants are very important, as radical oxygen species can cause irreversible oxidative damage to cellular organelles and DNA, which can impair the function of the individual and result in death. Urate accounts for up to 60% of total plasma antioxidant activity (Ames et al., 1981). This may help protect endothelial function, and combat oxidative stress associated with aging (Ames et al., 1981). It has also been implicated as being neuro-protective. Unusually low SU levels have been associated with diseases such as multiple sclerosis (Spitsin et al., 2001, Rentzos et al., 2006), Parkinson's disease (de Lau et al., 2005) and Alzheimer's disease (Kim et al., 2006). The brain is particularly vulnerable to oxidative damage because of its high metabolic rate and the high lipid content of brain tissue (Kutzing & Firestein, 2008). The antioxidant activity of urate seems an unlikely selective force specifically in the Pacific, as there is no reason to believe that people dwelling in the Pacific, or more precisely Polynesia, should face any more oxidative stress than any other island dwelling population. In addition, most of the diseases associated with low SU levels have impacts in post-reproductive periods of the lifecycle.

3.3.2 Urate and Hypertension

There is a strong association between hyperuricaemia, hypertension and cardiovascular risk (Cannon et al., 1966, Jossa et al., 1994, Johnson et al., 2003), which has led to the hypothesis that in the course of human evolution, urate may have played an important role in maintaining blood pressure in conditions of low salt ingestion (Watanabe et al., 2002). Through an animal experimental model, Watanabe et al (Watanabe et al., 2002) found that urate helps maintain

blood pressure both acutely, by stimulating the renin angiotensin system, and chronically, by inducing salt sensitivity.

There is evidence to suggest that during the Miocene epoch, which is when the series of mutations leading to the inactivation of the uricase gene in Hominids are thought to have occurred, salt consumption among our pre-human ancestors was considerably lower than in modern day societies (Watanabe et al., 2002). In this situation, the increase in blood pressure associated with raised serum urate levels was advantageous (Watanabe et al., 2002). However, again, with the ready availability of salt in Pacific Island environments, this seems an unlikely evolutionary force in the Pacific.

3.3.3 Uric Acid as a Physiological Alarm

(Johnson et al., 2009) have proposed that under conditions of environmental stress and starvation, urate may have had a beneficial effect as a physiological alarm. Studies have shown an increase of urate concentration under conditions of fasting (Gumaa et al., 1978) or starvation (Lennox, 1924), may have a number of beneficial effects, including increasing locomotor activity necessary for foraging, stimulating hypertriglyceridaemia, fatty liver and weight gain to help re-establish fat stores, and to increase salt sensitivity to help protect against dehydration (Johnson et al., 2009). They further suggest that the development of insulin resistance, which is also associated with many metabolic diseases, could also be beneficial, by reducing glucose uptake into skeletal muscle and adipose tissue, thus preserving glucose for utilisation by the brain where glucose uptake is insulin independent.

This potential role of urate has more credibility as a possible selective force in Pacific Island peoples. Island environments, such as those we see in the Pacific, are vulnerable to catastrophic events such as cyclones and tsunami, which are capable of wiping out not only food supplies but also sources of fresh, potable water on islands, causing periods of famine. It is possible that such events in the past have caused some selection for those with apparently enhanced genetic fitness. These sorts of catastrophic events can also result in population bottlenecking events, thus changing allele frequency. The widespread nature of the hyperuricaemic phenotype in Austronesian-speaking populations across Oceania and beyond, however, does suggest that any population bottlenecking to cause a hyperuricaemic phenotype would have had to occur early on prior to population expansion into ISEA and the Pacific for all of the descendant populations to be affected or else to have happened numerous times across the Pacific.

3.3.4 Urate as an Adjuvant

Urate plays a significant role in immune regulation (Shi et al., 2003). When crystallised, it has been found to activate innate host defence mechanisms in multiple ways and triggers a robust inflammatory response (Shi et al., 2010, Rock et al., 2013). For this reason, crystallised urate

(monosodium urate; MSU) is considered to be an important natural endogenous adjuvant and has been employed in vaccinations to help stimulate a host response against antigens which may otherwise be considered innocuous. Not only can MSU crystals stimulate phagocytes and monocytes, but also they can activate the NLRP3 inflammasome, both the classical and alternative complement pathways, interact with antibodies, as well as a number of other immune pathways (Martinon, 2010). Absence of urate inhibits the immune response associated with clearing debris from damaged cells.

As previously discussed, infectious disease is one of the strongest forces of selection (Sabeti et al., 2006). The genes associated with immunity are under selective pressure, as contact with an infectious agent requires an appropriate immune response. It is possible that urate-raising genetic variants may be selected for by infectious disease.

There are a number of events in the history and prehistory of the Pacific which may have resulted in the selection of genetic variants which could contribute to the inherently high mean urate levels throughout the region. Discussed below are two possible periods of selection for genetic variants contributing to inherently high serum urate levels, one deep in prehistory with the exposure of ancestral Polynesian populations to malaria prior to their arrival in the malaria-free islands of Polynesia, and the more recent exposure of these populations to novel diseases post-European contact.

3.3.4.1 Malaria as a means of selection

Malaria is possibly the most serious infectious disease currently known to humankind, killing on the order of 2 million people annually (Schofield & Grau, 2005). Malaria is not a new immunological challenge: humans and malaria-causing *Plasmodium* species parasites have had a long evolutionary host-parasite relationship (Conway et al., 2000). Malaria is known to have influenced the human genome, with disorders like sickle cell anaemia, alpha- and beta-thalassaemia, glucose-6-phosphate dehydrogenase (G6PD) deficiency and band 6 ovalocytosis having been attributed to malarial selective forces (Kwiatkowski, 2005). It is possible that the inherent hyperuricaemia seen in Polynesian populations is another such adaptation.

Malaria is a disease transmitted to humans by the bite of a *Plasmodium*-parasite bearing female mosquito of the genus *Anopheles.* The saliva of an infected mosquito contains malarial sporozoites, which travel through the blood vessels to the liver where they invade hepatocytes and reproduce asexually to produce thousands of merozoites. At this point, the malaria parasite is effectively immunologically invisible. The merozoites infect erythrocytes and undergo a number of asexual cycles of replication, producing yet more infective merozoites, before rupturing the cell and infecting yet more erythrocytes. Some of the merozoites develop into

immature gametocytes in preparation of infecting a mosquito host to allow the sexual phase of the *Plasmodium* lifecycle, and the eventual infection of more human hosts. It is during this cyclical blood phase of infection that the immune system detects the presence of the pathogen (Cowman et al., 2012).

Urate plays an important role in the innate immune response (Figure 3.1), and has been found to induce host inflammatory responses in malaria (Griffith et al., 2009, Shio et al., 2010). There appear to be a number of sources of urate during malaria infection, all of which occur during the final cyclical blood phase. Firstly, the malarial parasites sequester xanthine and hypoxanthine inside the erythrocytes at high concentration in order to make nucleic acids during parasite proliferation. When the infected erythrocytes are ruptured, this xanthine and hypoxanthine is released and broken down by extracellular xanthine oxidase to produce urate (Orengo et al., 2008, Orengo et al., 2009). Secondly, *in vitro* studies have found uric acid precipitate accumulations inside *Plasmodium falciparum*-infected erythrocytes (van de Hoef et al., 2013). When the erythrocytes burst, these precipitates are released into the extracellular matrix where they can be encountered by antigen presenting cells including dendritic cells and macrophages. van de Hoef et al. (2013) found that incubation of human dendritic cells with fractionated lysates from these infected erythrocytes (fractionated to remove haemozoin but to retain the uric acid precipitates) was found to induce maturation, with the upregulation of co-stimulatory molecules (CD80, CD86) and major histocompatibility complex class II molecules (human leukocyte antigen [HLA] in humans). Finally, haemozoin, a malarial pigment, also appears to directly stimulate the release of urate from the endothelial cells in a murine model (Griffith et al., 2009).

Allopurinol (a urate-lowering drug which inhibits xanthine oxidase) treatment in a mouse model of malaria lead to an increased parasite burden (Büngener, 1974), which indicates that urate may be critical in the development of an immune response against the parasite. Since an early and robust inflammatory response is critical for controlling the infection, inherently high serum urate levels may be advantageous for those living in malarial environments and could have been selected for, as fewer host erythrocytes would need to rupture before the innate immune response countering the malarial parasites was triggered, thus enhancing the rates of host survival.

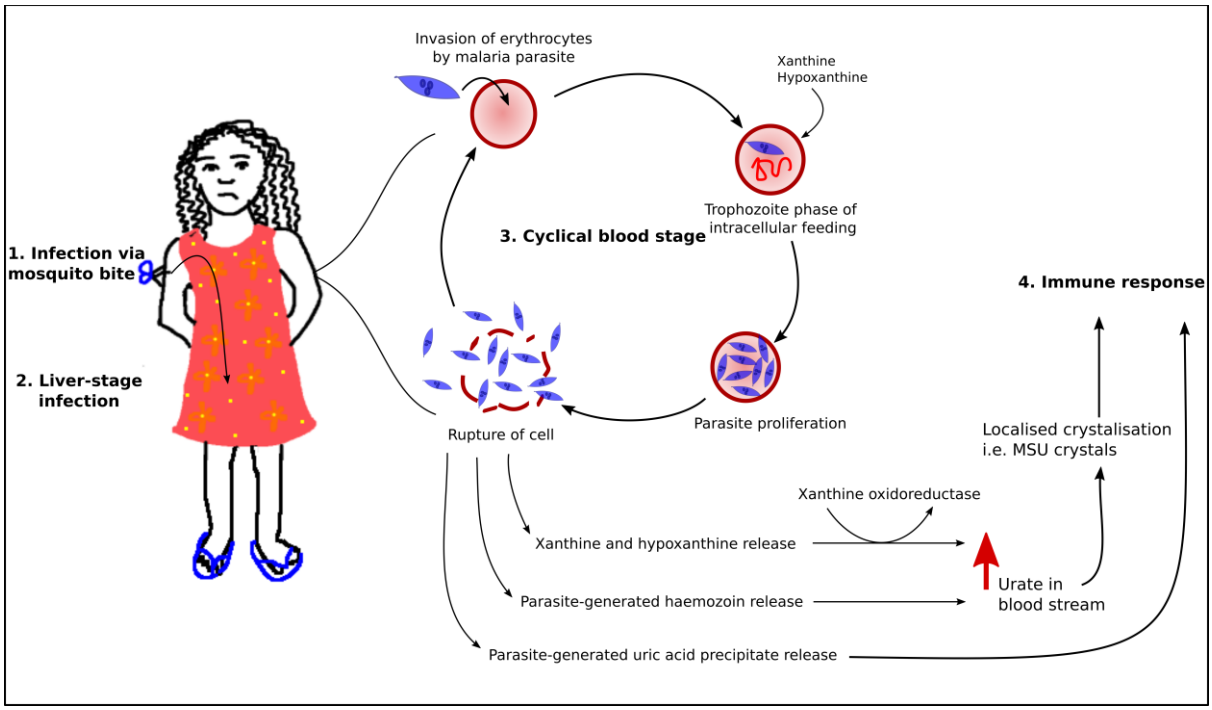


Figure 3.1: a role for serum urate in innate immune responses to malaria infection

While malaria is not present, nor has probably ever been present, in Polynesia, the ancestral populations passed through and may have originated in areas with endemic malaria (namely New Guinea, the Solomon Islands and Vanuatu) (Buckley, 2006). Malaria would also have been endemic in the East Asian/ISEA homeland of the original Austronesian-speaking populations from whom the Polynesians are ultimately descended (Hay et al., 2004). In fact, there is other evidence that transit through these malarial regions has shaped the genomes of modern Polynesians; there is an unusually high frequency of α-thalassaemia variants in Polynesians and particularly amongst East Polynesians (Figure 3.2) (Hill et al., 1985, Flint et al., 1986, Hill et al., 1987, Hill et al., 1989), especially for populations living in an area which does not have, and has probably never had, active malarial transmission (Clark & Kelly, 1993). Thus malarial selection could have operated either during the spread of populations through the region of Near Oceania, or in their place of origin in ISEA.

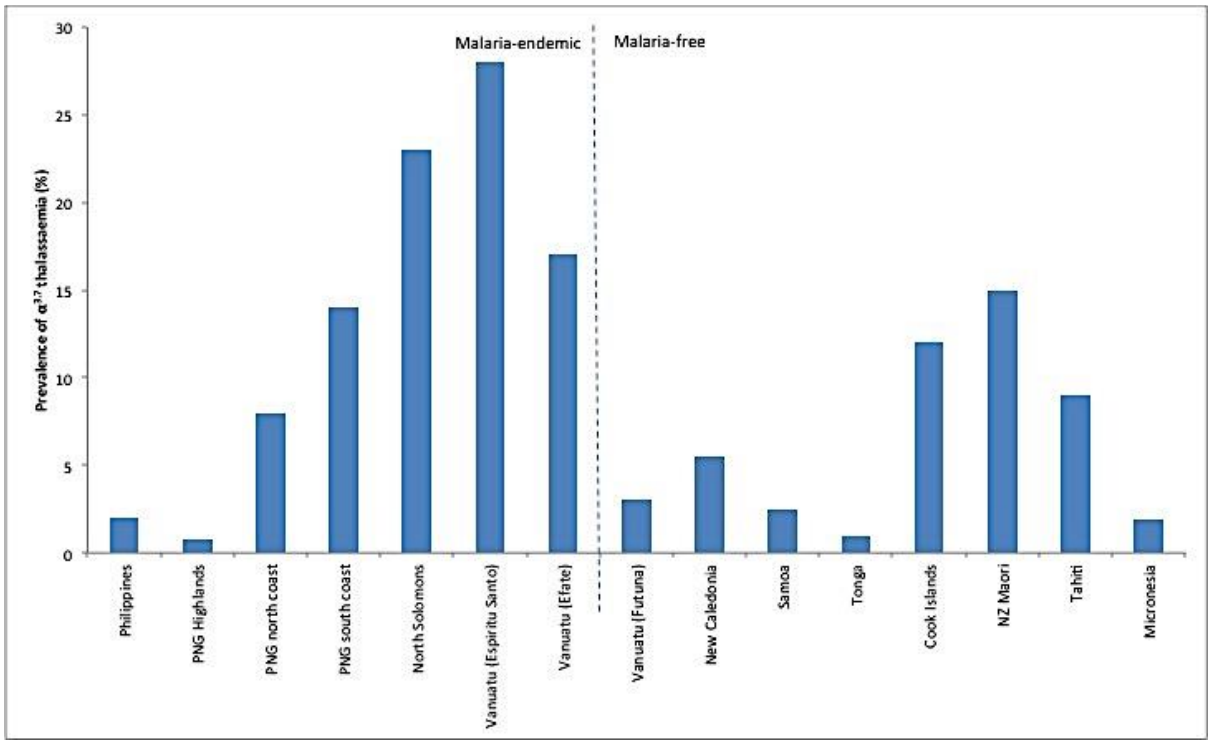


Figure 3.2: Prevalence of α-thalassaemia (α3.7III variant) in various malaria and non-malaria endemic Pacific populations

It is important to note that mean serum urate concentrations reported in populations from coastal Papua New Guinea, where malaria is endemic, are much lower than those observed in Polynesians, and indeed, Micronesians. For instance, male Papuans living on the Madang Coast (northern mainland Papua New Guinea) have serum urate levels of 4.8 mg/dL (Hornabrook et al., 1975), while males from Kalo village in the lowlands (Central Province, Papua New Guinea) were reported to have urate levels of 4.2 mg/dL (Wyatt et al., 1980). Malaria is thought to have been present in coastal regions for a substantial period of time – the early habitation (around 50,000 years ago) of the malaria-free Highland regions of New Guinea (Summerhayes et al., 2010b) may have been driven by the presence of the malarial parasite in the lowlands. As a result of this long exposure to malaria, a wide array of malaria-associated haemoglobinopathies, including the globin gene variant (α3.7III) seen in Polynesians, are observed in lowland New Guinean populations (Müller et al., 2003). Evidence of substantial genetic admixture between the Asian-derived Austronesians and the non-Austronesian original inhabitants of New Guinea is only significant in the Admiralty Islands and the Bismarck Archipelago, to the north of the mainland of New Guinea, particularly the New Ireland Province (Friedlaender et al., 2007). To date, there are only published data on the serum urate levels from mainland New Guinea populations. Because of the different genetic background of ancient Near Oceanic and Asian derived Austronesian populations, it is conceivable that different adaptive mechanisms

developed in response to malaria, explaining the absence of elevated serum urate in these populations.

3.3.4.2 Western diseases as a means of selection

The introduction of novel diseases and pathogens after European contact had a significant effect on the demographics of many Pacific populations (Kirch & Rallu, 2007). Prior to the exploration of the Pacific by Europeans, Oceania had long been isolated from most infectious diseases. The small-scale nature of most populations but particularly their isolation, protected these islands from influenza, measles, mumps, smallpox, tuberculosis, cholera, plague, typhoid, whooping cough and venereal disease (Denoon, 1995). These diseases were therefore alien to Pacific Islanders, leaving them immunologically naïve, and thus exposure to these diseases had the capacity to cause large-scale loss of life.

While it remains challenging for archaeological and historical demographers to assess the effect of the introduction of Western diseases on these island populations for a number of reasons, in part because of the lack of regular, systematic censuses on many islands and the uncertainty over the initial population sizes (McArthur, 1968), it is clear that introduced diseases did have a major impact. Some islands seem to have been more affected than others, but on some islands where records are available, it is possible to link epidemic outbreaks of diseases like whooping cough, measles and influenza to visits by certain European ships, and many of these epidemics caused significant mortality (Denoon, 1995). As previously mentioned, East Polynesia was hit particularly hard – much more so than West Polynesia – particularly during the initial introduction of these diseases which resulted in losses up to 75% of the population in some islands (McArthur, 1968, Harrison et al., 1988). This may go some way to explain some of the genetic differences between East and West Polynesia (Phipps-Green et al., 2010, Hill et al., 1989). Given the role of monosodium urate crystals in enhancing the innate immune response, it is possible that genotypes promoting increased urate levels were also positively selected for during these periods of massive depopulation. It is also likely, given the presence of archaeological evidence for gout in samples that are definitely from a pre-European era, that urate-raising variants were already at a higher prevalence prior to the introduction of European infectious diseases.

3.4 Conclusion

There have been a number of different hypotheses put forward to explain the high rates of metabolic disease among Pacific populations. The 'Thrifty Genotype' hypothesis, and variants of this, has been a common explanatory scenario. The lack of genetic evidence supporting this hypothesis, and because it does not fit well with current archaeological understandings surrounding the prehistory of the region, means that continuing to apply this hypothesis to the Pacific scenario is not helpful in understanding phenotypic variation between populations. Houghton's (1990) proposal that Bergmann's rule may account for the differences in body mass and susceptibility to disease is also unsupported by present archaeological data.

Raised serum urate levels are seen throughout the Pacific, particularly in Polynesia, Micronesia and amongst Taiwanese aborigines. Archaeological evidence suggests that this is not a new phenomenon (Buckley, 2011). The multifactorial aetiology of hyperuricaemia indicates that there are likely to be a number of contributing factors, including a genetic contribution. The localised heightened prevalence of hyperuricaemia and gout in Polynesian and Micronesian populations suggests that shared heredity may contribute. However, while potential reasons for this may be proposed, it remains difficult to disentangle how, when and why this hypothesised genetic contribution arose in these populations.

Processes of natural selection may have contributed to the phenotype which is observed in modern populations – in particular, infectious disease may have played a role in this process of selection; however, random genetic drift cannot be discounted. The colonisation process of the Pacific with serial founder effects, as well as catastrophic events in the fragile island environments, means that there have been ample opportunities for genetic drift. However, at the same time, given the high levels of hyperuricaemia and gout seen in populations who likely share at least some ancestry with the Polynesians and Micronesians, for instance, the Taiwanese aborigines (Chang et al., 1997, Chou & Lai, 1998, Chang et al., 2001), and the Malagasy highlanders (Capdevielle et al., 1980), the probability of random genetic drift operating in all of these populations to result in the same phenotype seems unlikely. Multiple processes are likely to have worked in tandem resulting in the genetic predisposition to hyperuricaemia.

Ancestry, itself, is not a trivial consideration. Examination of the available published literature indicate that elevated serum urate may be a trait shared by populations descended from the Austronesian-speaking peoples who departed from South East Asia around 8000 years ago. In light of the low serum urate levels seen in coastal populations in mainland Papua New Guinea, it would be interesting to investigate the state of serum urate levels among the Lapita-derived, Austronesian-speaking populations living in the Bismarck Archipelago, to the north-east of

mainland Papua New Guinea: are they inherently hyperuricaemic like Polynesian and Micronesian populations, or are there lower urate levels similar to the mainland?

There is significant scope for further investigations to determine the reasons for elevated serum urate, and indeed evolutionary reasons for other metabolic phenotypes, in populations in the Pacific region. While there have been efforts to understand prehistoric human migration in this region, disease has not been a major focus. The gulf between anthropological studies in the Pacific and genetic disease research has meant that genetic predisposition to conditions like hyperuricaemia has not been examined from an evolutionary perspective. Further genetic studies in Pacific populations are warranted and could assist in testing hypotheses, for example, the application of tools for detecting selection to the genomic sequence of genes involved in the regulation of urate.

It is also important to recognise that while the high serum urate levels seen throughout the Pacific are a useful marker to use to speculate about the origins of metabolic disorders like gout, high urate alone does not lead to disease. Conditions such as gout and type 2 diabetes have an auto-inflammatory component, where an inappropriate immune response is mounted and inflammation occurs, and as a result, there are likely other immune related genetic variants contributing to this susceptibility to disease. This is compatible with models of infectious disease contributing selective pressure as is discussed above.

4 NLRP3 inflammasome and metabolic disease

4.1 The NLRP3 inflammasome

As alluded to in the preceding chapter, the NLRP3 inflammasome constitutes an important part of the innate immune response countering malaria infection and has also been implicated in the inflammatory processes in gout. This observation underlies the proposal that exposure of ancestral Pacific populations to malaria may have resulted in positive selection for genetic variants contributing to elevated serum urate levels. Given the high prevalence of metabolic diseases among Polynesians, and emerging research implicating the NLRP3 inflammasome in a range of auto-inflammatory diseases, this chapter aims to review what is known about the role of this type of immune response to these disorders, and to give background into the reasons for exploring mitochondrial variation in the Polynesians.

The immune system in humans consists of two main branches: the innate and acquired immune systems. The innate immune system constitutes the first line of defence in the overall immune system (Akira et al., 2006). It is able to react to pathogens in a relatively generic way, recognising both pathogen-associated molecular patterns (PAMPs) such as microbial cell wall components but also damage-associated molecular patterns (DAMPs)(Table 4.1). DAMPs are largely endogenous, comprising certain common immune stimulatory molecules present inside most cells, but which are not usually exposed in the extracellular matrix where they may be encountered by antigen presenting cells such as dendritic cells or macrophages that are constantly on the lookout for such distress signals. When there is cellular damage or necrosis caused by pathogenesis or other means, these are released and recognised as danger signals. Therefore, these DAMPs can contribute not only to pathogen-associated immune responses, but also to sterile inflammation and autoimmunity. Notably, unlike the acquired immune system, the innate immune response does not confer long-lasting or protective immunity to the host: it is purely reactive. It is an ancient system among multicellular organisms protecting against attack by microbes, and is the dominant immune system found in not only vertebrates, but invertebrates, plants and primitive multicellular organisms (Kimbrell & Beutler, 2001).

There are multiple mechanisms for the innate immune response to detect perturbations potentially caused by exposure to pathogens. In mammals, humans included, cytokine-mediated inflammatory processes seem to be the dominant form of innate immunity.

Table 4.1: Common pathogen- and damage-associated molecular patterns which initiate innate immune responses

PAMPs	DAMPs
Microbial cell wall components	Extracellular ATP
i.e. Peptidoglycan, lipopolysaccharide, lipoteichoic acid	Extracellular host DNA (mtDNA and nuclear)
Flagellin	Hyaluronan
Pathogen RNA (especially double stranded)	Monosodium urate crystals
Pathogen DNA (especially unmethylated CpG motifs)	HMGB1
Toxins e.g. Nigericin, α-toxin	
Parasitic metabolites, e.g. Haemozoin	

It is becoming increasingly evident that inflammasomes are an important means of inducing of innate immune responses (Martinon et al., 2002). Inflammasomes are a relatively recently identified multi-protein complex which consist of caspase 1, a caspase recruitment domain (CARD), a pyrin domain and various other components (for instance, nucleotide-binding domains) depending on their activator. The NLRP3 inflammasome is one of a number of inflammasomes that have been identified, and has been found to respond to a wide range of PAMPs and DAMPs.

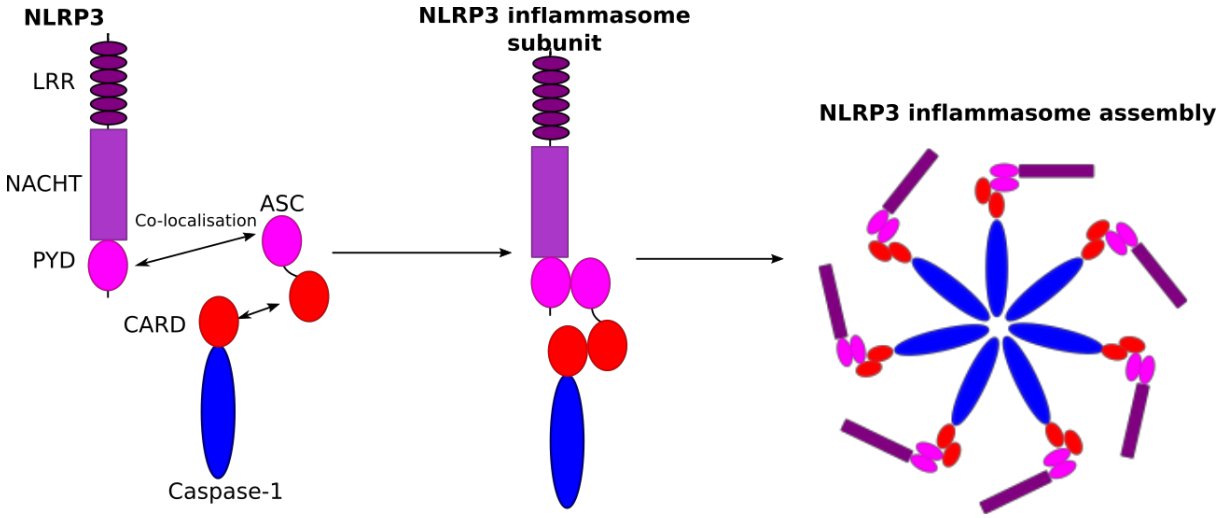


Figure 4.1: Components of the NLRP3 inflammasome. CARD, caspase-recruitment domain; LRRs, leucine-rich repeats; NACHT, NAIP, CIITA, HET-E and TP1; PYD, pyrin domain

Specifically, the NLRP3 inflammasome describes a multiprotein oligomer comprising Nod-like receptor protein 3, also known as nucleotide-binding domain, leucine rich repeat containing family, pyrin domain-containing 3 (NLRP3) protein, caspase-1 and apoptosis-associated speck-like protein containing a CARD (ASC) (Figure 4.1). Activation requires the NLRP3 protein and the ASC protein to come into proximity, a process dependent on other cellular processes.

NLRP3 inflammasome initiation is dependent on a two-signal activation system, which represents an important regulatory check-point to avoid inappropriate activation which would damage the host. The first activation signal triggers a signalling pathway which results in the stimulation of a protein complex named nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) via toll-like receptor 4 (TLR4) or toll-like receptor 2 (TLR2), transmembrane receptors highly expressed on the exterior of macrophages and dendritic cells, professional antigen-presenting cells whose sole job is to patrol the body looking for signs of abnormal cellular damage or out-of-place microbial cellular components. NF-κB is a transcription factor that is always present in cells in an inactive state, allowing for immune responses to be rapidly switched on when necessary. The triggering of signal 1 causes an upregulation of the production of NLRP3 protein, however without the second signal, inflammation will not proceed. As well as being triggered by foreign pathogenic entities, TLR2 and TLR4 can be stimulated by oxidised low density lipoprotein (LDL) (Chávez-Sánchez et al., 2010), a biomarker which is often found in elevated levels in individuals with metabolic dysfunction.

Table 4.2: - Known endogenous triggers of the NLRP3 inflammasome

Metabolic danger signal	Disease state	References
Amyloid beta	Alzheimer's disease	[1]
Calcium pyrophosphate dehydrate crystals	Pseudogout	[2]
Ceramides	Obesity	[3]
Cholesterol crystals	Atherosclerosis	[4]
Islet Amyloid Polypeptide	Type 2 diabetes progression	[5]
Monosodium urate crystals	Gout	[6]
Palmitate	Early type 2 diabetes	[7]

[1] (Halle et al., 2008); [2] (Martinon et al., 2006); [3] (Vandanmagsar et al., 2011); [4] (Duewell et al., 2010, Rajamäki et al., 2010); [5] (Masters et al., 2010); [6] (Martinon et al., 2006); [7] (Wen et al., 2011)

The second activation signal is known to be activated by a number of different danger signals, though its precise mechanism is still yet to be elucidated. There are several possibilities which are discussed later in the chapter. This second signal causes the co-localisation of the NLRP3 proteins with the ASC, a process in which mitochondria seem to play a central role (Zhou et al., 2011, Misawa et al., 2013), instigating the activation of caspase-1, which goes on to proteolyse the precursors of pro-inflammatory cytokines interleukin (IL)-1β and IL-18 (Martinon et al., 2002). IL-1β binds to the IL-1 receptor (IL-1R) and induces the formation of a ternary complex with the IL-1R accessory protein, resulting in a downstream signalling cascade involving the induction of more proinflammatory cytokines and chemokines and enticement of neutrophils and other immune effector cells to the region (Cullinan et al., 1998). This sort of response is invaluable when there is a pathogenic stimulus, indeed there is evidence for NLRP3 inflammasome involvement in the resolution of influenza (Allen et al., 2009) and malaria (Kalantari et al., 2014, Gazzinelli et al., 2014) infection. However, given that some of the danger signals recognised by the NRLP3 inflammasome are endogenous (Table 4.2), this opens up the possibility of auto-inflammatory diseases.

4.2 Links between NLRP3 and metabolic diseases

The causation of gouty pathology by monosodium urate (MSU) crystals inducing inflammation is something long established. As early as 1679, Anton van Leeuwenhoek, one of the pioneers of microscopy, described the appearance of crystals from a gouty tophus (McCarty, 1970). By 1859, Alfred Baring Garrod had identified these crystals as consisting of a “urate of soda” (Garrod, 1859). It has been a lot more recently that the specific inflammatory processes stimulated by urate have begun to be disentangled. Shi et al. (2003) found that eliminating uric acid *in vivo* inhibits the immune response associated with DAMPs, and Martinon et al. (2006) identified the specific involvement of the NLRP3 inflammasome in the inflammatory processes responsible for gout (Figure 4.2).

The NLRP3 inflammasome seems to be unique in that it can detect not only pathogen-associated molecular patterns (PAMPs) but also damage-associated molecular patterns (DAMPs) and other endogenous danger signals, particularly crystalline structures (Table 4.2). The NLRP3 appears to be playing a role in a range of other age-related auto-inflammatory diseases (Schroder et al., 2010), including obesity-induced insulin resistance, type 2 diabetes and atherosclerosis. This should perhaps be unsurprising given the fundamental and highly conserved and closely integrated natures of the immune and metabolic systems. Given that many of these conditions occur as co-morbidities (Li et al., 2013), it may be that an over-active (or more-easily stimulated) immune response of this sort (perhaps as a result of genetic predisposition) may

underlie disease – particularly in populations where there appears to be a higher prevalence of disease.

Below, the connections between a number of other metabolic conditions, which also occur at high frequency among Pacific peoples (see Chapter 2 regarding prevalence), and the links between these conditions and NLRP3 inflammasome activation are discussed.

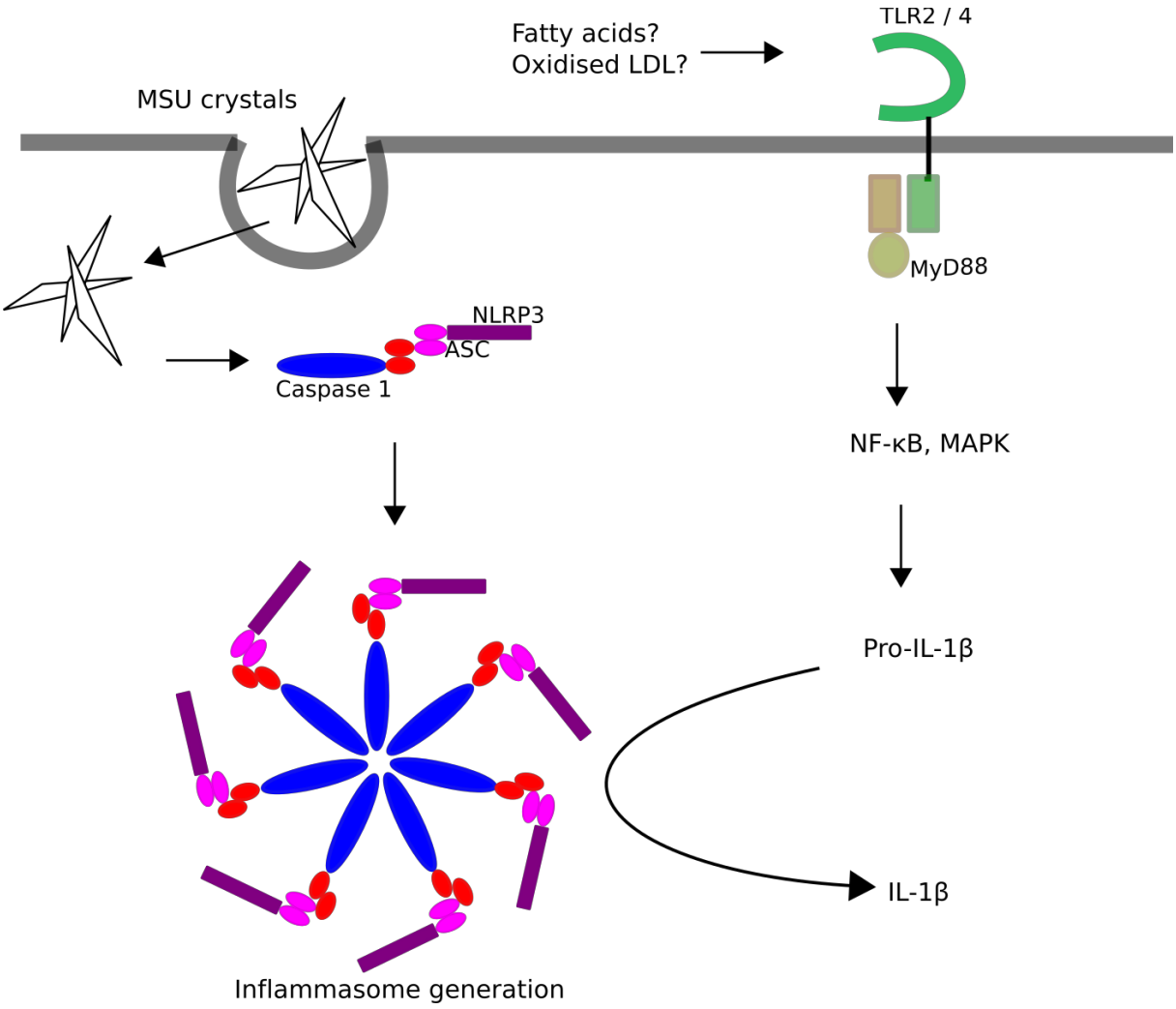


Figure 4.2: The NLRP3 inflammasome recognises monosodium urate crystals and is involved in the production of inflammatory cytokine IL-1b and IL-18 in gout

4.2.1 NLRP3 in Obesity

With the increasing rates of obesity among human populations worldwide, there has been a growing awareness of the accompanying health problems (Figure 4.3) – conditions including type 2 diabetes, cardiovascular disease, hepatic steatosis, neurodegeneration and biliary disease (Hotamisligil, 2006). Human obesity does not always result in disease, suggesting that there is a variable threshold for tolerable fat between individuals that is likely to be determined by environmental and genetic variables (Gregor & Hotamisligil, 2011). There is also some variation in terms of the total body fat to mass ratios, with populations with different ancestral backgrounds being shown to have subtly different body compositions (Lovejoy et al., 1996, Swinburn et al., 1999, Després et al., 2000, WHO, 2004) that may in turn affect their susceptibility to metabolic disease. The distribution of the fat also contributes variable risk; subcutaneous adipose tissue has subtly different cell size (Johnson & Hirsch, 1972), metabolic activity (Krotkiewski et al., 1983) and may play a different role in the development of insulin resistance (Gastaldelli et al., 2002) compared to abdominal or visceral fat. Indeed, visceral fat appears to be more pathogenic (Fujioka et al., 1987, Fox et al., 2007).

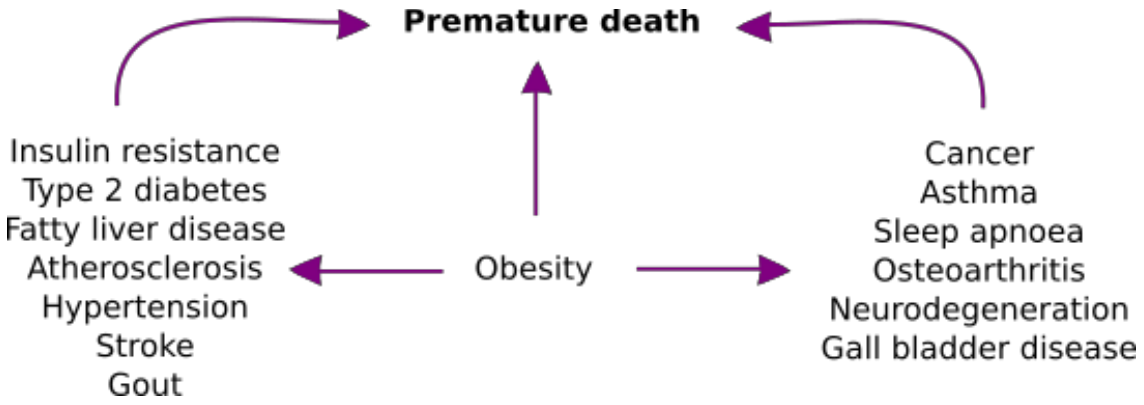


Figure 4.3: Clustering on metabolic diseases with obesity. The mechanisms behind the diseases on the right with obesity are less well understood than those on the left. (modified from Hotamisligil 2006).

Obesity is considered a risk factor for various other metabolic conditions. It may be that the low-grade inflammatory response which accompanies obesity underlies susceptibility to these other various metabolic diseases (Lumeng & Saltiel, 2011). The definition of this inflammatory state falls outside that of classical inflammation as defined by redness, swelling, heat and pain (the standard self-limited response to infection or injury), however the inflammation associated with obesity is chronic, arising in specialised cells such as adipocytes that are triggered by metabolic factors (for instance, the consumption of excess calories – pro-inflammatory cytokine levels have been found to fluctuate several hours after the consumption of food (Blackburn et al., 2006, Manning et al., 2008, Gregor & Hotamisligil, 2011)). The earliest studies detecting inflammation

in obesity (in a murine model) found increased levels of the pro-inflammatory cytokine TNF-α in adipose tissue (Hotamisligil et al., 1995), however since this finding a plethora of other cytokines have been detected including those with associated with NLRP3 inflammasome activity (Vandanmagsar et al., 2011). Adipose tissue is not the only affected tissue, with the liver (Cai et al., 2005), muscle (Saghizadeh et al., 1996, Fink et al., 2013) and pancreas (Maedler et al., 2002) also being subject to inflammation in presence of obesity. This chronic inflammatory response affects insulin resistance by interfering with insulin signalling in peripheral tissues. This may contribute to the development of type 2 diabetes.

Vandanmagsar et al. (2011) found that in mice, mRNA expression of *Il1b* and *Nlrp3* in visceral adipose tissue correlated with body weight and adiposity. Moreover calorie restriction and weight loss in humans resulted in decreased expression of these genes, which was accompanied by lower glycaemic levels and improved insulin sensitivity. *Nlrp3*$^{-/-}$ mice on a high fat diet had enhanced insulin sensitivity compared to wild-type mice, which suggests that the NLRP3 inflammasome is likely contributing to obesity-induced insulin resistance.

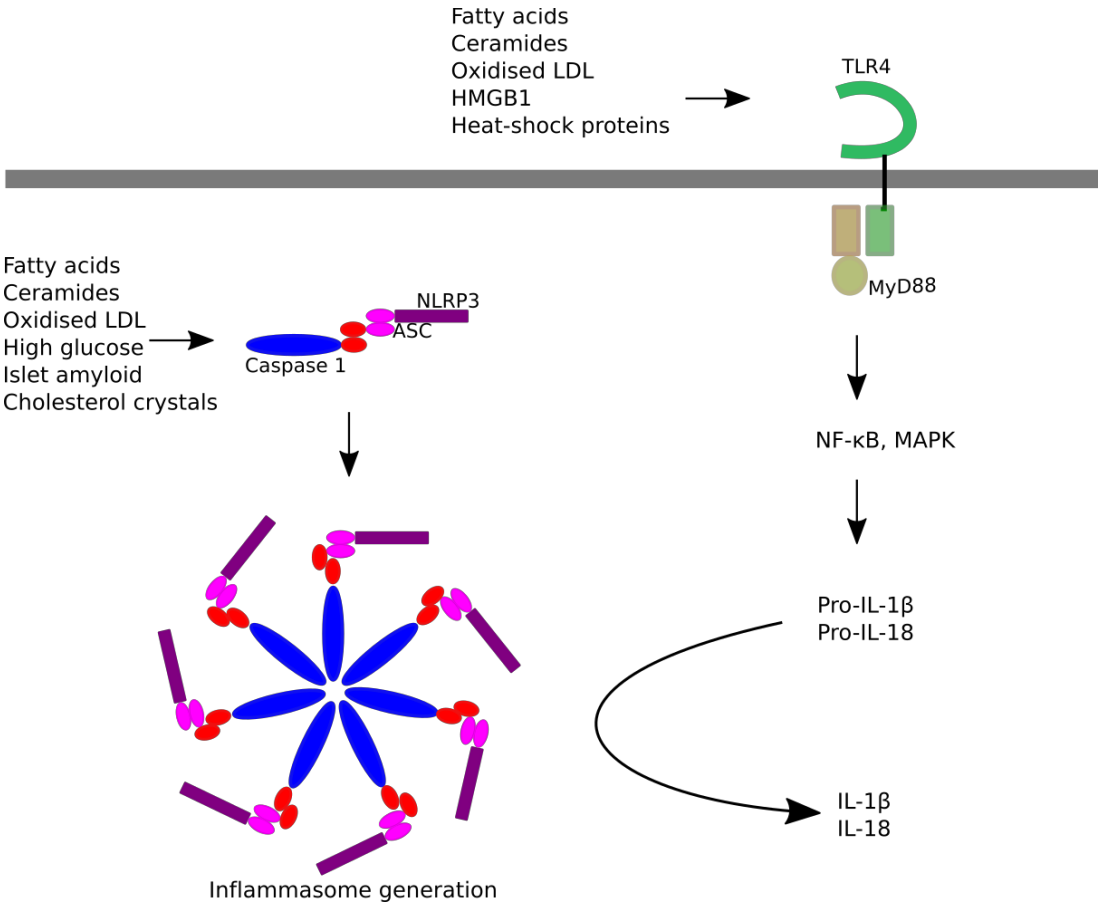


Figure 4.4: Activation of NLRP3 inflammasome in obesity. TLRs and NLRs can be activated by a variety of dietary factors and DAMPs in response to obesity-induced metabolic stress

There are a number of possible triggers for the NLRP3 inflammasome in obesity (Figure 4.4). The first trigger may be stimulated by saturated fatty acids such as palmitate, which have been shown to be able to induce inflammation via TLR4 (Shi et al., 2006, Davis et al., 2008, Milanski et al., 2009). Holland et al. (2011) found this TLR4-mediated insulin-resistance requires the biosynthesis of ceramides, which is compatible with research undertaken by Vandanmagsar et al. (2011) indicating that ceramides are able to stimulate the NLRP3 response (which therefore may constitute the second required signal for inflammasome activation). There may also be other factors contributing, considering that obesity is associated with hypoxia (Hosogai et al., 2007) and adipocyte death (Strissel et al., 2007), which likely also result in the release of pro-inflammatory markers. It is of note that hyperuricaemia is correlated with obesity, so MSU crystals are another potential trigger for the chronic nonspecific inflammation.

4.2.2 NLRP3 in Type 2 Diabetes

As just discussed, obesity-mediated inflammation often results in impaired insulin sensitivity, a state often considered as pre-diabetes. When this is accompanied by the inability to modulate insulin levels due to dysfunction of pancreatic islet β-cells, this is considered to be full-blown type 2 diabetes (Kahn et al., 2006). Pancreatic β–cells are responsible for releasing insulin to modulate glucose levels, so dysfunction can result in hyperglycaemia and complications associated with this.

The role of auto-inflammation in type 2 diabetes pathogenesis is well established and there are ample reports of various inflammatory markers associated with the disease (Pickup & Crook, 1998, Pradhan et al., 2001, Donath & Shoelson, 2011). Given the relatively recent discovery of inflammasome complexes in general (Martinon et al., 2002), it is unsurprising that the links found between the NLRP3 inflammasome and the pathological process of type 2 diabetes are still being uncovered (Wen et al., 2010, Vandanmagsar et al., 2011, Lee et al., 2013) (Figure 4.5).

Lee et al. (2013) observed up-regulated IL-1β maturation, IL-18 secretion and caspase-1 cleavage in monocyte-derived macrophages from drug-naïve type 2 diabetes patients, as well as increased expression of *Nlrp3, Asc* and *Il1b.* Interestingly, treating the cells with a mitochondria-targeting antioxidant to inhibit mitochondrial ROS production significantly reduced the IL-1β and IL-18 production (Lee et al., 2013), indicating that mitochondria may play a central role in this inflammasome generation. This is supported by other emerging research that will be discussed in more depth later in this chapter (Nakahira et al., 2011, Zhou et al., 2011).

As mentioned previously in relation to obesity-associated NLRP3 inflammasome activation, products of long chain saturated fatty acid metabolism such as palmitate and ceramide have also

been found to mediate NLRP3 inflammasome activation (Vandanmagsar et al., 2011, Wen et al., 2011). The resulting inflammatory response can cause damage to the pancreatic β-cells.

Further damage to β-cells results from the high glucose concentrations associated with hyperglycaemia that stimulate the disassociation of thioredoxin-interacting protein (TXNIP) from thioredoxin in a ROS-dependent manner (Zhou et al., 2010). TXNIP is a cytoplasmic protein that is able to shuttle into other cellular compartments including the nucleus and the mitochondria and plays an important role in maintaining reduced environment in the cell (Yoshihara et al., 2013) and regulating apoptosis (Saxena et al., 2010). Current evidence suggests that TXNIP can influence the development of type 2 diabetes in a number of ways. Firstly, while under normal physiological conditions, TXNIP is usually present in the nucleus of the pancreatic β-cells, under conditions of oxidative stress TXNIP shuttles into the mitochondria where is able to induce a mitochondrial pathway of apoptosis (Saxena et al., 2010). As well as stimulating this apoptosis pathway, when inside the mitochondria, TXNIP can cause mitochondrial dysfunction and ROS accumulation in mitochondria (Zhou et al., 2011), potentially part of the same inflammation-inducing process resulting in NLRP3 activation. Independently of this, TXNIP has also been found to bind directly with NLRP3 (Zhou et al., 2010). The ultimate result of all of these processes is further damage to β-cells, which then has other flow-on inflammatory effects.

Damage to the β-cells results in the dysregulation of insulin secretion, a process which helps modulate blood glucose concentrations. This can result in the aggregation of islet amyloid polypeptide (IAPP), a protein usually co-secreted with insulin, in the pancreas during type 2 diabetes. This can form amyloid deposits, which have also been shown to trigger NLRP3 inflammasome activation (Masters et al., 2010).

While there are other inflammatory pathways being stimulated in type 2 diabetes, much of the pathology can be linked back to an initial inappropriate NLRP3 inflammasome-mediated response to what could be a range of endogenous DAMPs. This is supported by evidence from type 2 diabetes drug therapies targeted at dampening NLRP3 activation that have been found to reduce hyperglycaemia and improve insulin sensitivity (Ehses et al., 2009). Some of these DAMPs which stimulate inflammation may stem from an underlying obese state, but other factors including dietary factors and underlying genetic susceptibility may also contribute.

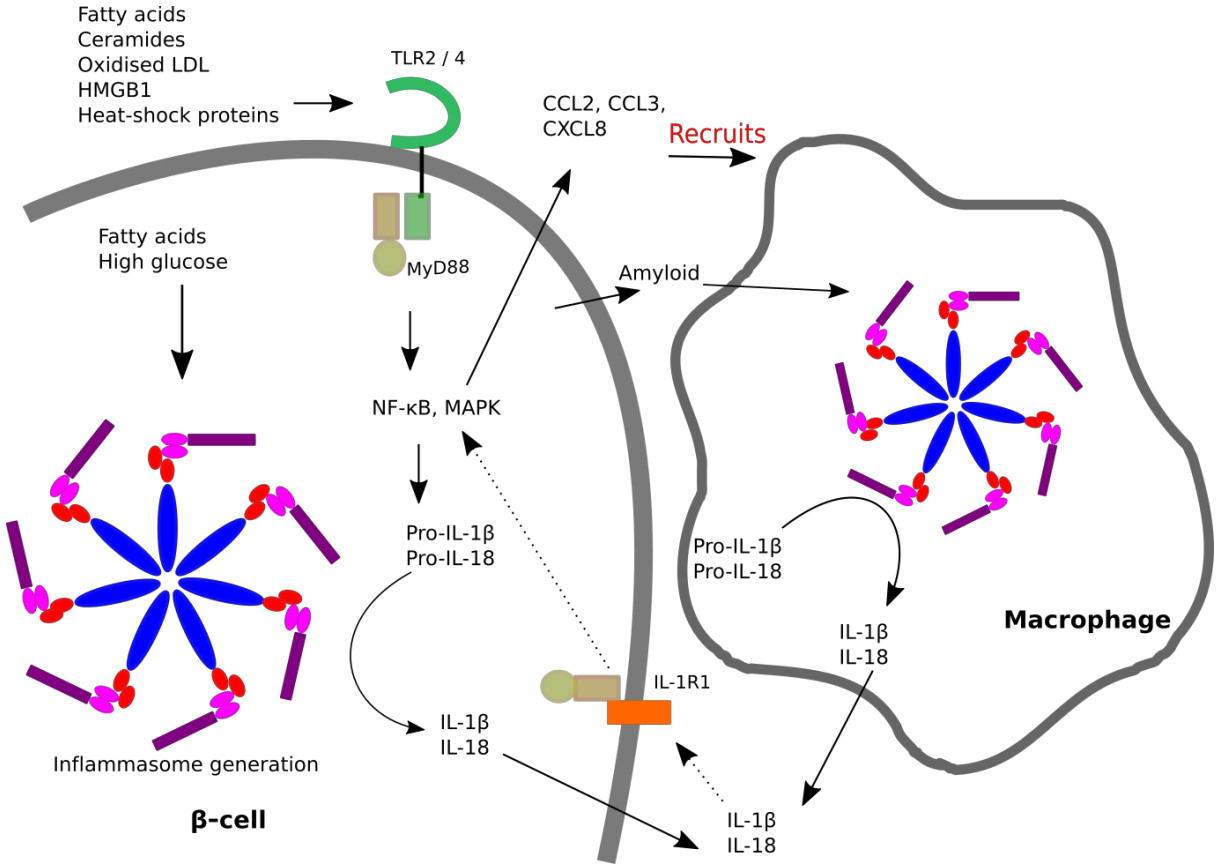


Figure 4.5: NLRP3 activation in type 2 diabetes involves NLRP3 activation in both macrophages and pancreatic beta-cells

4.2.3 NLRP3 in Atherosclerosis

Atherosclerosis is a condition wherein arterial walls thicken as a result of invasion and accumulation of white blood cells, including macrophages. It is a chronic disease that generally remains asymptomatic for such of the sufferers life, however in late phases of disease, rupture of atherosclerotic plaques may result in ischemic heart disease and stroke. Like other metabolic diseases, progression of disease is a complex and not fully understood process. Literature surrounding the development of the inflammatory response is summarised below.

The accumulation of immune cells is thought to be a result of the flux of LDL cholesterol into the endothelial cells of the arterial walls, where it can become oxidised and unable to migrate back into the arterial blood flow (Weber & Noels, 2011). Oxidised LDL has pro-inflammatory properties and is able to interact with TLR2 and TLR4, which is a priming signal for NLRP3 inflammasome activation (Chávez-Sánchez et al., 2010). The accumulation of lipids in the

vascular endothelium results in recruitment of macrophages to the site. Here they may become 'foam cells' resulting from their altered morphology following the uptake of cholesterol and its storage as lipid droplets. Duewell et al. (2010) have found that using microscopy, it is possible to detect cholesterol crystals present in the early stages of diet-induced atherosclerotic lesions, coinciding with the first appearance of inflammatory cells. Furthermore, Rajamäki et al. (2010) have shown that cholesterol crystals stimulate an inflammatory response in human macrophages, and that silencing of NLRP3 attenuates cholesterol crystal-induced IL-1β secretion. Further studies have confirmed the role of NLRP3 involvement in the secretion of IL-1β from foam cells using mouse (Usui et al., 2012) and cell-line (Jiang et al., 2012) models. Together, this suggests that the NLRP3 inflammasome underpins some of the inflammatory responses involved in atherosclerotic cardiovascular disease.

4.2.4 Implications of NLRP3 and its involvement in metabolic diseases

To summarise the above, all of these diseases piggyback on what is essentially the same inflammatory process. There may be slightly different triggers, for instance, monosodium urate crystals in gout (Martinon et al., 2006) or ceramide crystals in the stimulation of the inflammatory processes in obesity (Vandanmagsar et al., 2011), but at the core of it, it is the same process. This makes sense, as despite being a complex system with redundancies built in, evolution tends not to reinvent the wheel multiple times if it does not have to.

The high prevalence of disorders that are linked to NLRP3 inflammation, including not only gout (Winnard et al., 2012, Gosling et al., 2014), but also type 2 diabetes and obesity in Polynesians and other Pacific people (Gosling et al., 2015) may suggest that they may have over-reactive NLRP3 inflammatory responses. Atherosclerosis could also be added to this list as it is a major contributor to ischemic heart disease, a condition which occurs at a much higher frequency among Māori than among New Zealanders of European ancestry (Bramley et al., 2004, Tobias et al., 2006). While there are undoubtedly environmental factors contributing to this trend including diet (differences in dietary composition appears to affect an individual's immunological profile (Thorburn et al., 2014)), exercise levels and socio-economic class, genetic determinants are also likely to be influential. As suggested in previous chapters, immune responses have a strong genetic component (Cooke & Hill, 2001) and as such genes involved in survival of infectious diseases may be subject to strong selective pressure. There have been instances where selection by infectious disease exposure has had the opportunity to shape the genomes of the peoples who have since become Polynesians, for instance the proposed malarial influence in Chapter 2.

There are many components to the NLRP3 inflammasome that may be subject to genetic variation, which may downstream contribute to susceptibility to metabolic disease. In terms of gout, there has been much focus on identifying the urate transporters contributing to the underlying hyperuricaemia (Choi et al., 2010, Reginato et al., 2012, Merriman et al., 2014), however there has been minimal success in identifying genetic variation contributing to the immune response (Qing et al., 2013a, Qing et al., 2013b). Similarly, with other metabolic diseases including atherosclerosis (Lusis, 2012) and type 2 diabetes (Herder & Roden, 2011), genetic variation underlying the inflammatory processes have remained elusive. This will no doubt be addressed as the complex pathways underlying innate immunity are disentangled over the coming years and as more population-specific studies are undertaken, with consideration of the unique evolutionary histories of the populations in question.

Understanding of inflammasome-mediated disease is still the focus of intense study. While it is known that the NLRP3 inflammasome requires two distinct triggers for activation and a plethora of activating signals have been identified (Figure 4.2), the precise mechanisms by which these activators trigger the inflammasome are poorly understood. These known activators of NLRP3-mediated inflammation are structurally and chemically diverse which means that the mode of activation is probably more complex than the proposed mechanism for toll-like receptor activation where direct interaction between the agonist (PAMPs for instance) and the receptor appears likely (Tschopp, 2011). Instead it seems probable that a common cellular structure or organelle integrates the various activation signals and consequently initiates a signaling pathway that leads to inflammasome activation. Tschopp (2011) suggests that this could be mitochondria.

4.3 Mitochondria as an integral component of the NLRP3 inflammasome

4.3.1 What are mitochondria?

Mitochondria are organelles present within cells, usually in large numbers. They comprise a double-membrane-bound structure involved in a wide range of cellular processes (Figure 4.6). Mitochondria are unique among organelles in humans in that they have their own small circular genome, probably a remnant of their origin as a α-proteobacterium, which struck up a symbiotic relationship with proto-eukaryotic cells (Andersson et al., 2003). Through the course of evolution, they have become indispensible. Of most importance is the role of mitochondria in oxidative phosphorylation (OXPHOS). OXPHOS is the primary means of energy production, generating somewhere between 30 and 36 ATPs per glucose molecule. OXPHOS is achieved

through membrane potential differences, and the shuttling of electrons through the electron transport chain.

It is during this process that the mitochondria can also produce radical oxygen species (ROS) including superoxide and hydrogen peroxide, which can lead to mitochondrial dysfunction, cellular damage and correspondingly disease when left unchecked. ROS generation also constitute an important aspect of cellular signalling, including apoptosis (programmed cell death) (Fleury et al., 2002) and inflammasome activation (Tschopp & Schroder, 2010). Additionally, mitochondria are involved in calcium homeostasis and the biosynthesis of amino acids, lipids, nucleotides and haem (Gattermann et al., 2004, Ajioka et al., 2006).

It has been becoming increasingly clear that mitochondria play an important role in cellular defence and the immune response (Cloonan & Choi, 2013). As already discussed, there are many diverse activators of NLRP3-mediated inflammation as evidenced by its role in a range of metabolic conditions, and there is some indication that mitochondria are essential players in this process. Rather than the NLRP3 molecular complex itself detecting each of the diverse activator signals independently, it could instead monitor the activity of the mitochondrion, which may act as an integrator of danger signals, including those of metabolic origin (Tschopp, 2011).

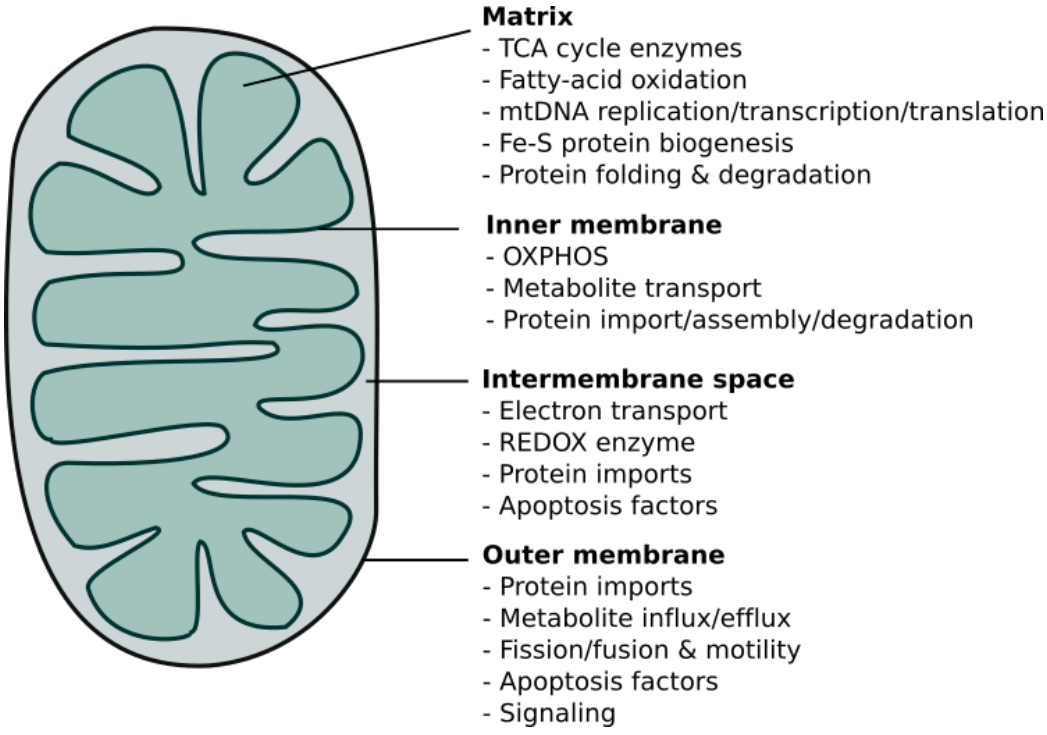


Figure 4.6: The mitochondrial sub-compartments and examples of compartment specific processes

Complex interplay between metabolism and innate immune processes appears to underpin the multitude of age-related metabolic diseases found in humans. Given that metabolic efficiency alters during pathogenic challenge, it is perhaps unsurprising that the innate immune response is hardwired to detect such perturbations and respond accordingly. The central role that mitochondria play in metabolism thus may act as a bridge linking these two vitally important processes together. Indeed, there is evidence to suggest that mitochondrially generated ROS (Zhou et al., 2011) and the release of mitochondrial DNA (Nakahira et al., 2011, Shimada et al., 2012) are important in inflammasome activation.

4.3.2 Proposed mechanisms for a role of mitochondria in NLRP3 inflammasome generation

Bearing in mind that this is still very much an area of active research and the details are yet to be elucidated, there have been a number of proposed mechanisms for NLRP3 inflammasome activation but no real consensus. Proposed mechanisms include potassium efflux (Perregaux & Gabel, 1994, Petrilli et al., 2007, Muñoz-Planillo et al., 2013), an increase in intracellular calcium and a decrease in cyclic AMP (Lee et al., 2012, Murakami et al., 2012), phagosomal destabilisation (Hornung et al., 2008, Dostert et al., 2008), pore-forming actions caused by bacteria or host (Pelegrin & Surprenant, 2006, Harder et al., 2009), changes to cell volume (Compan et al., 2012), and mitochondrial-associated dysfunction, including the release of mitochondrial ROS (Zhou et al., 2011), oxidised mtDNA (Nakahira et al., 2011, Shimada et al., 2012), the translocation of cardiolipin from the inner to the outer mitochondrial membrane (Iyer et al., 2013) or the expression of the mitochondria-associated adapter molecule, Mitochondrial Antiviral Signalling protein (MAVS) receptor on the mitochondrial outer membrane (Subramanian et al., 2013, Park et al., 2013). There is no reason to believe that any one of these is the only means of activating the inflammasome – it may well be a combination of signals.

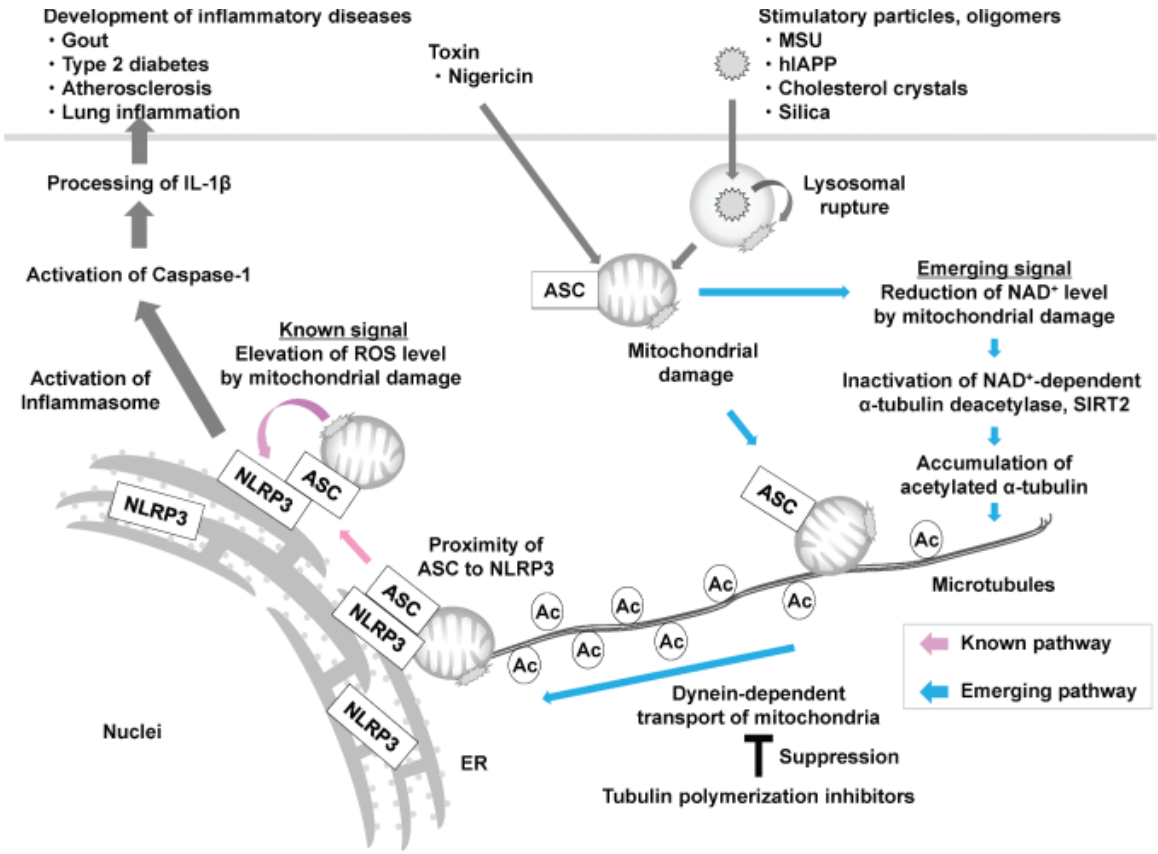


Figure 4.7: A role for the mitochondria in NLRP3 inflammasome activation. From (Akira et al., 2013)

The co-localisation of ASC and NLRP3 is vital for the induction of the NLRP3 inflammasome (Zhou et al., 2011, Misawa et al., 2013, Subramanian et al., 2013). Recent evidence suggests that the ASC is localised on the mitochondria, while the NLRP3 is present on the endoplasmic reticulum (Zhou et al., 2011, Misawa et al., 2013)(Figure 4.7). After stimulation with inducers of the NLRP3 inflammasome, mitochondria approach the endoplasmic reticulum at the peri-nuclear margin, resulting in the co-localisation of ASC and NLRP3, and the subsequent activation of caspase-1 and the generation of IL-1β. This appears to be mediated by acetylated α-tubulin in the form of microtubules (Misawa et al., 2013). This process does not seem to be affected by either potassium ion concentrations or by ROS, and seems to result from the inactivation of sirtuin 2 (SIRT2), a tubulin deacetylase sensitive to changes in the mitochondrial NAD+ gradient (Misawa et al., 2013), though there may also be other deacetylase inhibitors involved (Lee et al., 2015b). This co-localisation of NLRP3 and ASC was also observed in viral-mediated NLRP3 inflammasome activation via MAVS, which has been found to optimise this co-localisation process (Park et al., 2013, Subramanian et al., 2013).

It is unclear what triggers the co-localisation of the mitochondrion to the endoplasmic reticulum during the induction of the NLRP3 inflammasome. While Misawa et al. (2013) suggest that it

may be fluctuation of NAD^+ concentrations which inactivate SIRT2, it may equally be the mitochondrial-derived ROS (Zhou et al., 2011) or some other yet unidentified stimulus. Certainly, mitochondrial dysfunction and the blockade of mitophagy lead to an accumulation of ROS that go onto stimulate NLRP3 inflammasome initiation (Zhou et al., 2011).

4.3.3 Mitophagy and mitochondrial biogenesis

Mitophagy and mitochondrial biogenesis are two inter-related processes, and describe the degradation and the generation of mitochondria, respectively. These processes are essential to maintaining homeostasis. Mitophagy occurs via a process known as autophagy, wherein the mitochondrion becomes encapsulated by a double-membrane structure known as an autophagosome and is subsequently broken down and its components recycled by the cell (Youle & Narendra, 2011). This process is preceded by mitochondrial fission, which divides the mitochondria into smaller, more manageable units for encapsulation (Twig et al., 2008). Mitophagy is important for regulating mitochondria, in particular the disposal of those that have become dysfunctional, which may make the cell susceptible to inappropriate auto-inflammatory responses. There is evidence suggesting that alterations in mitophagy efficiency might contribute to pathology. OMM kinase *PINK1* (Valente et al., 2004) and *PARKIN* (Kitada et al., 1998), genes associated with autosomal recessive Parkinson's disease, encode proteins which mediate a signalling pathway inducing mitophagy in mammalian cells (Youle & Narendra, 2011). If cellular mitophagy processes breakdown and dysfunctional mitochondria are not being taken out of circulation, susceptibility to inappropriate NLRP3 inflammasome activation may ensue (Nakahira et al., 2011, Lazarou, 2015). Mitophagy has been found to negatively correlate with inflammation (Nakahira et al., 2011).

Mitochondrial biogenesis is equally important in maintaining normal homeostasis as if dysfunctional mitochondria are not replaced once they are taken out of circulation there may be energy imbalance. Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α; encoded by the gene *PPARGC1A*) seems to be involved in this process of biogenesis by modulating the transcriptional activity of nuclear-encoded mitochondrial genes (Scarpulla, 2011). Factors like exercise (Wright et al., 2007) and aging (Viña et al., 2009) have been found to affect *PPARGC1A* expression. Sahin et al. (2011) found that impaired mitochondrial biogenesis contributed to metabolic dysfunction. Interestingly, genetic variants in *PPARGC1A* have previously been associated with type 2 diabetes risk, insulin resistance and altered lipid profiles (Ek et al., 2001, Hara et al., 2002, Muller et al., 2003, Barroso et al., 2003). While the mechanism

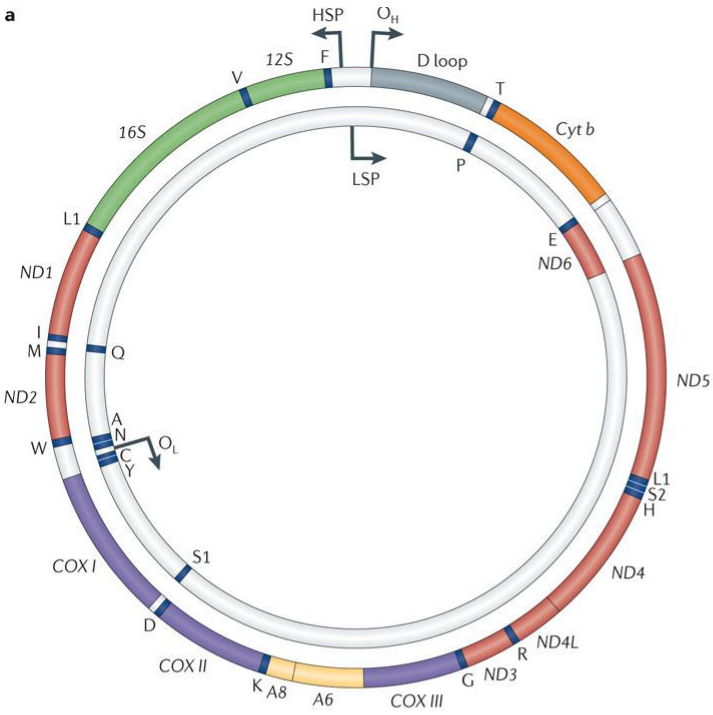
behind this remains unclear, genetic variants in *PPARGC1A* may be contributing to a reduction in mitochondrial turnover, contributing to a susceptibility to inflammation.

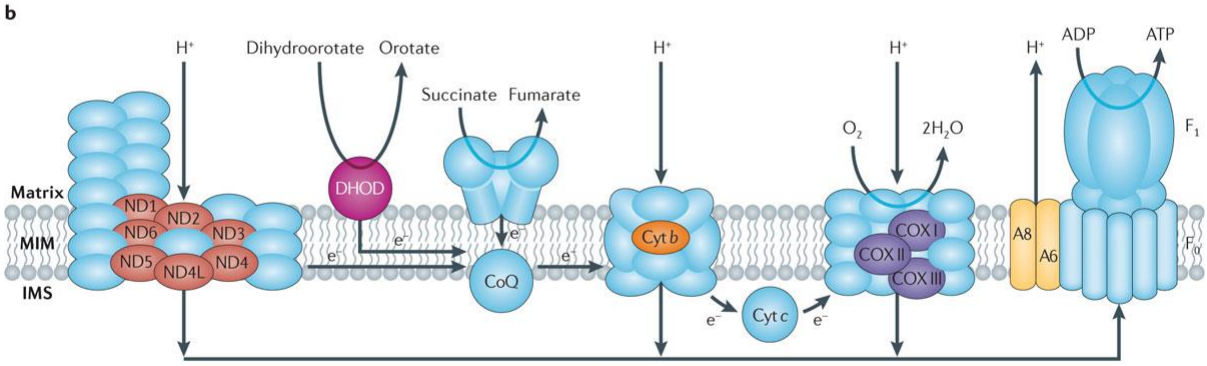
Consistent with the observation that *PPARGC1A* expression decreases with age (Viña et al., 2009), it has been subsequently found that the shortening of telomeres is accompanied by a down-regulation of *PPARGC1A* (Sahin et al., 2011). This may also be interpreted as reduced turnover of mitochondria, which in turn may result in the accumulation of dysfunctional mitochondria and contribute to age-related metabolic disease. Conditions such as gout, type 2 diabetes and ischemic heart disease all increase in prevalence with advanced age. Given that telomeres shorten with age (Hastie et al., 1990), these phenomena may be inter-related. This down-regulation in mitochondrial biogenesis with age is probably a normal part of the aging process, and possibly explains why there is an increased risk of complex disease with age (Fosslien, 2001, Petersen et al., 2003, Puddu et al., 2005, Lin & Beal, 2006).

4.3.4 Mitochondrial genetic variation

Much of the above, including OXPHOS (and the generation of ROS through this process) and mitochondrial biogenesis and autophagy, may be directly affected by mitochondrial genetic variation. Mitochondria are encoded by DNA contained not only within the nuclear genome but also in an accessory genome contained within the mitochondria themselves. Each mitochondrion possesses multiple copies of its own circular genome consisting of around 16.5 kB of DNA encoding 13 subunits of oxidative phosphorylation and 24 RNA components of the mitochondrial translational apparatus (2 rRNAs and 22 tRNAs) (Figure 4.8). The mitochondrial genome is reductionist, characterised by tight packaging of genetic material with a lack of intronic DNA – intergenic sequences are either absent or limited to a few bases. In some cases, genes overlap (e.g. *MT-ATP8/MT-ATP6* and *MT-ND4L/MTND4*). Recently, there has also been the identification of additional short open reading frames that can be translated into peptides (humanin and MOTS-c) with important biological functions (Guo et al., 2003, Lee et al., 2015a).

An additional 1500 genes are encoded by the nuclear genome – including all those that encode proteins responsible for mtDNA replication and repair, as well as all the proteins responsible for transcription and translation - which means that communication between the organelle and the nucleus is vital for coordinating mitochondrial biogenesis. Imbalances in this process likely lead to metabolic dysfunction (Michel et al., 2012, Horan & Cooper, 2014).





Polypeptides	Complex I	Complex II	Complex III	Complex IV	Complex V
mtDNA-encoded subunits	7	0	1	3	2
nDNA-encoded subunits	~39	4	10	10	~14
Assembly proteins	~11	~2	~9	~30	~3

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Figure 4.8: a) a human mitochondrial genome consisting of 37 mitochondrial genes. Encoded genes include seven subunits of complex I (ND1, 2, 3, 4, 4L, 5 and 6), one subunit of complex III (cytochrome *b;* Cyt b), three subsunits of complex IV (Cyt *c* oxidase (COX) I, II and III), two subunits of complex V (ATP6 and ATP8, labelled A6 and A8 on diagram), two rRNAs (12S and 16S) and 22 tRNAs. Also shown are the origins of replication of the heavy strand and the light strand (O_H and O_L, respectively), and their respective promoter regions (HSP and LSP) ; b) oxidative phosphorylation complexes, showing the location of the mitochondrially encoded subunits (coloured to reflect the different genes) . From (Schon et al., 2012)

It has been proposed that mitochondrial dysfunction, and alteration to mitochondrial genes, are key contributors to metabolic disease and may account for the differences in prevalence observed between populations (Wallace, 2013). The mitochondrial genome, for the most part, does not undergo recombination and is primarily transmitted through the maternal line (White et al., 2008). This strict pattern of inheritance means that the major mechanism of change to the genome sequence is the accumulation of sequential mutations over generations, which results in the emergence of phylogenetically new lineages. Changes to the genome sequence are tempered by the need to maintain the function of mitochondrial genes, thus the sequence evolutionary rate is generally slow enough to be of use in disentangling past human migrations (Cann et al., 1987). Despite this slow accumulation of nucleotide changes, the rate of mitochondrial genome evolution still far outpaces the nuclear genome (Brown et al., 1979), which suggests that it might provide some plasticity when encountering new environments or adverse conditions (Ruiz-Pesini & Wallace, 2006). There has been some evidence to suggest disparity in metabolic rates between mitochondrial haplogroups, which might be related to adaptation to new environments encountered by populations as they moved out of Africa (Mishmar et al., 2003, Ruiz-Pesini et al., 2004).

Mitochondrial genetic diversity has been the focus of a number of studies in the Pacific. These have largely emerged from the perspective of disentangling population histories and settlement of the region (Redd et al., 1995, Lum & Cann, 2000, Trejaut et al., 2005, Kayser et al., 2006, Pierson et al., 2006, Soares et al., 2011, Delfin et al., 2012, Duggan & Stoneking, 2013, Duggan et al., 2014, van Oven et al., 2014), but may also be relevant when considering disease epidemiology in the region given the role of the mitochondria in innate immunity. These studies have found that the range of mtDNA macrohaplogroups found in the Pacific, Polynesians in particular, are relatively limited compared to other geographic areas. This, in part, is likely due to the colonisation processes and the relative geographic isolation of island groups in this locality. There is a distinct cline as one moves east across the Pacific, with B4a1a1 lineages (also known as the 'Polynesian motif'), and derived sublineages, reaching frequencies of up to 96% in some Polynesian populations (Figure 4.9)(Kayser, 2010). Recent studies of whole mitochondrial genomes (as opposed to control region sequences, like many of the studies mentioned above) from the Pacific have shown that there is more diversity within the B4a1a1 haplogroup than previously thought (Benton et al., 2012, Duggan et al., 2014).

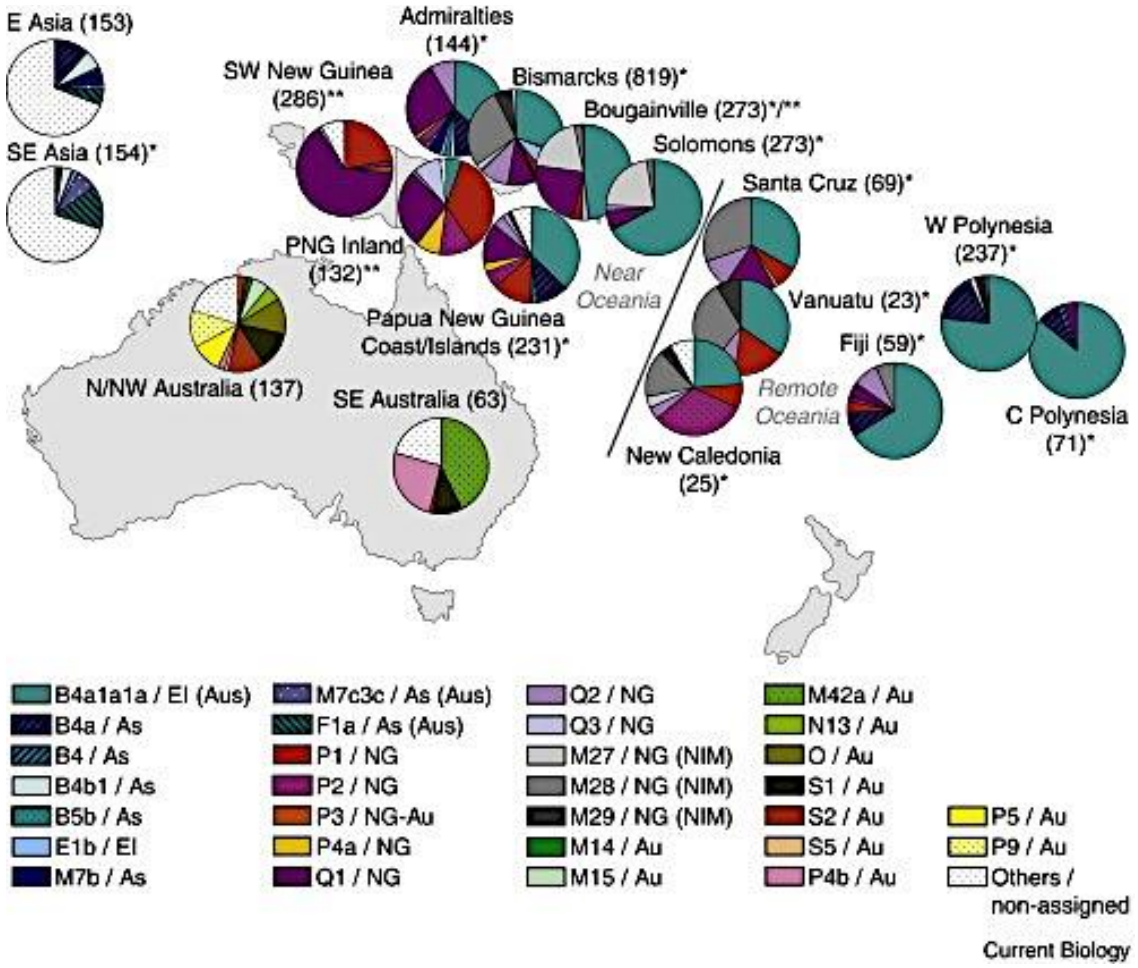

Figure 4.9: Mitochondrial haplogroup variation in the Pacific based on control region genotyping (Kayser 2010), note that the B4a1a1a lineage here has been downgraded to a B4a1a1 on the basis of the 16247G site which characterised this lineage has been found to be a mutational hotspot which as limited utility as a phylogenetic marker (Duggan & Stoneking, 2013).

4.4 Conclusion

Underlying the high rates of metabolic disease in Polynesian and other Pacific people there is a common immune pathway, the NLRP3 inflammasome. Given the shared ancestry of these populations, it may be that there is a shared genetic predisposition to an overactive NLRP3 immune response that may have developed in response to pathogen exposure prior to or during the settlement process. Studies of mitochondrial genetic variation among Polynesian populations have revealed a high frequency of haplogroups belonging the B4-lineage.

Given that mitochondria are emerging as a key player in innate immune responses, particularly their apparent involvement in the co-localisation of NLRP3 and ASC during the induction of NLRP3 inflammasome activation, mitochondrial genetic variation may influence the susceptibility to metabolic disease. This variation may be in the mitochondrial genome itself, or

within mitochondrial genes embedded in the nuclear genome responsible aspects of mitochondrial function, for instance mitochondrial biogenesis.

In the following two chapters, mitochondrial genetics and their potential impact on metabolic disease is going to be explored. Firstly, the role of the variation in the 16.5 kb mitochondrial genome itself and potential associations with disease among Polynesians will be investigated (Chapter 5). This will be followed up by a chapter looking at whether there may be differences in mtDNA copy number between those possessing metabolic diseases and those without, and exploring potential mechanisms for this difference (Chapter 6). There will be a more in depth discussion of some of the issues brought up in this chapter in these respective chapters.

5 Mitochondrial variation as a potential determinant for disease susceptibility in the Pacific

5.1 Introduction

As discussed in the previous chapter, mitochondrial genetic diversity has been the focus of a number of studies in the Pacific. What has been established in the past two decades of research is that there is relatively more mitochondrial variation in Near Oceania where there is a long history of human occupation. Present are lineages which were introduced by the earliest colonists who arrived in the region at least 49,000 years ago (Summerhayes et al., 2010b), including M27, M28, M29, P and Q haplogroups (Kayser et al., 2006, Friedlaender et al., 2007, Kayser et al., 2008, Duggan et al., 2014), but there are also more recent Asian-derived lineages which were likely introduced to the region with the expansion of Austronesian-speaking colonists. Within Remote Oceania, Polynesia in particular, the range of mtDNA macrohaplogroups is relatively limited, with the B4a1a1 lineage (also known as the 'Polynesian motif') (Melton et al., 1995, Redd et al., 1995, Kayser et al., 2006, Duggan et al., 2014), and derived sub-lineages, being ubiquitous. This distribution of mitochondrial genetic variation may be significant in terms of susceptibility to complex metabolic diseases. The mitochondrial genome is a functionally important entity and as such, this variation may be having a phenotypic effect.

There are a number of ways that mitochondrial genetic variation may affect susceptibility to metabolic disease. As discussed in the previous chapter, metabolism and immunity are inextricably intertwined, and mitochondria appear to play an integral role in the stimulation of the NLRP3 inflammasome, an important component of innate immunity. There is also the direct role that mitochondria play in the generation of ATP and cellular energetics that may contribute; with metabolic diseases being fundamentally a result of compromised metabolic function. Additionally, there is increasing evidence to suggest that heteroplasmy among mitochondrial populations within an individual may contribute to susceptibility to metabolic disease. Lastly, there is evidence for cross-talk between the mitochondrial and nuclear genomes, and it seems possible that variation in mtDNA may influence this inter-genome communication. Impaired communication between these genomes is also likely to effect organismal fitness. These are all themes that will be explored in this chapter.

5.1.1 Mitochondrial variation and energetics

Mitochondria play a vital role in energy production, so it is unsurprising that changes to the mtDNA sequence have been hypothesised to alter mitochondrial efficiency. As previously discussed in Chapter 4, the mitochondrial genome encodes 13 polypeptides involved in OXPHOS, therefore non-synonymous changes to nucleotide sequence may affect the structure of the subunits and ultimately the enzyme kinetics. Human mtDNA is variable and approximately one-third of sequence variants found in the general population may be functionally important (Wallace et al., 2010). As mentioned in Chapter 4, some of the variation that has arisen in the mitochondrial genome may be related to changing energy demands arising from altered environmental exposures (Mishmar et al., 2003, Ruiz-Pesini et al., 2004).

All manner of environments – tropical, temperate, glacial, high altitude, desert, coastal - have been inhabited by humans since the departure of human populations from Africa some 60,000 years ago, each environment possessing unique energetic demands, offering varying food sources, and with differing pathogen loads. The mitochondrial genome has been found to evolve much more rapidly than the nuclear genome (Brown et al., 1979), which may facilitate adaptive mitochondrial-nuclear co-evolution based on changing environmental demands (Levin et al., 2014). The idea that the mitochondrial genome is adaptive has major repercussions when it comes to the mitochondrial molecular clock and estimations of lineage divergence. If the rapidly changing mitochondrial genome, as Wallace (2013) suggests, is allowing for a certain degree of metabolic plasticity, it may be that certain sequence changes, in combination with modern diets and lifestyles are having an adverse effect on health.

This perspective remains contentious (Soares et al., 2009). There has been much debate over whether mtDNA is adaptive, and whether the observed variation is purely an artefact of the rapid dispersal of modern humans out of Africa and the population expansions that followed. This may have conflated what would otherwise be rare private mutations occurring at non-synonymous loci, as there may not been sufficient time for purifying selection to occur and remove all of these variants (Ingman & Gyllensten, 2007, Soares et al., 2009). However, emerging evidence for differences in the rate of nuclear genome evolution between populations resulting from different environmental stimuli, for instance UV exposure in Europeans (Harris, 2015), suggests that changes in environment may increase the rate of evolution. As such, the varying environments faced by anatomically modern humans as they departed Africa, or even within Africa itself, may have also provided selective pressures on the mitochondrial genome and played a role in adaptation.

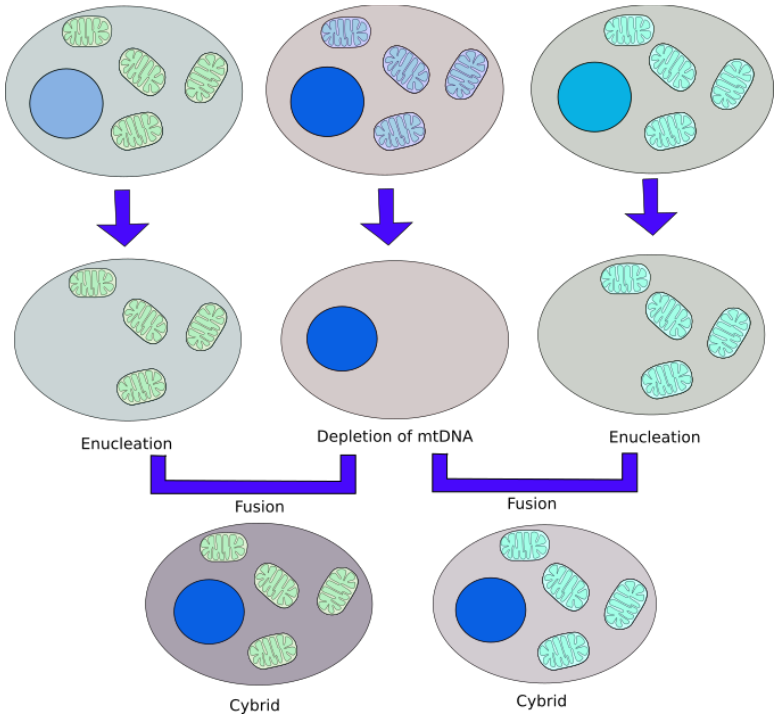

Figure 5.1: Cybrid: Cells depleted of mitochondria are merged with enucleated cells. The resulting cells have a common nucleus but differ in mtDNA

In support of some of these energetic differences resulting from variation in mtDNA sequence suggested by Mishmar et al. (2003), Ruiz-Pesini et al. (2004) and others, is evidence coming out of "cybrid" studies. A "cybrid" is a term that describes a cytoplasmic hybrid (Figure 5.1). This method is currently the best tool to dissociate the action of mitochondrial genomes from that of the nuclear genes. At present, it is impossible to genetically engineer or transform the mitochondrial genome *in vivo* or *in vitro*. Because the nuclear genome encodes a further 70 gene products involved in OXPHOS, as well as a number of mitochondrial regulatory elements, population-specific changes to the nuclear genome could mask the effect of mitochondrial variants. Gómez-Durán et al. (2010) were able to demonstrate that there were differences in mtDNA and mtRNA levels, mitochondrial protein synthesis, cytochrome oxidase activity and amount, normalised oxygen consumption, mitochondrial inner membrane potential and growth capacity between cybrids containing mitochondrial haplogroup H compared to haplogroup Uk. Furthermore, Mueller et al. (2012) were able to show that cells containing mitochondria belonging to haplogroup T had a higher growth rate than those belonging to haplogroup H, though the normalised activity rate of the OXPHOS enzymes in the respective mitochondria did not differ significantly. The haplogroup T cells were also found to be less susceptible to oxidative stress (Mueller et al., 2012).

Lin et al. (2012) prepared cybrids containing mtDNA from ethnically-Chinese Taiwanese. Cybrid B4b (a lineage closely related to the B4a-derived haplogroups prevalent among Polynesians)

was found to have a significantly lower oxygen consumption rate and higher mitochondrial membrane potential compared to F1a, B5, D4a, D5a, and N9a cybrids, however they were more susceptible to induced oxidative stress (Lin et al., 2012). This may be significant because of links between metabolic syndromes such as atherosclerosis, diabetes and hypertension and oxidative stress (Roberts & Sindhu, 2009).

5.1.2 Mitochondrial genetic variation and immunity

As discussed in the previous chapter, in addition to being a key player in metabolism, the mitochondrion also plays a vital role in the development of inflammation and the clearance of pathogens, being the largest producer of highly toxic radical oxygen species (ROS) in the cell. Indeed, there are data which suggest that not only NLRP3-mediated inflammation (as discussed in Chapter 4) but also retinoic acid inducible genes (RIG-1)-like receptor (RLR) and Toll-like receptor (TLR) signalling pathways are inextricably linked to mitochondrial function (Cloonan & Choi, 2013). These pathways all play an important role in innate immunity.

Cybrid studies have shown that mtDNA variation can affect the expression of nuclear genes involved in the innate immune response, particularly inflammatory and complement pathways (Kenney et al., 2014a, Kenney et al., 2014b). This could mean that mitochondrial variants are subject to selection mediated by exposure to infectious diseases. While most of the studies discussed above regarding adaptive mitochondrial genomes (e.g. Mishmar et al., 2003 and Ruiz-Pesini et al., 2004) suggest climatic stimuli contribute to selection, this could very well transfer over to different pathogenic exposures. There have been several studies that have shown that common mitochondrial variants can influence the likelihood of survival following acute sepsis (Baudouin et al., 2005, Jiménez-Sousa et al., 2015). Infectious disease has been shown to have a profound selective effect on the nuclear genome (Kwiatkowski, 2005). There is no reason to believe that the mitochondrial genome could not be similarly affected. If mitochondrial genomes have been subject to selection on the basis of phenotypic differences in immune function, this may also translate to susceptibility to auto-inflammatory and metabolic disease.

5.1.3 Mitochondrial-Nuclear Genome Cross-talk/Interaction

While variation in the mitochondrial genome may be having a direct effect on metabolism, as well as influencing the expression of genes involved in innate immunity, the cybrid studies discussed earlier show that the mitochondrial genome is having a more broad effect on gene expression in the cell. There is increasing evidence to suggest that the mitochondria can act as modifiers of nuclear gene function (Horan & Cooper, 2014). With over 1500 genes essential for

mitochondrial function being encoded by the nuclear genome rather than by the mitochondria itself (Lopez et al., 2000), inter-genome communication is essential for mitochondrial function, and indeed, the optimal functioning of the cell as a whole. The cell needs to know when to manufacture the protein subunits, which then need to be exported through the cytosol to the mitochondria, and because cells usually contain a high number of mitochondria, the cell also needs to get the products to the right mitochondrion. Signalling occurs in a number of ways.

Specific variants may induce compensatory signals between the nuclear and mitochondrial genomes and contribute to enhanced fitness in younger individuals that deteriorates with age, contributing to age-related disease. While there is little evidence for this in humans thus far, a study of *Drosophila* found that an amino acid change in the ATP6 protein of the electron transport system complex V in OXPHOS resulted in metabolic compensatory mechanisms within several metabolic pathways, which broke down as the individuals aged. The flies with the mutations were found to have enhanced fertility rates at a younger age but a shorter life expectancy with phenotypic defects that appeared with age (Ballard et al., 2007, Ballard & Melvin, 2011). While fruit flies clearly possess some significant differences in metabolic demands compared to humans, there may be a similar compensatory effect in humans where mtDNA mutations that enable an increase in performance in early life, contribute to disease in aged individuals (Ballard & Pichaud, 2014). An example of this may be mitochondrial haplogroup V, which appears to be overrepresented in some studies of endurance athletes (Nogales-Gadea et al., 2011), and also in individuals who suffer type 2 diabetes (Soini et al., 2012) and diabetes-related renal failure (Achilli et al., 2011).

As well as encoding thirteen subunits of oxidative phosphorylation, the mitochondrial genome also encodes in excess of 2000 noncoding RNAs (Mercer et al., 2011, Rackham et al., 2011) and also a number of small peptides (Guo et al., 2003, Lee et al., 2015a). These mitochondrial products may be affecting nuclear gene expression, and variation within these noncoding RNAs might influence the intergenomic cross-talk which must occur for normal cellular function. Additionally, this year, there has been the discovery of a short open reading frame encoding a small peptide, MOTS-c, which has been implicated in having a role in maintaining metabolic homeostasis (Lee et al., 2015a). This is not the first of such molecules to be discovered with humanin previously being identified and implicated in Alzheimer's disease (Guo et al., 2003). There have been further open-reading frames proposed (Faure et al., 2011), as well as the potential for functional anti-sense tRNAs (Seligmann, 2012), which may complicate the picture further should they be robust to rigorous testing. It is clear that the full complexity of the mitochondrial genome is not yet fully understood.

5.1.4 Mitochondrial heteroplasmy

Although each individual is generally characterised by a single mtDNA type, each individual actually possesses a population of mitochondrial genomes, and there may be multiple mtDNA types present. This is described by the term 'heteroplasmy'. Originally, heteroplasmy was thought to be quite rare in healthy individuals (Monnat & Loeb, 1985, Monnat & Reay, 1986), though it had been detected in those with mitochondrial disease (Holt et al., 1990, Shoffner et al., 1990, Boulet et al., 1992), however many non-disease related heteroplasmies have since been identified (Comas et al., 1995, Jazin et al., 1996, Elliott et al., 2008, Irwin et al., 2009). Advances in sequencing technology have lead to the ability to sequence mitochondrial genomes at higher resolution, thus allowing the detection of low-level heteroplasmy (Li et al., 2010, Sosa et al., 2012).

Mitochondrial heteroplasmy can be inherited – this is likely the basis of the divergence of different mitochondrial haplogroups – or it can develop in an individual over the course of their life. As discussed previously in Chapter 4, the inheritance of mtDNA is quite different from that of nuclear DNA. The mitochondrial genome does not undergo recombination and is solely transmitted through the maternal line. This continuity in maternal mtDNA transmission is amplified with mitochondria passing through an extreme bottleneck during postnatal folliculogenesis, reducing the variation in the mitochondria passed on to the next generation (Wai et al., 2008) (though it is perhaps notable that there is a correlation between advanced maternal age and an increase in heteroplasmies present in oocytes (Rebolledo-Jaramillo et al., 2014)). This process effectively limits the number of private mutations that may get passed onto the next generation and provides some stability in the mtDNA sequence. However, in somatic tissues, as a result of the relatively high turnover of mitochondria (even in post-mitotic cells), mtDNA is constantly going through replication, and thus there is plenty opportunity for small errors in DNA replication to occur during the lifetime of the individual and for changes to the nucleotide sequence to occur.

Assisting this process may be the existence of hypervariable sites that are hotspots of mutation (Stoneking, 2000); this is supported by a study that found three heteroplasmies occurring at the same position in two different individuals who belonged to not only different mitochondrial haplogroups but also different populations (Li et al., 2010). It is probable that these heteroplasmies were independent events, but it is highly improbable for them to occur at the same positions if mutations are random throughout the genome, suggesting that some positions are more variable than others. If these changes to the sequence are not overtly pathogenic, these may then get passed onto more mitochondria as further mitochondrial biogenesis occurs. Depending on where these changes happen, if enough of these mitochondria possessing mutant

mtDNA accumulate, there can be pathogenic effects (Stewart & Chinnery, 2015)(Figure 5.2). There is increasing evidence to suggest that many mitochondriopathies are a result of changes that occur during the life of the individual rather than inherited at birth, which may account for susceptibility to age-related disease in some (Ozawa, 1995, Bender et al., 2006, Li et al., 2015).

As previously mentioned, mitochondrial heteroplasmy has been implicated in disease. While being prevalent in the human population, mitochondrial heteroplasmy has been found to be overrepresented in disease-associated sites and specifically, heteroplasmies at non-synonymous and tRNA loci have been found to be highly pathogenic (Ye et al., 2014). *In vitro* studies of heteroplasmic mutations using cybrid cell lines (Koga et al., 1995), and studies using single cells isolated from tissue biopsy samples (Boulet et al., 1992), have shown that the proportion of mutated mtDNA must exceed a critical threshold level (~ 60-80%) before phenotypic changes can be detected using laboratory techniques. The threshold is likely to vary from tissue to tissue and mutation to mutation (Stewart & Chinnery, 2015). These differences in precise thresholds may contribute to clinical heterogeneity of mitochondrial disease caused by the same variants.

The precise reasons for high levels of heteroplasmy causing pathogenesis are not entirely understood, though it may be that it contributes to poor signalling between the mitochondrial and nuclear genomes. As previously discussed, this intergenome communication is vital for maintaining homeostasis, and perturbations to this cross-talk are thought to contribute to pathology (Horan & Cooper, 2014).

Given the pre-existing literature surrounding mitochondrial heteroplasmy and its potential pathogenicity, it may be worth exploring mitochondrial heteroplasmy in the context of gout and other metabolic traits among Polynesians.

Figure 5.2: mtDNA heteroplasmy threshold – the cell can usually tolerate a high threshold of heteroplasmic mtDNA. For pathogenic variants, there seems to be a threshold of >80% before phenotypic effects are detectable Modified from (Stewart & Chinnery, 2015).

5.1.6 Previous studies looking at mitochondrial variation and metabolic disease

Some of the earlier disease studies investigated the role of individual mtDNA SNPs rather than SNPs as a collective haplotype (Poulton et al., 1998, Poulton et al., 2002, Saxena et al., 2006). Such studies have yielded inconsistent results depending on the cohort used in the investigation. In genetically diverse populations with many different mitochondrial haplotypes, identification of variants associating with metabolic traits has largely been unsuccessful. For example, Saxena et al. (2006) studied mitochondrial variants in a cohort of 6,608 'white' subjects recruited from Scandinavia, Poland and North America. With participants in such studies being pooled across wide geographic areas with diverse population histories and with much mitochondrial diversity, SNPs associated with disease are likely to be masked by this inherent heterogeneity. Where less genetically diverse populations have been used, for instance Poulton et al. (1998), there is less background noise so variants associated with disease can be detected more easily. In this case, Poulton et al. (1998) were able to identify an association between metabolic disease and the T16189C variant in a cohort of 251 English men, predominantly of Anglo-Saxon-derived ancestry.

Evidence can be conflicting between studies. For instance, the European mtDNA haplogroup J has been associated increased risk of type 2 diabetes in populations of European descent (Mohlke et al., 2005, Crispim et al., 2006). Conversely, there have also been connections between this haplogroup and longevity (De Benedictis et al., 1999, Rose et al., 2001). This may suggest that there is some interaction with nuclear DNA variants or environmental factors – or indeed, that the associations are spurious – as metabolic conditions such as type 2 diabetes are not usually correlated with longevity. Likewise, A3243G, a locus which is thought to contribute to susceptibility to type 2 diabetes and insulin resistance, is underrepresented among Europeans belonging to mitochondrial haplogroup J (Pierron et al., 2008), which is interesting given the apparent susceptibility of people belonging to this haplogroup to diabetes. This may be because additional mutations to the haplotype increase the stress elicited by the molecule, which results in negative selection against this at the embryo or germ line stages (Pierron et al., 2008).

Exploring the effect of mitochondrial haplotypes as a whole and their association with obesity and other metabolic traits may offer insight into disease susceptible lineages. Because the mitochondrial genome does not recombine at an appreciable frequency and is subject to strong purifying selection, certain variants are often inherited together. This approach has also yielded variable results. A study of 2000 Danish participants was unable to find any association between mitochondrial haplogroup and type 2 diabetes (Li et al., 2014), while Liou et al. (2012) found that among 2010 unrelated Taiwanese of Han Chinese ancestry, the B4 haplogroup was

associated with type 2 diabetes while D4 derived lineages had a protective effect. This particular finding is interesting in light of the predominance of B4-derived lineages among Pacific populations.

5.1.7 Whole mitochondrial genome sequencing versus control region genotyping

The first complete mitochondrial genome was sequenced in 1981 (Anderson et al., 1981). Most early studies of mitochondria focused on the control region, a non-coding region of the mitochondrial genome that has been found to be a hotspot for variation (Aquadro & Greenberg, 1983). The non-coding nature of the region has allowed it to accumulate more mutations than the coding regions, though this variation is still constrained by the origin of replication of one of the strands and the origin of transcription for both strands being present in this region. Because of the variable nature of this region, it was soon realised that it was possible to identify population-based variation, which had had a lot of utility in disentangling past human migrations (Cann et al., 1987). Additional to this, restriction fragment length polymorphism (RFLP) analysis was employed to identify other features of the mitochondrial genome that varied from population to population. An example of this is the 9 base pair deletion that is one of the defining features of the B macrohaplogroup (Redd et al., 1995).

With the advent of massively parallel high throughput sequencing technology, in particular next generation sequencing technologies, it has been possible to sequence whole mitochondrial genomes relatively cheaply *en masse*. Prior to this, whole mitochondrial genome sequencing was costly and involved either primer-walking around the mitochondrial genome or shotgun sequencing methods with the use of molecular cloning (Burger et al., 2007), which was a time consuming and costly process. The capillary sequencing technology requires the use of only a single template in each sequencing reaction, and the readout is a consensus of the reaction within the samples. In contrast, next generation sequencing allows for the reading of many different molecules in a single sequencing run with the 'sequencing by synthesis' approach, where barcoded DNA libraries are dispersed on a plate, and emulsion PCR is carried out. This allows not only many molecules to be sequenced at once, but many individuals, as unique barcodes can be ligated to the DNA template enzymatically, allowing hundreds of individuals to be genotyped in a single sequencing operation. The ability to sequence whole mitochondrial genomes has a lot of utility, not only in contributing to our understanding of past human migrations, but to our understanding of human health.

5.1.9 Hypotheses

There are two separate hypotheses tested in this chapter.

1) Mitochondrial genetic variation may be contributing to differences in energetics, which may underlie the high prevalence of obesity among Polynesian populations. Here, BMI is used as a proxy for energy metabolism, being a phenotype controlled through energy homeostasis.
2) Mitochondrial genetic variation may be contributing to differences in immune response, which may contribute to the high prevalence of metabolic disease in Polynesians. Gout is used as a proxy for immune function, being an auto-inflammatory condition resulting from the activation of the NLRP3 inflammasome.

Given the mitochondrial diversity, or lack thereof, and the widespread occurrence of high BMI and gout among Pacific people, mitochondrial haplotype may be contributing to susceptibility to metabolic disease or high BMI. To address this, mtDNA sequencing of a cohort of Polynesians has been undertaken to test for over-representation of certain haplogroups and other mitochondrial variation.

5.2 Methods

5.2.1 Sample selection

The samples for this study were selected from a pre-existing cohort that was recruited from 2006 to 2013 for the study of metabolic diseases. These participants were recruited from throughout New Zealand; those with gout were predominantly recruited from rheumatology clinics, workplaces and community focal points from Auckland, Bay of Plenty, Wellington, Christchurch and Dunedin, while controls with no self-reported history of gout were sampled from workplaces and community focal points in Auckland. Ethical approval for the collection of the samples and subsequent analysis was obtained from the NZ Multi-Region Ethics Committee (MREC 05/10/130). Written consent was acquired from all subjects for collection of blood samples for DNA extraction and biochemical measurements. Alongside the informed consent, participants were asked to complete a questionnaire focusing on lifestyle factors and ancestry. As of 2015, this cohort comprised 2359 individuals who self-declare some portion of Polynesian ancestry, with varying amounts of admixture, predominantly with Europeans.

A subset of this cohort was selected with the criterion of an adjusted BMI calculation for Polynesians outlined by Swinburn et al (1999) which takes into account the inherently lower

body fat percentage Polynesian populations possess. This adjustment involves the following equation:

$$Body\ fat\ in\ kg\ (for\ males) = -30.56 + 1.817 \times BMI$$

The value that comes out gives a comparable body fat mass to European BMI calculations. For the East Polynesian cohort (comprised of NZ Māori, Cook Islanders and Tahitians living in NZ), 112 individuals with an adjusted BMI of greater than 33 and 118 individuals with an adjusted BMI less than 30 were selected for sequencing. For the West Polynesian cohort (comprised of Samoans, Tongans, Tokelauans, Niueans and Tuvaluans), 110 individuals with an adjusted BMI of greater than 33 and 105 individuals with an adjusted BMI of less than 31 were selected for genotyping. Other metabolic characteristics for the samples chosen can be viewed in Table 5.1. All individuals chosen were male, 40 years of age or greater and had self-reported at least 50% Polynesian ancestry, specifically with a Polynesian maternal grandmother. Individuals with greater proportions of Polynesian ancestry were targeted (assessed by looking at the number of self-declared Polynesian grandparents) during sample selection to try to mitigate the effect of non-Polynesian admixture.

Table 5.1: Mean metabolic characteristics of the 445 Polynesian men whose mitochondrial genomes were sequenced.

Variable	WP		EP		Combined	
	High BMI	Low BMI	High BMI	Low BMI	High BMI	Low BMI
n	110	106	113	121	223	227
Age	50.7±0.8	53.3±0.8	54.0±0.9	55.8±0.9	52.2±0.6	54.5±0.7
% Polynesian Ancestry	0.97±0.01	0.96±0.01	0.93±0.01	0.97±0.01	0.95±0.01	0.97±0.01
Adjusted BMI*	45.4±1.0	24.6±0.4	44.4±1.1	22.5±0.5	44.8±0.8	23.4±0.2
BMI (kg/m^2)	41.8±0.6	30.3±0.3	41.3±0.6	29.2±0.3	41.5±0.43	29.7±0.18
Triglycerides (mmol/L)	2.59±0.17	2.08±0.13	2.70±0.19	2.26±0.21	2.64±0.13	2.17±0.12
HDL (mmol/L)	0.95±0.03	1.08±0.03	1.02±0.04	1.19±0.04	0.98±0.02	1.13±0.02
Serum Urate (mmol/L)	0.43±0.01	0.42±0.01	0.44±0.01	0.41±0.01	0.44±0.01	0.42±0.01

5.2.2 Amplification of mitochondrial genome

Where the quality of the extracted DNA allowed, the complete mitochondrial genome was PCR amplified in two overlapping fragments of 8511 bp (LR_1) and 8735 bp (LR_2) (Clarke et al., 2014). Long range PCR was undertaken using two long range products: Roche's Expand Long Range dNTPack and the following reagent concentrations: 1x Long Range Buffer, 0.4 µM of each primer, 0.4 µM of each dNTP, 3% (v/v) DMSO (dimethyl sulfoxide) and 2 units of enzyme; and 5 Prime PCR Extender System with the following reagent concentrations: 1x Tuning Buffer, 0.4 µm of each primer, 0.5 µM of each dNTP, 1 unit of enzyme. 3 µL of DNA extract was added for a total reaction mix of 30 µL. The 5 Prime PCR Extender System product was used on difficult to amplify samples. PCR conditions for the reactions with Roche's Expand Long Range dNTPack

consisted of an initial denaturation period of 92°C for 2 minutes, followed by 10 cycles of 92°C for 10 seconds, 55°C for 10 seconds and 68°C for 8 minutes and 30 seconds. This was followed by 20 cycles of 90°C for 10 seconds, 55°C for 15 seconds 68°C for 8 minutes and 30 seconds, with an increase 20 seconds at 68°C each cycle. The cycling ended with a final extension at 68°C for 7 minutes. The conditions for the 5 Prime PCR expander system reactions consisted of an initial denaturation period of 93°C for 3 minutes, followed by 10 cycles of 93°C for 15 seconds, 55°C for 30 seconds and 68°C for 8 minutes. This was followed by 20 cycles of 93°C for 15 seconds, 55°C for 30 seconds and 68°C for 8 minutes, with an increase of 20 seconds at 68°C each cycle. The cycling ended with a final extension step 68°C for 10 minutes. PCR products were visualised under UV on SYBR Safe (Invitrogen) stained 2% agarose gels. The samples that could not be successfully amplified in two fragments were amplified in three or four fragments with primer pairs LR_1a, LR_1a, LR_2a and LR_2b.

Products were purified using the AMPure XP Kit (Beckman Coulter) and solid-phase reversible immobilisation (SPRI) technology. A PicoGreen quantification assay was used to determine the quantity of each PCR product to be pooled for fragmentation.

5.2.3 Fragmentation

PCR products from each individual were pooled in equimolar ratios, and 1 µg of DNA was fragmented by sonication using a PicoRuptor sonicator. A total of 12 samples could be sonicated at once for 8 cycles of 15 seconds on and 45 seconds off. Sonication was carried out in order to reduce the size of the fragments to less than 1000 base pairs.

5.2.4 Barcoding and library preparation for 454 Sequencing

Barcoding involved end-repair, the ligation of hairpin shaped barcodes, a fill-in step and subsequent restriction digest to cleave the hairpin, an approach known as parallel-tagged sequencing as described by Meyer et al. (2008). The barcoded DNA was subsequently library prepped for sequencing and sequenced on a GS FLX sequencer by the University of Otago High-Throughput Sequencing Unit as described by Clarke et al. (2014). Raw data processing also followed the pipeline presented by Clarke et al. (2014).

5.2.5 Library preparation for Illumina MiSeq Sequencing

An overview of the laboratory methods, beginning with the DNA extracts and ending with the ready to sequence libraries is outlined in Figure 5.3.

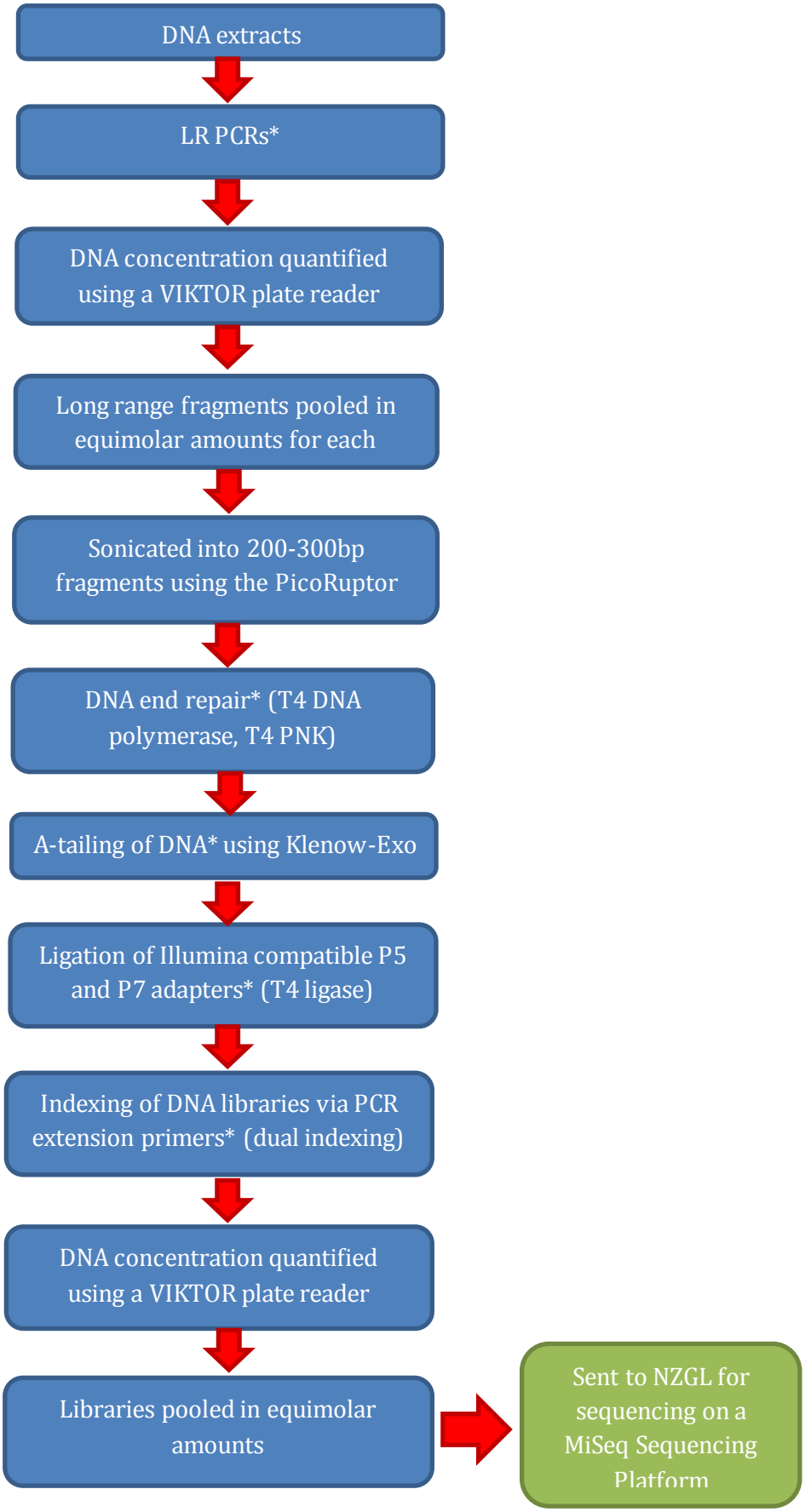

Figure 5.3: a summary of the laboratory methods required in prepping whole mt genomes for sequencing, starred steps indicated enzymatic reactions

The protocol and adapter sequences used in library preparation were adapted from the published protocols by Meyer & Kircher (2010) and Kircher et al. (2012). Library preparation involved end-repair steps (blunt-ending and 3' adenylation), ligation and the addition of index sequences using PCR. Each enzymatic step was followed by an AMPure purification as described earlier. The blunt-ending of the DNA fragments used the following reagent concentrations: 1x Buffer Tango, 100 μM of each dNTP, 1 mM ATP, 0.5 U/μL T4 polynucleotide kinase and 0.1 U/μL T4 polymerase. The total reaction volume was 40 μL, including 250 ng of template DNA, which was incubated in a thermocycler for 15 minutes at 12°C followed by 15 minutes at 25°C. Sticky ends were added to the DNA by A-tailing in an attempt to circumvent the problem of inefficient blunt ligation which was faced in the previous library preparation process for 454 sequencing. This reaction consisted of 1x NEBuffer 2, 0.1 mM dATP and 1 unit of Klenow fragment, and was incubated in a thermocycler for 30 minutes at 37°C.

Modified custom Solexa-compatible adapters (modified from (Meyer & Kircher, 2010) with an additional T added to reflect the A-tailed template) were next ligated to the DNA in a reaction with the following reagent concentrations: 1x T4 ligase buffer, 0.125 U/μL T4 ligase. The reaction was incubated for 1 hour at 22°C in a thermocycler. The adapter-ligated DNA template had dual-indexes added using a PCR step (Kircher et al., 2012), with 20 cycles of denaturation, annealing and extension. This PCR consisted of the entirety of the adapter-ligated DNA, 1x Kapa HiFi buffer, 0.3 μM of each primer, 0.3 μM of each nucleotide and 1 unit of Kapa HiFi Hot Start enzyme, in a volume of 50 μL. This step enriches the sample for template that had successfully ligated adapters, as well as giving each sample a unique combination of barcodes.
The barcoded libraries were then quantified using a PicoGreen quantification assay, prior to pooling in equimolar ratios. The pooled library was sent to New Zealand Genomic Limited (NZGL) for sequencing on an Illumina MiSeq Sequencing Platform using a 2x 250bp reagent kit.

5.2.6 TaqMan Genotyping of A6905G

TaqMan® assays are a quick and easy way of genotyping a single locus very rapidly. Here, TaqMan® genotyping was used to follow up a variant that was associated with BMI in the sequencing data produced above. A custom TaqMan® probe was designed for the 6905G variant using the Applied Biosystems web tool. A total of 1946 individuals who had Polynesian ancestry were genotyped using a Lightcycler 480 Real-Time PCR System. Of these, 446 had previously had their whole mitochondrial genomes sequenced, and thus were removed from subsequent analysis. Individuals who did not declare a Polynesian maternal grandmother were also

excluded from further analysis on the grounds of them being unlikely to have a Polynesian mitochondrial genome.

The data were then downloaded and statistical analyses were performed to test for associations with metabolic traits. Logistic regressions were performed in R Studio, as described later.

5.2.7 Bioinformatics processing of MiSeq Sequence Data

5.2.7.1 Raw data processing

The raw sequencing files were run through the pipeline described below. This pipeline was put together and is curated by James Boocock.

Sequence reads in the FASTQ format were aligned to the human reference genome (GRCH37/hg19) using the maximal exact matches (MEM) command of the Burrow's Wheeler Aligner (BWA) (v0.7.13) (Li, 2013). The full reference was chosen for alignment to reduce the impact of nuclear mitochondrial insertions (NUMTs). NUMTs that map to the mitochondrial reference (rCRS) (Andrews et al., 1999), but included in other sequences within the human reference genome (as many as 755 mitochondrial insertions into the nuclear genome have been detected in the build 37 of the Human Reference Genome (Ramos et al., 2011), some of which are close to whole mitochondrial genomes in length), will have a reduced mapping quality as a consequence of having multiple possible locations to map. This reduces the probability that NUMTs present in the reference genome will impact on downstream analyses. PCR duplicates were removed from the resulting BAM files using Picard's MarkDuplicates (v1.114) (http://broadinstitute.github.io/picard/). Read groups were added using Picard's AddOrReplaceGroups, and reads mapping to the mitochondrial reference sequence were extracted. Local realignment was performed using the Genome Analysis Toolkit's (GATK v 3.4.0) (McKenna et al., 2010) IndelRealigner with a set of mitochondrial insertions and deletions (INDELS) annotated in HmtDB (Rubino et al., 2012) and MITOMAP (Brandon et al., 2005). These realigned BAM files were then sorted and indexed using SAMtools (v0.1.19) (Li et al., 2009). All the above steps were run with GNU parallel (Tange, 2011) (a shell tool for executing jobs in parallel) improving the run-time of each step significantly. Coverage plots were created using the R programming language (v3.2.0) with the package ggplot2 (v1.0.1) (Hadley, 2009).

5.2.7.2 Variant calling and filtering

Homoplasmic single nucleotide polymorphisms (SNPs) and insertions and deletions (INDELs) were identified using the GATK HaplotypeCaller (v3.4.0) (DePristo et al., 2011) with the '-ploidy' option set to 1. This was justified because, although many copies of the mitochondria exist

within a single cell, usually only one dominant lineage is inherited. To simplify downstream association analyses the resulting variant call format (VCF) file was split into one containing only bi-allelic SNPs and INDELs, and one containing all other SNPs and INDELs. For the homoplasmic analyses only the former was considered. Both these files were filtered at the sample level to remove any SNP having a depth (DP) < 10, genotype quality (GQ) < 30, mapping quality (MQ) < 30, and a Phred scaled P-value for a Fisher's exact test for strand bias (FisherStrand) > 60. Furthermore, we calculated the number of bases that support the non-called allele, if this fraction was greater than 10% it was considered a putative heteroplasmic variant and was removed from this homoplasmic analysis.

To identify heteroplasmic variants, we used the GATK HaplotypeCaller (v3.4.0) with the '-ploidy' option set to 2. A variant was considered a candidate heteroplasmic variant, if at least two samples had heterozygote calls and the fraction of total bases supporting each of the alleles was greater than 10%. The 10% threshold was chosen to ensure that low frequency sequencing errors that are present when sequencing with the Illumina MiSeq were not identified as heteroplasmies. This VCF files was filtered for DP < 10, GQ < 30, MQ < 30, and FisherStrand > 60. In addition to the HaplotypeCaller, another variant caller freebayes (0.9.21) (Garrison & Marth, 2012) with the same set of filters was used to identify heteroplasmic variants. For downstream analysis, only variants present in both files were considered.

Annovar was used to annotate the gene(s) associated with each variant in the VCF file (Wang et al., 2010). This was done to facilitate burden analyses of genes .

5.2.7.3 Haplogroup assignment.

The VCF containing only bi-allelic SNPs and INDELS was converted to the Haplogrep (.hsd) format file using a locally developed python script (https://github.com/smilefreak/ancient_dna_pipeline/blob/master/python_scripts/vcf_to_fasta.py). The web-interface to Haplogrep, which utilises Phylotree version 16, was used to assign haplogroups to each sample. Haplogrep is a web-based application that assists with the assignment of haplogroup calls (Kloss-Brandstätter et al., 2011). The haplogroups are determined using a periodically updated database of worldwide mitochondrial genotypic variation named Phylotree (van Oven & Kayser, 2009). The software determines the ten most likely haplogroups matches based on the SNPs present in the input samples and shows why it has grouped the samples into these groups by compiling a list of the expected SNPs and any unexpected variants the individual possesses. The unexpected SNPs are classed as either local

private mutations, which are defined as variants observed in the sample but which are not associated with the haplogroup assignment, and global private mutations, which are mutations that have not previously been seen in Phylotree and thus, that the programmers have decided are likely the result of "inconsistent alignments, phantom mutations or point heteroplasmies", but which might represent real, novel SNPs. Sometimes these haplogroup calls need to be adjusted by eye because of discrepancies in how insertions and deletions are called, however this software is useful for grouping samples in a phylogenetic manner as it is able to rapidly compare the SNPs in each uploaded sample with known haplogroups and give the most probable match.

5.2.8 Statistical analyses

Statistical analysis was carried out in R-studio. Regression analysis was used to investigate relationships between mitochondrial variation and various phenotypic traits in this study. For binary variables, for instance gout status or stratified BMI (the grouping of individuals into two groups based on having BMI greater than or less than 34 kg/m^2), logistic regression was used. For continuous variables, such as the concentration of various biochemical markers in blood, a standard linear regression was performed. The main focus was gout status and BMI so as to test the two main hypotheses investigated in this chapter. The relationships of other metabolic markers (such as triglycerides, HDL levels and urate) were also explored because these have not been examined in relation to mitochondrial variation in Pacific people previously. In each test, age, East or West Polynesian ancestry and percentage Polynesian ancestry (as calculated on the basis of self-declared Polynesian grandparentage) were adjusted for. Accounting for degree of Polynesian ancestry is important because admixture may confound associations.

Associations between both haplogroup variation and individual SNP variants and various phenotypes were performed. For testing for associations with haplogroups, only those haplogroups previously identified in Phylotree possessing greater than 10 individuals were utilised. For associations between individual mitochondrial variants, there was a main focus on variants that occurred at a frequency greater than 0.05. For these common variants, linear and logistic regressions were undertaken (as described above). There was no adjustment for multiple testing such as a Bonferroni Correction performed for the reason that most of the variants were in linkage disequilibrium, which made determining the absolute number of independent tests challenging.

5.2.8.1 Heteroplasmies

When examining the common SNP variant data (MAF > 0.05), it was noticed that some variants were poly-allelic. Here hypervariable region I (HVR1) has been focused on. This region seems to

be highly variable. The region was visually inspected using the Broad Institute's Integrated Genomics Viewer (IGV) (Thorvaldsdóttir et al., 2013) and multiple haplotypes were possessed by some individuals. This led to this region to be explored more in depth.

Variant calling was redone using the GATK HaplotypeCaller (v3.4.0)(DePristo et al., 2011) with the '-ploidy' option set to 2, to produce diploid SNP calls. From individuals who had greater than 30 unique reads mapping in the region of interest, an allelic fraction was produced by dividing the number of reads mapping to alternate alleles by the total number of reads mapping at each variable position in this 16050-16189 mitochondrial region.

i.e.: $Allelic\ fraction = \frac{n\ alternate\ allele\ reads\ at\ position}{n\ of\ reads\ mapping\ to\ position}$

An allelic fraction threshold of 0.10 was used to subset the data into two groups, which represented those who possessed heteroplasmy at these positions at an appreciable frequency and those who did not. These groups were then statistically analysed using logistic regression to test for association with gout, adjusting for age, East versus West Polynesian ancestry and the proportion of self-declared Polynesian grandparentage.

Further statistical tests were undertaken to explore potential relationships between allelic fractions (as a measure of the amount of heteroplasmy in an individual) and among those possessing mtDNA haplogroup B lineages.

5.2.8.2 SKAT test for burden of mtDNA variants

Testing for associations between rare variants and disease is complicated by the nature of the variants being uncommon, which severely limits statistical power. Instead, a sequence kernel association test (SKAT) can be performed. This tests the association between genetic variants (common and rare) in a region and a continuous or discrete trait, allowing for the detection of a burden of variants rather than association between individual variants on their own (Wu et al., 2011). It does this by aggregating the individual score test statistics of SNPs in a SNP set and calculates SNP-set level *P*-values. SKAT tests were performed looking at the association of variants in mitochondrial genic regions and the dichotomous traits of gout status and stratified BMI. Age, East versus West Polynesian ancestry and percentage of self-declared Polynesian grandparental ancestry were adjusted for in these tests.

5.2.8.3 Testing for Associations with Maternal Grandparental Ancestry

Because there was a high concordance between self-reported maternal grandparental ancestry and haplogroup calling from the sequencing data (99.6% concordance), in addition to statistical analyses between the sequencing data and various phenotypes described above, further testing was undertaken to look at the association between self-declared maternal grandparentage and

disease status among those with three grandparents of Polynesian ancestry and one grandparent of European ancestry from the extended cohort described earlier that had not had mtDNA sequenced. This test was undertaken to try and detect any differences in inferred mitochondrial lineage and disease – with 96% of the participants belonging to the same mitochondrial macrohaplogroup, there are difficulties in assessing whether these individuals may all possess a predisposition to disease from possessing a mitochondrial lineage belonging to this haplogroup.

5.3 Results

5.3.1 454 Sequencing results

Sequencing was initially carried out in 4 sequencing runs on the 454 Junior Sequencer. Unfortunately, the quality of the sequence was not good enough to analyse. Many reads were not properly barcoded and despite efforts in the lab to reduce this loss of reads by increasing ligation times and reducing the number of individuals included in a run, there were still a significant proportion of reads which lacked barcodes making it impossible to assign them to an individual. As a result of this reduction of usable data, there were substantial numbers of individuals missing sequence coverage (Table 5.2). To re-run samples was going to be prohibitively expensive, so ultimately this approach was abandoned in favour of resequencing the same individuals on the Illumina MiSeq Platform.

Table 5.2: Attributes of the 454 Sequencing Runs

	No. Individuals in run	No. total reads	No. unbarcoded reads (%)	%individuals with missing coverage
Run 1	130	138871	77401 (56%)	48%
Run 2	112	95658	62604 (65%)	88%
Run 3	116	135067	20888 (15%)	24%
Run 4	117	161454	17527 (11%)	10%
TOTAL	475*			41%*

*NB. Some individuals were run on multiple runs in an attempt to improve their coverage. There were a total of 451 unique individual run across the four runs.

The problem with the approach taken here was the adherence to the PTS barcoding protocol (Meyer et al., 2008), wherein the samples are barcoded prior to library preparation for sequencing. This meant that if the barcoding process was inefficient and there was abundant unbarcoded DNA still present, this got carried through to the library preparation point and to sequencing. This would not necessarily have been such a big problem if the number of reads returned by the GS FLX Junior sequencer were reaching the levels that are returned from the Illumina MiSeq (though a different barcoding strategy was employed for the MiSeq sequencing),

however the relatively modest number of reads (albeit longer reads) returned by the GS FLX Junior did not compensate for this loss of usable reads. It is also of note that Roche are discontinuing their 454 sequencing platform in 2016.

5.3.2 MiSeq Sequencing

Of the 446 individuals sequenced, four had slightly less than 100% coverage, though these all had coverage above 92%. On average, the coverage was 774 ± 344 times, allowing for good confidence in base calls. The standard deviations for coverage were rather large because of the overlapping regions of the long range PCR products used, meaning that overlap regions had much higher coverage than the rest of the mitochondrial genome. Individuals who possessed less than 100% coverage were excluded from further analysis.

5.3.3 Haplogroups

There were 23 different haplogroups identified using the Haplogrep software. These haplogroups predominantly belonged to Asian-derived B4 lineages, though there were also a minority of people belonging to the M28a, M28b, Q1 and P1d lineages, which originated in Near Oceania (Table 5.3). Two individuals possessed European-derived mitochondrial haplogroups. These two individuals were excluded from subsequent analysis.

When comparing the mitochondrial sequences within the sample, it was possible to define novel haplogroups that had not previously been identified (Table 5.4) and to expand on previously described lineages. These new haplogroups were defined by the presence of new SNPs in four or more individuals who claimed ancestry from different island groups (to reduce the chance that these individuals were closely maternally related). Because the number of individuals belong to new lineages was limited, these were not incorporated into subsequent statistical analyses involving association with various phenotypes.

Table 5.3: Haplogroups from the genotyped samples split into the various high and low BMI cohorts, including novel haplogroups that are indicated an asterisk (these are described further in Table 5.4)

Haplogroup	Combined Polynesians High BMI (%)	Combined Polynesians Low BMI (%)	West Polynesians High BMI (%)	West Polynesians Low BMI (%)	East Polynesians High BMI (%)	East Polynesians Low BMI (%)	Total
	n = 219	**n = 223**	**n = 109**	**n = 104**	**n = 110**	**n = 119**	**n = 442**
R	211 (96.3%)	215 (96.4%)	102 (93.6%)	102 (98.1%)	109 (99.1%)	116 (95.0%)	426 (96.4%)
B4a1a1	65 (29.7%)	55 (24.7%)	50 (45.9%)	40 (38.5%)	15 (13.6%)	15 (12.6%)	120 (27.1%)
B4a1a1a	27 (12.3%)	22 (9.9%)	22 (20.2%)	22 (21.2%)	5 (4.5%)	…	49 (11.1%)
B4a1a1a1	…	1 (0.4%)	…	1 (1.0%)	…	…	1 (0.2%)
B4a1a1a1c*	1 (0.5%)	4 (1.8%)	1 (0.9%)	4 (3.8%)	…	…	5 (1.1%)
B4a1a1a8	2 (0.9%)	9 (4.0%)	2 (1.8%)	7 (6.7%)	…	2 (1.7%)	11 (2.5%)
B4a1a1a9	1 (0.5%)	1 (0.4%)	1 (0.9%)	1 (1.0%)	…	…	2 (0.5%)
B4a1a1a11	…	2 (0.9%)	…	2 (1.9%)	…	…	2 (0.5%)
B4a1a1a11a	1 (0.5%)	2 (0.9%)	1 (0.9%)	2 (1.9%)	…	…	3 (0.7%)
B4a1a1a13	1 (0.5%)	…	1 (0.9%)	…	…	…	1 (0.2%)
B4a1a1a14*	3 (1.4%)	7 (3.1%)	…	3 (2.9%)	3 (2.7%)	4 (3.4%)	10 (2.3%)
B4a1a1a15*	4 (1.8%)	5 (2.2%)	…	…	4 (3.6%)	5 (4.2%)	9 (2.0%)
B4a1a1a16*	1 (0.5%)	3 (1.3%)	1 (0.9%)	3 (2.9%)	…	…	4 (0.9%)
B4a1a1a17*	2 (0.9%)	6 (2.7%)	1 (0.9%)	…	1 (0.9%)	6 (5.0%)	8 (1.8%)
B4a1a1c	29 (13.2%)	24 (10.8%)	1 (0.9%)	…	28 (25.5%)	24 (20.2%)	53 (12.0%)
B4a1a1h	…	1 (0.4%)	…	…	…	1 (0.8%)	1 (0.2%)
B4a1a1k	2 (0.9%)	1 (0.4%)	2 (1.8%)	1 (1.0%)	…	…	3 (0.7%)
B4a1a1m	44 (20.1%)	40 (17.9%)	7 (6.4%)	6 (5.8%)	37 (33.6%)	34 (28.6%)	84 (19.0%)
B4a1a1n	7 (3.2%)	2 (0.9%)	1 (0.9%)	…	6 (5.5%)	2 (1.7%)	9 (2.0%)
B4a1a1r*	4 (1.8%)	10 (4.5%)	2 (1.8%)	1 (1.0%)	2 (1.8%)	9 (7.6%)	14 (3.2%)
B4a1a1x	…	1 (0.4%)	…	1 (1.0%)	…	…	1 (0.2%)
B4a1a1z*	5 (2.3%)	4 (1.8%)	1 (0.9%)	…	4 (3.6%)	4 (3.4%)	9 (2.0%)
B4a1a1aa*	3 (1.4%)	3 (1.3%)	3 (2.8%)	2 (1.9%)	…	1 (0.8%)	6 (1.4%)
B4a1a1ab*	4 (1.8%)	5 (2.2%)	1 (0.9%)	2 (1.9%)	3 (2.7%)	3 (2.5%)	9 (2.0%)
B4a1a1ac*	3 (1.4%)	4 (1.8%)	2 (1.8%)	1 (1.0%)	1 (0.9%)	3 (2.5%)	7 (1.6%)
B4b1a2	…	2 (0.9%)	…	2 (1.9%)	…	…	2 (0.5%)
P1d	2 (0.9%)	1 (0.4%)	2 (1.8%)	1 (1.0%)	…	…	3 (0.7%)
M	8 (3.7%)	8 (3.6%)	7 (6.4%)	2 (1.9%)	1 (0.9%)	6 (5.0%)	16(3.6%)
M28a	2 (0.9%)	2 (0.9%)	2 (1.8%)	2 (1.9%)	…	…	4 (0.9%)
M28b	1 (0.5%)	…	1 (0.9%)	…	…	…	1 (0.2%)
Q1	5 (2.3%)	6 (2.7%)	4 (3.7%)	…	1 (0.9%)	6 (5.0%)	11 (2.5%)

Table 5.4: Description of haplogroups belonging to B macrohaplogroup with defining polymorphisms, including the new lineages and where they occur. New lineages had to be present on more than one island group to be defined as a novel haplogroup and are indicated by an asterisk.

Haplogroup	n	Polynesian populations	Polymorphisms
B4a1a1	120	NZ Māori, Samoa, Tonga, Cook Island Māori, Pukapuka, Tokelau, Niue, Tahiti	A14022G
B4a1a1a	49	Niue, Samoa, Tonga, NZ Māori, Cook Island Māori,	A6905G
B4a1a1a1	1	Samoa	A13479G
B4a1a1a1c*	5	Samoa, Tonga	T152C, G12940A
B4a1a1a8	11	Niue, Samoa, Tonga, Cook Island Māori	G6261A, **A235G, T12468C**[1]
B4a1a1a9	2	Samoa	G2706A, A16163G
B4a1a1a11	2	Samoa, Tonga	C13943T
B4a1a1a11a	3	Samoa	T10966C
B4a1a1a13	1	Samoa	A4093G
B4a1a1a14*	10	Niue, NZ Māori, Cook Island Māori, Samoa	T3398C, G16384A
B4a1a1a15*	9	NZ Māori, Cook Island Māori	G9145A, A16051G
B4a1a1a16*	4	Samoa, Tonga	G13759A
B4a1a1a17*	8	NZ Māori, Cook Island Māori, Tonga	A14587G
B4a1a1c	53	NZ Māori, Cook Island Māori, Pukapuka, Tonga	C1185T, G4769A
B4a1a1h	1	NZ Māori	T318C, T10031C
B4a1a1k	3	Samoa, Tonga	A16399G
B4a1a1m	84	NZ Māori, Cook Island Māori, Pukapuka, Samoa, Tonga, Niue	C151T, A1692G, T2416C
B4a1a1n	9	Tonga, Cook Island Māori, NZ Māori	G8572A
B4a1a1r[2]	14	NZ Māori, Cook Island Māori, Samoa	T16126C
B4a1a1x	1	Tokelau	A15924G
B4a1a1z*	9	NZ Māori, Cook Island Māori, Tonga	T4314C
B4a1a1aa*	6	Niue, Samoa, Cook Island Māori	T414G
B4a1a1ab*	9	Samoa, NZ Māori, Cook Island Māori	T16092C
B4a1a1ac*	7	Samoa, Niue, Cook Island Māori, Pukapuka	C13692T
B4b1a2	2	Tonga, Tuvalu	**C16067T** (plus those normal for B4b1a2)

1) Bold text indicates additional defining SNPs for the lineage
2) B4a1a1r, defined by variants T16126C and C3909T, is a previously described lineage, however those in our cohort are missing one of the defining polymorphisms (C3909T). For this reason, we propose that individuals, who possess this C3909T variant, should belong to a new lineage named B4a1a1r1, making B4a1a1r defined solely on the presence of T16126C. This B4a1a1 + 16126C is found throughout Polynesia.

There was a difference in the distribution of certain haplogroups between East and West Polynesia, in particular when it came to the proportion of certain lineages in each population (Table 5.3). A breakdown of the haplogroup frequencies in various island populations is included in the Appendix 3 (Table 8.2). The B4a1a1c lineage, for instance, was common among East Polynesians, with 98% of those who belonged to this haplogroup coming from the Cook Islands or New Zealand (a single Tongan belonged to this lineage). Likewise, the B4a1a1a lineage and derived sub-lineages were less common in East Polynesia than West Polynesia, with 71% of those belonging to this lineage and sub-lineages coming from West Polynesia. In this way, the mitochondrial genome is able to show differences in population structure between what are often considered homogeneous populations and may be indicative of differences in nuclear genome variation also.

Table 5.3 shows no obvious signs of trends in haplogroup frequency contributing to high or low BMI status. Statistical analysis is carried out in an upcoming section. In Appendix 3, Table 8.1, the haplogroups are broken into gout status compared to healthy controls. Likewise there were few obvious trends between haplogroup and gout, although among the East Polynesians the lineage B4a1a1m was appreciably higher among individuals with gout than controls under visual inspection (48 versus 11). This proved statistically nominally significant when a Fisher's Exact test was utilised (OR 2.17 [1.01-5.05], P = 0.04). This statistical association was not evident in the combined East and West Polynesian dataset. No other haplogroup appeared overly inflated in either gout cases or healthy controls.

5.3.4 Non-synonymous and synonymous variants

As with most mitochondrial polymorphisms, most of the variants detected were transitions. There was also one lineage-defining transversion at position T414G, present in six individuals. Many of the more ancient lineage-defining mutations were synonymous rather than non-synonymous changes (Table 5.5). This is not unexpected because of the highly conserved nature of the mitochondrial genes. Most of the non-synonymous changes were relatively rare and associated with younger lineages so may not yet have been lost to purifying selection.

Table 5.5: Variants contributing to haplogroup calls and their context.

Variant	Lineage defining?	Coding/noncoding?	Syn/Non-syn	Frequency within sample
8261 9bp del	B	Noncoding		0.961
T16217C	B4	HVR		0.961
C16261T	B4a	HVR		0.957
C9123A	B4a	MT-ATP6	Syn	0.957
T5465C	B4a	MT-ND5	Syn	0.957
T10238C	B4a1	MT-ND3	Syn	0.957
T146C!	B4a1a	HVR		0.957
T6719C	B4a1a	MT-CO1	Syn	0.957
A15746G	B4a1a	MT-CYB	Non-syn	0.957
C12239T	B4a1a	MT-TS2 (tRNA)		0.957
14022G	B4a1a1	MT-ND5	Syn	0.957
(A16247G)	B4a1a1	HVR		0.840
A6905G	B4a1a1a	MT-CO1	Syn	0.235
A13479G	B4a1a1a1	MT-ND5	Syn	0.013
C13943T	B4a1a1a11	MT-ND5	Non-syn	0.011
T10966C	B4a1a1a11a	MT-ND4	Syn	0.007
A4093G	B4a1a1a13	MT-ND1	Non-syn	0.02
G16384A	B4a1a1a14*	HVR		0.022
T3398C[1]	B4a1a1a14*	MT-ND1	Non-syn	0.022
G16051G	B4a1a1a15*	HVR		0.02
G9145A	B4a1a1a15*	MT-ATP6	Non-syn	0.02
G13759A	B4a1a1a16*	MT-ND5	Non-syn	0.009
A14587G	B4a1a1a17*	MT-ND6	Syn	0.018
T152C	B4a1a1a1c*	HVR		0.011
G12940A	B4a1a1a1c*	MT-ND5	Non-syn	0.011
A235G	B4a1a1a8	HVR		0.025
G6261A	B4a1a1a8	MT-CO1	Non-syn	0.025
T12468C	B4a1a1a8	MT-ND5	Syn	0.025
G2706A	B4a1a1a9	MT-RNR2		0.004
G4769A	B4a1a1c	MT-ND2	Syn	0.119
C1185T	B4a1a1c	MT-RNR1		0.119
T318C	B4a1a1h	HVR		0.002
T10031C	B4a1a1h	MT-TG (tRNA)		0.002
A16399G	B4a1a1k	HVR		0.007
C151T	B4a1a1m	HVR		0.197
A1692G	B4a1a1m	MT-RNR1		0.197
T2416C	B4a1a1m	MT-RNR2		0.197
G8572A	B4a1a1n	MT-ATP8/MT-ATP6	Syn/Non-syn	0.02
T16126C	B4a1a1r**	HVR		0.031
T4314C[2]	B4a1a1z*	MT-TI (tRNA)		0.02
T414G	B4a1a1aa*	HVR		0.013
T16092C	B4a1a1ab*	HVR		0.02
C13692T	B4a1a1ac*	MT-ND5	Syn	0.016

* Designates the novel lineages identified in this study

[1]) Previously been found to be associated with Gestational Diabetes Mellitus and a few other disorders (Jaksch et al., 1996, Chen et al., 2000, Tang et al., 2010)

[2]) Previously reported as a possible hypertension factor (Zhu et al., 2009)

5.3.5 Associations between mitochondrial haplogroup and BMI and gout

Logistic regression was used to test for associations between mitochondrial haplogroup and BMI. BMI was transformed into a binomial factor by splitting into two groups – in this study, 'high BMI' is defined as BMI greater than 34 kg/m^2, while 'low BMI' is less than 34 kg/m^2. This cut-off is used in part because of the high proportion of high BMI individuals among Pacific people, but also in recognition of the differences in body composition that have been noted by

(Swinburn et al., 1999) and others. Because relatively few individuals belonged to certain haplogroups (Table 5.3), haplogroups with less than ten individuals were excluded from the statistical analysis. From the haplogroups where there were sufficient numbers of individuals, it was only possible to detect a statistical association by logistic regression with B4a1a1a8 (OR 0.18 [0.03-0.72], $P = 0.03$), which appeared to have a protective effect against BMI (Table 5.6). This haplogroup was relatively rare, with only 11 individuals possessing it. No other associations were detected for any of the other haplogroups. This lack of detected association between mitochondrial haplogroup and BMI held true when the data were divided into East and West Polynesian groups, though very few mitochondrial haplogroups possessed enough individuals once stratified into sub-populations.

Gout was also tested for association with haplogroup. There were no associations (Table 5.6). The association detected in the raw data tables with the B4a1a1m lineage and gout risk among East Polynesians (OR 2.17 [1.01-5.05], $P = 0.04$), was not supported in this analysis. Further traits were tested including urate, HDL cholesterol and triglycerides, and also yielded no associations. These are reported in Appendix 3, Table 8.3.

Table 5.6: Logistic regression predicting stratified BMI and gout from haplogroup, adjusted for age, East versus West Polynesian ancestry and proportion of self-declared Polynesian grandparents

Population	**Haplogroup**	***n***	***BMI***		***Gout***	
			OR (CI)	***P***	**OR (CI)**	***P***
All	B4a1a1+16126	13	0.46 (0.11-1.57)	0.23	1.04 (0.21-2.84)	0.60
(n = 396)	B4a1a1a	78	0.70 (0.40-1.23)	0.22	1.00 (0.43-1.58)	0.55
	B4a1a1a8	11	0.18 (0.03-0.72)	0.03*	0.99 (31-9.03)	0.74
	B4a1a1c	53	1.46 (0.72-2.99)	0.30	0.95 (0.34-1.62)	0.45
	B4a1a1m	83	1.18 (0.66-2.11)	0.59	1.05 (0.59-2.37)	0.65
	Q1	11	0.83 (0.22-2.96)	0.77	1.12 (0.15-2.19)	0.35
EP	B4a1a1a	27	0.94 (0.36-2.44)	0.89	1.04 (0.29-2.13)	0.62
(n = 188)	B4a1a1c	52	1.19 (0.52-2.70)	0.68	1.00 (0.38-2.11)	0.81
	B4a1a1m	59	1.16 (0.53-2.56)	0.70	0.99 (0.80-4.88)	0.14
WP	B4a1a1a	51	0.57 (0.28-1.15)	0.12	1.04 (0.38-2.21)	0.82
(n = 171)	B4a1a1m	24	1.01 (0.40-2.61)	0.98	1.00 (0.15-1.31)	0.12

5.3.6 Associations between individual mitochondrial variants and BMI

Further analysis was undertaken with individual variants, including SNPs and small insertions and deletions which had a minor allele frequency of greater than 0.05. While the SNPs define the haplogroups in an additive manner, by looking at the SNPs in isolation of the haplogroup call, it can increase the number of individuals in some of the groups that get stratified further as more peripheral haplogroups are called. Additionally, some SNP variants are not used in the assignment of haplogroups because they are considered too variable and switch backwards and forwards too rapidly to be reliable phylogenetic markers. Such variants may confer risk to disease.

Most of the mitochondrial variants are biallelic, so present as either the same as the revised Cambridge Reference Sequence at the position or as a single alternate allele, however there were two positions (302 and 16179) where it was possible to detect multiple different alleles. At position 302, there is a variable length insertion, while at position 16179, there is a variable length deletion. Both of these positions are located close by extended tracts of consecutive cytosine bases. Below, the results for statistical analyses of both simple bi-alleleic variants and these poly-allelic variants are reported.

5.3.6.1 Bi-allelic variants

Here, BMI was tested in a stratified manner, splitting the cohort into the high and low BMI groups previously described. The only mitochondrial SNP that was found to associate with stratified BMI was A6905G (Table 5.7), which appeared to be having a protective effect against high BMI (OR 0.57 [0.35-0.90], P = 0.02)(Figure 5.4A). Because the 6905G variant is more common among West Polynesians, the association was also tested among West Polynesians alone, which also yielded statistically significant results (OR 0.42 [0.23-0.79], P = 0.007)(Table 5.7).

However, when the sample size was increased to 1811 individuals using TaqMan SNP genotyping, of which 1019 remained after removing the individuals who had already been genotyped via whole mitochondrial genome sequencing, and those who did not declare a Polynesian maternal grandmother. Most unusually, the direction of the effect switched in this replication cohort (Figure 5.4B), with the 6905G allele appearing to contribute a 39% greater risk of high BMI than the reference A allele (OR 1.39 [1.00-1.94], P = 0.05)(Table 5.8). Again, the association was driven by West Polynesians, where the 6905G allele appeared to be contributing risk of obesity (OR 1.83 [1.16-2.95], P = 0.01), in particular West Polynesian men (OR 2.54 [1.45-4.56], P = 0.001).

Table 5.7: The results for logistic regression predicting stratified BMI by mitochondrial variants. Results adjusted for age, East versus West Polynesian ancestry and proportion of Polynesian grandparental ancestry.

		Combined Polynesians (n=442)			*East Polynesians (n = 213)*			*West Polynesians (n = 229)*		
Variant	**Alleles Ref/Alt**	**Alt Allele Freq High/Low BMI**	**OR (CI)**	***P***	**Alt Allele Freq High/Low BMI**	**OR (95% CI)**	***P***	**Alt Allele Freq High/Low BMI**	**OR (95% CI)**	***P***
C151T	C/T	0.20/0.19	1.41 (0.70-1.85)	0.59	0.25/0.22	1.18 (0.62-2.24)	0.61	0.16/0.16	1.00 (0.49-2.07)	0.99
310T/TC	T/TC	0.92/0.91	1.10 (0.55-2.19)	0.78	0.94/0.89	2.13 (0.78-6.45)	0.15	0.90/0.94	0.67 (0.23-1.78)	0.43
C1185T	C/T	0.14/0.11	1.42 (0.77-2.64)	0.26	0.19/0.16	1.11 (0.55-2.33)	0.77	0.10/0.06	1.11 (0.43-2.33)	0.77
A1692G	A/G	0.19/0.18	1.21 (0.73-2.00)	0.47	0.23/0.21	1.10 (0.56-2.14)	0.78	0.10/0.06	1.22 (0.58-2.58)	0.60
T2416C	T/C	0.20/0.18	1.23 (0.75-2.03)	0.41	0.24/0.22	1.11 (0.57-2.13)	0.76	0.16/0.15	1.28 (0.61-2.70)	0.50
A4679G	A/G	0.86/0.89	0.65 (0.35-1.21)	0.17	0.80/0.84	0.84 (0.40-1.73)	0.63	0.91/0.95	0.55 (0.18-1.55)	0.27
T5465C	T/C	0.95/0.95	1.17 (0.48-2.93)	0.73	0.97/0.93	2.89 (0.79-14.0)	0.13	0.94/0.97	0.46 (0.10-1.75)	0.28
T6719C	T/C	0.95/0.95	1.17 (0.48-2.93)	0.73	0.97/0.93	2.89 (0.79-14.0)	0.13	0.94/0.97	0.46 (0.10-1.75)	0.28
A6905G	A/G	0.20/0.28	0.57 (0.35-0.90)	0.02*	0.20/0.22	0.95 (0.48-1.90)	0.89	0.20/0.35	0.42 (0.23-0.79)	0.007*
G9123A	G/A	0.95/0.95	1.17 (0.48-2.93)	0.73	0.97/0.93	2.89 (0.79-13.98)	0.12	0.94/0.97	0.46 (0.10-1.75)	0.28
T10238C	T/C	0.95/0.95	1.28 (0.53-3.14)	0.59	0.97/0.92	3.36 (0.94-16.07)	0.08	0.94/0.97	0.46 (0.10-1.75)	0.28
C12239T	C/T	0.95/0.95	1.04 (0.43-2.53)	0.92	0.96/0.93	2.07 (0.62-8.20)	0.26	0.94/0.97	0.46 (0.10-1.75)	0.28
A14022G	A/G	0.95/0.95	1.28 (0.53-3.14)	0.59	0.97/0.92	3.36 (0.94-16.07)	0.08	0.94/0.97	0.46 (0.10-1.75)	0.28
A15746G	A/G	0.95/0.95	1.17 (0.48-2.93)	0.73	0.97/0.93	2.89 (0.79-13.98)	0.13	0.94/0.97	0.46 (0.10-1.75)	0.28
A16183C	A/C	0.93/0.91	1.39 (0.68-2.89)	0.36	0.93/0.88	2.09 (0.80-5.95)	0.15	0.93/0.95	0.83 (0.26-2.50)	0.73
T16189C	T/C	0.95/0.95	1.10 (0.46-2.69)	0.83	0.96/0.93	2.34 (0.68-9.46)	0.19	0.94/0.97	0.47 (0.10-1.78)	0.29
T16217C	T/C	0.95/0.95	0.92 (038-2.23)	0.86	0.96/0.93	2.34 (0.68-9.46)	0.19	0.93/0.98	0.29 (0.04-1.23)	0.13
A16247G	A/G	0.84/0.84	1.00 (0.59-1.68)	0.99	0.82/0.83	1.04 (0.50-2.16)	0.92	0.85/0.86	0.97 (0.45-2.07)	0.94
C16261T	C/T	0.94/0.94	1.20 (0.54-2.70)	0.65	0.96/0.92	2.52 (0.77-9.92)	0.15	0.92/0.95	0.65 (0.19-1.99)	0.46

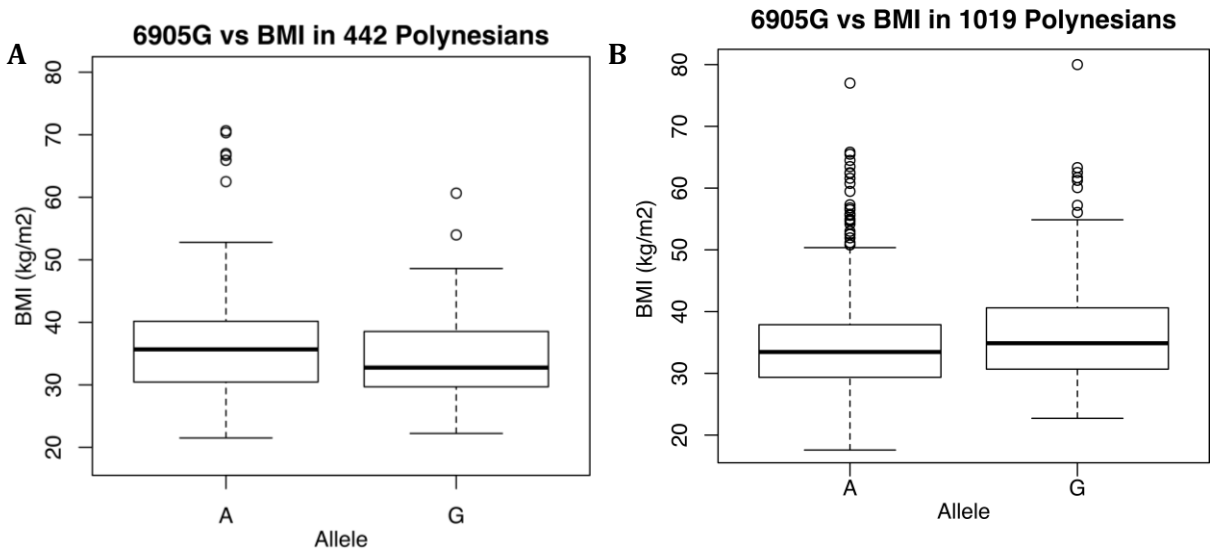

Figure 5.4: Box plot showing differences in BMI based on variation at mtDNA position 6905. A) Individuals possessing the 6905G variants have on average a lower BMI in the 442 Polynesian men sequenced in this study; B) Testing in a larger cohort (1019 Polynesians) lead to the trend switching direction.

To ensure that the alleles had been correctly coded in the results of the TaqMan assay, a Pearson correlation test was performed between the whole mitochondrial genome data and the TaqMan genotyping data for the 440 individuals who overlapped between datasets. There was a correlation of 0.97 ($P < 2.2 \times 10^{-16}$), indicating that only one individual was incorrectly genotyped. Furthermore, using the TaqMan data for these 440 individuals, the original result (and its direction of effect) was replicated. This either suggests that there is something fundamentally different about the make-up of the sample sets (which seems unlikely), or that the conflicting findings are a random statistic sampling effect.

When the data from both the genotyping and the whole mitochondrial genome groups were merged, there was no longer a statistically significant association between the 6905G and stratified BMI (OR 1.04 [0.80-1.36], $P = 0.76$) (Table 5.8).

Table 5.8: Logistic regression predicting stratified BMI (High BMI > 34, low BMI < 34) by 6905G. Results have been adjusted for sex, age, East versus West Polynesian ancestry and grandparental ancestry as covariates. Analysis only includes individuals who self-reported Polynesian maternal ancestry (so who should therefore possess Polynesian mitochondrial lineages).

Population	N	N High/Low	MAF High/Low	OR (CI)	*P*
Discovery					
All	442	219/223	0.20/0.28	0.57 (0.35-0.90)	0.02*
EP	213	102/111	0.20/0.22	0.95 (0.48-1.90)	0.89
WP	229	119/110	0.20/0.35	0.42 (0.23-0.79)	0.007*
Replication					
All	1019	484/535	0.22/0.15	1.39 (1.00-1.94)	0.05*
Males	529	248/282	0.30/0.15	1.98 (1.27-3.13)	0.003*
Females	490	236/253	0.16/0.16	0.88 (0.53-1.44)	0.60
EP	747	317/430	0.12/0.11	1.11 (0.70-1.76)	0.65
Males	284	105/179	0.10/0.09	1.16 (0.50-2.60)	0.72
Females	392	177/215	0.10/0.08	0.83 (0.43-1.57)	0.57
WP	353	208/145	0.41/0.28	1.83 (1.16-2.95)	0.01*
Males	252	147/105	0.45/0.25	2.54 (1.45-4.56)	0.001*
Females	101	61/40	0.33/0.38	0.89 (0.37-2.12)	0.78
Merged					
All	1449	696/753	0.22/0.19	1.04 (0.80-1.36)	0.76
Males	954	457/497	0.25/0.20	1.14 (0.83-1.57)	0.41
EP	899	390/509	0.12/0.12	0.93 (0.60-1.41)	0.72
Males	505	211/294	0.11/0.08	1.04 (0.59-1.82)	0.89
WP	550	306/244	0.36/0.34	1.08 (0.76-1.54)	0.66
Males	449	246/203	0.37/0.34	1.15 (0.78-1.60)	0.49

5.3.6.2 Poly-allelic variants

Logistic regression analysis showed that the CAA variant at position 16179 appeared to have a protective effect against high BMI (OR 0.48 [0.30-0.77], *P* = 0.002)(Table 5.9). This remained consistent when splitting the cohort into East and West Polynesian sub-populations (OR 0.37 [0.18-0.75], *P* = 0.008; OR 0.52 (0.27-1.01), *P* = 0.05, respectively).

None of the variants at postion 302 showed association with BMI status until split into subpopulations, where two of the non-reference alleles appeared to contribute to risk of high BMI in East Polynesians (ACC variant: 2.40 [1.03-5.90], *P* = 0.05; AC variant: 2.80 [1.15-7.19], *P* = 0.02).

5.3.7 Associations between individual mitochondrial variants and Gout

5.3.7.1 Bi-allelic variants

When the mitochondrial variants with a MAF > 0.05 were tested for association with gout, the variant A16183C was found to be associated with nearly four-fold higher disease risk (OR 3.80 [1.86-7.83], P = 0.0002) (Table 5.10). When East and West Polynesian subgroupings were analysed separately, this association was maintained, though the effect appeared to be greater among West Polynesians (EP: OR 4.15 [1.43-12.93], P = 0.01; WP: OR 4.73 [1.67-13.63], P = 0.003).

There were also population-specific SNPs detected (Table 5.10). Among East Polynesians, A1692G and T2416C were found to contribute risk to gout. These SNPs are found in linkage disequilibrium, thus had the same effect size (OR 2.14 [1.05-4.66], P = 0.04). The C151T SNP that these variants are in linkage disequilibrium with (making up the mitochondrial haplogroup B4a1a1m) was also trending towards a significant association in the same direction (OR 1.86 [0.93-3.97], P = 0.09). These SNPs are less common among West Polynesians, and this same trend was not detected. However, among West Polynesians, the variant T16189C was found to contribute to risk to gout (OR 3.92 [1.13-13.40], P = 0.03). This was not detected among East Polynesians.

5.3.7.2 Poly-allelic variants

Logistic regression analysis of the poly-allelic variants found no association with gout status for variation at position 302, however one of the variants (the CA variant of the deletion at position 16179) was found to contribute to gout risk (OR 2.70 [1.17-7.32], P = 0.03)(Table 5.9). Given that this variant is physically close to the biallelic variant 16183C, it seems likely that these variants are in linkage disequilibrium and the same effect is being detected in both tests. This was confirmed using a Pearson Correlation test, which found a correlation of 0.81 between the two variants ($P < 2.2 \times 10^{-16}$). The variant did not retain significance when the combined Polynesian group was split into East and West Polynesian subgroups.

Table 5.9: The results for logistic regression predicting stratified BMI and gout status by poly-allelic mitochondrial variants. Case status refers to individuals with BMI > 34 or participants with gout respectively, while control status refers to individuals with BMI < 34 or non-gout control, respectively. Results adjusted for age, East versus West Polynesian ancestry (were appropriate) and proportion of Polynesian grandparental ancestry.

		Combined Polynesians (n=442)			*East Polynesians (n = 211)*			*West Polynesians (n = 226)*		
Position	**Alleles Ref/Alt**	**Alt Allele Freq Case/Control**	**OR (95% CI)**	***P***	**Alt Allele Freq Case/Control**	**OR (95% CI)**	***P***	**Alt Allele Freq Case/Control**	**OR (95% CI)**	***P***
BMI										
302	A/ACC	0.51/0.49	1.55 (0.89-2.75)	0.13	0.53/0.53	2.40 (1.03-5.90)	0.05*	0.53/0.53	1.11 (0.50-2.42)	0.80
	A/AC	0.30/0.28	1.56 (0.86-2.88)	0.15	0.28/0.26	2.80 (1.15-7.19)	0.02*	0.25/0.28	1.06 (0.44-2.53)	0.90
	A/ACCC	0.06/0.05	1.58 (0.61-4.14)	0.34	0.07/0.05	1.67 (0.39-7.108)	0.48	0.07/0.05	1.58 (0.42-6.34)	0.50
16179	CAAA/C	0.005/0.005	1.38 (0.13-30.4)	0.79	0.01/0	-	-	0.01/0.01	0.52 (0.02-13.49)	0.65
	CAAA/CAA	0.10/0.14	0.48 (0.30-0.77)	0.002*	0.15/0.29	0.37 (0.18-0.75)	0.008*	0.18/0.28	0.52 (0.27-1.01)	0.05*
	CAAA/CA	0.05/0.07	0.78 (0.42-1.43)	0.43	0.11/0.10	1.22 (0.17-6.45)	0.92	0.10/0.15	0.66 (0.28-1.54)	0.34
Gout										
302	A/ACC	0.50/0.52	1.13 (0.60-2.07)	0.70	0.53/0.53	1.11 (0.50-2.42)	0.80	0.53/0.53	1.24 (0.50-2.92)	0.64
	A/AC	0.31/0.22	1.75 (0.86-3.52)	0.12	0.28/0.26	1.06 (0.44-2.53)	0.90	0.28/0.22	1.48 (0.53-4.10)	0.45
	A/ACCC	0.05/0.08	0.76 (0.25-1.89)	0.44	0.07/0.05	1.58 (0.42-6.34)	0.50	0.06/0.06	1.33 (0.31-7.07)	0.71
16179	CAAA/C	0.003/0.02	0.18 (0.01-1.94)	0.17	0.02/0	-	-	0/0.04	-	-
	CAAA/CAA	0.24/0.20	1.41 (0.83-2.47)	0.22	0.23/0.20	1.26 (0.061-2.77)	0.54	0.24/0.20	1.47 (0.67-3.42)	0.35
	CAAA/CA	0.13/0.05	2.70 (1.17-7.32)	0.03*	0.13/0.05	2.90 (0.91-12.84)	0.10	0.14/0.06	2.36 (0.74-1.05)	0.19

Table 5.10: The results for logistic regressions predicting gout status by mitochondrial variants. Results adjusted for age, East versus West Polynesian ancestry and proportion of Polynesian grandparental ancestry.

		Combined Polynesians (n=442)			*East Polynesians (n = 211)*			*West Polynesians (n = 226)*		
Variant	**Alleles Ref/Alt**	**Alt Allele Freq Gout/Control**	**OR (95% CI)**	***P***	**Alt Allele Freq Gout/Control**	**OR (95% CI)**	***P***	**Alt Allele Freq Gout/Control**	**OR (95% CI)**	***P***
C151T	C/T	0.21/0.17	1.38 (0.77-2.51)	0.27	0.31/0.20	1.86 (0.93-3.97)	0.09	0.12/0.14	0.70 (0.28-1.91)	0.46
310T/TC	T/TC	0.93/0.89	1.51 (0.70-3.09)	0.27	0.91/0.90	1.17 (0.39-3.14)	0.77	0.94/0.88	1.80 (0.58-5.16)	0.89
C1185T	C/T	0.11/0.17	0.65 (0.34-1.27)	0.20	0.23/0.28	0.77 (0.39-1.56)	0.46	-	-	-
A1692G	A/G	0.19/0.15	1.47 (0.82-2.77)	0.21	0.32/0.18	2.14 (1.05-4.66)	0.04*	0.08/0.12	0.54 (0.20-1.63)	0.25
T2416C	T/C	0.20/0.16	1.41 (0.79-2.60)	0.26	0.32/0.18	2.14 (1.05-4.66)	0.04*	0.10/0.14	0.54 (0.21-1.63)	0.22
A4679G	A/G	0.88/0.84	1.25 (0.63-2.43)	0.51	0.77/0.72	1.29 (0.64-2.53)	0.46	0.99/1.00	-	-
T5465C	T/C	0.96/0.94	1.75 (0.64-4.41)	0.25	0.97/0.97	1.22 (0.17-6.45)	0.82	0.94/0.90	2.42 (0.68-7.82)	0.15
T6719C	T/C	0.96/0.94	1.75 (0.64-4.41)	0.25	0.97/0.97	1.22 (0.17-6.45)	0.82	0.94/0.90	2.42 (0.68-7.82)	0.15
A6905G	A/G	0.24/0.25	0.89 (0.53-1.51)	0.65	0.13/0.16	0.74 (0.33-1.77)	0.49	0.33/0.35	1.00 (0.51-2.01)	0.99
G9123A	G/A	0.96/0.94	1.75 (0.64-4.41)	0.25	0.97/0.97	1.22 (0.17-6.45)	0.82	0.94/0.90	2.42 (0.68-7.82)	0.15
T10238C	T/C	0.95/0.94	1.62 (0.60-4.02)	0.32	0.97/0.97	0.97 (0.14-4.67)	0.98	0.94/0.90	2.42 (0.68-7.82)	0.15
C12239T	C/T	0.96/0.93	1.99 (0.77-4.87)	0.14	0.97/0.97	1.87 (0.36-8.77)	0.43	0.94/0.90	2.42 (0.68-7.82)	0.15
A14022G	A/G	0.95/0.94	1.62 (0.60-4.02)	0.32	0.97/0.97	0.97 (0.14-4.67)	0.98	0.94/0.90	2.42 (0.68-7.82)	0.15
A15746G	A/G	0.96/0.94	1.75 (0.64-4.41)	0.25	0.97/0.97	1.22 (0.17-6.45)	0.82	0.94/0.90	2.42 (0.68-7.82)	0.15
A16183C	A/C	0.95/0.84	4.11 (1.99-8.62)	0.0001*	0.96/0.85	4.15 (1.43-12.93)	0.01*	0.95/0.82	4.73 (1.67-13.63)	0.003*
T16189C	T/C	0.96/0.93	1.99 (0.77-4.87)	0.14	0.96/0.97	0.81 (0.12-3.66)	0.81	0.95/0.86	3.92 (1.13-13.40)	0.03*
T16217C	T/C	0.95/0.94	1.56 (0.57-3.88)	0.36	0.96/0.97	0.81 (0.12-3.66)	0.81	0.95/0.90	2.47 (0.68-8.29)	0.15
A16247G	A/G	0.84/0.85	1.00 (0.53-1.80)	1.00	0.89/0.89	1.01 (0.37-2.49)	0.98	0.80/0.80	1.04 (0.45-2.27)	0.92
C16261T	C/T	0.94/0.94	1.18 (0.45-2.79)	0.72	0.97/0.97	0.98 (0.14-4.69)	0.98	0.92/0.90	1.32 (0.40-3.79)	0.63

5.3.8 Associations between individual mitochondrial variants and other metabolic markers

Other metabolic markers were tested for association with mitochondrial variants (Table 5.11). Results for East and West Polynesian sub-populations are included in the Tables 8.4 and 8.5 in Appendix 3. Interestingly, the two variants that appeared to be associated with urate levels albeit with miniscule effect sizes (variants C1185T: β-coefficient 0.03, $P = 0.05$; A4769G: β-coefficient -0.03, $P = 0.04$) are those that define the B4a1a1c lineage. The effect of the A4769G variant is flipped because the variant defining the B4a1a1c lineage is a reversion to the ancestral A allele. Based on this analysis, people possessing these SNPs have a slight tendency toward higher serum urate levels. Because this lineage primarily presents among East Polynesians, association was also tested for among this subset of individuals, the association did not hold and the direction of the effect changed, appearing to reduce urate levels (C1185T: β-coefficient -0.02, $P = 0.23$; A4679G: β-coefficient 0.02, $P = 0.27$). This indicates that this finding is not likely to be a real effect, particularly when problems associated with multiple testing (which have not been corrected for in this analysis) are considered.

Table 5.11: The results for linear regressions predicting various metabolic markers by mitochondrial variants. These have been adjusted by age, East versus West Polynesian ancestry and proportion of Polynesian grandparental ancestry.

Variant	*Triglycerides* β (std error)	*P*	*HDL* β (std error)	*P*	*Urate* β (std error)	*P*
C151T	-0.36 (0.22)	0.11	0.08 (0.04)	0.07	0.00	0.99
310T/TC	0.60 (0.35)	0.08	-0.03 (0.07)	0.61	0.00 (0.02)	0.84
C1185T	0.09 (0.27)	0.75	0.00 (0.05)	0.94	0.03 (0.02)	0.05*
A1692G	-0.32 (0.23)	0.16	0.07 (0.04)	0.10	0.01 (0.01)	0.69
T2416C	-0.33 (0.23)	0.14	0.07 (0.04)	0.11	0.01 (0.01)	0.69
A4769G	-0.09 (0.27)	0.75	0.02 (0.05)	0.74	-0.03 (0.02)	0.04*
T5465C	-0.55 (0.41)	0.18	0.01 (0.08)	0.86	0.02 (0.02)	0.46
T6719C	-0.55 (0.41)	0.18	0.01 (0.08)	0.86	0.02 (0.02)	0.46
A6905G	0.25 (0.21)	0.25	-0.03 (0.04)	0.49	0.00 (0.01)	0.69
G9123A	-0.55 (0.41)	0.18	0.01 (0.08)	0.86	0.02 (0.02)	0.46
T10238C	-0.55 (0.41)	0.18	0.01 (0.08)	0.86	0.02 (0.02)	0.46
C12239T	-0.62 (0.40)	0.12	0.03 (0.08)	0.74	0.01 (0.02)	0.75
A14022G	-0.55 (0.41)	0.18	0.01 (0.08)	0.86	0.02 (0.02)	0.46
A15746G	-0.55 (0.41)	0.18	0.01 (0.08)	0.66	0.02 (0.02)	0.46
A16183C	-0.12 (0.34)	0.73	-0.01 (0.07)	0.89	0.02 (0.02)	0.24
T16189C	-0.46 (0.41)	0.26	0.00 (0.08)	0.95	0.02 (0.02)	0.48
T16217C	-0.45 (0.42)	0.29	-0.01 (0.08)	0.87	0.02 (0.02)	0.53
A16247G	-0.44 (0.24)	0.07	0.01 (0.05)	0.81	0.00 (0.01)	0.98
C16261T	-0.21 (0.96)	0.40	-0.02 (0.07)	0.73	0.01 (0.02)	0.48

5.3.9 Heteroplasmies

All SNPs that were identified as being associated with a disease phenotype were visually checked using the Integrated Genomics Viewer (IGV) (Thorvaldsdóttir et al., 2013). By doing this, it was possible to see that in some places, there are multiple haplotypes present (Figure 5.5). The region where the variants that were found to associate with gout (i.e. 16179CA and 16183C) was found to be particularly complex, with some individuals possessing multiple haplotypes in this region. This heteroplasmy seems to be exclusive to those who possess mitochondrial genomes belonging to the B macrohaplogroup in this dataset. There are three main haplotypes detectable (Figure 5.5):

1) A single base pair deletion at position 16179 in linkage disequilibrium with 16183C;
2) A two base pair deletion at the same position that co-occurs with the reference A alleles at positions 16182 and 16183 (as opposed to the derived C alleles);
3) No deletion at position 16179 in these individuals belonging to the B macrohaplogroup is usually associated with the presence of the alternate C allele at positions 16182 and 16183.

Most people possess variable levels of these three different co-existing haplotypes. In contrast, individuals with Q1, P1d or M28-derived mtDNA appear to be homoplasmic at these positions (Figure 5.5c). Statistical analysis for eleven detected heteroplasmic positions in hypervariable region I (HVR1) from positions 16051 to 16189 was undertaken to determine whether possessing heteroplasmy in this region contributed risk to developing gout (Table 5.12). Heteroplasmic variants from 16179 to 16182 (which are likely in linkage disequilibrium) were all found to be significantly associated with gout, contributing an around three-fold higher risk of gout (i.e. at positions 16179 and 16180: OR 3.28 [1.32-8.05], P= 0.009; at position 16181: OR 3.87 [1.96-7.64], P = 0.00009; at position 16182: OR 3.43 [1.43-8.18], P = 0.05). Position 16183 was not significantly associated but had an odds ratio that was trending in the same direction (OR 2.24 [0.80-5.87], P = 0.11).

None of the upstream heteroplasmic positions were found to be associated with gout. These other variants all occurred at low frequency compared to the heteroplasmic variants from 16179 to 16183, which as previously mentioned were only observed among those individuals possessing a mitochondrial genome belonging to the B macrohaplogroup.

When only individuals belonging to the B-derived lineages were statistically analysed (n= 399: 308 gout cases, 91 controls), this association between variants at 16179 and 16183 became difficult to test. This was because heteroplasmy in this region is almost ubiquitous. At only two positions, 16181 (AAAC>GAAC), and 16182 (A>C) were the statistical associations detectable

(OR 6.28 [2.52-16.60] *P* = 0.0001 and OR 19.50 [2.68-396.62], *P* = 0.01, respectively). It was not possible to determine whether or not the degree of heteroplasmy may be affecting the risk to gout with the dataset presented here, as once filtering for those with adequate read depth (i.e.. more than 30 reads mapping to the region), there were too few gout-free control individuals to do perform reliable statistical analysis.

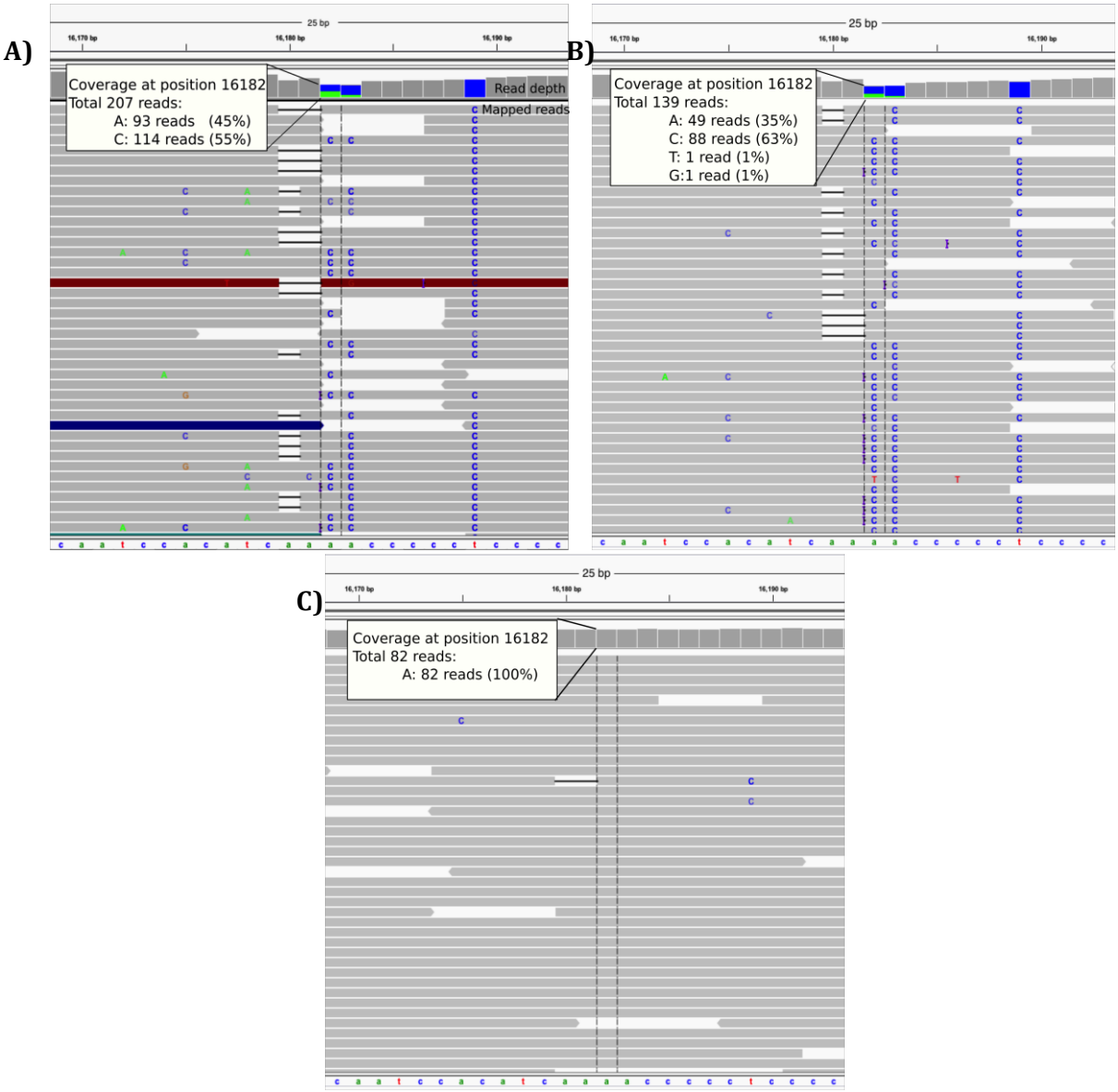

Figure 5.5: Screenshots from IGV showing different proportions of calls at position 16182 (and the surrounding region). 5.5a & b belong the B4 derived lineages, while 5.5c belongs to haplogroup Q1. The heteroplasmic indels at this position appear to occur almost exclusively in B4 related lineages. In 5.5a & b, it is possible to see linked variants. A 2 bp deletion at position 16180 always occurs with the reference A allele, a 1bp deletion at position 16180 almost always co-occurs with a C at positon 16183, while no deletion at this position is almost always accompanied by a C at position 16182. Clearly this is a very complex region.

Table 5.12: The results for logistic regressions predicting gout status by allelic fraction of presence of heteroplasmy at positions within HVR1. Results adjusted for age, East versus West Polynesian ancestry and proportion of Polynesian grandparental ancestry. N= 420

Position	Alleles Reference/Alternate	Heteroplasmy freq Gout/Controls	OR (95% CI)	P
16051	A/G	0.02/0.05	0.38 (0.10-1.43)	0.14
16092	T/C	0.02/0.02	1.34 (0.31-9.17)	0.72
16126	T/C	0.03/0.04	0.79 (0.25-3.02)	0.71
16129	G/A	0.04/0.06	0.72 (0.27-2.10)	0.51
16144	T/C	0.02/0.04	0.59 (0.17-2.31)	0.41
16148	C/T	0.03/0.06	0.52 (0.19-1.58)	0.22
16179	CAAA/C/CAA/CA	0.96/0.91	3.28 (1.32-8.05)	0.009*
16180	A/G	0.96/0.91	3.28 (1.32-8.05)	0.009*
16181	AAAC/GAAC	0.94/0.84	3.86 (1.96-7.64)	0.00009*
16182	A/C	0.96/0.91	3.43 (1.43-8.18)	0.005*
16183	A/C/AGC/ACCC	0.96/0.93	2.24 (0.80-5.87)	0.11

5.3.10 Mitochondrial rare variants and phenotypic variation?

Rare variants are difficult to test for because they are by nature uncommon. SKAT tests were undertaken to test for an over representation of common and rare variants in mitochondrial genic regions. Table 5.13 shows that no statistically significant associations (as indicated by a *P* value less than 0.05) were detected in this cohort between a burden of variants in any mitochondrial gene and the phenotypes of BMI (>34 kg/m^2) or gout status.

Table 5.13: The results of a SKAT test for a burden of rare variants in genic regions contributing to stratified BMI and gout, adjusted for age, East versus West Polynesian ancestry and percentage of Polynesian ancestry based on self-declared Polynesian grandparentage.

Gene	Total number of rare variants	Number variants tested	BMI *P*	Gout *P*
ATP6	19	19	0.89	0.55
ATP6/ATP8 intersect	5	5	0.11	0.90
ATP8	1	1	0.50	0.59
COX1	31	29	0.32	0.80
COX2	12	12	0.78	0.77
COX3	17	17	0.63	0.33
CYTB	38	32	0.92	0.50
ND1	16	15	0.55	0.30
ND2	18	18	0.57	0.65
ND3	6	6	0.81	0.34
ND4	20	18	0.50	0.64
ND4L	2	1	0.55	0.20
ND5	38	35	0.37	0.45
ND6	9	9	0.32	0.37
RNR1	13	11	0.92	0.50
RNR2	12	11	0.83	0.69
tRNA-Ala	2	1	0.87	0.40
tRNA-Asn	1	1	0.45	0.63
tRNA-Asp	1	1	0.34	0.08
tRNA-Cys	2	2	0.32	0.78
tRNA-Gln	1	1	0.43	0.63
tRNA-Glu	1	1	0.37	0.72
tRNA-Gly	1	1	0.30	0.07
tRNA-His	1	1	0.34	0.61
tRNA-Ile	1	1	0.59	0.25
tRNA-Lys	3	3	0.10	0.29
tRNA-Met	2	2	0.79	0.65
tRNA-Phe	1	1	0.32	0.49
tRNA-Ser	2	2	0.97	0.20
tRNA-Thr	2	2	0.31	0.18
tRNA-Thy	1	1	0.82	0.36

5.3.11 Maternal grandparentage and disease?

Because 96% of the cohort sequenced here belong to the B4 macrohaplogroup that has been previously suggested to be susceptible to disease, it is difficult to determine whether this lineage may in itself be more susceptible to disease – though the heteroplasmy results are certainly suggestive of this. Because there was a high concordance (99.6%) between self-declared maternal grandparentage and mitochondrial haplogroup, and because 96% of the Polynesians sequenced here belong to B4-derived sublineages, it may be assumed that most of those declaring Polynesian maternal grandparental ancestry belong to a B4-derived mitochondrial lineage, while those who claim to have a maternal grandmother of European ancestry are likely to possess a European-derived mitochondrial genome. On the basis of these assumptions, individuals with three self-declared Polynesian grandparents (so as to compare individuals with similar levels of European-admixture) were analysed to see whether there was any phenotypic

effect arising from having a Polynesian or a European maternal grandmother. Unfortunately, few people met these criteria (only 224 out of the total 2359 people with Polynesian ancestry for whom there is phenotypic data for) meaning there is very little power to detect associations.

Table 5.14 shows the results of the statistical tests. A statistically significant association between maternal grandparental ancestry and gout was found only with no adjustment for sex, mainly because the number of males possessing a European maternal grandmother and three Polynesian grandparents was very small in this sample (12 men). Having a maternal grandmother of European ancestry appeared to confer a protective effect against gout (unadjusted OR 2.24 (1.07-4.97), $P = 0.02$). Individuals with a Polynesian maternal grandmother were twice as likely to have gout than those with a grandmother of European ancestry. This effect remained consistent after adjusting for sex, age and East versus West Polynesian ancestry, however the significant result did not remain. As mentioned earlier, this is likely an artefact of the small sample size, in particular, the limited number of men with European maternal grandmothers. This may suggest that Polynesian individuals possessing a Polynesian mitochondrial genome are more likely to have gout than those possessing a European mitochondrial genome.

For testing BMI, the cohort was stratified into two groups, for high and low (BMI greater or less than 34 kg/m^2). There was no significant association detected between maternal grandparental ancestry and BMI.

Table 5.14: The relationship between gout status and stratified BMI and having a maternal grandmother of Polynesian or European ancestry (as a proxy of Polynesian- versus European- derived mitochondrial lineages) in 224 Polynesians with three grandparents of Polynesian ancestry and one of European ancestry. Presented here are the results of logistic regressions. The adjusted results have been adjusted for sex (where appropriate), age and East versus West Polynesian ancestry. For BMI, the case/control status refers to individuals with a BMI > 34 as a case, and individuals with a BMI < 34 as a control.

		Polynesian	European	Unadjusted		Adjusted	
	n	Case/Control	Case/Control	OR (95% CI)	*P*	OR (95% CI)	*P*
Gout							
All	224	91/96	11/26	2.24 (1.07-4.97)	0.02*	1.69 (0.67-4.46)	0.27
Males	121	74/35	8/4	1.05 (0.27-3.60)	0.93	1.54 (0.37-5.68)	0.52
Females	103	16/61	3/22	2.04 (0.61-9.35)	0.29	1.97 (0.52-9.89)	0.36
BMI							
All	224	86/87	19/15	0.78 (0.37-1.63)	0.51	0.79 (0.37-1.67)	0.54
Males	121	52/52	6/6	1.00 (0.29-3.39)	1.00	1.09 (0.31-3.77)	0.89
Females	103	34/35	13/9	0.67 (0.25-1.76)	0.42	0.67 (0.24-1.79)	0.42

5.4 Discussion

5.4.1 Mitochondrial variation and implications for settlement history

As previously discussed in Chapter 2, understanding population history is important in understanding the prevalence of chronic metabolic disease. The high prevalence of metabolic disease among Pacific peoples is undoubtedly due to a combination of environmental and genetic factors. As was discussed earlier in the chapter, mitochondria have been found to play an important role in the immune system and variation in mtDNA may contribute to immune function. For this reason, undertaking a study assessing mitochondrial variation among Polynesians and its potential associations with disease may help disentangle reasons for the high frequency of conditions such as gout.

When this study was designed, it was prior to the recent update of Phylotree and the reported variation within Polynesia was restricted to a handful of whole mitochondrial genomes – several Samoans (Pierson et al., 2006), twenty whole mitochondrial genomes from New Zealand Māori (Benton et al., 2012), and a single ancient whole mitochondrial genome from an ancient Māori (Knapp et al., 2012). All other studies had focussed on short control region fragments. As such, the degree of variation within the cohort was not anticipated. The 2014 update of Phylotree to Build 16 saw at least an additional 30 descendent lineages of the B4a1a1 sub-lineage added onto the phylogenetic tree. These were primarily identified from the mtDNA of Solomon Islanders, Tuvaluans and Cook Islanders sequenced by Duggan et al. (2014). The sequencing effort described here is the largest to date for Polynesia exclusively and thus identified previously undetected variation.

The haplogroups identified in this study belong predominantly to sub-lineages of the previously described 'Polynesian motif', B4a1a1 (Figure 5.6). Geography has an effect on the frequency of the haplogroups among the various sub-populations (Figure 5.7), but this is unsurprising given the rapid mode of colonisation and putative founder effects. The variation fits into a wider Pacific-wide pattern, where moving east from Papua New Guinea, through the Solomon Islands and beyond sees a reduction in the frequency of non-B related lineages. Because of the recent nature of this colonisation, with West Polynesia being settled only 3000 years ago (Burley et al., 2012), and East Polynesia less than 1000 years ago (Wilmshurst et al., 2011), and with West Polynesia being a putative source of these East Polynesian colonists, the similarities seen in the lineages in each region with different proportions of the respective lineages in each population are somewhat surprising (Appendix 3, Table 8.2). One particularly striking difference in haplogroup frequency is B4a1a1c, which presented in 22.7% of the East Polynesian cohort reported in this study (though as shown in Figure 5.7, there are also archipelago-specific

differences in frequency, with this lineage being more common among New Zealand Māori than Cook Island Māori), compared to 0.03% of the West Polynesian cohort (only one individual – a Tongan). Similarly, the lineage B4a1a1m occurs at a frequency of 31% in this East Polynesian cohort compared to 6.1% among West Polynesians. In other datasets, the B4a1a1c lineage has been proven to be rare in West Polynesian populations, with Duggan et al. (2014) reporting a frequency of 4% and 2% in Niue and Futuna respectively. The B4a1a1m lineage is less rare, though it still occurs at modest frequencies in West Polynesians, Fijians (Duggan et al., 2014) and Polynesian outlier islands in the Solomon Island archipelago (Delfin et al., 2012).

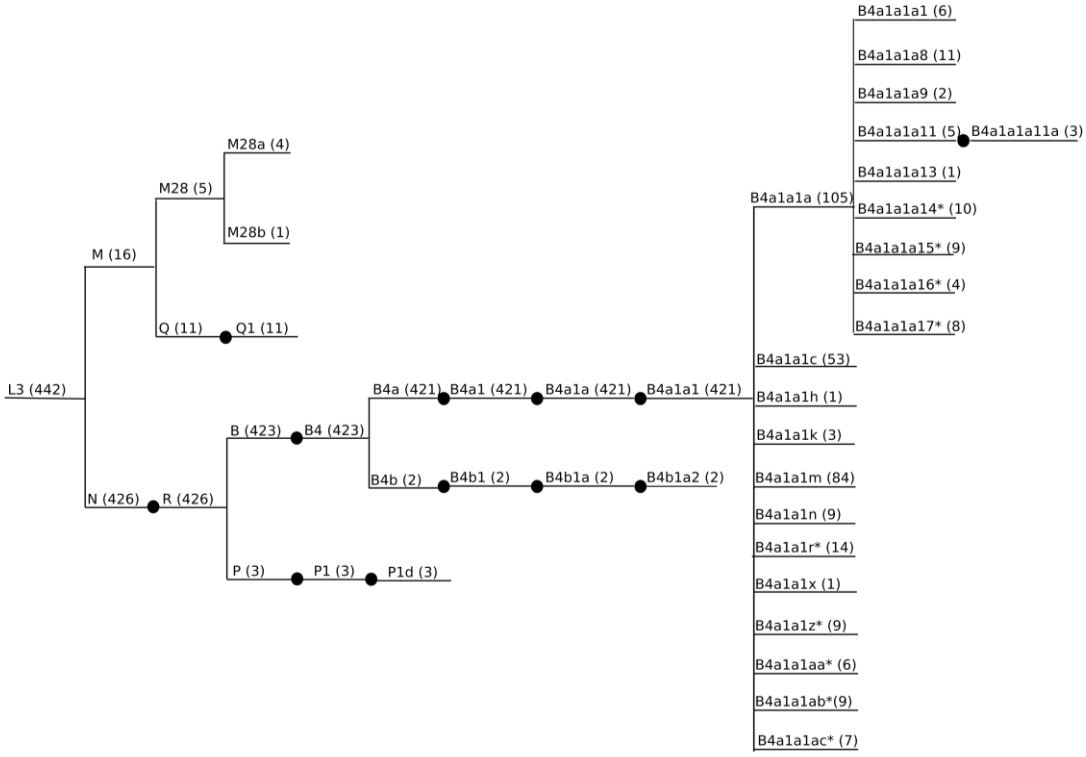

Figure 5.6: A tree showing the divergence of the groups. Note that most of the variation occurs at the very tips of the tree, meaning that most of the haplogroups differ by only two or three SNPs.

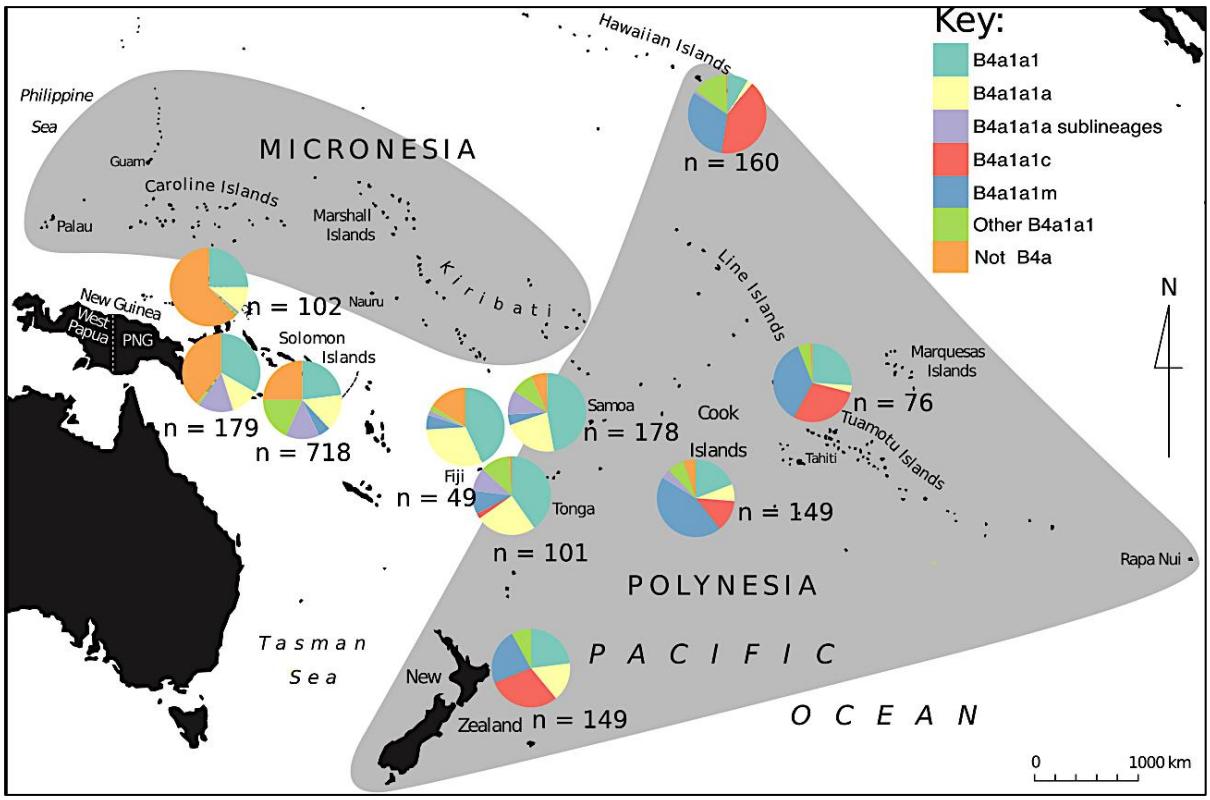

Figure 5.7: Frequency of mtDNA haplogroups between Pacific populations (data from this study, also Delfin et al. (2012): Solomons data, Duggan et al. (2014): New Britain, Bougainville, Fiji, and some additional Tongan, Samoan and Cook Island data, Kim et al. (2012): Hawaiian data, and some unpublished data from the Society, Tuamotu, Gambier, Austral and Marquesas archipelagos

This may have some interesting implications regarding the settlement of the region. The coalescence time for the B4a1a1c haplogroup has recently been calculated at 4.94 kya (95% HPD: 2.02-8.28) (Rieux et al., 2014). This estimate clearly long predates the settlement of East Polynesia, and indeed, West Polynesia, which may indicate problems with the calibration of the molecular dating. The ultra low frequency of the B4a1a1c haplogroup among modern West Polynesians may raise some questions about the putative homeland of this lineage. This lineage is clearly very rare outside of East Polynesia, something indisputable given the large number of non-Polynesian Oceanic populations which have undergone mitochondrial sequencing (Delfin et al., 2012, Duggan et al., 2014). However, there are a few conspicuous gaps in sampling within the region, with no data from Micronesia, New Caledonia or Vanuatu being available to date.

As well as giving insight to settlement history, these differences in mitochondrial lineage frequencies may indicate differences in population structure that may well have an impact on the differences in frequency of disease between island groups. Even if the mitochondrial haplogroups and genetic variation themselves seem mostly to be having only a subtle effect on phenotype, they may be themselves associated with nuclear variation that contributes to subtle differences in disease susceptibility. There have been indications from studies of nuclear variation that different allele frequencies of variants occur between East and West Polynesia,

and that these variants may be contributing different degrees of risk or protection against the development of disease (Phipps-Green et al., 2010). Table 5.1 shows that there are subtle differences in mean BMI and HDL cholesterol levels between groups.

Another factor regarding population stratification that needs considering is the amount of European admixture that has occurred since European contact, as this will undoubtedly have an effect on population structure. Among the sub-populations in this study, there are differences in the amount of admixture. For instance, NZ Māori have a higher proportion of admixture compared to Tongans, fully unsurprising considering the histories of these localities and the relative numbers of people of European ancestry living in each country respectively. While there was a high concordance (99.6%) in the self-reported maternal grandparental ancestry among those who were sequenced with only two of the 447 men whose mtDNA was sequenced belonging to European-derived mitochondrial lineages, there is still likely genome-wide scale European admixture introduced.

There is a possibility that this admixture in itself may be introducing a tendency towards disease, potentially interrupting mito-nuclear coevolution that has developed over the generations since each population diverged (Osada & Akashi, 2012). There is some evidence to suggest that the influence of a specific mitochondrial haplotype may be dependent on the autosomal background (Dunbar et al., 1995), thus this admixture and the disruption of mitochondrial-nuclear crosstalk pathways may contribute to pathology (Gershoni et al., 2014).

5.4.2 B4a1a1-derived lineages and disease susceptibility?

The observed diversity among the B4a1a1-derived lineages, and paradoxically, this lack of diversity, with few non-B derived lineages present, makes it difficult to assess the role of the mitochondrial genome in contributing to BMI and other metabolic traits in Polynesians. There were not enough non-B lineages present in order to test whether metabolic conditions were overrepresented among those possessing mitochondrial genomes belonging to B lineages, as 96% of the individuals genotyped were found to possess B4-derived lineages. As noted previously, the B4 haplotype has been previously found to be associated with type 2 diabetes in a more genetically diverse Asian population from Taiwan (Wang et al., 2009, Liou et al., 2012), and a number of the variants which define the lineage, including T16189C (Poulton et al., 2002, Liou et al., 2007, Park et al., 2008), C16261T and T16519C (Weng et al., 2013) have found to be associated with metabolic conditions (type 2 diabetes, abdominal obesity and hypertension respectively).

As with many association-type studies, the evidence supporting association with T16189C is contested by a number of studies that were unable to replicate these findings among several

different ancestral groups (Gill-Randall et al., 2001, Chinnery et al., 2005, Zhong et al., 2014). In their study, Zhong et al. (2014) concede that the B macrohaplogroup does seem to have a propensity towards type 2 diabetes (which holds up to statistical significance prior to correcting for multiple testing), which may be associated with other mitochondrial variants. Given the almost homogeneous inheritance of the B4-related lineages in our cohort, we simply do not have the power to detect this association. In other words, the modest success in detecting mtDNA differences within the Polynesian mitochondrial lineages that explain differential susceptibility to disease may be in part because the predominant lineage in Polynesia is inherently more susceptible to disease than other lineages (or the lineage may be equally be marking nuclear variation which has this effect).

This is supported by the crude analysis of self-reported maternal grandparentage, which shows that individuals who had three Polynesian grandparents (so who would have similar levels of European ancestry and thus European-derived nuclear DNA) with a Polynesian maternal grandmother (and therefore likely a Polynesian mitochondrial genome) are more susceptible to gout than those with a maternal grandmother of European ancestry (unadjusted OR 2.24 (1.07-4.97), P = 0.02). This result should be considered tentatively because of the small sample size, however it hints to potential differences in the propensity toward developing auto-inflammatory disease on the basis of mitochondrial lineage. This result is supported by the indication that the presence of heteroplasmy in the region 16179-16183 is associated with gout (e.g. at position 16179: OR 5.08 [1.74-14.14], P = 0.002). Given that this heteroplasmy is ubiquitous among those belonging to B4 derived lineages, this may be interpreted as this lineage being particularly prone to gout compared to M28, P1d and Q1 lineages who typically do not possess heteroplasmic sequences at this position, though once again, there are sample size issues which will be discussed further later.

The high prevalence of metabolic disease in Polynesia and other Oceanic populations, in combination with the high incidence of derivations of the B4 haplotype make a compelling argument for the susceptibility of this lineage to adiposity and metabolic problems. Indeed, it was difficult to select samples with a 'low BMI' to make up a control group – particularly for the West Polynesian cohort. Table 1 shows that even once the adjusted BMI was calculated, taking into consideration the differing body fat to mass ratio of Polynesians (Swinburn et al., 1999), the mean adjusted BMI was close to being in the overweight range. This could be a result of a genetic propensity to higher BMI driven in part by mitochondrial background, though environmental and lifestyle-related factors will certainly be a major contributing factor.

5.4.3 A6905G and BMI?

There was only one SNP among those with an allele frequency of greater than 0.05, which was found to associate with BMI among the Polynesian whole mitochondrial genomes genotyped in this project (Figure 5.4). The A6905G variant is relatively common with an allele frequency of 0.24 and defines the B4a1a1a lineage and its corresponding sub-lineages. The derived allele appeared to be having a protective effect against obesity, with those possessing it having (on average) a lower BMI than those without. Interestingly, this lineage is more common among West Polynesians, who generally have a higher BMI than East Polynesians (Table 5.1).

As discussed in the results section, this association did not hold out after further genotyping in a larger Polynesian cohort – in fact, the replication cohort showed an altered direction of effect, with the 6905G variant seeming to contribute a 40% higher risk of having a BMI exceeding 34 kg/m^2 (OR 1.39 [1.00-1.94], P = 0.05). Again, this effect was driven by West Polynesians, who had a higher allele frequency of the variant, and the effect appeared to be stronger in West Polynesian men. Because of this apparent complete switch in direction of the effect of the allele in a slightly different cohort, and the flattening of the result when the original cohort was added to the analysis, it may be that the variant has no real effect on BMI and what was detected here was a statistical anomaly arising from small datasets. The association is likely underpowered, as it is unlikely that any real major differences exist between the cohort chosen for whole genome sequencing and those in the replication cohort. Problems with replication of phenotype-genotype associations are a well-documented phenomenon, colloquially known as 'Winner's Curse', where associations with a disease phenotype may be identified in a small, underpowered cohort and not replicated in a larger cohort (Chanock et al., 2007).

5.4.4 Association of mitochondrial SNPs with gout

Only one variant was found to associate with gout in both East and West Polynesian populations. Those possessing the 16183C variant have a four-fold higher risk of gout than those with the A variant (OR 4.11 [1.99-862], P = 0.0001). The 16183C variant did not appear to be having a role in modulating urate levels (Table 5.11) suggesting that it was somehow involved in inflammatory processes contributing to the gout phenotype. Given the putative role of the mitochondria in NLRP3 inflammasome generation (Akira et al., 2013), genetic variation within the mitochondrial genome could be contributing to this. Closer inspection of the variant showed while the variant was bi-allelic, it was also present within a heteroplasmic region, which makes it difficult to assess what is causing the increase in gout risk – the variant itself, or the heteroplasmy. Those who were heteroplasmic at the 16183 position, were also found to have a similarly high risk of gout (5.54 [1.87-15.73], P = 0.001), which is probably due to the same effect is being detected in both statistical tests.

The A16183C variant is present within hypervariable region I (HVR1), thus is considered non-coding. The site is considered highly variable and is not used as a lineage-defining marker. It has not been previously associated with metabolic disease but was found over-represented among Caucasian melanoma patients (Ebner et al., 2011). This may indicate that the region may influence immune responses, as cancers are known to manipulate the immune system to avoid destruction. A transition at this position would contribute to a poly-cytosine (poly-C) tract, something previously suggested to contribute to defective mtDNA replication, which may affect mtDNA copy number and susceptibility to disease (Liou et al., 2010). mtDNA copy number and its effect on gout is explored in the next chapter.

A second variant found to trend with risk of gout, though among West Polynesians exclusively, was at position T16189C (OR 3.92 [1.13-13.40], P = 0.03), which also contributes to the generation of a poly-C tract. It is of note that this variant is very close by the 16183 variant, and as will be discussed later, these variants appear to be at least partly in linkage. This 16189C variant has, as discussed earlier, previously been associated with metabolic disease including type 2 diabetes and obesity in a number of populations (Poulton et al., 2002, Liou et al., 2007, Park et al., 2008) (though this has not necessarily been consistently replicable (Gill-Randall et al., 2001, Chinnery et al., 2005, Zhong et al., 2014)). This would be the first such association in gout, largely because mitochondrial variation has not previously been explored in relation to this disease. If the poly-C tract is somehow affecting copy number, this may have implications when it comes to mitochondrial biogenesis and mitochondrial function, and resulting in impaired mitochondrial turnover, something which has been found to be pro-inflammatory (Nakahira et al., 2011).

Among East Polynesians, it was possible to detect further association between a number of linked SNPs and risk of gout. Variants C151T, A1692G and T2416C are those that define the mitochondrial haplogroup B4a1a1m, which is not uncommon among East Polynesians (in this dataset, it was present at a frequency of 31% in East Polynesia making it the most common single lineage). Of the East Polynesians belonging to this haplogroup, 81.4% of them had gout (though this is skewed by an imbalance of gout versus control patients in this cohort). In the whole haplogroup analysis, this haplogroup did not appear to be associated with gout (OR 0.99, P = 0.14; Table 5.6), while the analyses of 1692G and 2416C in East Polynesians showed a two-fold higher risk of gout among those with these variants (OR 2.14 [1.05-4.66], P = 0.04) which is intriguing, but may point to some problems with using the whole haplogroup in analysis rather than the SNPs themselves, though it may also be a result of a relatively underpowered study.

These variants are located in the hypervariable region (C151T), and in the 16S rRNA gene (A1692G and T2416C). It is not clear how such variants may contribute to disease, but the presence of variants within the 16S rRNA gene may result in subtle differences in the secondary structure of these ribosomal subunits, which may have flow on effects to pathology. Genetic variation in 16S rRNA has previously been observed in Alzheimer's and Parkinson's disease (Shoffner et al., 1993, Tanaka et al., 2010), mitochondrial encephalomyopathy, lactic acidosis, and stroke like episode (MELAS) (Hsieh et al., 2001) and myopathy (Coulbault et al., 2007), so it is not unreasonable to consider that such variation may play a role in auto-inflammatory diseases like gout.

5.4.5 Heteroplasmy and disease

It was possible to detect what appeared to be a lineage specific heteroplasmic region, where there were three main haplotypes. This variation occurs in a region which has previously been identified as being highly variable, with length heteroplasmy from positions 16184-16193 having been detected in association with the SNP variant 16189C (Bendall & Sykes, 1995), a variant which is near universal in our cohort (allele frequency 0.95). The presence of this heteroplasmy appeared to associate strongly with gout risk when including other haplogroups in the analysis, conferring up to a three-fold higher risk of gout (e.g. at position 16179: OR 3.28 [1.32-8.05], P = 0.009; at position 16181: OR 3.87 [1.96-7.64], P = 0.00009; at position 16182: OR 3.43 [1.43-8.18], P = 0.05).

The previously mentioned 16189C variant contributes to a poly-C tract between 16184 and 16193, and there is accumulating evidence that homo-nucleotide tracts such as this may induce polymerase slippage – something that has certainly been observed *in vitro*, but which may also be occurring *in vivo* (Verscheure et al., 2015). The 16189C variant is prevalent among those possessing a mitochondrial genome belonging to the B macrohaplogroup, however what is interesting is this variant itself was not consistently found to be associated with gout risk – though it was trending in the right direction (for all Polynesians: OR 1.99 [0.77-4.87], P = 0.14; for West Polynesians: OR 3.92 [1.13-13.40], P = 0.03). It is also in linkage disequilibrium with variants indicative of the heteroplasmy – a Pearson correlation of 0.81 was calculated between the 16189C variant and the 16183C variant ($P < 2.2 \times 10^{-16}$). The heteroplasmy may be a direct result of the variant.

Consistent with the observation that 16189C is associated with length variation heteroplasmies, the heteroplasmic variants observed in this data appear to alter the length of a poly-C tract in HVR1, close by the origin of replication. This extension of the poly-C tract may further amplify this apparent polymerase slippage effect and have downstream functional consequences.

Alterations to poly-C tracts have previously been linked to cancer (Ha et al., 2002). If replication is slowed down, this may reduce the rate of mitochondrial biogenesis, an important cellular process that has been previously shown to reduce inappropriate auto-inflammatory stimulation (Nakahira et al., 2011).

The near universal nature of the heteroplasmy among those possessing B-derived mtDNA, made it difficult to conclude that the heteroplasmy itself was contributing to susceptibility to gout, or whether it was possessing a B-derived mitochondrial genome more generally. What is clear is that those with heteroplasmy at these positions (who all happen to belong to the B macrohaplogroup) are three times more susceptible to developing gout rather than those who do not have the heteroplasmy. Those belonging to B lineages who did not have gout had less diversity in the heteroplasmic haplotypes (i.e. they were less likely to have all three of the three main haplotypes in this region) – though this is purely observational and something that has not been quantified as it is difficult to quantify the precise proportions of these haplotypes using the crude quantificative measures of heteroplasmy that were applied here (which only tested whether the presence of heteroplasmy, not the amount of heteroplasmy, was correlated with gout). The statistically significant associations seen when analysing only individuals possessing a B mitochondrial lineage (i.e. at position 16181 [AAAC>GAAC]: OR 6.288 [2.52-16.60], P = 0.0001; position 16182 [A>C]: OR 19.50 [2.68-396.62], P = 0.01) may be detecting this potential difference. Further analysis looking at the proportions of haplotypes would be something worthwhile doing.

5.4.6 Concluding remarks

This study is a testament to how whole mitochondrial DNA sequencing is required in order to assess not only disease associations within a population but also when considering phylogenetic questions. This was the first mitochondrial project of its scale among Polynesians, and it was possible to detect novel variation, some of which was region specific. While an in depth phylogenetic study was not carried out here, it was possible to detect differences in mitochondrial lineages between East and West Polynesia, which may relate to the settlement processes of the region. Furthermore, variants associating with both BMI and gout were detected, which supports the multifaceted role of mitochondria, even if was not immediately obvious what functional effect the variants were having. The modest size of the dataset (in terms of medical genetic studies) means that there are certainly some limitations, and that follow-up work is necessary to confirm these associations – in particular, the heteroplasmic findings. This in itself comes with complications given sampling constraints, and when the effect may be more subtle or absent in more highly admixed individuals.

6 Mitochondrial Copy Number Variation & Metabolic disease

6.1 Introduction

6.1.1 Why mitochondrial DNA copy number?

As established in the previous chapter, it was not possible to detect a clear relationship between mitochondrial DNA haplogroup and disease in the genotyped individuals. What was found was an indication that those belonging to the B macrohaplogroup may be more susceptible to gout, and that heteroplasmies in the poly-cytosine (poly-C) tract in hyper-variable region I (HVR1) may contribute to this. The putative mechanism involved polymerase slippage that may slow mtDNA replication, and ultimately mtDNA copy number and mitochondrial biogenesis. Altered mtDNA copy number has been implicated in a range of human diseases, including certain cancers (Lee et al., 2005, Wu et al., 2005, Wang et al., 2006), type 2 diabetes (Wong et al., 2009), neurodegenerative conditions (Baron et al., 2007, Coskun et al., 2012), and aging (Cree et al., 2008, Mengel-From et al., 2014, van Leeuwen et al., 2014).

Lee et al. (1998) were the first to detect a lower mtDNA content in peripheral blood mononuclear cells (PBMCs) in patients with type 2 diabetes compared to healthy controls. In their admittedly small cohort of 55 type 2 diabetes patients and 29 age- and sex-matched controls, they found a 35% lower amount of mtDNA in individuals with type 2 diabetes compared to healthy controls using slot blot analysis (Lee et al., 1998), a DNA quantification method that is less sensitive than quantitative PCR (qPCR) methods (Bosio et al., 2004). This corresponds well with the finding that reduced mtDNA content associates with earlier age of type 2 diabetes onset, though this result only held in patients without diabetes complications (Wong et al., 2009).

Lower mtDNA levels were reported in peripheral blood of offspring of diabetes patients (Song et al., 2001), which was thought to predispose the offspring to developing metabolic disease themselves. Given the hereditary component of metabolic diseases like type 2 diabetes, this assumption was not unreasonable. The result was brought into question when Singh et al. (2007) were unable to replicate the findings in a larger cohort. In contrast to these findings of reduced mtDNA copy number, Weng et al. (2008) found progressive increases of mtDNA copy number in patients with impaired fasting glucose and type 2 diabetes compared with healthy controls.

Given the commonalities in the inflammatory processes underlying type 2 diabetes and gout, differences in copy number may be associated with gout. This chapter explores mtDNA copy number variation in individuals with gout and healthy controls.

As previously discussed in Chapter 4, the mitochondria, and thereby mtDNA content, is usually stringently regulated. Mitochondria are far from static entities and through ongoing processes of fission, fusion, and motility, their shapes and sizes are constantly changing (Youle & van der Bliek, 2012). As such, the arrangement of the mtDNA within the mitochondrion is very pertinent when considering this fluidity of mitochondrial morphology, and indeed, questions of mtDNA copy number variation. Unlike most illustrations in biology textbooks, mtDNA is not a free-floating entity within the mitochondrial matrix. Rather it has been shown that mtDNA is protein-coated and packaged into bacterial-like nucleoid structures that are attached firmly at regular intervals to the inner membrane of the mitochondrion (Chen & Butow, 2005)(Figure 6.1). The number of mtDNA molecules within each of these nucleoids is debated, with a recent study suggesting an average of 1.4 copies (Kukat et al., 2011). If mtDNA copy number within these nucleoids gets too high, the structure becomes enlarged and corresponds with defective transcription (Ylikallio et al., 2010). These nucleoids provide some important organisation to mitochondria, in particular, ensuring that during the frequent fusion and fission events that occur between mitochondria during normal homeostasis, sufficient mtDNA and proteins involved in transcription and DNA replication are retained in each mitochondrial unit (Clay Montier et al., 2009).

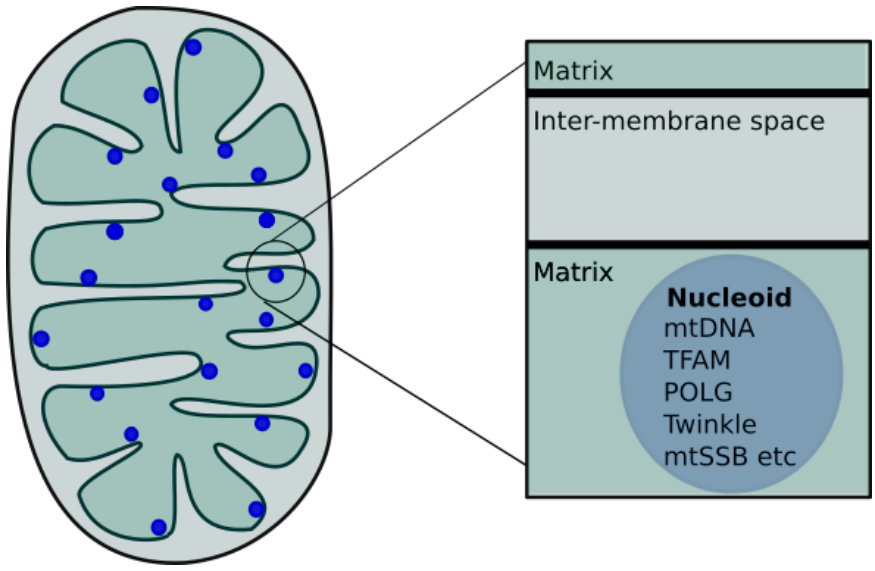

Figure 6.1: mtDNA is packaged into nucleoid structures which are anchored onto the inner surface of the inner mitochondrial membrane. These nucleoids contain sometimes multiple copies of mtDNA as well as enzymes which are involved in transcription and replication, for instance mitochondrial transcription factor A (TFAM), DNA polymerase gamma (POLG), human mitochondrial helicase (Twinkle), mitochondrial single-stranded binding protein (mtSSB)

Mitochondria, and thereby mtDNA, are present in different amounts in different tissues, depending on their respective energy requirements. Spermatozoa contain around five mitochondria (San Gabriel et al., 2012), while oocytes may contain more than 500,000 (Reynier et al., 2001). Not all cells require mitochondria or mtDNA – erythrocytes do not possess mitochondria at all, while eosinophils and neutrophils can eject their mtDNA into the extracellular environment as an anti-microbial defense and remain viable (Brinkmann et al., 2004, Yousefi et al., 2008, Yousefi et al., 2009). In any given tissue, normal mtDNA copy number is a range rather than a set figure, and in apparently healthy individuals, the amount of mtDNA can vary significantly; Stringer et al. (2013) found in their study of mtDNA copy number in statin-induced myopathy, that the calculated mtDNA:nuclear DNA ratios varied from 753–7762 among their control cohort. Additional to this normal variation, there is also a general reduction in copy number with age, with lower amounts of mtDNA among the elderly. This reduction in mtDNA is not necessarily accompanied with a reduction of fitness and increased pathology (Cree et al., 2008, Mengel-From et al., 2014, van Leeuwen et al., 2014). However, altered mtDNA levels have been detected in various pathologies so there clearly are some consequences of extreme amounts of mtDNA. Reduced mtDNA copy number may contribute to pathology when other genetic and environmental conditions are met. Alternatively, the pathological conditions themselves may be influencing mtDNA copy number by some other mechanism.

6.1.2 Regulation of mtDNA copy number

As previously mentioned, mitochondrial mass affects the number of mitochondria, and thereby mtDNA copy number. Regulation of mitochondrial mass is something which is not entirely understood, though it is affected by factors like mitochondrial stress and metabolism. Mitochondrial mass is governed largely by the co-dependent processes of biogenesis and mitophagy, as well as the on-going fission and fusion of mitochondria. Fission and fusion occur frequently and may allow for the rescuing of damaged mitochondrion by co-localising damaged portions, before undergoing fission, allowing all of the damaged components to be located in a single unit to be broken down via autophagy (Youle & van der Bliek, 2012).

Nitric oxide, PGC1α (encoded by the gene *PPARGC1A,* as discussed in Chapter 4), nuclear receptor co-repressor-1 (NCOR1) and their co-activators have been implicated as regulators of mtDNA copy number through their effects on mitochondrial biogenesis (Shokolenko & Alexeyev, 2015). A study carried out in *Saccharomyces cerevisiae* yeast strains by Zhang & Singh (2014) identified 102 nuclear genes whose deletions resulted in complete mtDNA loss. These genes had

diverse functions, though 55% were involved in mitochondrial protein biosynthesis. Of the human orthologs of these genes identified by Zhang & Singh (2014), 47 of them, when deleted from the genome, resulted in the depletion of mtDNA in human cell lines, and eight (*MRPL3, MRPS12, MRPS14, MSH3, POLG, PPOX* and *AFG3L2*) had previously been identified as being associated with mitochondrial disease in humans. There have been other genes identified associating with mtDNA depletion syndromes (Nogueira et al., 2014), which suggests that there are a wide range of nuclear genes which contribute to maintaining mtDNA copy number. Genome-wide linkage analysis of quantitative trait loci has also indicated that males and females may have subtly different genetic mechanisms controlling mtDNA copy number, though mtDNA levels among males and females does not differ significantly (López et al., 2012).

A decrease in mtDNA copy number may also represent mitochondria stress or dysfunction. There is increasing evidence to suggest that mitochondria play an essential role in the generation of the NLRP3 inflammasome response that is important in innate immunity, and that this immune response plays a key role in auto-inflammatory diseases including type 2 diabetes and gout.

There has been some indication that there is some heritability of mtDNA copy number. López et al. (2012) calculated that genetic heritability of mtDNA levels was 0.33 ± 0.09 ($p = 1.8 \times 10^{-5}$). Interestingly, there is some indication that mtDNA variation may affect mtDNA copy number, with Liou et al. (2010) suggesting that the variant 16189C is associated with a reduction in mtDNA copy number. Their study cohort consisted of 837 healthy Taiwanese adults (of Han Chinese ancestry). Given that this variant is one of those that define the B macrohaplogroup and the high frequency of B-derived haplogroups in Polynesia, it may be that Polynesians possess lower copies of mtDNA, which may put them at higher risk of developing metabolic disease such as type 2 diabetes. Liou et al. (2010) suggest that the mechanism in play here may be something to do the with Poly-C tract which is caused by the variant 16189C affecting the association of proteins involved in the replication of DNA at this position in the mitochondrial D-loop. Given that it was possible to find variants in this region which associated with gout among Polynesians, most of whom belong to this haplogroup, this may potentially contribute to a susceptibility to reduced mtDNA levels. Because a high proportion of Polynesians belong to the B mitochondrial haplogroup, it is unlikely to be possible to detect differences in mtDNA copy number associated with differences in mtDNA sequence variation, however given that mtDNA copy number regulation is not fully understood, there may be other mechanisms at play and perturbations in mtDNA copy may be contributing to, or possibly resulting from, susceptibility to disease.

6.1.3 Purpose of this chapter

Given the indication that mitochondrial mass and mtDNA copy number may influence other metabolic diseases, mtDNA copy number was assessed in individuals with gout compared to healthy controls to determine whether there is an association with disease.

6.2 Methods

Two main approaches were used to assess the effect of mtDNA copy number on gout and metabolic disease. Gout will be primarily focussed on in the analysis, as the cohorts used in this study were collected for the primary purpose of exploring the genetics of gout and hyperuricaemia.

6.2.1 Sample selection

The *in silico* portion of the analyses presented here utilises two next generation sequencing (NGS) datasets which have been produced by the Merriman laboratory throughout their on-going studies of the genetics of gout. These data have been produced from DNA from the pre-existing cohort of individuals with gout and healthy controls who have been recruited for the study of metabolic disease (described previously in Chapter 5). To date, genetic samples from 2359 individuals of Polynesian ancestry have been collected. To take full advantage of this study cohort, whole genome sequencing was undertaken to identify possible Polynesian-specific genetic variation. For this purpose, the Merriman laboratory have sequenced 82 low coverage genomes, of which 75 belong to people of Māori and Pacific ancestry. The participants sequenced were chosen on the basis of their urate levels – both gout cases and asymptomatic hyperuricaemic controls were selected for sequencing, everyone having high serum urate levels.

The second NGS resource is the recently produced Urate Locus Resequencing dataset, which comprises 374 people of Māori and Pacific ancestry (excluding individuals who also have whole genome data and those with significant European ancestry), again, chosen largely for belonging to various extremes of gout/hyperuricaemia and normouricaemia. The gout cases comprised 151 participants with the highest serum urate measures (average 0.55 $mmolL^{-1}$), additional to the requirement that their gout fulfilled the 1977 American Rheumatology Association preliminary classification criteria for gout by clinical examination (Wallace et al., 1977), while the control group consisted of 222 participants who had self-reported no history of gouty arthritis and who had low serum urate levels (average 0.33 $mmolL^{-1}$). The Resequencing dataset targeted predominately urate-transporter genes, but also ancestry informative regions and loci

associated with other metabolic traits such as BMI and renal disease from throughout the genome.

The quantitative PCR (qPCR) used to validate the *in silico* findings utilised a subset of samples from the same cohort of 2359 individuals. These comprise Māori and Pacific participants with gout and healthy controls. This expanded cohort has lower on average percentage of Polynesian ancestry (based on self-declared grandparental ancestry), with the mean proportion of Polynesian ancestry among these individuals being 0.82, compared to 0.89 and 0.92 for the Whole Genome and the Urate Locus Resequencing groups respectively (Table 6.1).

Table 6.1 comprises the mean metabolic characteristics of each of these datasets used in testing. Some of these characteristics differ between groups, in particular between the Whole Genome and Resequencing cohorts compared to the qPCR validation cohort. 588 individuals were tested with qPCR including 128 who had previously been sequenced in the Whole Genome or Resequencing, and 460 additional individuals. The table below reports only individuals whose results passed the quality control thresholds (described in the qPCR methods section) and whose data were analysed further.

Table 6.1: Mean metabolic characteristics of the cohorts tested

	Whole Genome Cohort (n=75)		**Resequencing Cohort (n = 373)**		**qPCR validation (n= 484)**	
	Gout (32)	**Controls (43)**	**Gout (151)**	**Controls (222)**	**Gout (243)**	**Controls (241)**
Males/Females	30/2	43/0	133/18	99/123	207/36	129/112
Age	45.4	44.2	47.4	44.9	50.2	40.9
BMI (kg/m^2)	36.7	34.9	37.5	32.3	36.1	33.0
Triglycerides (mmol/L)	2.35	2.44	2.54	1.87	2.47	1.98
HDL (mmol/L)	1.07	0.99	1.09	1.26	0.96	1.16
Serum Urate (mmol/L)	0.49	0.45	0.55	0.33	0.42	0.38
%Polynesian Ancestry	0.91	0.87	0.90	0.93	0.85	0.79

6.2.2 *In silico* testing

The process of preparing DNA for whole genome sequencing involves the fragmentation of genomic DNA through a process such as sonication into short fragments, followed by the ligation of sequencing adapters, similar to what was previously described in Chapter 5 for the sequencing of the whole mitochondrial genomes (the only difference being that the whole mitochondrial genome sequencing involved prior amplification of the mtDNA). Because there is no amplification of DNA prior to the ligation of sequencing adapters, it can be assumed that the

relative number of reads mapping to various parts of the genome after sequencing represents the relative amounts of DNA.

The whole genome data had previously undergone raw data processing (again, similar to what was described previously for the whole mitochondrial genomes), which meant that .bam files that contain aligned reads could be utilised to calculate how many reads map to various loci within the genome. The Samtools count function (Li et al., 2009) was used to count the reads localised to each of the mitochondrial genome and to chromosome 2 (Figure 6.2). A ratio of the reads mapping was generated for each individual by dividing the total number of reads mapping to the mitochondrial genome by the reads mapping to chromosome 2. This gives a relative mtDNA copy number per individual.

These data were then imported into R Studio and statistically analysed. Logistic regression was used to test for an association between the relative amount of mtDNA and gout status. This was adjusted for sex, age, East versus West Polynesian ancestry and percentage of Polynesian ancestry (calculated by assessing self-declared grandparental ancestry).

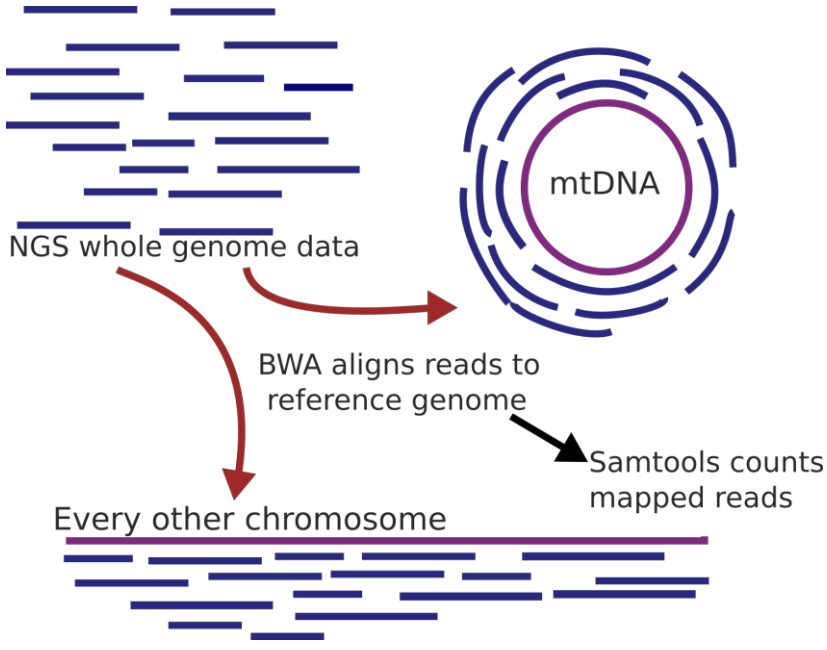

Figure 6.2: How ratios were produced from the NGS sequence data. The number of reads mapping to the mitochondrial genome calculated by Samtools was divided by the number of reads mapping to nuclear chromosomal regions. The resulting number reflects the relative amount of mtDNA possessed by an individual

This was repeated for other chromosomes to make sure the trend was consistent. The relative mtDNA amount was highly reproducible when using the reads mapping to other chromosomal regions. For instance, a Spearman correlation of 1.00 ($P < 2.2 \times 10^{-16}$) was calculated when testing for a correlation between the ratio produced from the reads mapping to Chromosome 2 compared to a ratio of mitochondrial reads produced from the reads mapping to Chromosome 12.

When a statistically significant association was found, validation was sought using the second dataset – the Urate Locus Resequencing Cohort. This dataset was produced in a slightly different manner than that of the whole genome data. After the ligation of sequencing adapters, a hybridisation method was used to enrich the DNA for certain parts of the nuclear genome (mostly loci thought to be involved in urate control). This hybridisation was not 100% efficient in enriching only for reads mapping to regions of interest, and some DNA from other parts of the genome also got sequenced. Because of the way this dataset was produced, with certain parts of the genome being actively targeted, the mitochondrial reads in this dataset were off-target reads. So as to produce similar ratios as those produced from the whole genome data, it was necessary to extract reads mapping to regions of the genome that had not been enriched in the sequencing approach. Bedtools (Quinlan & Hall, 2010) was used to pull out the off-target reads mapping to chromosomal regions. After these regions were extracted, the same process of counting reads mapping the mitochondrial and chromosome 2 regions using Samtools was employed, and the ratio of reads was determined. Again, a logistic regression in R Studio was used to determine whether the same trends were detectable. This again was adjusted for sex, age, East versus West Polynesian ancestry and percentage of self-reported Polynesian grandparents.

6.2.3 Validation using Quantitative PCR

To validate the findings from the *in silico* analyses of the pre-existing datasets, experimental validation was required. Quantitative PCR (qPCR) is an accepted method for confirming suspected copy number variable regions (D'haene et al., 2010). Here, a TaqMan® copy number assay was used to quantify the relative amounts of mitochondrial to nuclear DNA in Polynesian gout cases and controls using dual hybridisation probes. The probes used target a portion of the MT-ATP8 gene (Applied Biosystems, catalogue no. 433118, assay ID Hs02596863_g1) and the standard nuclear RNase P reference assay (Applied Biosystems catalogue no. 4403328). The mitochondrial probe was designed by Applied Biosystems for the purposes of gene expression assays (i.e. qPCR of RNA transcripts), however after consultation with a technical application

scientist from Applied Biosystems, it was decided that this probe would likely work for the purposes of testing the amount of mtDNA present.

TaqMan® copy number assays are single tube assays, which allow the detection of the relative amount of both the mitochondrial target and the standard reference. The assay consists of primers and assay probes which anneal to the specific sequences targeted by the primers. The TaqMan copy number assay uses fluorescence resonance energy transfer (FRET) probes, which have a fluorescent dye attached at one end and a quencher dye at the other. When the probe is intact, the proximity of the quencher to the reporter dye causes the signal to be undetectable.

During the course of PCR, the Taq polymerase cleaves the fluorescent dye off the probe, thereby dissociating it from the quencher, allowing the signal to be detected by the Lightcycler instrument (Figure 6.3). Because the two probes have different dyes attached [Fam and Vic respectively] the LightCycler can detect the relative amount of template DNA present based on the relative fluorescence for each dye after each PCR cycle. Duplex qPCR is preferable for this type of assay, as it means that there is less chance for detected differences between target and reference fluorescence to be due to differences in starting template volume.

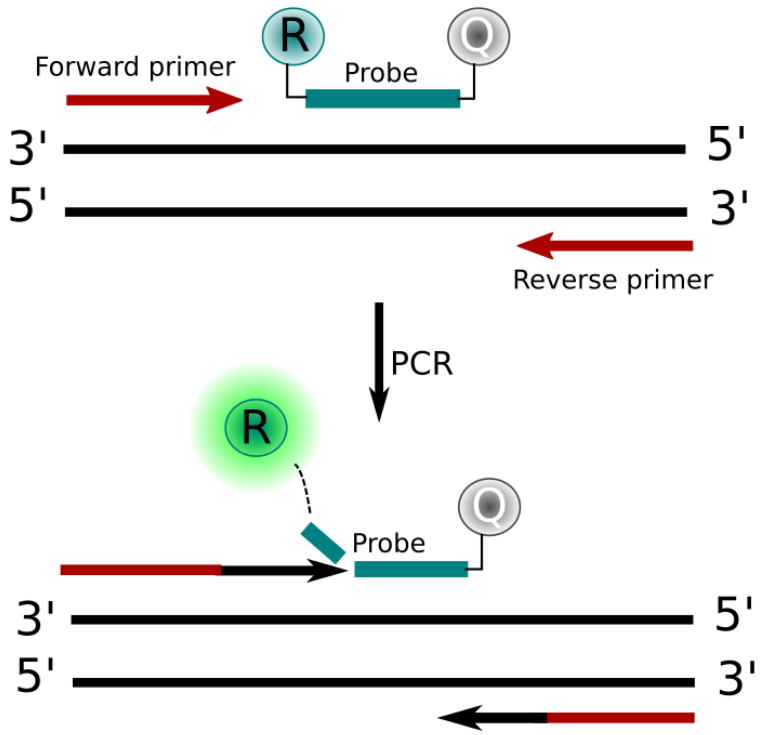

Figure 6.3: qPCR and detection of target DNA sequences using FRET probes

6.2.3.1 Testing of samples

The qPCR setup involved 16-20 ng of genomic DNA pipetted in 2 µL volumes in triplicate onto a 384 well plate, containing both gout cases and non-gout controls, allowing 128 samples to be run simultaneously. Cases and controls were plated together, spaced across the plate (Figure 6.4), in order to minimise any instrument-introduced bias and batch effects which could lead to inconsistencies in the fluorescence contributing to misleading results. Five plates were done in total. The first plate replicated samples from the Whole Genome and Resequencing Cohorts in order to validate the *in silico* analytical approach. The following four plates consisted of samples blindly plated from pre-existing boxes of DNA from gout cases and healthy controls. As such some of the individuals within these plates were replicates from the Whole Genome and Resequencing groups, though the overlap was minimal.

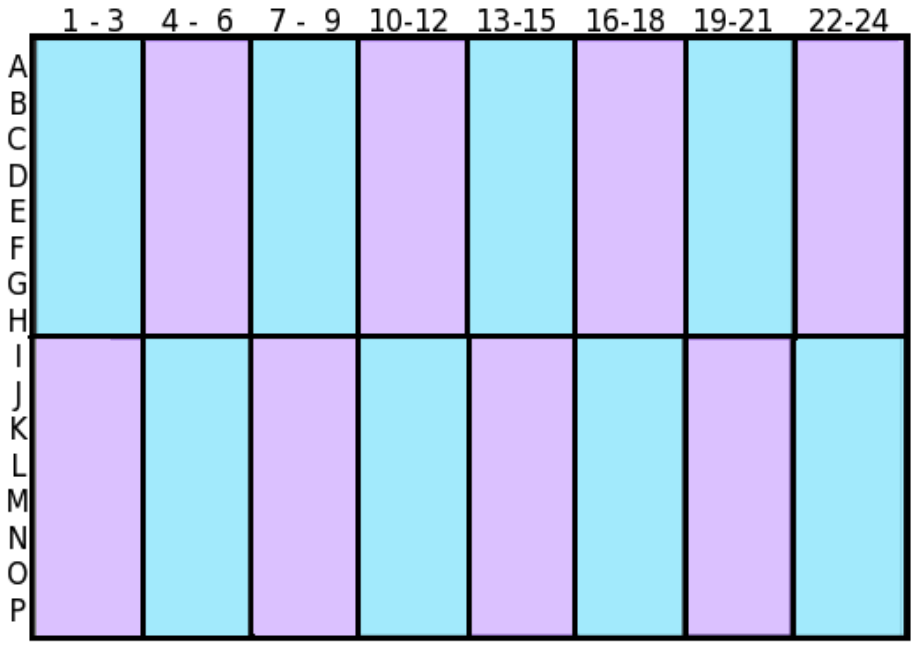

Figure 6.4: 384 well plate set-up. Samples were plated in triplicate, alternating between cases and controls (purple and blue sections) across the plate to reduce instrument-related biases

Very subtle differences in the amount of DNA was detectable by the qPCR (as evidenced by large standard deviation values between PCR replicates), which meant that manual single-channelled pipetting of samples was necessary as plating samples using a multichannel pipette was not precise enough. The DNA was dried (i.e. the plate was left covered but not sealed overnight in order for the liquid portion to evaporate) in order to reduce any inconsistencies that might arise from subtle differences in reaction volume. The total volume for each individual reaction was 10 µL and consisted of 1x mtDNA probes, 1x RNase P probes and 1x TaqMan Genotyping Master

mix. The plates were centrifuged prior to being placed into the Lightcycler 480 Real-Time PCR System. The cycling conditions involved a 10 minute incubation at 95°C to activate the hot-start AmpliTaq Gold® DNA polymerase, followed by 40 cycles of a 15 second incubation at 95°C followed by a 60 second incubation at 60°C. At the end of each cycle, the instrument recorded the fluorescence.

The Lightcycler software maps this fluorescence data against the number of cycles, allowing for the calculation of C_t (threshold cycle). C_t is the intersection between an amplification curve and a threshold line. The threshold line usually used in the analysis of qPCR is at the very beginning of the exponential increase phase of the PCR reaction. In this study, the C_t values used are those calculated by the Lightcycler software using the Second Derivative Maximum for absolute quantification (Rebrikov & Trofimov, 2006). The Second Derivative Maximum is automatically calculated by the computer, and is the point at which the rate of change of fluorescence is fastest. This usually occurs in the cycle where the sample fluorescence can first be distinguished from background fluorescence. The relative amount of mtDNA was ascertained by calculating ΔC_t using the following equation:

$$\Delta Ct = Ct(mtDNA) - Ct(RNase\ P)$$

For samples with a standard deviation of ΔC_t values of the three replicates greater than 0.4, where an outlier replicate could be detected, this was removed. If the standard deviation of the ΔC_t remained above 0.4, the sample was excluded completely from further analysis. These data were exported to R Studio for statistical analysis. Linear and logistic regressions were conducted to test for association between ΔC_t (i.e. relative amount of mtDNA) and various covariates including gout status, age, sex and ethnicity.

It should be noted that there is also a $\Delta\Delta C_t$ method, which was not employed in this study as the reference assay was not working at optimum efficiency as is shown below in the results section.

6.2.3.2 Standard Curve to test Primer Efficiency

In qPCR, standard curves are used to test the efficiency of the primers. This is particularly important when using relative quantification qPCR methods. Primer efficiency indicates whether there is 100% doubling of the target DNA per cycle during the exponential phase of the PCR reaction. Generally, an efficiency between 90 and 110% is considered acceptable. Primer efficiency is important as a sample amplified under low and high efficiency conditions affects the C_t values for the sample and affects repeatability. To assess PCR efficiency, a standard curve is produced from the serial dilution of template, so as to test the predictability of the primers over

a range of concentrations. The standard curve comprised a 1:5 dilution series that covered 5 logs to ensure the accurate calculation of the efficiency of the probes. The resulting Ct values were plotted against the log of the concentration of the DNA, and the slope was calculated. The efficiency of the primers is calculated using the following equation:

$$E = 10^{-\frac{1}{slope}} - 1$$

As discussed in the results section below, there were some issues with primer efficiency. A range of different conditions were tested in an attempt to optimise the reaction, including trialling different concentrations of mitochondrial probe. Standard curves from assays containing 1x, 0.5x, 0.25x and 0.125x mitochondrial probes were produced.

6.3 Results

6.3.1 *In silico* testing

An association between gout and mitochondrial DNA copy number was detected in the Whole Genome Sequencing cohort, which was replicated in the Urate Resequencing cohort (Table 6.2). The data show that lower ratios of mtDNA:chromosome 2 (i.e. the relative amount of mtDNA) are seen in patients with gout compared to controls without gout (Figure 6.5).

Figure 6.5 shows significant overlap between the relative amounts of mtDNA in participants with gout versus healthy controls, however, on average, the ratios are lower in those with gout. This is reinforced by the statistically significant association calculated when a logistic regression is performed, despite the relatively small numbers of individuals in the study.

Table 6.2: Results from logistic regression testing association between gout status, ratio of mtDNA: chr2 reads, sex, age and grandparental ancestry in two cohorts

Cohort	n	Estimate (SE)	Pr (>|z|)
In Silico whole genome sequencing	72	-391.66 (134.36)	0.004* *
In Silico Resequencing	374	-263.10 (78.78)	8.38×10^{-4} ***

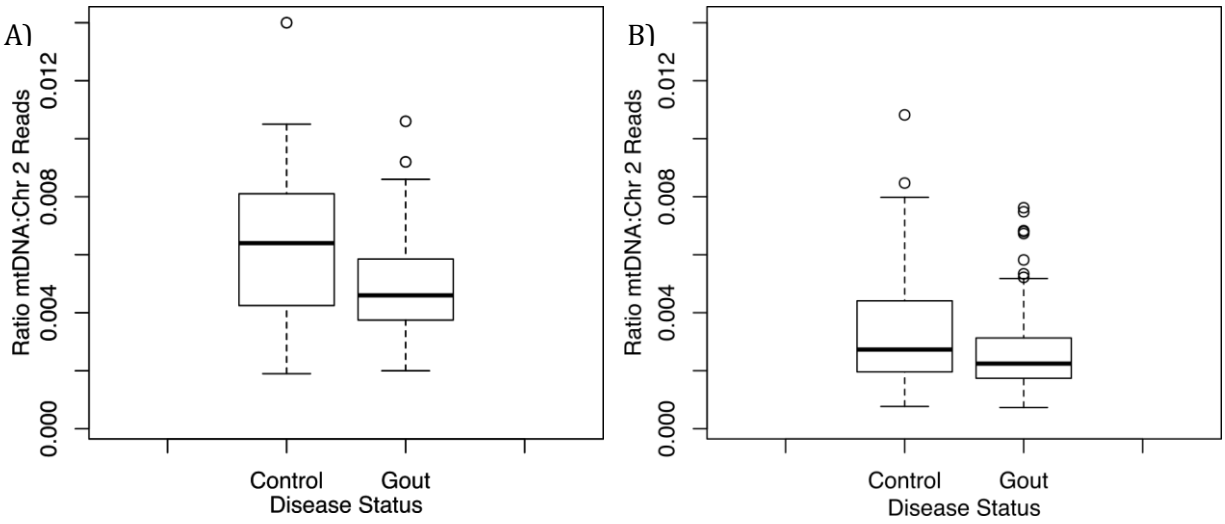

Figure 6.5: Box plots showing the difference in ratio of mtDNA to nuclear DNA among NZ Māori and Pacific gout cases and controls. A) Shows the ratio differences in the whole genome dataset, while B) shows the ratio differences in the resequencing cohort.

6.3.2 Quantitative PCR

6.3.2.1 qPCR of samples

Firstly, a plate replicating samples from the Whole Genome and Resequencing cohorts was run to validate the *in silico* approach. Of the 75 Sequencing cohort individuals, 64 of them had a standard deviation of the C_t replicates below 0.4. The remaining 11 individuals were excluded from the analysis. When a Spearman Correlation was performed, testing for similarity between the *in silico* results for the Sequencing Cohort with the qPCR results, a correlation of -0.58 ($P = 5.67 \times 10^{-7}$) was found (Figure 6.6a). The correlation coefficient is negative because of the way in the ΔC_t values were calculated (i.e. subtracting the C_t for the mitochondrial probe from the C_t for the reference probe) – because the mtDNA is relatively more abundant than the reference RNase P DNA, the more negative the ΔC_t, the more mtDNA is present. Similarly, for the Resequencing cohort, 65 individuals were run, of which 58 individuals remained after filtering. A Spearman Correlation of -0.81 ($P = 1.21 \times 10^{-14}$) was found between the qPCR results and the results obtained from the NGS data (Figure 6.6b).

The significant correlations between the *in silico* data and from those obtained through the qPCR show that the differences that were observed in the NGS data were replicable real differences.

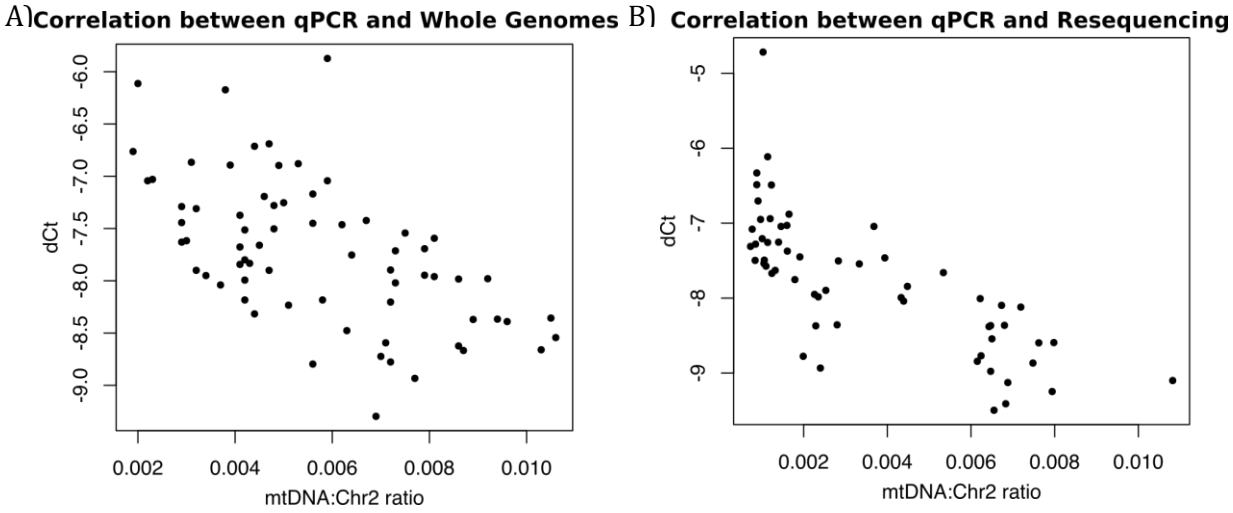

Figure 6.6: plot showing correlation between the experimental and the *in silico* results for A) The Whole Genome Cohort; and B)the Urate Resequencing Cohort. Note that the more negative the ΔC_t value, the higher the mtDNA copy number

Because the qPCR data correlated well with the *in silico* data, further samples were assayed to increase the sample size to allow for testing further associations. A further 480 individuals were assayed using qPCR. After filtering to a standard deviation of less than 0.4, and removing individuals who had been assayed multiple times (choosing the ΔC_t value with the lowest standard deviation between technical replicates), 484 individuals remained. Statistical analyses were carried out for these individuals, regressing gout status against the ΔC_t value calculated, and adjusting for various parameters (sex, age, ethnicity, qPCR batch and percentage of self-declared Polynesian grandparentage) (Table 6.3). A significant association was found between gout status and ΔC_t (OR 1.33 (1.03-1.75), $P = 0.03$). This means that per ΔC_t unit increase, people have a 33% higher chance of having gout, and given that a higher ΔC_t value reflects the individual possessing less mtDNA, this amounts to individuals with gout possessing less mtDNA, on average (Figure 6.7a).

When the cohort was divided on the basis of ancestry (East versus West Polynesian), it was found that the trend was stronger among East Polynesians than West Polynesians (Figure 6.7b-c). This was reflected in the results of the statistical models (Table 6.3), where a statistically significant association between reduced mtDNA and gout was found among the East Polynesians (OR 1.44 (1.03-2.03), $P = 0.03$), but not the West Polynesians (OR 1.25 (0.79-2.02), $P = 0.34$), though the odds ratio was still trending in the same direction for the West Polynesians.

The same trend was significant among males where an increase in ΔC_t was associated with gout risk (OR 1.50 (1.09-2.10), $P = 0.01$), but not females (OR 0.98 (0.58-1.66), $P = 0.95$). This is possibly in part because of the low number of female gout cases.

When the asymptomatic hyperuricaemic individuals (those possessing serum urate levels above 0.42 mmolL^{-1}) in the cohort were used as controls to test for the association of gout with ΔC_t, not only was a statistically significant association was detected between reduced mtDNA copy number and gout, but there was also a stronger effect size detected, where for each unit increase of ΔC_t, there was a two-fold higher risk of gout (OR 2.14 (1.22-3.90), $P = 0.01$)(Figure 6.7d). This indicates that the mechanism is likely something immunological rather than resulting from an association between the mtDNA copy number and the elevated serum urate levels.

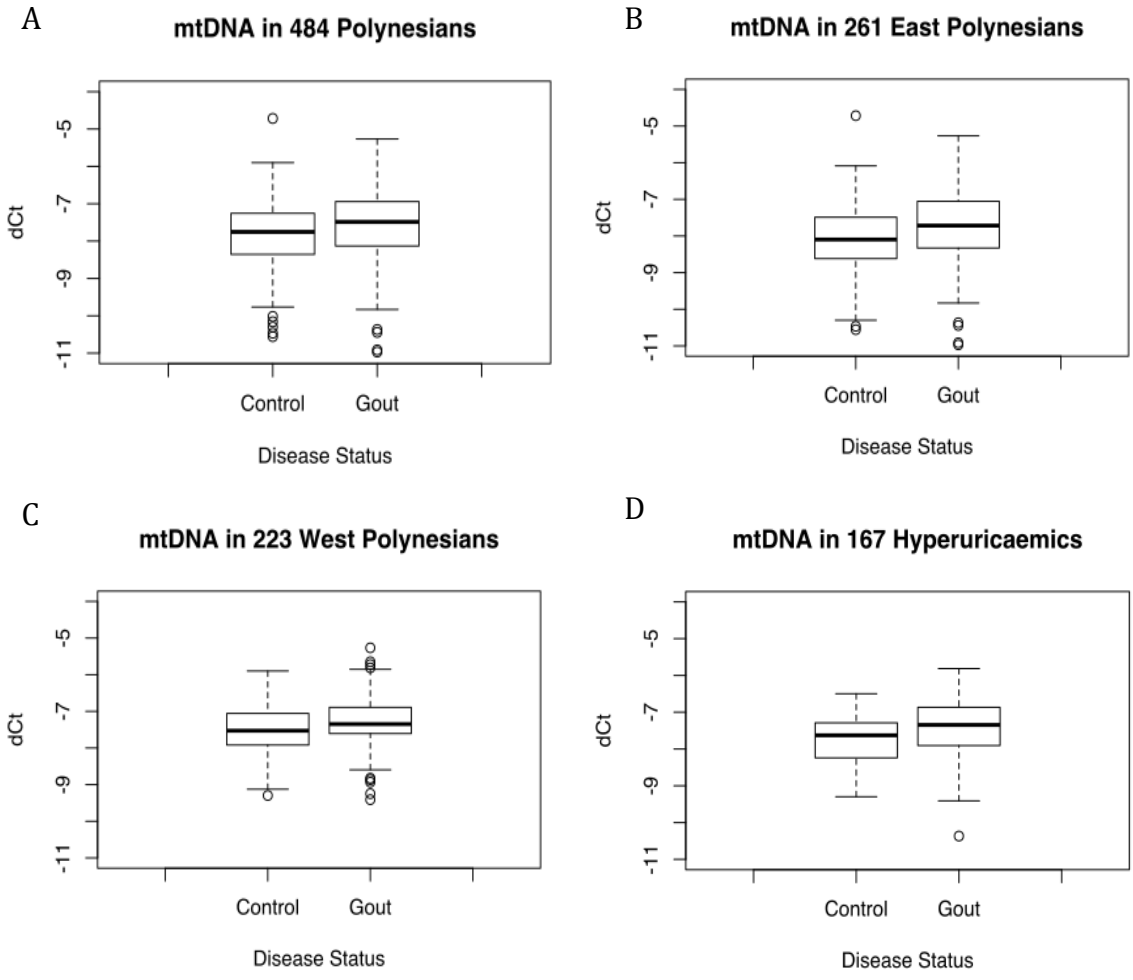

Figure 6.7: Box plots showing the differences in dCt values between gout cases and healthy controls from: a) 484 Polynesians; b) 261 East Polynesians; c) 223 West Polynesians; d) 167 Hyperuricaemics. Note that a more negative dCt relates to more mitochondrial DNA

Table 6.3: Results from logistic regression testing association between gout status, ΔC_t from qPCR. Adjusted results include sex, age, ethnicity, qPCR batch and percentage self-declared Polynesian grandparental ancestry as covariates.

Samples	n	Gout/Controls	OR (95% CI)	Pr (>|z|)
All Polynesians	484	243/241	1.33 (1.03-1.75)	0.03*
East Polynesians	261	138/123	1.44 (1.03-2.03)	0.03*
West Polynesians	223	105/118	1.25 (0.79-2.02)	0.34
Males	336	207/129	1.50 (1.09-2.10)	0.01*
Females	148	36/112	0.98 (0.58-1.66)	0.95
Hyperuricaemics	167	109/58	2.14 (1.22-3.90)	0.01*

6.3.2.2 Standard Curve to test Primer Efficiency

Standard curves were generated to test the efficiency of the probe sets used in the quantification of mtDNA (Figure 6.8). Unfortunately, the standard curve was not run at the beginning of the experimentation. It was assumed that because the probes were predesigned by Applied Biosystems and had therefore been tested extensively for specificity and efficiency, that running a standard curve was not immediately important, which in hindsight was a mistake.

The PCR efficiency from a standard curve produced from a 1:5 dilution series (Figure 6.5) was 100% and 160% for the mitochondrial probe and the reference probe respectively. Ideally, efficiency should be 100%, though a range of 90-110% is generally accepted. Clearly, the efficiency of the reference probe falls outside this range. A high efficiency calculation is usually indicative of some kind of inhibition inside the PCR reaction, rather than the reaction being extra efficient (it is impossible for the DNA to more than double per cycle, so "high" efficiency actually indicates a problem with the assay) – in this case this may result from the huge disparity between the mitochondrial and nuclear target, with the mitochondrial reaction out-competing the reference and causing a reduction in efficiency.

As such, further experiments were carried out with reduced concentrations of mitochondrial probe. A standard curve produced with 0.25x mitochondrial probe and 1x reference yielded the best results, with 93% and 114% reaction efficiency respectively (see appended material). This did not ablate the problem as this falls outside the accepted 90-110% range. Likewise, when the probes were run in single-plex, the mitochondrial probe did not have efficiency falling within this 90-110% range.

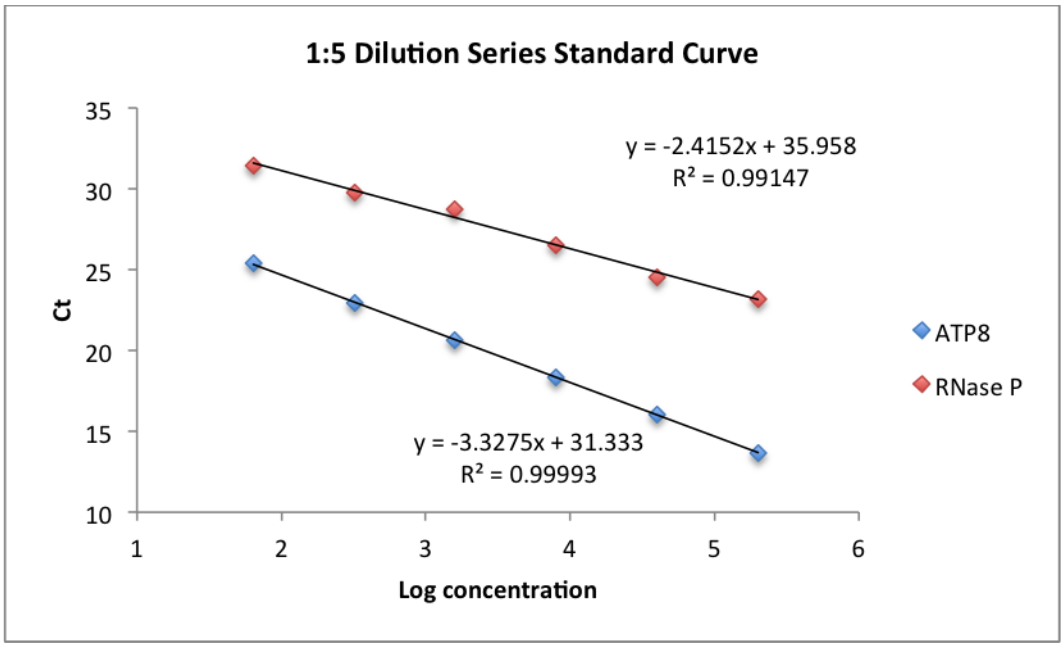

Figure 6.8: Standard curve assessing assay efficiency, efficiency calculated for mtDNA probe 99.8%; efficiency of the RNase P probe 159.4%

This has implications for the assays that were carried out and means that relative quantification using the ΔΔCt method could not be undertaken as the mitochondrial DNA is being amplified at a different rate than the nuclear DNA. Despite this, the data from the assay should still show differences in copy number between samples. The concentration of DNA in samples used in the assay were normalised to around 8 ng/μL, thus the relative amounts of starting material should be around the same. The systemic problem of the different priming efficiencies may in fact amplify the differences in mtDNA between samples, so if more mtDNA is present in a given sample, there may be slightly more inhibition of the reference assay compared to those with less mtDNA. However, despite these problems, there was a strong correlation between the qPCR results and the *in silico* results (Spearman rank correlations of -0.58 (P = 5.67 x 10^{-7}) and -0.81 (P = 1.21 x 10^{-14}) for the Whole Genome and Resequencing data respectively) which suggests that the results are robust despite these efficiency problems.

6.4 Discussion

6.4.1 *In silico* testing methods

There have been a number of methods published recently regarding the extraction of mtDNA copy number from NGS data (Chu et al., 2012, D'Erchia et al., 2015, Ding et al., 2015, Fragouli et al., 2015). D'Erchia et al. (2015) even published a study which looked at the amount of mtDNA using off-target reads, much in the manner which was done in this thesis, with the data from the Urate Resequencing Cohort. Given that three of these methodologies have been published this year (2015), there has clearly been a global trend in maximising what information can be obtained from NGS datasets. In line with this are emerging tools for investigating nuclear genome copy number variation, by examining regions where there are higher proportions of reads mapping (Zhao et al., 2013). In effect, the approach taken in this thesis is a crude version of this.

Use of relative mapping coverage to calculate "mtDNA copy number", much in the manner of Ding et al. (2015) for the Whole Genome and Resequencing data was undertaken and a range of 55-411 and 21-308 copies of mtDNA was calculated for each dataset. However, because of the low coverage nature of the whole genome data (1-3x mean coverage), and the low proportion of off-target reads in the Resequencing data, it is unclear how reliable these assignments are. It was decided that using the ratios of mitochondrial to nuclear reads, which in effect communicate the same information but do not assume to make absolute calls, was the best approach.

6.4.2 qPCR Issues

The issue with the qPCR and the differences in the efficiency of the primers is not a trivial matter. It does raise concerns surrounding the data produced from the assay presented here. However, given the correlation with the Whole Genome Sequencing and the Urate Resequencing cohorts (-0.60 and -0.81 respectively), the differences seen between copy number among gout cases and controls are consistent with the trends detected in these datasets, and thus are robust. This is a significant, novel finding which may elucidate processes surrounding the disease development.

A number of strategies to improve the assay have been undertaken, including reducing the amount of mitochondrial probe which appears to be significantly outcompeting the nuclear probe, trying the assay in single-plex, and trying an alternative master-mix. There has been some

success in improving the assay, which will have to be repeated prior to the publication of these results. Due to time constraints, it was not possible to optimise the experiment prior to writing up this thesis.

Numerous studies using qPCR to assess mitochondrial copy number that have been carried out in the literature use SYBR® Green assays rather than TaqMan® assays (e.g. (Amaral et al., 2007, Burgueño et al., 2013, Menezes et al., 2013, Xie et al., 2013, Zhou et al., 2014)). The chemistry behind these assays is quite different. SYBR® green dye binds to double stranded DNA, so as the PCR reaction progresses and the total amount of DNA increases, the fluorescence also increases accordingly. Because the dye binds to any double-stranded DNA non-specifically, the test and the reference assays need to be done separately, rather in duplex. This makes the method prone to subtle differences in amounts of starting template, and thus is less sensitive.

In contrast, the TaqMan® copy number assays use a fluorogenic probe specific to the target gene and reference, and are able to detect the fluorescence of both of these in a single tube. This specificity has definite advantages, however, clearly there are some issues with getting both probes working at similar efficiency in a duplex reaction. There is variability in the literature as to how people employ these probes and there is little standardisation as to how to quantify. Even with TaqMan® probes, there are some laboratories that run the reactions singly (Grady et al., 2014), presumably to avoid problems with competition between the abundant mtDNA and the nuclear templates. Other laboratory groups appear to have got similar duplex (or indeed, multiplex, with two mitochondrial probes and a nuclear marker (Phillips et al., 2014)) assays working (Ashar et al., 2015), though with different mitochondrial probes and nuclear references. This indicates that with additional experimental modification, it is likely that the assay can be improved.

In the meantime, the data presented here are interesting, especially in the context of other recent findings surrounding the role of the mitochondria, and mtDNA, in the establishment of an NLRP3 inflammatory response.

6.4.3 Reasons for reduced mtDNA copy number in individuals with gout

It was found that mtDNA copy number was reduced in people with gout compared to healthy controls. This trend was significant in males (OR 1.50 [1.09-2.10], $P = 0.01$), but not females (OR 0.98 [0.58-1.66], $P = 0.95$), which may relate to differences in urate handling between males and females (hormonal buffering in pre-menopausal females contributes to lower serum urate levels

and a reduced risk of gout (Hak & Choi, 2008)). Interestingly, it was possible to detect a stronger effect when testing for an association between gout status and mtDNA copy number in a subset of individuals possessing elevated serum urate levels. There was a two-fold higher risk of gout for each unit increase in ΔC_t (OR 2.14 [1.22-3.90] $P = 0.01$) among individuals with serum urate levels equal to or exceeding 0.42 $mmolL^{-1}$, compared to a 30% increase in gout risk in the unfiltered cohort which includes individuals with lower serum urate levels (OR 1.33 [1.03-1.75] $P = 0.03$). An increase of ΔC_t represents a decrease in the amount of mtDNA. This indicates that the mechanism is likely something immunological rather than resulting from the elevated serum urate levels which predispose to gout. Given the role of mitochondria in the stimulation of the NLRP3 inflammasome, being directly involved in the co-localisation of NLRP3 inflammasome components (as discussed previously in Chapter 4, but also see (Misawa et al., 2013)), this reduction of mtDNA may be indicative of processes contributing to the inflammation itself.

Given the regular distribution of nucleoid structures in the mitochondrial inner membrane, a reduction in mtDNA copy number may be indicative of a reduction of total mitochondria or total mitochondrial mass (since mitochondria come in a range of sizes) (D'Erchia et al., 2015). There are several different scenarios that could explain the decrease in mtDNA copy number in the individuals who have gout:

a) The process of the disease itself is causing the decreased copy number. Under this scenario, a reduction in mtDNA copy number could be considered to be a marker of disease.
b) Reduced mtDNA copy number results in susceptibility to disease, perhaps via reduced mitochondrial biogenesis and mitophagy.
c) Reduced mtDNA copy number is unrelated directly to disease, but is related to confounding factors that contribute to susceptibility to gout.

This study lacks data that could disentangle which of these scenarios is predominantly occurring – this would require more than just the genetic data presented here. One approach that could support our preliminary results would be the analysis of cell lines. However despite this, each of these possibilities will be discussed below.

6.4.5 Disease-induced mtDNA reductions?

Reduced mtDNA among individuals with gout may in part be mediated by the inflammatory processes in gout, in particular the stimulation of the NLRP3 inflammasome. This may occur either by direct damage to the mtDNA of the peripheral blood mononuclear cells (PBMCs) from which the mtDNA was prepared, during the stimulation of the inflammasome with increased ROS production, or perhaps by the recruitment of neutrophils during the inflammatory process.

IL-1β is one of the major cytokines produced as a result of the induction of the NLRP3 inflammasome. This cytokine has been shown to recruit neutrophils and eosinophils (Mitroulis et al., 2011), which have been shown to react to MSU crystals by ejecting their mtDNA in a neutrophil extracellular trap (NET) (Yousefi et al., 2008, Yousefi et al., 2009, Schorn et al., 2012a). NETs have been found to be part of the innate immune response against bacteria (Brinkmann et al., 2004), and can comprise nuclear DNA (complete with pro-inflammatory histones)(Saffarzadeh et al., 2012) or mtDNA (Yousefi et al., 2009). Their induction by MSU crystals suggests that they may be playing a role in gout (Schorn et al., 2012b). These NETs aggregate and effectively immobilise and degrade cytokines and chemokines, assisting in the localisation and resolution of inflammation (Farrera & Fadeel, 2013, Schauer et al., 2014). This may mean that this mechanism is important in the resolution of the inflammatory responses in gout. Interestingly, NETs have also been found in patients with atherosclerosis (Megens et al., 2012, Borissoff et al., 2013), another NLRP3 inflammatory condition, which may lend credence to this hypothesis.

Another possibility is that individuals with gout (and perhaps also other metabolic conditions) may have altered mitochondrial structure resulting from the pathology. There have been a number of studies that have found changes in mitochondrial conformation in pathological individuals (Widlansky et al., 2010, Sobenin et al., 2013). Widlansky et al. (2010) noted that while the number of mitochondria in type 2 diabetes patients remained consistent, mitochondrial mass decreased, with the mitochondria in the individuals with type 2 diabetes being significantly smaller and spherically shaped compared to healthy controls (Figure 6.9).

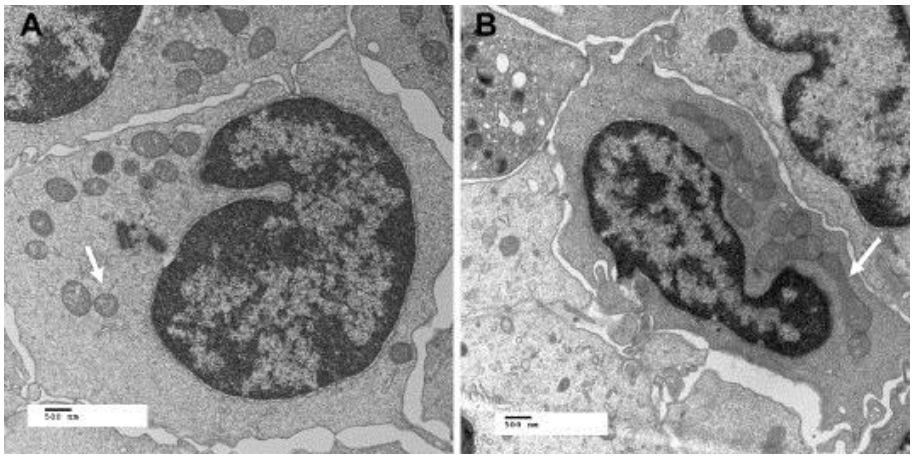

Figure 6.9: Differences in mitochondrial morphology between patients with type 2 diabetes compared to healthy controls. The mitochondria from the participants with type 2 diabetes (A) are generally smaller and rounder compared with healthy controls (B). The white arrows point to representative mitochondria in each image (Widlansky et al., 2010)

Furthermore, Sobenin et al. (2013) found structural abnormalities of the inner membrane of mitochondria belonging to patients with carotid atherosclerosis compared to healthy individuals (Figure 6.10). In the patients with atherosclerosis, there was a decreased mitochondrial inner-membrane and fewer cristae, and in some cases, there were disruptions to the inner membrane entirely. A reduction in the number of mitochondrial cristae may also contribute to reduced amounts of mtDNA, given that it is this inner membrane to which the nucleoid structures containing the mtDNA are anchored.

Given the connections between type 2 diabetes, atherosclerosis and the NLRP3 inflammasome (discussed in Chapter 4), it may be that other conditions with an NLRP3-mediated inflammatory response also have altered mitochondrial structure. Either reduced mitochondrial mass (as observed in type 2 diabetes patients (Widlansky et al., 2010)) or a disrupted inner membrane (exhibited by the reduced cristae in carotid atherosclerosis patients (Sobenin et al., 2013)) may account for the reduced mtDNA levels which are observed in people with gout in this study.

Another thing that remains unclear is whether the changes in mitochondrial morphology observed in the patients with type 2 diabetes and atherosclerosis described above are a result of the pathology or if they are actually driving it. This is something that is very difficult to disentangle.

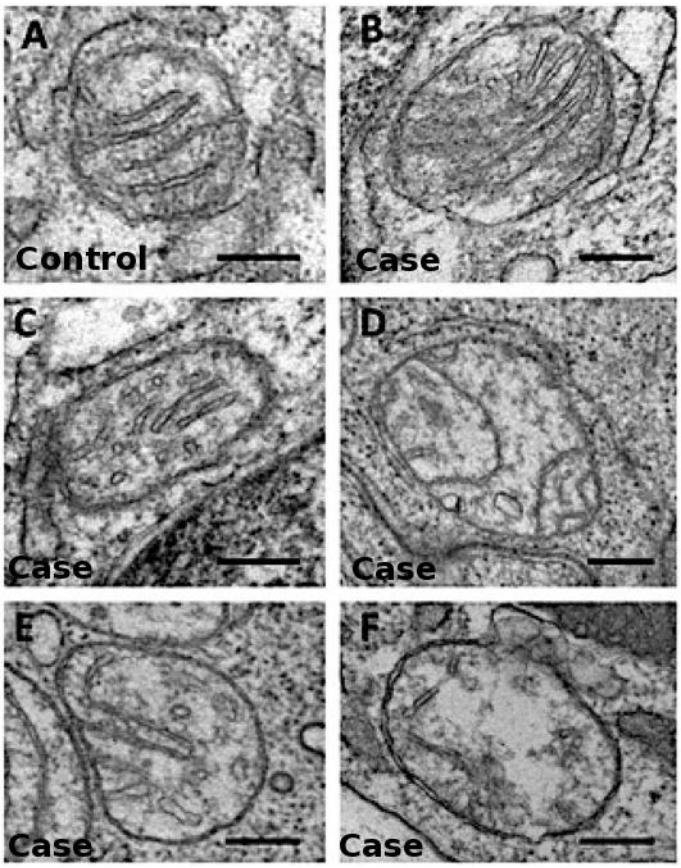

Figure 6.10: Different ultrastructural appearances in leukocytes obtained from healthy volunteers and patients (A) with carotid atherosclerosis (B-F). Note the well-defined cristae and well-preserved surrounding membranes in the mitochondria from the healthy volunteer (A). This contrasts with the reduced numbers of cristae and the oedema of the mitochondrial matrix observed in patients with carotid atherosclerosis (Image modified from Sobenin et al, 2013)

The role of mitochondria in the induction of the NLRP3 inflammasome, and the importance of the production of radical oxygen species (ROS) as a means of cell signalling, might very well account for lower mtDNA copies (and therefore lower numbers of mitochondria) in individuals suffering from gout. Mitochondrial ROS production and oxidative stress (Tschopp & Schroder, 2010, Tschopp, 2011) have been observed to correlate with NLRP3 activation. Mitophagic processes, removing damaged mitochondria from circulation, are required to prevent mitochondrial ROS and excessive NLRP3 signalling (Nakahira et al., 2011, Zhou et al., 2011). Uncontrolled production of ROS by the mitochondria may result in damage to mtDNA and therefore reduction in the amount measurable.

6.4.6 Reduced mtDNA contributes to disease susceptibility?

The reduction in mitochondrial mass, and thereby mitochondria, may be contributing to the susceptibility to metabolic disease in itself. While the data are somewhat contradictory, there

have been studies that suggest reduced amounts of mtDNA precede the onset of type 2 diabetes (Lee et al., 1998).

This certainly may be in line with a study that has suggested that the variant 16189C causes alteration of mtDNA copy number in human blood cells (Liou et al., 2010). As discussed in the previous chapter, this variant is one that defines the B macrohaplogroup, and was possessed by 95% of the individuals whose mitochondrial genomes were sequenced in this study. Unfortunately there is only an overlap of 120 individuals between the whole mitochondrial genome dataset and the qPCR that has been carried out to assess mitochondrial DNA copy number, which is not enough individuals to robustly statistically assess whether mitochondrial genetic variants may be contributing to differences in mtDNA copy number, or whether the B macrohaplogroup has a reduced amount of mtDNA compared to other haplogroups. However, it is reasonable to speculate given the high frequency of this variant among Pacific peoples, that this may contribute to the high burden of metabolic disease – however, an inter-population study (or even an inter-mitochondrial haplogroup study) looking at relative amounts of mitochondria in peripheral blood has not yet been undertaken.

As discussed earlier, reduced mtDNA copy number may come about through the reduction of mitochondrial biogenesis and mitophagy. The governance of mitochondrial mass is something which is not entirely understood, though there have been some contributing factors identified. Inhibition of mitochondrial autophagy has been found to have a pro-inflammatory effect (Nakahira et al., 2011).

On the other hand, given that gout, and also many other metabolic diseases, are associated with age (Smith et al., 2014), and mitochondrial copy number is known to decrease with age, it may be that it is the reduction of mitochondria, which is contributing to the susceptibility to disease. There have been a number of compelling papers on the subject of telomere dysfunction and telomerase reverse transcriptase (TERT) deficiency and mitochondrial impairment (Haendeler et al 2009; Kovalenko et al 2010, Sahin et al 2011). Sahin et al (2011) found that in a TERT-deficient murine model, there was a repression of the peroxisome proliferator-activated receptor gamma, coactivator 1 alpha and beta genes (*PPARGC1A* and *PPARGC1B*), which encode proteins important in mitochondrial biosynthesis, and therefore which may affect mitochondrial copy number.

If a reduction of mitochondrial mass (as evidenced by the reduction in mtDNA copy number) is contributing to the development of gout, peroxisome proliferator-activated receptor gamma (PPARγ) agonists such as pioglitazone, which have been used in type 2 diabetes therapy (Bogacka et al., 2005), may be an effective therapeutic option. Pioglitazone treatment has been

shown to significantly increase mitochondrial copy number, as well as the expression of factors involved in biogenesis such as *PPARGC1A* and mitochondrial transcription factor A (*TFAM*) (Bogacka et al., 2005).

In a gouty rat model, pioglitazone was found to reduce cytokine expression (Wang & Jiang, 2014). This finding was supported by the work of Hong et al. (2015), who found that treatment with pioglitazone significantly reduced MSU crystal-induced NLRP3 inflammasome activation and IL-1β production in a HK-2 cell line.

Likewise, a study of women suffering polycystic ovary syndrome found that upon treatment with pioglitazone, there was an up-regulation of OXPHOS genes, mediated by an increase in *PPARGC1A* expression (Skov et al., 2008). Granted, that the sample size in this study was small (10 cases and 13 healthy controls), treatment improved the insulin-stimulated glucose metabolism and plasma adiponectin, and reduced fasting serum insulin.

As discussed previously in Chapter 4, mitochondrial biogenesis is protective of inflammation. If it is indeed the reduced number of mitochondria that is contributing to susceptibility to gout, it may mean that upregulation in mitochondrial biogenesis via therapies such as pioglitazone may prove to be a therapeutic option in treating gouty disease. Among the hyperuricaemics tested in the expanded cohort (Table 6.3), it was found that there was a significantly lower amount of mtDNA among the participants with gout compared to the asymptomatic hyperuricaemic controls.

6.4.7 Reduced mtDNA is not directly related to gout, but instead related to lifestyle factors that contribute to gout?

Other factors are known to affect mitochondrial mass/copy number as well, including exercise – it was nearly 50 years ago that Holloszy (1967) first observed that exercise training affects the biosynthesis of mitochondrial proteins. More recently, it has been shown that this is accompanied by an increase in mitochondrial volume (Irrcher et al., 2003, Wright et al., 2007, Geng et al., 2010). The general decline in physical activity is one environmental factor that has changed markedly in the last 50 years, and this increase in inactivity is probably related to an increased prevalence of a multitude of chronic metabolic diseases (Poulsen et al., 2001). Low cardio-respiratory metabolic fitness (generally measured as the maximum volume of oxygen that an individual can use, i.e. VO_{2max}) is a strong independent risk factor for not only several cardiovascular diseases but also other metabolic conditions and indeed, all-cause mortality (Wei et al., 1999, Myers et al., 2002, Church, 2009). This is significant, as reduced skeletal muscle

mitochondrial content has been observed in individuals with metabolic disease. This is not necessarily the total number of mitochondria, but certainly in their relative size. Type 2 diabetes and obese subjects have been found to possess smaller skeletal muscle mitochondria compared with those in lean controls (Kelley et al., 2002). While what has been discussed here relates to mitochondrial content of skeletal muscle, it has been shown that exercise can similarly affect mitochondrial biogenesis in peripheral blood (Lim et al., 2000), which is the tissue used in this study.

As such, exercise may be a confounding factor – one that could not be accounted for because of insufficient data collected surrounding the activity habits of the study participants. There has been evidence to suggest that more active men are at less risk of developing gout (Williams, 2008), however there is also the converse problem where minor damage associated with sports injuries may induce the onset of a gout flare (Baker & Synnott, 2010). Given the severe pain associated with gout, this may discourage gout-prone individuals from partaking in voluntary physical activity which may, in turn, account for these reduced amounts of mtDNA that are observed among this gout cohort.

6.4.8 Concluding remarks

While it is not possible to disentangle whether the reduction of mtDNA content among the participants with gout is causal of disease, a result of it or just some coincidental bystander, the finding may be significant in understanding the pathological processes in disease. For instance, as suggested previously, if the reduction in mtDNA and mitochondrial mass is playing a causal role in disease, drug therapies that induce mitochondrial biogenesis may be something that could be explored. This finding may provide valuable insight into the mechanisms that underlie the inflammatory processes in gout.

7 Conclusion

7.1 Summary

This thesis had three main aims, all relating to understanding how the evolutionary and population history of Pacific peoples may have contributed to the high metabolic disease burden present among contemporary Polynesians.

Gout is shown to be a disease with quite some antiquity in the Pacific, dating back to the earliest inhabitants of the region (Buckley, 2011). Through historical research it is established that these reports of gout from archaeological sites is unlikely to be coincidental. The condition is likely to have been common throughout time and space, not just a result of changing diets and lifestyles with western influence (or in the archaeological cases, a reaction to high purine diets among the earliest settlers prior to the establishment of horticultural food crops). This points to a genetic predisposition, probably predating the settlement of the region.

A case for considering population history as being important when it comes to formulating hypotheses to explain the burden of genetic disease is also something that is stressed throughout this thesis. The archaeological and historic findings fall into a wider context wherein elevated serum urate levels can be seen in many Pacific populations, particularly those who speak Austronesian-languages and are descendants of a population expansion out of Asia that began some 5000 years ago. It is likely that these colonists possessed an innate genetic predisposition to high urate levels, which may contribute to the high burden of metabolic disease.

With this background information, the 'Thrifty Genotype' hypothesis, a commonly applied explanation for metabolic disease susceptibility, was assessed in the context of the Pacific and found wanting. Not only is there no evidence to support the 'Thrifty Genotype' hypothesis among Pacific populations, but it is also not supported on a more global scale. There has been no genetic data that support it, despite it being more than 50 years since the hypothesis was first proposed and the wide-scale application of the hypothesis to many diverse populations. Other environmental exposures, such as infectious disease, have been established to have shaped the genome, so are more obvious causal factors to explore. Thus with consideration of the history of the Pacific and the island South East Asian homeland of the Austronesian-speaking populations that went on to colonise the wider Pacific region, a new hypothesis was put forward to explain

the high prevalence of hyperuricaemia, gout and other related metabolic conditions. This proposed that exposure to malaria may have resulted in genetic selection for variants contributing to an up-regulated NLRP3 inflammatory response, which may manifest in part with elevated serum urate levels. It is important to be upfront that this hypothesis was not tested in this thesis, though it may be something worth exploring further in future.

In light of the recent immunological findings surrounding the probable involvement of the mitochondria in the development of the NLRP3 inflammasome response, an immune pathway involved in not only the resolution of infectious diseases but a multitude of chronic auto-inflammatory conditions, mitochondrial genetic variation may contribute susceptibility to disease development. Mitochondria more generally have also been suggested to play a role in the development of metabolic disease through imbalances in energy generation, given the mitochondria's central role in oxidative phosphorylation and ATP production. Thus, complete mitochondrial genomes of 442 Polynesians were sequenced and associations between obesity (as a proxy for impaired mitochondrial energetics) and gout (as a proxy for inappropriate inflammatory responses) were tested. The mitochondrial genetic variation among Polynesians is relatively constrained, however it was possible to detect some associations between mitochondrial variation and gout. The SNP variant T16183C was found to associate with gout in this population and is likely to be a marker for a heteroplasmy in hypervariable region 1 (HVR1) spanning from positions 16179-16183. This heteroplasmy was found to associate with gout. Indeed this 16183C variant was in linkage disequilibrium with the variant 16189C, which had previously been associated with metabolic diseases in other populations (Poulton et al., 2002, Liou et al., 2007, Park et al., 2008). This variant contributes to a poly-cytosine tract which might be directly responsible for the heteroplasmy seen in this region as it is hypothesised that extended homo-nucleotide tracts may result in polymerase slippage (Verscheure et al., 2015).

These variants may fall into a the wider genetic predisposition of those carrying the B4 mitochondrial macrohaplogroup as being more prone to disease, something that was unable to be tested using this cohort because of the 96% frequency of the lineage among those genotyped. Previous studies of mitochondrial variation among Taiwanese have suggested that the B4 lineage is more susceptible to type 2 diabetes than many other common mitochondrial lineages (Liou et al., 2007). Cybrid studies have suggested that this lineage may be more susceptible to oxidative stress (Lin et al., 2012), which may lend credence to this link.

The distribution of mitochondrial variation in the Pacific appears to correlate with the Austronesian expansion, and the differences in derived B4 haplotypes may be indicative of population structure. Certain lineages, B4a1a1c and B4a1a1m, are very common among East

Polynesian populations and occur at very low frequencies among West Polynesians. These sorts of differences in allele frequency may both provide evidence for population origins and settlement history and may further suggest different frequencies of risk alleles between populations.

While only a few mitochondrial variants appear to be associated with an increase in risk of gout, a previously unreported decrease in mtDNA copy number in individuals with gout was found in this study. It is unclear as to whether this is the result of the disease processes i.e. mitochondrial damage sustained during the stimulation of the NLRP3 inflammasome, or whether it is providing risk in itself.

This finding is significant in a number of ways, though clearly further research is necessitated. If the reduction in mitochondrial copy number precedes the onset of gout, it may prove useful as a diagnostic measure for those susceptible to developing gout. It also opens up possibilities for gout therapy, meaning that drugs that stimulate mitochondrial biogenesis, such as pioglitazone, may have some utility.

7.2 Limitations of study

Putting aside the obvious methodological problems from Chapter 6, which still require more work to optimise the qPCR, there are a few limitations to this thesis. A wider sampling of populations throughout the Pacific region would have improved what could be said regarding the evolutionary history of the region. For instance, there is the possibility that the B mitochondrial lineage in itself is contributing to risk of metabolic disease, something that due to the limited diversity among the Polynesians could not be tested. This is where a more genetically diverse study cohort would be useful.

Furthermore, while evolutionary explanations for the high prevalence of metabolic disease among Pacific Islanders, largely the 'Thrifty Genotype' hypothesis, are critiqued, these are not explicitly tested in this work using quantitative methods. This could be done by looking for signatures of genetic selection at loci which have been associated with metabolic disease among Polynesians, using statistics such as Extended Haplotype Homozygosity (EHH) (Sabeti et al., 2002) and the integrated haplotype score (iHS) (Voight et al., 2006), though a genome-wide approach would be more ideal. Bioinformatic testing for evidence of selection is underway by another postgraduate student in the Merriman laboratory that will shed further light on the likelihood of selection having occurred in these populations. Despite this, the 'Thrifty Genotype'

hypothesis still remains an improbable driver of selection for the reasons presented in Chapter 3. It remains an example of where the evolutionary history of the region has been overly simplified and not properly considered before the application of a ready-made hypothesis. Likewise, the malaria hypothesis proposed here, is not explicitly tested either, however it remains a tenable possibility for further investigation.

7.3 Future directions

A number of possibilities for further research arise from this thesis. Most notably, the case study of the 'Thrifty Genotype' hypothesis and its inappropriate usage (and over usage) really emphasises the need to explicitly test hypotheses. It was one of the aims when beginning this research to test the malaria hypothesis that is proposed in Chapter 3, however this did not end up occurring because of delays in getting research permits. If malaria had indeed been a selective pressure for elevated serum urate levels, it may be expected to see elevated serum urate in ancestrally-related Lapita-descendent populations still living in malarial regions – populations in which there are very little data on in the medical literature. Much time and effort has been put into acquiring funding, ethical approval and research permits from the Papua New Guinea Institute of Medical Research to undertake such research in Kavieng in the New Ireland Province of Papua New Guinea. Kavieng is the regional capital and has a major hospital and large resident and semi-resident populations from the off-shore islands. The people of Emirau, Mussau, Tabar and similar islands located off the coast of New Ireland are ideal candidate populations to test this malaria hypothesis. These islands are known to have been first colonised by Lapita peoples 3350 years ago, and have been occupied continuously since this time, so the current inhabitants are likely to be Lapita-descendants (i.e. ancestrally related to modern Polynesians). Malaria rates for the New Ireland region are some of the highest recorded for New Guinea (PNG National Department of Health, 2013).

The research permits ultimately took too long to come through for the time frame of this thesis, which led to the need to explore other questions. Despite these bureaucratic problems with doing research in developing countries, it is important for research to be done in such regions, not only to shed light on evolutionary hypotheses like the ones discussed in this thesis, but also potentially highlighting chronic diseases among remote communities which may have gone unreported. Gout, and indeed type 2 diabetes, are debilitating diseases, which are increasingly becoming a problem in developing countries. Undertaking research in such communities can

assist in public education and to help develop strategies for dealing with such burdens of disease.

Expanding the mitochondrial study into a more genetically diverse population (such as that from Kavieng, Papua New Guinea) is another interesting possibility. As shown here, it was possible to detect some variants within the mitochondrial genome that may be playing a role in the development of auto-inflammatory conditions like gout, though our cohort was admittedly small and further investigation is still required. While there have been an increasing number of studies of mitochondrial diversity in the Pacific region, and even more worldwide, few have focused on the implications of the mitochondria being a functionally important unit, despite this having been discussed by researchers like Douglas Wallace in the literature since the early 1990s. Anthropological and medical research do not have to be done in a mutually exclusive manner, and indeed, acknowledgement of the population history and cultural particulars of a population may enhance medical studies, particularly medical genetics studies, markedly.

The observed reduction of mtDNA in participants with gout and the recent immunological evidence surrounding a role for mitochondria in the generation of a NLRP3 inflammatory response is certainly something that requires further research and may be indicative of the processes underlying disease. This reduced mtDNA content may be indicative of a reduction in the size and/or number of mitochondria. As discussed in Chapter 4, mitochondria play an important role in the activation of the NLRP3 inflammasome, with the physical translocation of mitochondria to the endoplasmic reticulum via tubulin-mediated transport. The finding of reduced mtDNA copy number has several implications and opens up a number of possible novel therapeutic options – in both cases where reduced mitochondrial mass contribute to the development of disease and where disease contributes to reduced mitochondrial mass. For the former, therapies that increase the rate of mitochondrial biogenesis (such as pioglitazone) may be valuable (Bogacka et al., 2005). While the latter may indicate that therapies aimed at reducing the physical translocation of mitochondria to the endoplasmic reticulum during the induction of the NLRP3 inflammasome, for instance the targeting tubulin deacetylases such as HDAC6 or Sirtuins, may be of some utility (Akira et al., 2013). Indeed, the treatment colchicine for acute inflammatory gout is thought to be a tubulin deacetylase inhibitor (Misawa et al., 2013).

In an evolutionary sense, the finding of the reduced mtDNA among the participants with gout may also suggest a potential mechanism underlying the high rates of gout and other metabolic diseases among Pacific peoples. Up-regulation of a tubulin deacetylase inhibitor, allowing for the more ready activation of the NLRP3 inflammasome, may be a trait susceptible to selection by an infectious disease like malaria, suggested earlier as a mechanism for explaining the high

prevalence of hyperuricaemia in Pacific peoples. Recent research from the Merriman laboratory has found copy number variation at a locus putatively thought to be involved with tubulin polymerisation and organisation into microtubules, which associates with gout risk. This has not yet been explored among Pacific peoples. Given the past exposure of populations in this geographic region to this pathogen, it may have resulted in associated variants to occur at higher frequency in certain populations.

The reduced mtDNA finding is not only relevant to gout, but possibly to other NLRP3-associated auto-inflammatory diseases which occur at high frequency among Pacific peoples, including type 2 diabetes and cardiovascular disease. It would be worth investigating whether a similar reduction of mtDNA can be detected in individuals suffering these conditions, and whether there are accompanying alterations in the physical structure of the mitochondrion.

8 Appended material

8.1 Appendix 1: Published works

Gosling, AL., Matisoo-Smith, E. and Merriman, TR. 2014. Gout in Māori. *Rheumatology* 54(5):773-774

Gosling, AL., Matisoo-Smith, E. and Merriman, TR. 2014. Hyperuricaemia in the Pacific: why the elevated serum urate levels? *Rheumatology International* 34(6): 743-757

Gosling, AL., Buckley, HR., Matisoo-Smith, E. and Merriman, TR. (2015). Pacific populations, metabolic disease and 'Just-So Stories': a critique of the 'Thrifty Genotype' hypothesis in Oceania. *Annals of Human Genetics* 79(6): 470-480

8.1.1 Gout in Māori. *Rheumatology* 54(5):773-774

RHEUMATOLOGY

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Editorial

Gout in Māori

Modern affliction or ancestral trait?

EDITORIAL

There is an established viewpoint originating from Rose [1] that the high prevalence of gout in contemporary New Zealand Māori is attributable primarily to modern diet. However, recent bioarchaeological studies of prehistoric Māori have identified bony lesions consistent with gout [2, 3]. Of the 42 individuals studied from a 13th century South Island site, Wairau Bar, six males (14%) possessed these lesions [2]. Eleven of the 53 (21%) adult individuals from a 17th century Ihumatao site in Mangere, Auckland, showed evidence of gout [3]. The tendency towards males possessing the lesions and the location of the lesions predominantly on the margins of the first metatarsophalangeal joint in both assemblages are consistent with gout. Urate has not been recovered from the lesions, however, this is probably a result of post-excavation treatment of the assemblages. For instance, the Wairau Bar remains were curated by the Canterbury Museum for years prior to analysis and repatriation. The individuals from Wairau Bar were likely part of an immigrant population, making them among the first New Zealanders [2], whereas the Ihumatao site dates to a period [3] when Māori populations were well established and had developed a horticultural diet. Recent evidence that the Māori suffered from gout prior to contact with Europeans challenges the view that the elevated prevalence of gout in modern Māori is a recent phenomenon. It is important that this is reconciled in order to better understand the causes of gout in Māori, both pre- and post-European contact.

A confounding factor in the reporting of gout in New Zealand during the early 19th century could be co-existing schools of medical thought. Westernized medicine, placing the causation of disease on a biological basis and the pre-existing Māori perception that there was no natural disease, illness was a result of either transgression against an *atua* (god) by the breaking of *tapu* (law) or the result of *makutu* (witchcraft) [4]. Illnesses were considered either mate Māori (traditional Māori conditions) or mate Pakeha (European-introduced diseases), influencing who Māori consulted for treatment [5]. Gout was therefore less likely to come to the attention of British physicians because Māori considered it an inherent disease.

The earliest enquiry into Māori health was by the physician Fairfowl in 1821 [6]. Gout was not mentioned in his report, although rheumatism was reported as prevalent [6]. This apparent high frequency of rheumatism was also commented on by others, including Thomson [7], but again, no mention of gout was made, with Thomson stating that gout was unknown among Māori [7]. However, there were reports of gout in Māori men in regional newspapers (http://paperspast.natlib.govt.nz/cgi-bin/paperspast). For example, the northern chief Paora (Paul) Tuhaere was repeatedly reported to suffer from gout. In 1879 he was part of an official party and was 'mounted on a charger, as he suffer[ed] acutely from gout' (http://paperspast.natlib.govt.nz/cgi-bin/paperspast), and in 1884 a further attack of gout forced him to abandon the notion of visiting England (http://paperspast.natlib.govt.nz/cgi-bin/paperspast). This account states that the natives believed that the government prevented Tuhaere's visit by employing other Māori to curse him and give him rheumatism and gout (http://paperspast.natlib.govt.nz/cgi-bin/paperspast). This supports the idea that Māori may have perceived gout as caused by more spiritual means.

Rose [1] argues that because gout was familiar to European colonists, they would have recognized it in Māori and the historical accounts denying its presence among Māori are therefore credible. However, since the perception at the time was that gout was a disease caused by affluent lifestyles, gouty pathologies observed in uncivilized countries may not have been regarded as the same disease and were described instead as rheumatism. Garrod, who was a contemporary authority on gout stated 'among nations in an uncivilized state . . . gout . . . is entirely unknown . . . but in our own country [England], and in many other parts of the civilized world, the case is far otherwise' [8]. In 1914 Wohlmann observed that 'nowhere else in medicine is there such confusion of terminology. Thus the unqualified names gout, rheumatism and rheumatoid arthritis are almost without meaning, for they may bear absolutely different interpretations to different readers' [9].

Indeed, this perceived link between gout and social status is evident in the contemporary literature from New Zealand; in a description of court proceedings published in 1865, the link between gouty pathology and social status was made in conjunction with a Māori chief, with the reporter commenting that the chief, Winiata, was probably 'aware of this complaint being a sign of good blood' (http://paperspast.natlib.govt.nz/cgi-bin/paperspast). An attending physician, Dr Nicholson, was quoted as saying that 'it was the only case he had ever met with among the natives, and is conclusive evidence that we can boast of the best blood among our Kaipara [region in northern New Zealand] natives' (http://paperspast.natlib.govt.nz/cgi-bin/paperspast). Newman stated that 'not one of all the descendants of the

canoes that came from Hawaiki ever suffered from gout. Imagine an old Māori Chief suffering from rich gout, when he had for food irregular and scanty allowances of fern root, dried eels…' [10]. Clearly the Māori lifestyle did not align with British expectations of gout, thus they may not have diagnosed it on these grounds, ascribing any gouty episodes to the broader term of rheumatism.

There is no way to determine whether the prevalence of gout in Māori has been previously understated due to misdiagnosis. However, the underrepresentation of gout as a serious pathology in Māori as reported in historic documents is likely related to a combination of factors, including a lack of comprehensive epidemiological studies of populations prior to the mid-20th century, the Māori viewpoint on the cause of gout and the perceived prerequisite lifestyle conditions for the development of gout, leading to a misdiagnosis of the condition as rheumatism in Māori. Therefore we argue that rather than being attributed primarily to the adoption of a westernized diet, Māori, like other Oceanic populations, have a long history of gout. While their diet has changed considerably since European contact, with the introduction of alcohol and, more recently, fructose-containing beverages, it is likely that the pre-contact Māori diet included seasonal consumption of high fructose foods such as the *ti* (New Zealand cabbage tree; *Cordyline australis*) rhizomes and the drupes of the *karaka* tree (*Corynocarpus laevigata*), as well as year-round consumption of purine-rich seafoods, all of which contribute to increased serum urate levels. The prehistoric presence of gout has implications in our understanding of the risk of disease, and wider recognition of this would allow better focus on the range of factors contributing to gout in Māori, including inherited genetic variants.

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8.1.2 Hyperuricaemia in the Pacific: why the elevated serum urate levels? *Rheumatol. Int.* 34: 748-757

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REVIEW

Hyperuricaemia in the Pacific: why the elevated serum urate levels?

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Abstract Pacific Island populations, particularly those of Polynesian descent, have a high prevalence of hyperuricaemia and gout. This is due to an inherently higher urate level among these populations with a demonstrated genetic predisposition. While an excess of urate can cause pathology, urate is also important for human health. It has been implicated as an antioxidant, has a neuroprotective role and is involved in innate immune responses. This paper provides a brief review of urate levels worldwide, with a particular focus on island Southeast Asia and the Pacific. We then present possible evolutionary explanations for the elevated serum urate levels among Pacific populations in the context of the physiological importance of urate and of the settlement history of the region. Finally, we propose that ancestry may play a significant role in hyperuricaemia in these populations and that exposure to malaria prior to population expansion into the wider Pacific may have driven genetic selection for variants contributing to high serum urate.

Keywords Hyperuricaemia · Serum urate · Pacific Islanders · Migration · Selection · Malaria

Elizabeth Matisoo-Smith and Tony R. Merriman have contributed equally to this study.

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Introduction

In humans and the other great apes, urate is the end product of purine catabolism. In most other mammals, it is further degraded into the more soluble allantoin by the enzyme uricase and can thus be easily excreted from the body in urine [1]. Due to a series of mutational silencing events in this gene during hominoid evolution, humans, and their great ape relatives, do not have a functional uricase [2]. This results in urate levels that are much higher in humans (averaging between 4.0 and 6.0 mg/dL) compared to other mammals who generally have a range of 0.5–2.0 mg/dL [3].

Urate is clinically important because high concentrations in the blood (a condition known as hyperuricaemia; usually defined as >7.0 mg/dL in men and >6.0 mg/dL in women) can result in nucleation, forming crystals of monosodium urate (MSU). These crystals can trigger inflammation [4], which, depending on the location of the crystal deposition, may result in pathology—gout if in joints and nephrolithiasis if in the kidney. Diseases associated with hyperuricaemia in humans include hypertension, chronic kidney disease and type 2 diabetes [5]. Interestingly, low levels of serum urate have also been found to be associated with diseases of neurological origin, such as Parkinson's disease [6], multiple sclerosis [7, 8] and Alzheimer's disease [9]. Thus, urate is clearly of some biological importance to the physiology of humans—this is supported by the observation that there seem to be multiple pathways resulting in these heightened serum urate levels. Not only is there dysfunction of the uricase gene in humans, but 90 % of the uric acid filtered by the kidneys is resorbed, further raising the levels of circulating urate in the blood [10]. The regulation of urate is complex, with main causal factors of

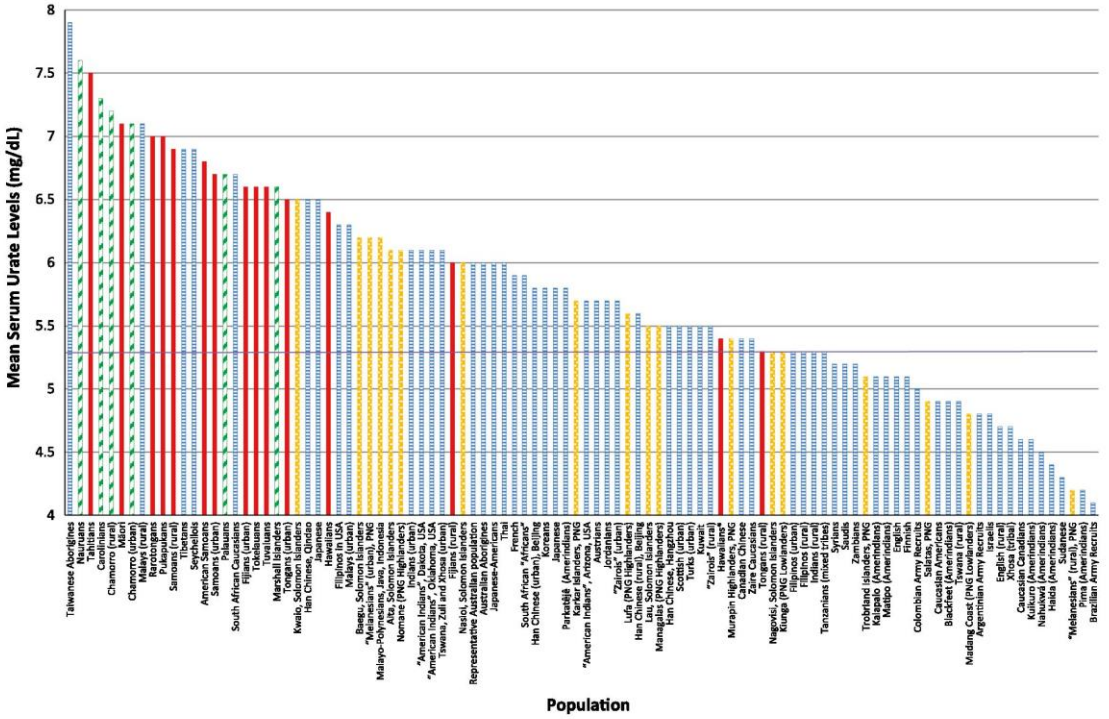

Fig. 1 Mean serum urate concentrations (measured in mg/dL) worldwide, *red bars* indicate Polynesian populations, *green bars* indicate Micronesian populations, *yellow bars* indicate Melanesian populations, non-Pacific populations indicated by *blue bars*. The intersecting *blue line* indicates the mean urate levels worldwide, as calculated from the collated data in the appended material. The Hawaiian data indicated by the *asterisk* are unexpectedly low, and as discussed in text, probably anomalous

hyperuricaemia being diet and genetic variants of renal urate transporters [11].

Hyperuricaemia in the Pacific

In order to evaluate the phenomenon of elevated serum urate in the Pacific, these data need to be situated in a wider geographical or worldwide population context (Fig. 1). To this end, a summary of the published data available on the mean serum urate levels worldwide is provided in "Appendix". Only male data, unless otherwise stated, have been presented because of the confounding effect of hormones on urate levels in pre-menopausal women [12].

Examination of these data show that most Pacific Island populations (Polynesians and Micronesians) and their possible ancestral populations (Taiwanese aboriginals) in particular have higher rates of hyperuricaemia, with few other populations showing high mean serum urate levels (in the 6.5 mg/dL + range). Most populations have average serum urate concentrations of between 4 and 6 mg/dL, below the hyperuricaemic threshold. While some of this variation could relate to differences in lifestyles, subsistence and environment, ancestry is also a likely contributing factor. Given the varied evolutionary experiences of populations worldwide since the expansions out of Africa, it is feasible that higher serum urate concentrations may have been positively selected under certain environmental conditions, leading to the variability which we see in modern populations globally.

Though Polynesian populations tend to show high levels of serum urate, there is reported variation (Fig. 2). For instance, the urate levels from a small sample of 49 Polynesians in Hawaii (5.4 mg/dL), none of which showed any gouty disease, are unexpectedly low [13]—this has been used to argue that this population may be metabolically different than other Polynesian populations [14]. These unusual results have not been revisited in the literature despite the study being carried out nearly 50 years ago. Currently there is a relatively high disease burden of gout in Indigenous Hawaiians (Pers. Comm.: K. Chong-Hanssen), and gout has been identified in skeletal remains from ancient Hawaiians [15]. A high disease burden of gout can be taken

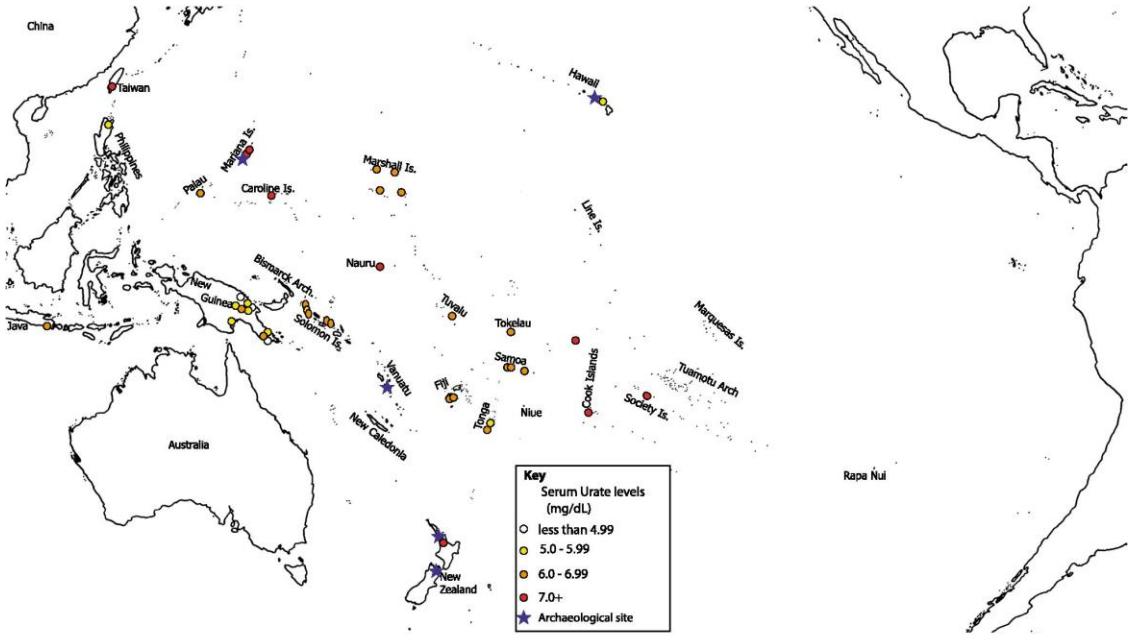

Fig. 2 Mean serum urate levels in the Pacific, compiled from the published literature (for references, see appended material)

as an indication of a high rate of hyperuricaemia in Indigenous Hawaiians.

Environment clearly also has an effect on serum urate levels; a number of studies have sampled from both urban and rural cohorts from within the same population, presumably to help understand the effects of urbanised living [16–23]. Higher urate levels were generally observed in those inhabiting an urban environment. For instance, Tongan men from urban areas had mean serum urate levels of 6.5 mg/dL compared with 5.3 mg/dL in those living in rural areas [21]. Similar trends were seen in Papua New Guinea, with an urban Melanesian population living in Port Moresby having mean serum urate levels of 6.2 ± 1.3 mg/dL, compared to 4.2 ± 1.3 mg/dL in a rural cohort [19]. In general, living in an urban environment exacerbates the tendency towards elevated serum urate levels, concomitant with the increased consumption of foodstuffs (such as sugar-sweetened beverages and alcohol) that increase urate [24–26]. However, the fact that even those living rurally and with more traditional lifestyles in Polynesia have high rates of hyperuricaemia, compared to other populations worldwide, suggests a genetic predisposition.

Hyperuricaemia may be an Austronesian phenomenon

Polynesian and Micronesian peoples speak languages belonging to the Austronesian language family. Austronesian languages are thought to have originated in Island Southeast Asia (ISEA), perhaps Taiwan, around 5,000 years ago before being dispersed throughout ISEA, Oceania and beyond [27]. Non-Pacific Austronesian-speaking populations also have elevated heightened serum urate levels. Taiwanese aborigines have particularly high serum urate concentrations [28, 29], while Filipinos also seem to have elevated serum urate levels [16]. The Merina population in the Highlands of Madagascar, who have also been ancestrally linked to an Austronesian expansion out of ISEA [30], have likewise been observed to have high rates of gout and hyperuricaemia [31]. Similarly, the Seychellois, who likely have an ancestral genetic contribution from Austronesian-speaking colonists [32] exhibit elevated serum urate concentrations [33].

Mitochondrial DNA analyses indicate that Polynesian and many other Austronesian-speaking populations have a significant genetic contribution from Southeast Asia [34], where mean SU levels range from 5.5 to 6.5 mg/dL (Appended data). Y-chromosome STR analyses of Polynesians show that there is also a significant ancestral contribution from ancient Melanesian or Near Oceanic, non-Austronesian-speaking populations [34]. Figure 2 shows that the hyperuricaemic phenotype does not seem to be present in many Melanesian populations on the mainland of New Guinea and in the Solomon Islands, where sampled populations show mean SU concentrations ranging from 4.2 to

6.2 mg/dL, compared with Polynesians whose mean SU range from 5.3 to 7.1 mg/dL. Near Oceanic populations are not genetically homogenous [35], so these data should not be taken to be representative of all populations living in this area. However, the raised urate levels as seen in other Austronesian-speaking populations predominate over the lower serum urate concentrations in non-Austronesian speakers. This suggests that there may have been some form of selection to a more hyperuricaemic phenotype in Austronesian populations.

Possible explanations for heightened serum urate in Pacific Islanders

Modern epidemiology has demonstrated that raised serum urate levels have a multifactorial aetiology, with both environmental and genetic factors contributing. Lifestyle, diet in particular, is known to heavily influence the concentration of SU—more specifically the consumption of purine and fructose-rich foods [24–26]. The last five decades in particular have seen radical changes in dietary habits in the Pacific. This is most evident in urban settings, but these changes are also occurring in many rural communities. There is now a reliance on the importation of foreign foodstuffs, because population sizes have reached points where the islands are now no longer self-sustainable [36]. Exacerbating this shift in subsistence practices is the decreasing popularity of traditional production practices and diets [37, 38]. Many of the imported foods are high in fat and sugar, leading to the coining of terms such as "dietary colonisation", "Coca-colonisation" and "dietary genocide" [36].

Many theorists writing on the topic have attributed the high rates of hyperuricaemia (and indeed, gout) in the Pacific to this change away from traditional diets and lifestyles, stating that prior to the twentieth century gout was unknown [39–43]. However, recent skeletal evidence of gout from prehistoric archaeological sites in New Zealand [44, 45], Hawaii [15], Vanuatu [46] and Micronesia [47, 48], in addition to the nineteenth century reporting and diagnosis of gout [49], suggest that hyperuricaemia and gout in Pacific Island populations may not be as modern a phenomenon as previously supposed.

Traditional Pacific diets are thought to have been to be relatively healthy, with subsistence dominated by the horticultural production of starchy food crops, including various combinations of taro, yams, breadfruit and kumara, among other domesticated plants [36, 37, 50]. The starchy staple was generally served accompanied with a lesser quantity of some form of protein, often fish or shellfish. It should be noted that seafood consumption has been associated with the development of gout [24]. Given the perception of the healthy prehistoric Pacific diet, with few obvious dietary urate-raising factors, other factors, such as a genetic predisposition to elevated serum urate, are likely to have played a role in prehistoric Pacific hyperuricaemia. Genes encoding proteins involved in renal and gut excretion of uric acid have a strong effect on serum urate levels, though genes involved in glycolysis are also associated [51]. There have been several studies carried out on Māori and Pacific Island people living in New Zealand, which have found that at least some of these genetic variants contribute to the risk of gout [52–54].

Collectively, the evidence suggests an inherent biological (genetic) predisposition to hyperuricaemia in Polynesians. How might this have occurred? There are a number of processes by which genetic variants contributing to heightened serum urate concentrations may have increased in frequency at the population level, including ancestry, random genetic drift and natural selection. The circumstances under which these may have operated will be discussed below.

Colonisation processes and Oceanic voyaging: bottleneck effects?

Pacific populations, at least in part, are descended from peoples associated with the archaeologically defined Lapita cultural complex. The Lapita culture first appeared in the Bismarck Archipelago approximately 3,350 years ago, and people carrying this culture rapidly sailed eastwards colonising the Reef Santa Cruz (Southeast Solomon Islands), Vanuatu and New Caledonia within a few 100 years. Lapita expansion stopped at the edge of Polynesian Triangle, in Tonga, which was settled by 2,830–2,846 years before present (BP), and Samoa, settled at a similar time [55]. The most recent dates for settlement of East Polynesia suggest that around 1,200–1,500 years later, further migrations were undertaken from Samoa into Central and East Polynesia, the Marquesas and the Cook and Society Islands, and even later into the more remote islands, with settlement of Rapa Nui at 800 BP, Hawaii between 800 and 1,200 BP, and Aotearoa New Zealand around 700 BP [56].

The settlement of Polynesia, and hence modern Polynesians, is thus the end product of a voyaging process, which occurred over many thousands of years. Often founder effects are employed to explain an apparent lack of genetic variation in Polynesian populations and the apparent dissimilarity phenotypically between Polynesia and Melanesia [57]—though continuous contact and voyaging would have nullified these effects. The basis of the underlying idea of limited genetic diversity in the Pacific resulting from founder effects lies in the observation that on average, 94 % of the mitochondrial variation in Polynesian populations is derived from East Asian haplogroups specifically those derived from the B4a1a1 haplotype [58]. However,

to suggest that these founder effects have led to the chance emergence of a hyperuricaemic phenotype in Polynesians is simplistic and unlikely. Also, when the data from the wider region are reviewed, the Polynesian serum urate levels are not out of context (Fig. 2) because the phenotype seems to be present in other populations in the homeland region, Taiwanese aboriginals, for instance, who were unlikely to have had the same demographic founder alterations resulting from serial migration.

Natural selection

Another mechanism by which genetic variants contributing to elevated serum urate may have come to prominence is natural selection. In order to assess the possibility of selection for heightened serum urate concentrations, it is necessary to discuss the different biological roles of urate in humans. What follows is a brief description of these roles and how these may have provided a selective advantage to those with hyperuricaemia.

Antioxidant effect of urate

Urate can function as an antioxidant [59, 60]. Antioxidants are very important, as radical oxygen species can cause irreversible oxidative damage to cellular organelles and DNA, which can impair the function of the individual and result in death. Urate accounts for up to 60 % of total plasma antioxidant activity [59]. This may help protect endothelial function, and combat oxidative stress associated with ageing [59]. It has also been implicated as being neuro-protective. Unusually low SU levels have been associated with diseases such as multiple sclerosis [7, 8], Parkinson's disease [6] and Alzheimer's disease [9]. The brain is particularly vulnerable to oxidative damage because of its high metabolic rate and the high lipid content of brain tissue [61]. The antioxidant activity of urate seems an unlikely selective force specifically in the Pacific, as there is no reason to believe that people dwelling in the Pacific, or more precisely Polynesia, should face any more oxidative stress than any other island dwelling population. In addition, most of the diseases associated with low SU levels have impacts in post-reproductive periods of the lifecycle.

Hypertension

There is a strong association between hyperuricaemia, hypertension and cardiovascular risk [62–64], which has led to the hypothesis that in the course of human evolution, urate may have played an important role in maintaining blood pressure in conditions of low salt ingestion [65]. Through an animal experimental model, Watanabe et al. [65] found that urate helps maintain blood pressure both acutely, by stimulating the renin angiotensin system and chronically, by inducing salt sensitivity.

There is evidence to suggest that during the Miocene epoch, which is when the series of mutations leading to the inactivation of the uricase gene in Hominids are thought to have occurred, salt consumption among our pre-human ancestors was considerably lower than in modern-day societies [65]. In this situation, the increase in blood pressure associated with raised serum urate levels was advantageous [65]. However, again, with the ready availability of salt in Pacific Island environments, this seems an unlikely evolutionary force in the Pacific.

Uric acid as a physiological alarm

Johnson et al. [66] have proposed that under conditions of environmental stress and starvation, urate may have had a beneficial effect as a physiological alarm. Studies have shown an increase in urate concentration under conditions of fasting [67] or starvation [68], may have a number of beneficial effects, including increasing locomotor activity necessary for foraging, stimulating hypertriglyceridaemia, fatty liver and weight gain to help re-establish fat stores, and to increase salt sensitivity to help protect against dehydration [66]. They further suggest that the development of insulin resistance, which is also associated with many metabolic diseases, could also be beneficial, by reducing glucose uptake into skeletal muscle and adipose tissue, thus preserving glucose for utilisation by the brain where glucose uptake is insulin independent.

This potential role of urate has more credibility as a possible selective force in Pacific Island peoples. Island environments, such as those we see in the Pacific, are vulnerable to catastrophic events such as cyclones and tsunami, which are capable of wiping out not only food supplies but also sources of fresh, potable water on islands, causing periods of famine. It is possible that such events in the past have caused some selection for those with apparently enhanced genetic fitness. These sorts of catastrophic events can also result in population bottlenecking events, thus changing allele frequency. The widespread nature of the hyperuricaemic phenotype in Austronesian-speaking populations across Oceania and beyond, however, does suggest that any population bottlenecking to cause a hyperuricaemic phenotype would have had to occur early on prior to population expansion into ISEA and the Pacific for all of the descendant populations to be affected or else to have happened numerous times across the Pacific.

Urate as an adjuvant

Urate plays a significant role in immune regulation [69]. When crystallised, it has been found to activate innate

host defence mechanisms in multiple ways and triggers a robust inflammatory response [5, 70]. For this reason, crystallised urate (monosodium urate; MSU) is considered to be an important natural endogenous adjuvant and has been employed in vaccinations to help stimulate a host response against antigens, which may otherwise be considered innocuous. Not only can monosodium urate crystals stimulate phagocytes and monocytes, but they can activate the NLRP3 inflammasome; both the classical and alternative complement pathways interact with antibodies, as well as a number of other immune pathways [71]. Absence of urate inhibits the immune response associated with clearing debris from damaged cells.

Infectious disease is one of the strongest forces of selection [72]. The genes associated with immunity are under selective pressure as contact with an infectious agent requires an appropriate immune response. It is possible that urate-raising genetic variants may be selected for by infectious disease.

There are a number of events in the history and prehistory of the Pacific which may have resulted in the selection of genetic variants, which could contribute to the inherently high mean urate levels throughout the region. Discussed below are two possible periods of selection for genetic variants contributing to inherently high serum urate levels, one deep in prehistory with the exposure of ancestral Polynesian populations to malaria prior to their arrival in the malaria-free islands of Polynesia, and the more recent exposure of these populations to novel diseases post-European contact.

Malaria as a means of selection Malaria is possibly the most serious infectious disease currently known to humankind, killing on the order of 2 million people annually [73]. Malaria is not a new immunological challenge: humans and malaria-causing *Plasmodium* species parasites have had a long evolutionary host–parasite relationship [74]. Malaria is known to have influenced the human genome, with disorders such as sickle cell anaemia, alpha- and beta-thalassaemia, glucose-6-phosphate dehydrogenase (G6PD) deficiency and band 6 ovalocytosis having been attributed to malarial selective forces [75]. It is possible that the inherent hyperuricaemia seen in Polynesian populations is another such adaptation.

Urate plays an important role in the innate immune response and has been found to induce host inflammatory responses in malaria [76–78]. The urate is released with the lysis of erythrocytes during the cyclical blood stage of malarial infection. It is during this time that the host immune system finally detects the presence of the parasite. Since an early and robust inflammatory response is critical for controlling the infection, inherently high serum urate levels may be advantageous for those living in malarial environments and could have been selected for, as fewer host erythrocytes would need to rupture before the innate immune response countering the malarial parasites was triggered, thus enhancing the rates of host survival.

While malaria is not present, nor has probably ever been present, in Polynesia, the ancestral populations passed through and may have originated in areas with endemic malaria (namely New Guinea, the Solomon Islands and Vanuatu) [79]. Malaria would also have been endemic in the East Asian/ISEA homeland of the original Austronesian-speaking populations from whom the Polynesians ultimately descended [80]. In fact, there is other evidence that transit through these malarial regions has shaped the genomes of modern Polynesians; there is an unusually high frequency of α-thalassaemia variants in Polynesians and particularly among East Polynesians (Fig. 3) [81–84], especially for populations living in an area which does not have, and has probably never had, active malarial transmission [85]. Thus, malarial selection could have operated either during the spread of populations through the region of Near Oceania or in their place of origin in ISEA.

It is important to note that mean serum urate concentrations reported in populations from coastal Papua New Guinea, where malaria is endemic, are much lower than those observed in Polynesians and, indeed, Micronesians (Fig. 2). For instance, male Papuans living on the Madang Coast (northern mainland Papua New Guinea) have serum urate levels of 4.8 mg/dL [86], while males from Kalo village in the lowlands (Central Province, Papua New Guinea) were reported to have urate levels of 4.2 mg/dL [19]. Malaria is thought to have been present in coastal regions for a substantial period of time—the early habitation (around 50,000 years ago) of the malaria-free Highland regions of New Guinea [87] may have been driven by the presence of the malarial parasite in the lowlands. As a result of this long exposure to malaria, a wide array of malaria-associated haemoglobinopathies, including the globin gene variant (α3.7III) seen in Polynesians, are observed in lowland New Guinean populations [88]. Evidence of substantial genetic admixture between the Asian-derived Austronesians and the non-Austronesian original inhabitants of New Guinea is only significant in the Admiralty Islands and the Bismarck Archipelago, to the north of the mainland of New Guinea, particularly the New Ireland Province [35]. To date, there are only published data on the serum urate levels from mainland New Guinea populations. Because of the different genetic background of ancient Near Oceanic- and Asian-derived Austronesian populations, it is conceivable that different adaptive mechanisms developed in response to malaria, explaining the absence of elevated serum urate in these populations.

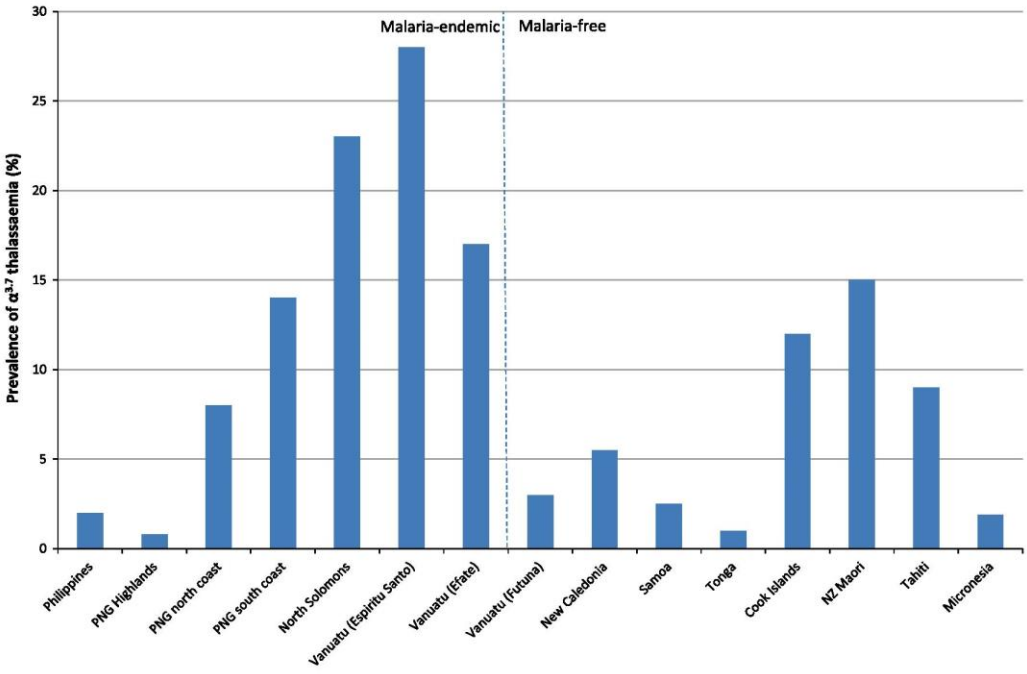

Fig. 3 Prevalence of alpha-thalassaemia (caused by the $-\alpha^{3.7}$ deletion) in Pacific Island populations. Data from [84]

Western diseases The introduction of novel diseases and pathogens after European contact had a significant effect on the demographics of many Pacific populations [89]. Prior to the exploration of the Pacific by Europeans, Oceania had long been isolated from most infectious diseases. The small-scale nature of most populations but particularly their isolation protected these islands from influenza, measles, mumps, smallpox, tuberculosis, cholera, plague, typhoid, whooping cough and venereal disease [90]. These diseases were therefore alien to Pacific Islanders, leaving them immunologically na ve, and thus exposure to these diseases had the capacity to cause large-scale loss of life.

While it remains challenging for archaeological and historical demographers to assess the effect of the introduction of Western diseases on these island populations for a number of reasons, in part because of the lack of regular, systematic censuses on many islands and the uncertainty over the initial population sizes [91], it is clear that introduced diseases did have a major impact. Some islands seem to have been more affected than others, but on some islands where records are available, it is possible to link epidemic outbreaks of diseases such as whooping cough, measles and influenza to visits by certain European ships, and many of these epidemics caused significant mortality [90]. East Polynesia was hit particularly hard—much more so than West Polynesia—particularly during the initial introduction of these diseases, which resulted in losses up to 75 % of the population in some islands [92]. This may go some way to explain some of the genetic differences between East and West Polynesia [53, 84].

Given the role of monosodium urate crystals in enhancing the innate immune response, it is possible that genotypes promoting increased urate levels were positively selected for during these periods of massive depopulation. It is also likely, given the presence of archaeological evidence for gout in samples that are definitely from a pre-European era, that urate-raising variants were already at a higher prevalence prior to the introduction of European infectious diseases.

Conclusion

Raised serum urate levels are seen throughout the Pacific, particularly in Polynesia, Micronesia and among Taiwanese aborigines. Archaeological evidence suggests that this is not a new phenomenon. The multifactorial aetiology of hyperuricaemia indicates that there are likely to be a number of contributing factors, including a genetic contribution. The localised heightened prevalence of hyperuricaemia and gout in Polynesian and Micronesian populations suggests that shared heredity may contribute. However, while

potential reasons for this may be proposed, it remains difficult to disentangle how, when and why this hypothesised genetic contribution arose in these populations.

Processes of natural selection may have contributed to the phenotype, which is observed in modern populations—in particular, infectious disease may have played a role in this process of selection; however, random genetic drift cannot be discounted. The colonisation process of the Pacific with serial founder effects, as well as catastrophic events in the fragile island environments, means that there have been ample opportunities for genetic drift. However, at the same time, given the high levels of hyperuricaemia and gout seen in populations who likely share at least some ancestry with the Polynesians and Micronesians, for instance, the Taiwanese aborigines [28, 29, 93], and the Malagasy highlanders [31], the probability of random genetic drift operating in all of these populations to result in the same phenotype seems unlikely. Multiple processes are likely to have worked in tandem resulting in the genetic predisposition to hyperuricaemia.

Ancestry, itself, is not a trivial consideration. Examination of the available published literature indicates that elevated serum urate may be a trait shared by populations descended from the Austronesian-speaking peoples who departed from Southeast Asia around 8,000 years ago. In the light of the low serum urate levels seen in coastal populations in mainland Papua New Guinea, it would be interesting to investigate the state of serum urate levels among the Lapita-derived, Austronesian-speaking populations living in the Bismarck Archipelago, to the north-east of mainland Papua New Guinea: are they inherently hyperuricaemic like Polynesian and Micronesian populations, or are there lower urate levels similar to the mainland?

There is significant scope for further investigations to determine the reasons for elevated serum urate in populations in the Pacific region. While there have been efforts to understand prehistoric human migration in this region, disease has not been a major focus. The gulf between anthropological studies in the Pacific and genetic disease research has meant that genetic predisposition to conditions like hyperuricaemia has not been examined from an evolutionary perspective. Further genetic studies in Pacific populations are warranted and could assist in testing hypotheses, for example, the application of tools for detecting selection to the genomic sequence of genes involved in the regulation of urate.

Appendix: Mean SU levels and prevalence of gout and hyperuricaemia in different adult male populations worldwide summarised from the published literature

Location	Population	*n*	Age range	Mean SU		Prevalence		References
				mg/dL	mmol/L	HU (%)	Gout (%)	
Oceania								
New Zealand	Māori	388	>15 years	7.1 ± 1.5	0.42 ± 0.09	49	–	[94]
New Zealand	Māori	215	20–64 years	–	–	27.8	18.4	[95]
New Zealand	NZ "non-Māori"	125	20–64 years	–	–	15.3	4.0	[95]
Cook Islands	Rarotongans	243	>20 years	7.0 ± 1.4	0.41 ± 0.08	44	2.5	[96]
Cook Islands	Pukapukans	188	>20 years	7.0 ± 1.1	0.42 ± 0.07	48.5	5.4	[96]
Papeete, Tahiti	Tahitians	179	20–50 years	7.5	0.45	–	–	[97]
Hawaii	Hawaiians	49	22–72 years	5.4 ± 1.1	0.32 ± 0.07	–	–	[13]
Kaneohe, Hawaii	Hawaiians	11	"Adult"	6.4 ± 1.4	0.38 ± 0.08	–	–	[98]
Apia, Samoa	Samoans (urban)	325	>20 years	6.7 ± 1.4	0.40 ± 0.08	36.4	7.2	[20]
Samoa	Samoans (rural)	358	>20 years	6.9 ± 1.2	0.41 ± 0.07	43.3	6.2	[20]
American Samoa	American Samoans	71	–	6.8 ± 1.2	0.40 ± 0.07	–	–	[99]
Nuku'alofa, Tonga	Tongans (urban)	177	20–69 years	6.5 ± 0.1	0.39 ± 0.00	–	2.7	[21]
Foa Island, Tonga	Tongans (rural)	199	20–69 years	5.3 ± 0.0	0.32 ± 0.00	–	0	[21]
Suva, Fiji	Fijians (urban)	401	>20 years	6.6 ± 1.2	0.39 ± 0.07	32.4	0.002	[22]
Fiji	Fijians (rural)	242	>20 years	6.0 ± 1.0	0.36 ± 0.06	16.9	0	[22]
Tokelau	Tokelauans	433	35–74 years	6.6 ± 1.3	0.39 ± 0.08	–	–	[100]
Tuvalu	Tuvaluans	189	>20 years	6.6	0.39	32.3	0	[101]
Nauru	Nauruans	217	>20 years	7.6 ± 1.6	0.45 ± 0.10	64	6.9	[102]
Marshall Islands	Marshall Islanders	188	>15 years	6.6 ± 1.3	0.39 ± 0.08	–	1.7	[103]
Rongelap, Marshall Islands	Marshall Islanders	37	>15 years	6.1 ± 1.0	0.36 ± 0.06	–	–	[103]
Utirik, Marshall Islands	Marshall Islanders	68	>15 years	6.5 ± 1.4	0.39 ± 0.08	–	–	[103]

continued

Location	Population	*n*	Age range	Mean SU		Prevalence		References
				mg/dL	mmol/L	HU (%)	Gout (%)	
Beijing, China	Han Chinese (rural)	558	40–58 years	5.6 ± 1.3	0.33 ± 0.08	11.3	–	[114]
Beijing, China	Han Chinese (urban)	1,062	40–58 years	5.8 ± 1.3	0.34 ± 0.08	15.4	–	[114]
Qingdao, China	Han Chinese	903	20–74 years	6.5	0.39	32.1	–	[115]
Hangzhou, China	Han Chinese	186	"adults"	5.5 ± 1.2	0.33 ± 0.07	11	–	[116]
Vancouver, Canada	Canadian Chinese	100	–	5.4 ± 1.1	0.32 ± 0.06	–	–	[117]
Korea	Koreans	22,698	30–77 years	5.8 ± 1.6	0.35 ± 0.10	–	–	[118]
Nagano Prefecture, Japan	Japanese	15,712	>15 years	5.8 ± 1.1	0.35 ± 0.06	–	–	[119]
Okinawa, Japan	Japanese	6,163	18–89 years	6.5 ± 1.3	0.39 ± 0.08	34.5	–	[120]
Hawaii, USA	Japanese Americans	7,971	45–69 years	6.0 ± 1.5	0.36 ± 0.09	–	–	[121]
Thailand	Thai	376	–	6.0 ± 1.4	0.36 ± 0.08	18.4	–	[122]
Americas								
Vancouver, Canada	Caucasian Canadians	200	–	4.6 ± 1.0	0.27 ± 0.06	–	–	[117]
Canada	Haida (Amerindians)	236	–	4.4 ± 1.0	0.26 ± 0.06	–	–	[117]
Tecumeseh, Michigan, USA	Caucasian Americans	2,987	>3 years	4.9 ± 2.0	0.29 ± 0.12	7.5	–	[123]
New England, USA	Caucasian Americans	534	>20 years	6.4 ± 1.4	0.38 ± 0.08	–	–	[124]
NHANES Cohort, USA	Americans of multiple ancestral backgrounds	8,816	>20 years	6.1	0.36	21.2	5.9	[125]
Montana, USA[a]	Blackfeet (Amerindians)	1,018	–	4.9 ± 1.2	0.29 ± 0.07	7.17	–	[126]
Arizona, USA[a]	Pima (Amerindians)	949	–	4.2 ± 1.2	0.25 ± 0.07	3.27	–	[126]
Arizona, USA	"American Indians"	–	>18 years	5.7 ± 1.4	0.33 ± 0.08	–	–	[127]
Dakotas, USA	"American Indians"	–	>18 years	6.1 ± 1.5	0.36 ± 0.09	–	–	[127]
Oklahoma, USA	"American Indians"	–	>18 years	6.1 ± 1.4	0.36 ± 0.08	–	–	[127]
USA	American Army Recruits	2,084	–	4.9	0.29	–	–	[128]
Colombia	Colombian Army Recruits	481	–	5.0	0.30	–	–	[128]
Argentina	Argentinian Army Recruits	491	–	4.8	0.29	–	–	[128]
Brazil	Brazilian Army Recruits	1,990	–	4.1	0.24	–	–	[128]
Brazil	Parkat j (Amerindians)	53	20–80 years	5.8 ± 0.9	0.34 ± 0.05	5.6	–	[129]
Brazil	Kalapalo (Amerindians)	53	20–80 years	5.1 ± 1.3	0.30 ± 0.07	3.2	–	[130]
Brazil	Kuikuro (Amerindians)	54	20–80 years	4.6 ± 1.0	0.27 ± 0.06	0	–	[130]
Brazil	Matipo (Amerindians)	5	20–80 years	5.1 ± 0.9	0.30 ± 0.05	0	–	[130]
Brazil	Nahukw (Amerindians)	13	20–80 years	4.5 ± 1.0	0.27 ± 0.06	0	–	[130]
Europe								
Birmingham and Dorset, England	English	512	>15 years	5.5 ± 1.0	0.33 ± 0.06	6.6	–	[131]
Cotwolds, England	English	1,727	>35 years	5.1 ± 1.4	0.30 ± 0.08	6.75	1.68	[132]
Watford, England	English	158	>15 years	5.0 ± 1.2	0.30 ± 0.07	–	–	[124]
Wensleydale, England	English (rural)	435	>15 years	4.7 ± 0.9	0.28 ± 0.05	–	–	[124]
Glasgow, Scotland	Scottish (urban)	337	>15 years	5.5 ± 1.0	0.33 ± 0.06	8.0	–	[131]
Finland	Finnish	3,295	40–69 years	5.1 ± 1.2	0.30 ± 0.07	6.6	–	[133]
France	French	23,923	20–55 years	5.9 ± 1.2	0.35 ± 0.07	17.6	–	[134]
Austria	Austrians	83,683	>18 years	5.7 ± 1.4	0.34 ± 0.08	–	–	[135]
Izmir, Turkey	Turks (urban)	63	"Adult"	5.5 ± 1.3	0.33 ± 0.07	19.0	–	[136]
Middle East								
Israel	Israelis	9,909	>40 years	4.8 ± 1.0	0.28 ± 0.06	–	–	[137]
Jordan	Jordanians	96	18–60 years	5.7 ± 1.1	0.35 ± 0.07	–	–	[138]
Syria	Syrians	1,042	20–60 years	5.2 ± 1.2	0.31 ± 0.07	–	–	[139]
Kuwait	Kuwait	110	20–40 years	4.7 ± 0.9	0.28 ± 0.05	–	–	[140]
Kuwait	Kuwait	79	>20 years	5.5 ± 1.4	0.33 ± 0.08	–	–	[141]

continued

Location	Population	*n*	Age range	Mean SU		Prevalence		References
				mg/dL	mmol/L	HU (%)	Gout (%)	
Saudi Arabia	Saudis	278	14–65 years	5.2 ± 1.0	0.31 ± 0.06	–	–	[142]
Saudi Arabia	Saudis	250	14–83 years	5.2 ± 1.5	0.31 ± 0.09	8	–	[143]
Africa								
Phokeng, South Africa	Tswana (rural)	128	14–84 years	4.9 ± 1.9	0.29 ± 0.11	–	–	[144]
Soweto, South Africa	Tswana, Zuli and Xhosa (urban)	144	15–90 years	6.1 ± 1.4	0.36 ± 0.08	–	–	[145]
Transkei, South Africa	Xhosa (tribal)	80	>15 years	4.7 ± 1.1	0.28 ± 0.06	–	–	[146]
South Africa	"Africans"	87	"Adult"	5.9 ± 1.5	0.35 ± 0.09	–	–	[147]
South Africa	Caucasians	121	"Adult"	6.7 ± 1.6	0.40 ± 0.10	–	–	[147]
Zaire[a]	"Zairois"	75	–	5.6 ± 1.1	0.33 ± 0.06	–	–	[148]
Kinshasa, Zaire[a]	"Zairois" (urban)	100	–	5.7 ± 1.2	0.34 ± 0.07	–	–	[148]
Kasongo-Batetela, Zaire	"Zairois" (rural)	33	–	5.5 ± 1.1	0.33 ± 0.06	–	–	[148]
Zaire[a]	Caucasians	19	–	5.4 ± 1.1	0.32 ± 0.06	–	–	[148]
Zambia	Zambians	50	–	5.2 ± 1.5	0.31 ± 0.09	–	–	[149]
Mbeya, Tanzania	Tanzanians (mixed tribes)	137	19–48 years	5.3	0.32	–	–	[150]
Seychelles	Seychellois	482	25–64 years	6.9 ± 2.8	0.41 ± 0.17	35.2	–	[33]
Sudan	Sudanese	16	20–22 years	4.3 ± 0.2	0.26 ± 0.01	–	–	[67]

[a] Mean SU value and prevalence of hyperuricaemia is for a mixed male and female sample

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8.1.3 Pacific Populations, Metabolic Disease and 'Just-So Stories': A Critique of the 'Thrifty Genotype' Hypothesis in Oceania. *Ann. Hum. Genet.* 79: 470-480

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Pacific Populations, Metabolic Disease and 'Just-So Stories': A Critique of the 'Thrifty Genotype' Hypothesis in Oceania

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Summary

Pacific populations have long been observed to suffer a high burden of metabolic disease, including obesity, type 2 diabetes and gout. The 'Thrifty Genotype' hypothesis has frequently been used to explain this high prevalence of disease. Here, the 'Thrifty Genotype' hypothesis and the evolutionary background of Pacific populations are examined. We question its relevance not only in the Pacific region but more generally. Not only has the hypothesis not been explicitly tested, but most archaeological and anthropological data from the Pacific fundamentally do not support its application.

Keywords: Obesity, type 2 diabetes, metabolic disease, polynesians, thrifty genes

Introduction

In 1962, the American geneticist, James Neel, put forth a revolutionary hypothesis that the increasing prevalence of type 2 diabetes among many indigenous populations was a result of an evolutionary adaptation to periods of famine (Neel, 1962). Thus, the 'Thrifty Genotype' hypothesis, which also relates to other metabolic conditions, was born. The hypothesis was an important first step in employing evolutionary explanations for differences in disease prevalence between populations. Over the following 50 years, the hypothesis was modified in conjunction with advances in understanding of the complexity of metabolic disease and the anthropology of hunter-gatherer and early Neolithic cultures and their subsistence patterns (Neel, 1982, 1999; Corbett et al., 2009).

The premise of the original hypothesis was that contemporary type 2 diabetes was caused by a disjunction between genes and environment. Specifically, variation in genes which facilitated survival during periods of famine in preindustrialised societies, by allowing for the more efficient storage of energy during periods of plenty, were at odds with a modern, industrialised environment of plenty (Neel, 1962). Consequent to the replacement of traditional diets with a Westernised diet, these so-called 'thrifty genes' were proposed to contribute to the onset of diabetic symptoms. Following the publication of the hypothesis, other possible explanations relating to reasons for metabolic disease were discussed in the literature. These include the 'Thrifty Phenotype' hypothesis wherein inadequate nutrition early in life predisposes an individual to type 2 diabetes (Hales & Barker, 1992), and the 'Drifty Genotype' hypothesis which postulates that the removal of heavy predation pressure resulting from increased social behaviour, the use of fire and invention of weapons removed selective pressures permitting genetic drift which has allowed for genetic variants causal of obesity and type 2 diabetes to accumulate (Speakman, 2008).

Despite many critiques of the hypothesis (Benyshek & Watson, 2006; Speakman, 2006; Paradies et al., 2007; Beil, 2014; Sellayah et al., 2014), and a general lack of genetic evidence, for instance, the detection of selection signatures in metabolic genes (Helgason et al., 2007; Ayub et al., 2014; Steinthorsdottir et al., 2014), the 'Thrifty Genotype' hypothesis continues to be cited as a reason for disparities in metabolic health between populations. In their seminal paper, Gould and Lewontin (1979) warned against the acceptance of evolutionary narratives without due testing and critical thought, and this sentiment remains as true now as it did in 1979 (Nielsen, 2009).

Here, we argue that the 'Thrifty Genotype' hypothesis is another 'Just-So Story' (to borrow the analogy used by

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Gould and Lewontin in their critique of the 'adaptationist programme' (Lewontin, 1991; Gould, 1997), or 'how the human got his fat' as Speakman (2013: 305) put it, referring to the fantastical fables of Rudyard Kipling). It is a possible explanation but one for which there are little or no supporting data. This is particularly true for Pacific Island populations, for whom the 'Thrifty Genotype' hypothesis has been a recurring theme in describing reasons for the high rates of type 2 diabetes, obesity and other metabolic disorders; Pacific Island populations, along with Pima Indians, have long been considered archetypal examples of a 'Thrifty Genotype' in action (Zimmet et al., 1990; Bindon & Baker, 1997; Diamond, 2003; Myles et al., 2007). Other populations, such as Australian aborigines (O'Dea, 1992), Asian Indians (Mohan et al., 2007), sub-Saharan Africans (Van Der Sande, 2003) and Latin Americans (Filozof et al., 2001), have also been subject to the hypothesis. It seems that the 'Thrifty Genotype' hypothesis has been applied in such a way that one of the few populations that seems exempt from these 'thrifty genes' are European-derived populations. As Allen and Cheer (1996) pointed out, it may be that the Europeans themselves were subject to some selection process which caused this discrepancy. But does the 'Thrifty Genotype' hypothesis fit the genetic and anthropological data?

Perhaps a place to begin is the observation that there are indeed high rates of obesity, type 2 diabetes (Fig. 1) and hyperuricaemia (Gosling et al., 2014) among many populations throughout the Pacific both among those still living in their ancestral homelands, and those who have migrated to urban centres in places such as New Zealand, the United States and Australia. While the prevalence of disease is higher among those living in more westernised contexts, the occurrence of metabolic disease in those living a more traditional lifestyle (pre-mid-twentieth century and prehistoric) suggests a genetic contribution to disease. This is supported by skeletal indicators of metabolic disorders (gout and diffuse idiopathic skeletal hyperostosis in particular) in bioarchaeological assemblages from the region (Buckley, 2007, 2011; Buckley et al., 2010) and the epidemiological evidence from the 1950s through to the 1970s (Prior, 1981; Prior et al., 1966). Although it is reasonable to expect that indigenous Pacific populations will have diabetes and obesity risk alleles at a higher prevalence and penetrance, we note that there are currently no systematic genome-wide association studies on genetics of diabetes and obesity in any Pacific population that could illuminate this possibility. More importantly, the theory that this is due to selection as a result of food deprivation is not supported when one considers the population history, settlement process and environment of the Pacific in more depth.

The Migratory History of the Pacific

Understanding the evolutionary history of the region and the colonisation process is instrumental to place the 'Thrifty Genotype' hypothesis in a better context when applied to Oceanic populations. There have been multiple movements of people into the Pacific region throughout the 50,000-year history of human occupation. Given this time depth and population diversity, it might be expected to see significant differences in the metabolic disease frequencies in various populations. People first arrived on the continent of Sahul, which became New Guinea and Australia with subsequent sea-level changes, at least 49,000 years ago (Summerhayes, Leavesley et al., 2010). Genetic analyses have shown that there were probably multiple populations involved in these Late Pleistocene and Early Holocene migrations out into New Guinea and the Solomon Islands, a region known as Near Oceania (Rasmussen et al., 2011). Subsequently, there have been several influxes of people from the Island Southeast Asia region into the islands of the Bismarck Archipelago and beyond as early as 5000 years ago but certainly by some 3350 years ago (Kirch et al., 1989; Summerhayes, Matisoo-Smith et al., 2010). These people, often referred as Austronesians based on their linguistic affiliations, mixed with resident coastal populations prior to expanding out into the wider Pacific where they were the initial colonists on the previously unoccupied islands of Remote Oceania. Thus, Polynesians and Micronesians have been found to be more genetically similar to certain Asian populations than to the general Near Oceanic populations which are highly heterogeneous (Friedlaender et al., 2008). Because of these differences in ancestry, there are both genetic and phenotypic differences between Pacific populations, even those within a reasonably close geographical distance to one another. The implication of this is that population history should be a major consideration when discussing differences in disease prevalence in the Pacific. For instance, while rates of type 2 diabetes and obesity are high among Polynesian and Micronesian populations, the rates are much lower in Papua New Guinea (Fig. 1), whose general population has a larger proportion of their ancestry being derived from the earliest inhabitants of the region than the later Austronesian-speaking colonists. Some of this disparity between prevalence of obesity and type 2 diabetes in Polynesia and Micronesia and populations in Papua New Guinea could be explained by the differential penetrance of western nutritional influences in this region. A proponent of the 'Thrifty Genotype' hypothesis might suggest other explanations for this phenomenon – for instance, the selection for 'thrifty genes' occurred after moving through this region, but these arguments are inconsistent when examined in context of the Pacific archaeology and anthropology.

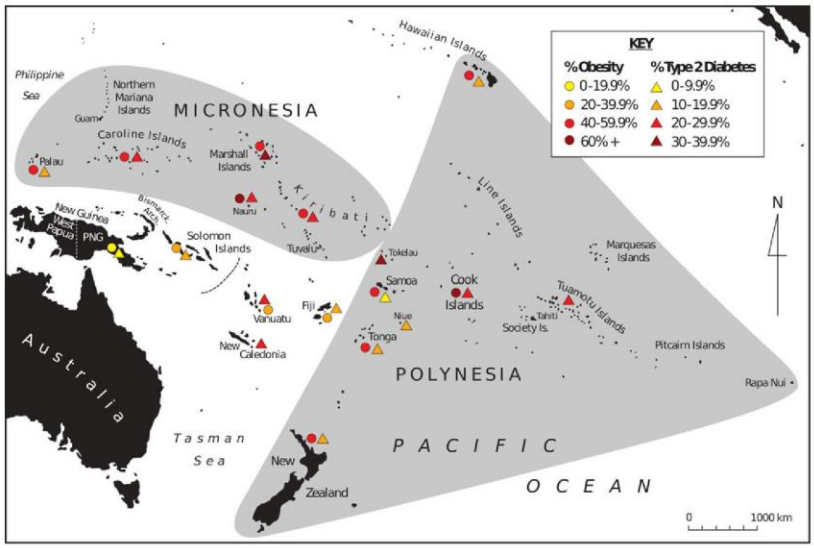

Figure 1 Prevalence of type 2 diabetes and obesity in the Pacific (for references, see Table S1). The dashed line indicates the division between near and remote Oceania.

'Thrifty Genotypes' and Oceanic Voyaging

One of the inherent weaknesses of the 'Thrifty Genotype' hypothesis in the context of the Pacific is the reliance on selective mortality or variation in reproductive success mediated by restricted food supply. A number of mechanisms describing how the hypothesis might operate in the Pacific have been put forth. The scenario laid out by Bindon and Baker (1997) is that the selection for these 'thrifty' traits occurred during the ocean voyages involved in settlement of the Pacific or once people arrived on their new island. This is based on the assumption that the voyaging and island settlement process was perilous and that there was a reasonably high mortality rate. Diamond (2003) takes this a step further by stating that 'in many attested examples of such lengthy voyages, many or most of the canoe occupants died of starvation, and only those who were originally the fattest survived'. No citations were given for this claim. However, given that colonisation voyages are not something that have happened for at least a couple of centuries (Irwin, 1989), it is likely that Diamond is referring to more recent examples of people who have not intentionally ventured out and are not necessarily prepared for long periods in a boat in the middle of the ocean (for instance, fishermen lost at sea). It is certainly not in line with studies which have found that the process of colonising the Pacific is likely to have involved safe, systematic and planned exploration prior to the colonisation (Hiroa, 1954; Irwin, 1994).

Archaeological sites associated with the Lapita culture, the first colonists into islands beyond the Solomon Islands, first appeared in the Bismarck Archipelago approximately 3350 years ago (Summerhayes, Matisoo-Smith et al., 2010). Within a few hundred years, new settlements were established in the Reef Santa Cruz (Southeast Solomon Islands), Vanuatu, Fiji and New Caledonia (Bedford et al., 2006). Lapita expansion halted in Tonga which was settled by 2830–2846 years ago (Burley et al., 2012), and Samoa which was settled at a similar time (Spriggs, 2011). There was an interval close to 2000 years between the settlement of Western and Eastern Polynesia.

The settlement of Eastern Polynesia, from the Samoa/Tonga Polynesian homelands, was similarly rapid, with the occupation of all of the main island groups within a 300-year window between AD 1025 and 1290 (Wilmshurst et al., 2011). The speed at which successful settlement of the wider Pacific region occurred could simply not be sustained if there was great loss of life. While there was some doubt during the 1950s and 1960s about the deliberate nature of Pacific voyaging and navigation, with theorists such as Sharp (1956) arguing for 'accidental voyaging', most scholars now agree that preliminary scouts were sent out on two-way exploratory voyages prior to the departure of a well-provisioned colonisation party who were travelling to a known destination with known resources and for a known period of time (Irwin, 1989; Kirch, 2000; Fitzpatrick, 2007; Montenegro et al., 2014). These colonisation events may have been assisted by

weather patterns such as El Niño (Goodwin et al., 2014), which would have made the voyaging times faster.

Starving at sea is not the only voyaging-related proposal put forth to explain genetic diversity in the Pacific. Houghton (1990, 1996) and Bindon and Baker (1997) have suggested that the increased body mass among New Zealand Māori and other Polynesian populations is a result of selection due to the cold temperatures faced during open ocean voyaging, the premise being that higher body mass would insulate one against wind chill and ocean spray (an adaption of a hypothesis known as Bergmann's rule). This increase in body mass includes a shift in the muscle mass to fat ratio; Polynesians possess less fat per unit weight when compared to Europeans (Swinburn et al., 1999; Rush et al., 2009). More recently, this hypothesis has also been subject to criticism, as the premise that larger mass may be selected for by exposure to cold where a lower surface-to-volume ratio as more heat conserving has been challenged by the fact that larger individuals also need to consume more food; meaning that there is little difference in the absolute energy balance (McNab, 2002). The implication of this observation is that it would be more beneficial to take smaller people voyaging if there was a fixed food supply as their consumptive needs would be lower. To support his hypothesis, Houghton (1996) devised sophisticated calculations to show the likelihood of survival for 10 days voyaging on open ocean at various latitudes and indicated that larger body masses were required for survival under his simulated scenarios.

There are several problems with this hypothesis. First, if selection for large body size was the result of surviving cold temperatures during voyaging, we would expect that populations that inhabit the most southerly or most distant islands (e.g. New Zealand or Rapa Nui) and would have therefore had the longest and coldest voyages, would be the most robust, having the strongest selective pressures – or that East Polynesians would be more robust than Tongans and Samoans, given the greater distances involved in the settlement of East Polynesia. This pattern is not indicated in any data from skeletal or modern populations (Howells, 1970; Houghton, 1996; Pietrusewsky, 1996). Another problem is that Houghton's calculations assume naked bodies that have no cold protection. This does not fit with knowledge of Polynesian voyaging skill and evidence for protective clothing (Hiroa, 1924) or structures in the canoe allowing for some weather protection (Van Dijk, 1991).

Similarly, these simulations are also undermined by the recent reinterpretations of archaeological radiocarbon dates, with the imposition of stricter chronometric hygiene measures, supporting a general shortening of East Polynesian prehistory from the previously accepted dates of AD 410–1270 to AD 1025–1290 (Wilmshurst et al., 2011). For rapid, successive population movements, a larger founding population would be expected, which in turn would require voyaging to have a much reduced mortality rate than this particular application of Bergmann's rule. This is supported by a recent study investigating the cold-induced vasodilation response in a number of populations worldwide, which has indicated that the cold adaptation seen in Polynesian populations is likely an ancestral trait which evolved in Asia prior to the start of the Pacific colonisation process (Wilberfoss, 2012).

There is also archaeological evidence to support extensive sailing between archipelagos (Collerson & Weisler, 2007), at least in the early periods of colonisation, though this interaction dropped off in later prehistory (Irwin, 1994). This reduction in interisland contact may be due to the development of greater sociopolitical complexity in some of these island groups, possibly triggered by climatic change (Field & Lape, 2010). The focus of the island communities turned inwards, rather than maintaining strong links with distant islands. Thus, given this evidence of return voyaging and continued contact within the Polynesian Triangle for at least the first few hundred years, it seems improbable that the voyaging process would be a significant selective pressure. The mortality rates described to select for the postulated 'thrifty' traits indicate a lack of appreciation for the sophistication of sailing technology and expertise of early Pacific Islanders (Hiroa, 1954). Increased mortality rates cannot explain the uniformity of so-called 'thrifty' traits across both Polynesia and Micronesia and are inconsistent with the speed of settlement based on the archaeological record.

Fragile Island Environments

It has been argued that Pacific Island populations might be subject to more famine events than continental populations because of their relative isolation and their susceptibility to cyclonic weather patterns and tsunami (Zimmet et al., 1990; McGarvey, 1994). This might result in the destruction of horticultural crops and impact staple foods such as shellfish and other marine foods, which were mainstays to many prehistoric Pacific Island populations. As evidenced by the devastation caused by these sorts of phenomena in modern times (e.g. the Samoan tsunami in 2009), it is not unreasonable to assume that such occurrences have been persistent but intermittent and relatively localised problems for many island populations since initial colonisation. Indeed, skeletons from archaeological sites in the region show skeletal pathology consistent with nutritional deficiencies (Snow, 1974; Buckley, 2000; Buckley et al., 2014). The palaeopathology record can indicate other skeletal and dental changes reflecting growth disruption for assessing periods of food insufficiency in the past (Goodman et al., 1984). However, these signs of growth disruption may also be a response to infection, especially in a tropical

environment where pathogen loads are high (Buckley, 2006) and they indicate recovery from the stressful event. Therefore, these growth disturbances are generally considered to be a reflection of nonspecific or more generalised stress during growth.

This permutation of the hypothesis does not appreciate the variation in subsistence patterns across the Pacific, partly as a result of the different geological origins of the inhabited islands. Pacific peoples have taken to inhabiting atolls, volcanic and continental islands (Neall & Trewick, 2008), and these different island types offer different subsistence opportunities due to variation in ecological diversity (Kirch, 2000). Factors such as water availability and soil composition alter what horticultural domesticates are likely to grow, and any surpluses which might be produced. As such, different islands are likely to have had different levels of vulnerability when faced with natural disasters. The distribution of obesity-prone populations throughout the Pacific, under this model, would require multiple independent episodes of selection for a 'Thrifty Genotype'. This is unlikely to have occurred. Rather, the broad patterning observed in metabolic disease prevalence among modern Pacific populations is more likely a result of the genetics of founding populations, in addition to differential founder effects and drift.

The high prevalence of obesity throughout the Pacific (Fig. 1) seems indicative of increased body mass index (BMI) being an ancestral trait, which was already present among the colonising population rather than selection for 'thrifty' traits *in situ*. Selective mortality or reproductive success once islands were colonised was unlikely to be a factor that would drive selection or 'thriftiness' uniformly across the broad region and diverse environments of island Polynesia.

Genetic Studies of 'Thrifty Genes' in the Pacific

It seems telling that despite multiple genetic studies (Ohashi et al., 2007; Åberg et al., 2009; Deka et al., 2009; Furusawa et al., 2010; Myles et al., 2011), there is no direct evidence for 'thrifty genes' among Pacific Islanders or other related populations, as manifest by signatures of selection at known diabetes and obesity loci. Furthermore, loci that have been strongly associated with obesity among European populations, for instance, intronic variants in the *FTO* gene (Dina et al., 2007; Frayling et al., 2007), have been found to have allele frequencies consistent with what is seen among Asian populations, so have been argued to be unlikely 'thrifty' candidates (Ohashi et al., 2007). Supporting this assertion, Karns et al. (2012) found that there were no significant associations between common variants in *FTO* with obesity-related phenotypes among Samoans.

The Gln223Arg variant of *LEPR* has also been suggested as a 'thrifty gene' on the basis of differences in allele frequency across Oceania (Furusawa et al., 2010). *LEPR* encodes a protein that is the receptor for the hormone leptin which is released predominantly from fat cells to regulate satiety, and is associated with BMI (Park et al., 2006). An elevated allele frequency, as observed by Furusawa et al. (2010), would be expected in specific populations if the locus had indeed been subject to selection. However, there are other explanations for differences in allele frequency, such as genetic drift. This variation in common allele frequency could very well be related to serial founder effects – as indicated by other genetic markers, for instance, common mitochondrial genome haplogroups which show a similar gradient across the Pacific (Kayser et al., 2006). This, indeed, seems more likely than selection when one considers the lack of evidence for a functional effect of alleles at this particular locus (Stratigopoulos et al., 2009).

Some variants have been associated with metabolic traits in certain populations and not others, for instance, the Gly482Ser variant in *PPARGC1A,* a gene involved in energy metabolism, has been found to associate with high BMI among Tongans but not New Zealand Māori (Myles et al., 2011) although this is not necessarily indicative of this being a 'thrifty gene' *per se*. A recent study has demonstrated no evidence for a selection signature at the *PPARGC1A* locus, nor was the association with BMI in Tongans able to be replicated (Cadzow and Merriman, unpublished data). The higher allele frequency of the *PPARGC1A* variant (Myles et al., 2011) in islands colonised relatively late, such as New Zealand (670–720 BP; Wilmshurst et al., 2011) compared with Tonga (2838 ± 68 BP; Burley et al., 2012), one of the first Polynesian islands colonised, is consistent with the manifestation of serial founder populations, though inconsistent with a population model of return voyaging and sustained contact between populations which has been suggested through the archaeological evidence.

The higher allele frequency of the Gly482Ser variant in *PPARGC1A* has also been cited as a potential 'thrifty gene' on the basis of its higher allele frequency among Tongans compared with Han Chinese and Papua New Guinean Highlanders (Myles et al., 2007). This same study suggested that this locus has been subject to selection based on the application of F_{ST} analyses on these populations – a rather high F_{ST} value was observed when comparing Highland New Guineans with Tongans (0.703). However, no recognition is given to the fact that these are populations with vastly different ancestral backgrounds. Polynesian populations have been found to have a higher degree of Asian ancestral contribution to their autosomal DNA than Near Oceanic (New Guinea; Wollstein et al., 2010). Given their dissimilar histories, it would therefore be expected to observe genetic differences between these populations – this is made particularly clear in a recent review of genetic diversity in Oceania which emphasises this

need to consider population history (Duggan & Stoneking, 2014). Additionally, the small sample sizes used in this study are unlikely to capture the genetic variation in any of the populations – data from only 23 Polynesians (9 Cook Islanders, 8 Samoans, 4 Tongans and 2 Niueans), 23 Highland New Guineans and 19 Han Chinese were used (Myles et al., 2007). A better approach for detecting more recent selection (i.e. selection consistent with the 'Thrifty Genotype' hypothesis in the Pacific) is probably the haplotype-based extended haplotype heterozygosity concept (Sabeti et al., 2002).

There is suggested evidence for founder effects in Remote Oceania, in Polynesians in particular (Kayser, 2010), though the sampling of Polynesian populations, in particular East Polynesian populations, is limited. This has significant implications when it comes to impact on genome-wide variation and may complicate the detection of signatures of selection if relying on statistics such as F_{ST} which detect changes in allele frequency. Focused, large-scale genetic studies of populations worldwide with known founder effects, for instance, Ashkenazi Jews and Icelandic populations, have assisted in identification of important susceptibility loci associated with other complex phenotypes (Helgason et al., 2005; Steinthorsdottir et al., 2007; Guha et al., 2012). The only such study, which has been carried out in the Pacific, was a genome-wide association study of a cohort from Kosrae, an isolated island located in the Federated States of Micronesia (Lowe et al., 2009). This study found a relatively genetically homogenous population and showed that a majority of the common variants contributing predisposition to disease in Europeans have little effect on Kosraens. Novel disease associations were identified which further underlines the need for studies focused on populations with well-defined ancestry and population history.

The 'Thrifty Genotype' Hypothesis Worldwide

The lack of support for the 'Thrifty Genotype' hypothesis is a pattern which has emerged from other studies of populations worldwide, including Europeans and Asians (Helgason et al., 2007; Southam et al., 2009; Ayub et al., 2014; Koh et al., 2014; Steinthorsdottir et al., 2014). Ayub et al. (2014) critically evaluated the hypothesis by testing for signatures of selection at type 2 diabetes loci ('thrifty genes'). They examined 65 loci and found no experiment-wide evidence for signatures of selection. Furthermore, Koh et al. (2014) looked for signatures of selection at loci associated with obesity and type 2 diabetes identified in a genome-wide association study of an East Asian cohort and similarly did not find consistent support for the 'Thrifty Genotype' hypothesis. Together, the findings of these studies indicate that the 'Thrifty Genotype' hypothesis should be reassessed as a way of considering evolutionary explanations for higher rates of obesity and type 2 diabetes among various populations.

The 'Thrifty Genotype' hypothesis does not account for the fact that famines are likely to have been a periodic problem for all human populations even before the emergence of our species from other hominins (Prentice, 2005), or that there is little evidence to suggest that famines have ever been a very important mechanism of selection (Speakman, 2013). Mathematical modelling suggests that if 'thrifty genes' did exist, given the number of famine events faced by *Homo sapiens* since the divergence from our hominin ancestors these alleles would likely have reached fixation (where the advantageous allele becomes homozygous; Speakman & Westerterp, 2013). This is in line with the recent suggestion that we should perhaps be looking deeper in human evolution for these 'thrifty genes'. Uricase has been highlighted as a putative 'thrifty gene' (Kratzer et al., 2014), but given that the inactivation of the gene occurred during the Miocene, prior to the divergence of humans from the ape lineage, uricase is also unlikely to account for differences in metabolic health between modern populations – nor is there any direct evidence that the loss of uricase function was driven by periods of famine. In other words, it 'assume[s] rather than demonstrate[s] the operation of natural selection' (Pigliucci & Kaplan, 2000). Given the multiple roles of urate (as an antioxidant, an adjuvant and in maintaining blood pressure; Gosling et al., 2014), there may have been selective forces at work other than nutritional stress.

This is not to say that nutritional exposures have not led to the selection of certain genetic variants (Stover, 2006). Variation in allele frequencies for enzymes involved in the digestion of lactose (Bersaglieri et al., 2004), starch (Perry et al., 2007) and the metabolism of alcohol (Eng et al., 2007) and fructose (Ali et al., 1998) have been detected between populations. That some populations have locally adapted genetically to optimise their nutrient uptake is unsurprising given the role of food in health and well-being, and indeed survival in general. The array of different adaptations is symptomatic of the diversity in subsistence patterns and nutritive resources available in different geographical regions. Some might argue that these variants themselves could be classed as 'thrifty genes', since the ability to digest lactose from cow's milk or to better break down starches from tubers and grains would undoubtedly be advantageous in situations of famine, though being advantageous is not the same as being 'thrifty'.

However, there is also an important distinction to be made between genetic variants which may be potentially advantageous in situations of hypothetical famine, and those actually being advantageous in an actual famine. Given that few famines involve a complete absence of food (Speakman, 2006), an ability to monopolise food resources is likely to be more critical for survival than level of adiposity, for example, via social factors like status and wealth. Certainly, in

a number of Polynesian populations, chiefs are able to restrict access to certain food resources such as fishery stocks by imposing a *rahui* (Best, 1904). It should be noted that *rahui* is not necessarily based on status and wealth, and the purpose of restricting access to food resources is usually to allow the stocks to replenish themselves in order to avoid future food shortages. In such a way, cultural processes may circumvent natural selective forces such as those assumed under the 'Thrifty Genotype' hypothesis. Furthermore, the burden of mortality in famine usually lies among the elderly and the young and both groups are irrelevant as far as the transmission of 'thrifty genes', as the elderly are likely to have already reproduced and the children are usually not variable enough in terms of adiposity for theoretical 'thrifty genes' to confer any particular survival advantage (Speakman, 2007). Therefore, famines may not impose as strong a selective force as assumed by the hypothesis. While beyond the scope of this review, there is growing evidence that individuals with higher BMI can better survive infectious disease (Van Der Sande et al., 2004; Hanrahan et al., 2010; Corrales-Medina et al., 2011). This possibility should be considered in future work on reasons for the increased obesity in modern Pacific populations.

Concluding Remarks

The 'Thrifty Genotype' hypothesis overlooks other important factors that are likely to have had more significant impacts on populations and their genetic diversity, such as migration and disease epidemics (O'Rourke, 2012) or social/cultural selection for particular phenotypes (Van Dijk, 1991). As discussed previously in relation to genetic differences between New Guinean and Tongan populations, genetic ancestry and the specific evolutionary history of a population has a significant impact on the particular variants present within a population and indeed the relative proportions of various alleles. Founder effects are clearly something that require consideration in Oceanic populations, as serial migrations have played a large part in the settlement of the area. Admixture, and the degree and nature of that admixture, is another factor which impacts the genetic variability of a population.

Infectious disease is one of the strongest drivers of genetic change (Karlsson et al., 2014): major human pathogens such as malaria (Kwiatkowski, 2005) and HIV (Schliekelman et al., 2001; Nkenfou et al., 2013) have shaped the genome in exposed populations. Given the links that are increasingly being made between metabolic diseases and innate immunity (Pickup & Crook, 1998; Lumeng & Saltiel, 2011; Robbins et al., 2014), genetic selection resulting from infectious disease exposure may contribute to an underlying susceptibility of certain populations to metabolic diseases. An example of the striking effect that infectious disease can have on a population is the introduction of Western diseases including measles, whooping cough and influenza to the Pacific during the 19th century; it is thought that these introductions caused up to 75% mortality in some East Polynesian populations (Harrison et al., 1988). Clearly this is likely to have had a large impact on the genetic diversity in the modern population.

For its time, the 'Thrifty Genotype' hypothesis was revolutionary. It reflected one of the first efforts to integrate our knowledge of modern epidemiology with human evolution. However, the hypothesis does not recognise other factors that are likely to have a greater impact on the development of genetic traits. In the 53 years since its introduction, contributions by archaeology and biological anthropology to our understanding of colonisation processes and prehistoric demography have remained overlooked in genetic studies and other reviews exploring the possibility of 'thrifty genes' among Pacific Islanders – and indeed, among other populations globally. When the evolutionary and cultural history of Pacific people is considered, the application of the 'Thrifty Genotype' hypothesis is no longer useful; the scenario certainly does not mesh with our current understandings. The continued reiteration of the 'Thrifty Genotype' hypothesis in general and in its application to Pacific populations in particular gets in the way of our formulating alternative, better supported and testable hypotheses based on our knowledge of the context and histories of the populations in question. It is our suggestion that alternative hypotheses, such as selection by infectious disease exposures (Gosling et al., 2014), need to be explored.

Conflict of Interests Disclosure

The authors declare no conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Type 2 diabetes prevalence estimates for 2013 for Pacific Island populations as reported by the International Diabetes Federation (http://www.idf.org/atlasmap/atlasmap) and age-standardised prevalence of obesity (BMI $\geq$30 kg/m^2) in Pacific Island populations as of 2008 as reported by the WHO (http://apps.who.int/gho/data/view.main.2450?lang=en). New Zealand Māori and Pacific data as reported by NZ Ministry of Health (http://www.health.govt.nz/system/files/documents/publications/health-of-new-zealand-adults-2011-12-v2.pdf). Data for Native Hawaiians as reported by the University of Hawaii (http://www.oha.org/health/downloads/2011NativeHawaiianHealthFactSheet.pdf).

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8.2 Appendix 2: Historic Newspaper articles regarding gout

Excerpts from regional newspapers in New Zealand from the 19th and early 20th centuries mentioning gout in Maori persons, assessed via Papers Past (http://paperspast.natlib.govt.nz/cgi-bin/paperspast)

New Zealand Herald, 28 June 1865	Another chief, Winiata, appeared to afford some amusement to his own *confreres*, by the fact of his having to come to Court with a long pole and his boot off, actually troubled by that peculiar disease only claimed by English aristocracy as their hereditary right - the "gout". Probably Uiniata [sic.] was aware of this complaint being a sign of good blood, for in spite of the twinges by which he evidently suffered during his stay in Court, he evidently bore the "chaffing" of his friends with the greatest nonchalance, and almost looked proud of being the possessor of such a proof of his descent. Dr Nicholson informed me it was the only case he ever met with among the natives, and is conclusive evidence that we can boast of the best blood among our Kaipara natives as well as amongst the Pakehas.
Wellington Independent, 1 July 1872	When one hears of these Māoris purchasing steamers, owning flour mills, drawing large incomes from royalties on gold mines, and engaging in commerce and agriculture with intelligence and success, and when one sees the artistic and technical ability which many of them exhibit in the construction and decoration of their houses and canoes, as well as in their carvings of wood, bone and ivory, one is driven to acknowledge that here is a people whom it would be an act of folly to despise, and whom it would be a flagitious crime to exterminate, even supposing we could do so in fair and open warfare. There is lying before me at this moment a photographic portrait of a chief at Timaru, an elderly man with white hair, moustache, and Dundreary whiskers, whom I would be easy to mistake for an English gentleman farmer, with a remarkably fine head on his shoulders, and just a touch of gout in his feet...
New Zealand Herald, 3 May 1879	Paul Tuhaere, of Orakei, is here, but ill with rheumatic gout. Sir George visited him this afternoon.

The Press, Volume XXXI, 3 May 1879	Sir G. Grey and Mr Sheehan and five or six other Europeans arrived at 3 o'clock in the steamer Lily. They were received with three cheers by the crowd, and Te Wheoro's Natives assembled on the bridge……. Further on another assemblage of Paul's tribe from Auckland and Kaipara, gave a similar welcome. Minister's paid a visit to Paul, who has been suffering from an attack of gout.
Thames Advertiser, 5 May 1879	On their arrival to Te Kopua the canoes were met by the chief Paul, of Orakei, and other friendlies, who greeted Sir George Grey from the river bank……. Paul, of Orakei, whose people were guests on the occasion, was mounted on a charger, as he suffers acutely from gout.
New Zealand Herald, 1 December 1882	They were also entitled to the consideration that in what they had done or what they had alleged to have done, they were acting under the instigation of their chief, Henare Potae, who was equally if not more to blame than the defendants, although, from his being an infirm man, a martyr to gout and dropsy, he had kept in the background himself.
New Zealand Herald, 1 April 1884	The reason why Paora Tuhaere, of Orakei, abandoned the idea of going to England with Tawhaio and party was a sudden access [sic.] of rheumatism and gout. It is said that the natives are not all satisfied on that head, and are under the belief that the Government in order to prevent Paul from going, have employed other Māoris to makutu him, and give him rheumatism and gout. The old leaven of superstition has evidently not yet been got out of the Māori mind.
Wanganui Herald, 24 July 1884	The Māori party are very diverse in their appearance, although they are all able to maintain the same impenetrable reserve…… Topai Turoa, a chief of high rank, is the oldest and most infirm member of the embassy. He is afflicted with rheumatic gout, leans heavily on a stick, and moves about with some difficulty.
Grey River Argus, 22 October 1885	Suicide of Ihaia Tainui [West Coast Times] An inquest was held at the Māori School House yesterday (Tuesday 20th) morning, before J. Giles, Esq., Coroner, and a jury of six, over the body of Ihaia Tainui…….Dr King stated he had examined the body, and deposed that death was caused by asphyxia, induced by strangulation. He had attended Tainui recently for gout, and thought it likely that an attack of this might, in first instance, have caused some despondency and gloomy forebearings….
Evening Post, 6 December 1886	We regret to have to chronicle the death of the influential chief Ihaia Porutu, who passed away last night at his residence, Pipitea Point, at the age of 69. The deceased has been subject to chronic rheumatic gout for some twenty years, and to this and a

	complication of other disorders, his dissolution has been due.
Taranaki Herald, 8 December 1886	Isaiah Porutu, the most important chief of the Ngatiawa tribe, and the firm friend, ally, and protector of the early colonists died last week. His family originally came from Taranaki, and he was a near relation of Te Whiti, his father having been a brother of Te Whiti's mother..... He had been an invalid for about 20 years, and for the last few years had been almost bedridden, except that on fine days he was wheeled in a chair about the town. He suffered from chronic rheumatic gout, and a complication of other disorders.
Dominion, 30 September 1911	The House met at 2.30 pm. Mr A.E. GLOVER (Auckland Central) moved: "That fourteen days' leave be granted to Mr Kaihau, member for the Western Māori district on account of illness." ..... Mr Glover said that he had visited Mr Kaihau in Auckland a few days ago, and found that the member was suffering from a severe attack of gout, from which he would not recover for some months..... The desired leave was granted on the voices.
Ashburton Guardian, 2 October 1911	Mr Glover (Auckland West) moved to grant fourteen days' leave of absence to the Western Māori member (Mr H. Kaihau), on account of illness..... Mr Kaihau was suffering from gout and a kind of rheumatic fever.
Ellesmere Guardian, 27 April 1921	There was one man of 70 who had been crippled with gout for 15 years. He got about with the aid of two crutches and was doubled up with his affliction. Ratana took his crutches away from him and told the patient to come to him.....
NZ Truth, 13 August 1921	Two of his patients at the Pa claim that Ratana's work was successful in relieving them from their bodily ills. One of these was a venerable Māori of Rapaki - by the name of Hone Tikau. For many years he had been a martyr to gout and rheumatism, and latterly had been a victim to insomnia. He saw Ratana on the Sunday during the week-end visit of the Māori healer..... The old Māori who has not been able to travel for thirteen years, went on with Ratana to Temuka.

8.3 Appendix 3: Supplementary tables from Chapter 5

Table 8.1: Distribution of haplogroups between gout cases and healthy controls. NB. The total number of individuals is lower than the total number of samples sequenced because some individuals had incomplete gout data

	Combined Polynesians		West Polynesians		East Polynesians		
Haplogroup	**Gout (%)**	**No gout(%)**	**Gout (%)**	**No gout(%)**	**Gout (%)**	**No gout(%)**	**Total (%)**
	n = 327	n=112	n = 177	n= 50	n = 150	n = 62	n = 439
R	317 (96.9%)	106 (94.6%)	144 (96.6%)	46 (92.0%)	146 (97.3%)	60 (96.8%)	423 (96.4%)
B4a1a1	94 (28.7%)	26 (23.2%)	74 (41.8%)	16 (32.0%)	20 (13.3%)	10 (16.1%)	120 (27.3%)
B4a1a1a	35 (10.7%)	9 (8.0%)	35 (19.8%)	9 (18.0%)	…	…	44 (10.0%)
B4a1a1a1	1 (0.3%)	…	1 (0.6%)	…	…	…	4 (0.9%)
B4a1a1a1c*	…	3 (2.7%)	…	3 (6.0%)	…	…	3 (0.7%)
B4a1a1a8	9 (2.8%)	2 (1.8%)	7 (4.0%)	2 (4.0%)	2 (1.3%)	…	11 (2.5%)
B4a1a1a9	1 (0.3%)	1 (0.9%)	1 (0.6%)	1 (2.0%)	…	…	2 (0.5%)
B4a1a1a11	2 (0.6%)	…	2 (1.1%)	…	…	…	2 (0.5%)
B4a1a1a11a	3 (0.9%)	…	3 (2.0%)	…	…	…	3 (0.7%)
B4a1a1a13	1 (0.3%)	…	1 (0.7%)	…	…	…	1 (0.2%)
B4a1a1a14*	6 (1.8%)	3 (2.7%)	1 (0.7%)	1 (2.0%)	5 (3.3%)	2 (3.2%)	9 (2.1%)
B4a1a1a15*	5 (1.5%)	4 (3.6%)	…	…	5 (3.3%)	4 (6.5%)	9 (2.1%)
B4a1a1a16*	3 (0.9%)	1 (0.9%)	3 (1.7%)	1 (2.0%)	…	…	4 (0.9%)
B4a1a1a17*	5 (1.5%)	2 (1.8%)	1 (0.6%)	…	4 (2.7%)	2 (3.2%)	7 (1.6%)
B4a1a1c	36 (11.0%)	17 (15.2%)	1 (0.6%)	…	35 (23.3%)	17 (27.4%)	53 (12.1%)
B4a1a1k	2 (0.6%)	1 (0.9%)	2 (1.1%)	1 (2.0%)	…	…	3 (0.7%)
B4a1a1m	65 (19.9%)	18 (16.1%)	17 (9.6%)	7 (14.0%)	48 (32%)	11 (17.8%)	83 (18.9%)
B4a1a1n	6 (1.8%)	2 (1.8%)	1 (0.6%)	…	5 (3.3%)	2 (3.2%)	8 (1.8%)
B4a1a1r**	9 (2.8%)	4 (3.6%)	3 (1.7%)	…	6 (4.0%)	4 (6.5%)	13 (3.0%)
B4a1a1x	…	1 (0.9%)	…	1 (2.0%)	…	…	1 (0.2%)
B4a1a1z*	5 (1.5%)	4 (3.6%)	1 (0.6%)	…	4 (2.7%)	4 (6.5%)	9 (2.1%)
B4a1a1aa*	6 (1.8%)	…	5 (2.8%)	…	1 (0.7%)	…	6 (1.4%)
B4a1a1ab*	7 (2.1%)	2 (1.8%)	2 (1.1%)	1 (2.0%)	5 (3.3%)	1 (1.6%)	9 (2.1%)
B4a1a1ac*	5 (1.5%)	2 (1.8%)	3 (1.7%)	2 (4.0%)	2 (1.3%)	…	7 (1.6%)
B4b1a2	2 (0.6%)	…	2 (1.1%)	…	…	…	2 (0.5%)
P1d	2 (0.6%)	1 (0.9%)	2 (1.1%)	1 (2.0%)	…	…	3 (0.7%)
M	10 (3.1%)	6 (5.5%)	6 (3.4%)	4 (8.0%)	4 (2.7%)	2 (3.2%)	16 (3.6%)
M28a	2 (0.6%)	2 (1.8%)	2 (1.1%)	2 (4.0%)	…	…	4 (0.9%)
M28b	1 (0.3%)	…	1 (0.6%)	…	…	…	1 (0.2%)
Q1	7 (2.1%)	4 (3.6%)	3 (1.7%)	2 (4.0%)	4 (2.7%)	2 (3.2%)	11 (2.5%)

Table 8.2: Frequency of haplogroups in various Pacific sub-populations included in this study

Haplogroup	NZ Maori	Cook Islands	Pukapuka	Samoa	Tonga	Niue
B4a1a	-	-	-	4 (0.03)	2 (0.04)	1 (0.04)
B4a1a1	23 (0.16)	8 (0.12)	-	53 (0.40)	17 (0.39)	8 (0.31)
B4a1a1a	10 (0.07)	2 (0.03)	-	36 (0.27)	6 (0.12)	5 (0.19)
B4a1a1a1c*	-	-	-	4 (0.03)	2 (0.04)	-
B4a1a1a5	-	-	-	1 (<0.01)	-	-
B4a1a1a8	-	2 (0.03)	-	2 (0.02)	1 (0.02)	6 (0.23)
B4a1a1a9	-	-	-	2 (0.02)	-	-
B4a1a1a11	-	-	-	2 (0.02)	-	-
B4a1a1a11a	-	-	-	2 (0.02)	1 (0.02)	-
B4a1a1a13	-	-	-	1 (<0.01)	-	-
B4a1a1a14*	7 (0.05)	2 (0.03)	-	-	-	-
B4a1a1a15*	-	-	-	3 (0.02)	1 (0.02)	-
B4a1a1a16*	6 (0.04)	1 (0.01)	-	-	1 (0.02)	-
B4a1a1c	43 (0.30)	9 (0.13)	1 (0.07)	-	1 (0.02)	-
B4a1a1k	-	-	-	1 (<0.01)	2 (0.04)	-
B4a1a1m	32 (0.23)	28 (0.41)	11 (0.73)	4 (0.03)	7 (0.14)	2 (0.08)
B4a1a1n	4 (0.03)	3 (0.04)	-	-	1 (0.02)	-
B4a1a1r*	8 (0.06)	2 (0.03)	-	3 (0.02)	-	-
B4a1a1x	-	-	-	-	-	-
B4a1a1z*	6 (0.04)	2 (0.03)	-	-	1 (0.02)	-
B4a1a1aa*	-	1 (0.01)	-	2 (0.02)	-	4 (0.14)
B4a1a1ab*	3 (0.02)	1 (0.01)	-	2 (0.02)	-	-
B4a1a1ac*	-	2 (0.03)	2 (0.13)	2 (0.02)	-	-
B4b1a2	-	-	-	-	1 (0.02)	-
M28a	-	-	-	2 (0.02)	2 (0.04)	-
M28b	-	-	-	-	1 (0.02)	-
P1d	-	-	-	1 (<0.01)	2 (0.04)	-
Q1	-	6 (0.09)	1 (0.07)	4 (0.03)	-	-
Total	142	69	15	131	49	26

Table 8.3: Results of linear regression testing for association between triglycerides, HDL, serum urate levels and mitochondrial haplogroup. Adjusted for age, East versus West Polynesian ancestry and self-declared proportion of Polynesian grandparents

Population	Haplogroup	*n*	*Triglycerides* β (std error)	*P*	*HDL* β (std error)	*P*	*Urate* β (std error)	*P*
All	B4a1a1+16126	13	-0.52 (0.58)	0.37	0.04 (0.11)	0.73	0.01 (0.03)	0.73
(n = 394)	B4a1a1a	78	0.17 (0.26)	0.51	-0.002 (0.05)	0.98	0.01 (0.02)	0.56
	B4a1a1a8	9	1.51 (0.60)	0.01*	-0.009 (0.12)	0.94	-0.03 (0.04)	0.38
	B4a1a1c	53	-0.05 (033)	0.89	-0.05 (0.07)	0.46	0.05 (0.02)	0.01*
	B4a1a1m	83	-0.31 (0.27)	0.24	0.04 (0.05)	0.40	0.02 (0.02)	0.19
	Q1	11	0.89 (0.57)	0.12	0.12 (0.11)	0.30	0.02 (0.03)	0.65
EP	B4a1a1a	27	0.07 (0.37)	0.86	0.03 (0.08)	0.76	0.003 (0.02)	0.91
(n = 188)	B4a1a1c	52	-0.44 (0.38)	0.24	-0.03 (0.09)	0.70	0.003 (0.02)	0.91
	B4a1a1m	59	-0.56 (0.34)	0.10	0.06 (0.08)	0.46	0.02 (0.02)	0.37
WP	B4a1a1a	51	0.22 (0.27)	0.42	-0.02 (0.06)	0.75	0.01 (0.02)	0.52
(n = 171)	B4a1a1m	24	0.003 (0.32)	0.99	-0.04 (0.11)	0.70	0.01 (0.02)	0.54

Table 8.4: The results for linear regression predicting various metabolic markers by mitochondrial variants in 213 East Polynesian men. These have been adjusted by age and proportion of self-declared Polynesian grandparental ancestry

Variant	Alt Allele Freq	*Triglycerides* β (std error)	*P*	*HDL* β (std error)	*P*	*Urate* β (std error)	*P*
C151T	0.28	-0.58 (0.34)	0.09	0.06 (0.06)	0.30	0.01 (0.02)	0.47
310T/TC	0.91	0.71 (0.56)	0.21	-0.01 (0.10)	0.89	-0.01 (0.03)	0.65
C1185T	0.24	-0.25 (0.39)	0.51	-0.01 (0.07)	0.83	-0.02 (0.02)	0.23
A1692G	0.28	-0.52 (0.35)	0.14	0.07 (0.06)	0.29	0.01 (0.02)	0.49
T2416C	0.28	-0.50 (0.35)	0.16	0.06 (0.06)	0.36	0.01 (0.02)	0.44
A4769G	0.76	0.27 (0.38)	0.48	0.02 (0.07)	0.68	0.02 (0.02)	0.27
T5465C	0.97	-0.90 (0.61)	0.14	-0.13 (0.11)	0.24	-0.005 (0.03)	0.88
T6719C	0.97	-0.90 (0.61)	0.14	-0.13 (0.11)	0.24	-0.005 (0.03)	0.88
A6905G	0.14	0.48 (0.37)	0.19	-0.02 (0.07)	0.78	-0.005 (0.03)	0.81
G9123A	0.97	-0.90 (0.61)	0.14	-0.13 (0.11)	0.24	-0.005 (0.03)	0.88
T10238C	0.97	-0.90 (0.61)	0.14	-0.13 (0.11)	0.24	-0.005 (0.03)	0.88
C12239T	0.97	-0.98 (0.58)	0.10	-0.09 (0.10)	0.37	-0.02 (0.03)	0.51
A14022G	0.97	-0.90 (0.61)	0.14	-0.13 (0.11)	0.24	-0.005 (0.03)	0.88
A15746G	0.97	-0.90 (0.61)	0.14	-0.13 (0.11)	0.24	-0.005 (0.03)	0.88
A16183C	0.93	-0.37 (0.49)	0.45	-0.07 (0.09)	0.42	0.01 (0.03)	0. 55
T16189C	0.96	-0.99 (0.61)	0.11	-0.08 (0.11)	0.44	-0.004 (0.03)	0.91
T16217C	0.96	-0.99 (0.61)	0.11	-0.08 (0.11)	0.44	-0.004 (0.03)	0.91
A16247G	0.89	-0.57 (0.38)	0.14	0.00 (0.07)	1.00	-0.01 (0.02)	0.61
C16261T	0.97	-0.62 (0.57)	0.27	-0.10 (0.10)	0.31	0.004 (0.03)	0.90

Table 8.5: The results for linear regression predicting various metabolic markers by mitochondrial variants in 210 West Polynesian men. These have been adjusted by age and proportion of self-declared Polynesian grandparental ancestry

Variant	Alt Allele Freq	*Triglycerides* β (std error)	*P*	*HDL* β (std error)	*P*	*Urate* β (std error)	*P*
C151T	0.12	-0.14 (0.28)	0.63	0.11 (0.06)	0.08	-0.01 (0.02)	0.51
310T/TC	0.99	0.47 (0.40)	0.24	-0.04 (0.09)	0.66	0.03 (0.03)	0.36
A1692G	0.09	-0.13 (0.29)	0.65	0.09 (0.06)	0.14	0.00 (0.02)	0.97
T2416C	0.11	-0.18 (0.28)	0.53	0.10 (0.06)	0.12	0.00 (0.02)	0.90
T5465C	0.93	-0.09 (0.54)	0.86	0.21 (0.12)	0.08	0.04 (0.04)	0.25
T6719C	0.93	-0.09 (0.54)	0.86	0.21 (0.12)	0.08	0.04 (0.04)	0.25
A6905G	0.33	0.10 (0.24)	0.67	-0.04 (0.05)	0.45	0.00 (0.02)	0.56
G9123A	0.93	-0.09 (0.54)	0.86	0.21 (0.12)	0.08	0.04 (0.04)	0.25
T10238C	0.93	-0.09 (0.54)	0.86	0.21 (0.12)	0.08	0.04 (0.04)	0.25
C12239T	0.93	-0.09 (0.54)	0.86	0.21 (0.12)	0.08	0.04 (0.04)	0.25
A14022G	0.93	-0.09 (0.54)	0.86	0.21 (0.12)	0.08	0.04 (0.04)	0.25
A15746G	0.93	-0.09 (0.54)	0.86	0.21 (0.12)	0.08	0.04 (0.04)	0.25
A16183C	0.92	0.28 (0.46)	0.55	0.09 (0.10)	0.37	0.03 (0.03)	0. 34
T16189C	0.94	0.24 (0.54)	0.65	0.11 (0.12)	0.37	0.04 (0.04)	0.27
T16217C	0.94	0.35 (0.57)	0.53	0.10 (0.13)	0.44	0.04 (0.04)	0.33
A16247G	0.80	-0.32 (0.30)	0.28	0.03 (0.07)	0.70	0.00 (0.02)	0.82
C16261T	0.91	0.02 (0.44)	0.96	0.06 (0.10)	0.52	0.02 (0.03)	0.46

Table 8.6: Mean metabolic characteristics for the 1019 Polynesians who were genotyped for position 6905 for replication

Variable	WP		EP		Combined	
	High BMI	**Low BMI**	**High BMI**	**Low BMI**	**High BMI**	**Low BMI**
n	233	173	251	362	484	535
Sex (Male/Female)	150/83	116/57	98/153	165/197	248/236	281/254
Age	43.2±0.9	38.4±1.1	49.9±0.9	48.5±0.9	46.7±0.7	45.2±0.7
% Polynesian Ancestry	0.94±0.01	0.90±0.01	0.86±0.01	0.83±0.01	0.90±0.01	0.85±0.01
BMI (kg/m²)	40.6±0.5	30.0±0.2	41.0±0.5	28.8±0.2	40.8±0.3	29.2±0.2
Triglycerides (mmol/L)	2.31±0.11	2.10±0.11	2.43±0.11	2.11±0.08	2.37±0.08	2.11±0.06
HDL (mmol/L)	1.06±0.02	1.15±0.03	1.12±0.03	1.22±0.02	1.09±0.02	1.20±0.02
Serum Urate (mmol/L)	0.44±0.01	0.40±0.01	0.40±0.01	0.37±0.01	0.42±0.01	0.38±0.00

8.4 Appendix 4: Supplementary material from Chapter 6

8.4.1 MtDNA qPCR standard curves

After it became clear that the probes did not appear to be working optimally after creating a standard curve with a 1:2 dilution series (Figure 8.1), further standard curves were run with concentrations expanding over a wider log range that gives a more accurate calculation of PCR efficiency.

Apparently high PCR efficiency usually results from some kind of inhibition going on in a PCR reaction. In this case, it seems that there may be competition going on between the mitochondrial assay and the reference assay – especially given that the mitochondria exist in such high copy number per cell compared to the nuclear genome. With an exponential increase in DNA, the reaction is likely to be quickly overrun with mtDNA product.

To explore this problem further, standard curves were carried out with the mitochondrial and the references probes separately, as well as with a variety of dilutions of the mitochondrial probe to test whether this impacts the efficiency of the reference assay. Decreasing the amount of mitochondrial probe did seem to improve the assay down to a concentration of 0.25x, however past this point, the assay seemed to decrease in efficiency.

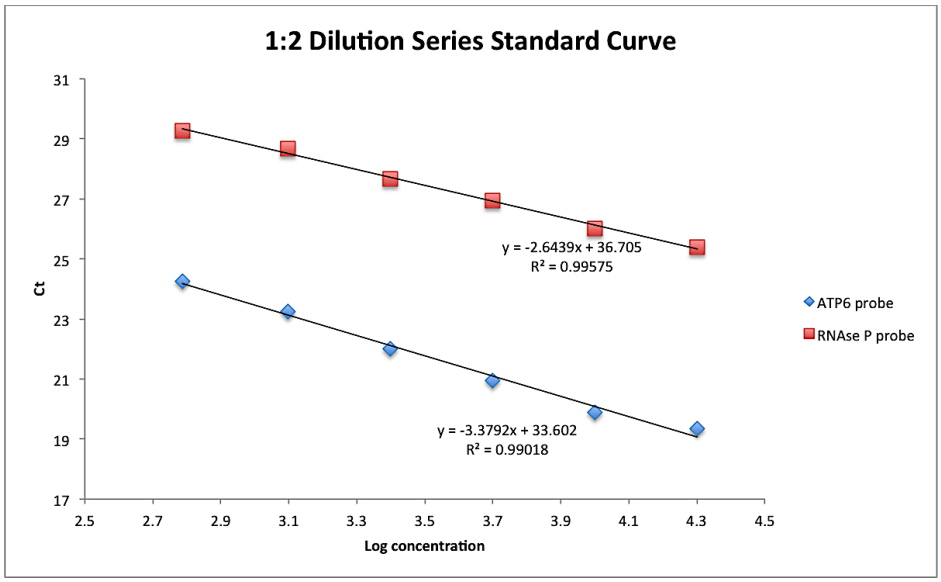

Figure 8.1: 1:2 dilution series standard curve, efficiency calculated for mtDNA probe 97.6%; efficiency of the RNase P probe 138.9%

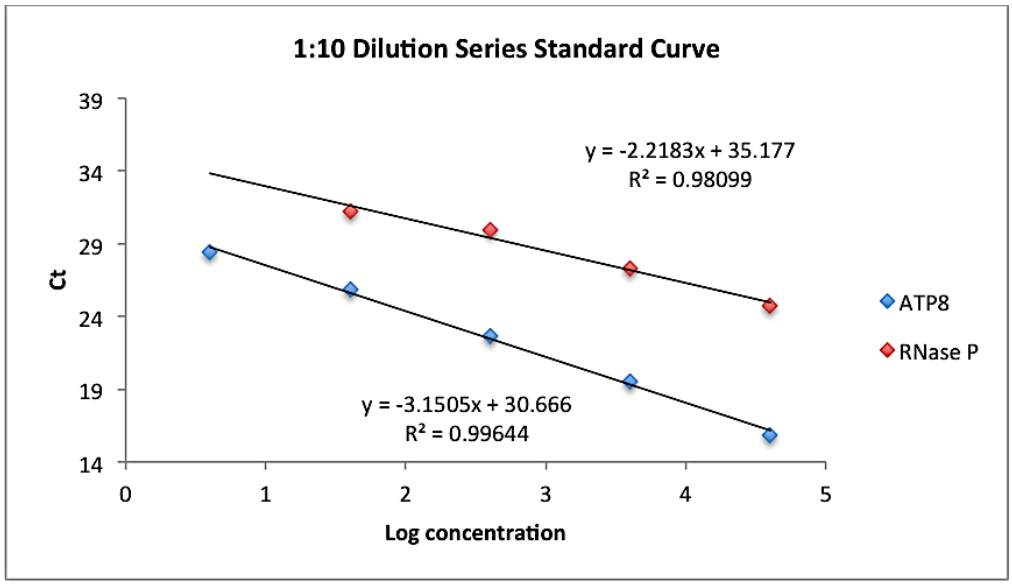

Figure 8.2: 1:10 dilution series standard curve, efficiency calculated for mtDNA probe 100.3%; efficiency of the RNase P probe 182.4%

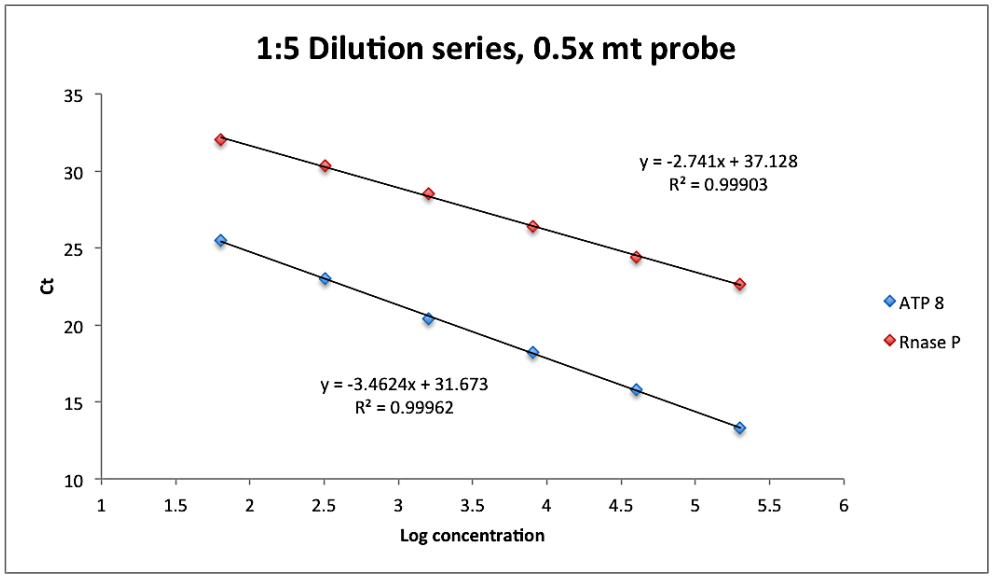

Figure 8.3: 1:5 dilution series standard curve with 0.5x mitochondrial probe, efficiency calculated for mtDNA probe 94%; efficiency of the RNase P probe 132%

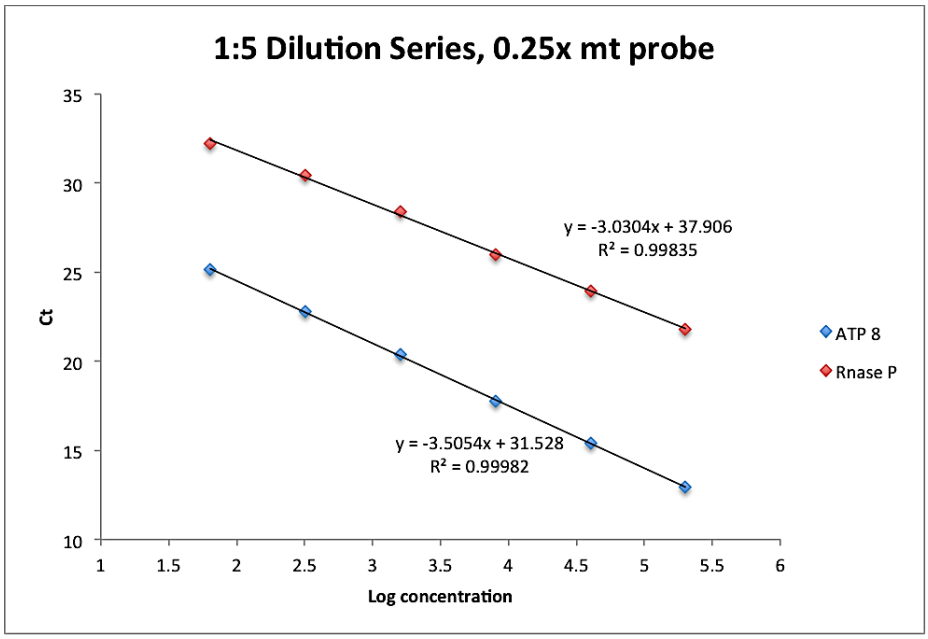

Figure 8.4: 1:5 dilution series standard curve with 0.25x mitochondrial probe, efficiency calculated for mtDNA probe 93%; efficiency of the RNase P probe 114%

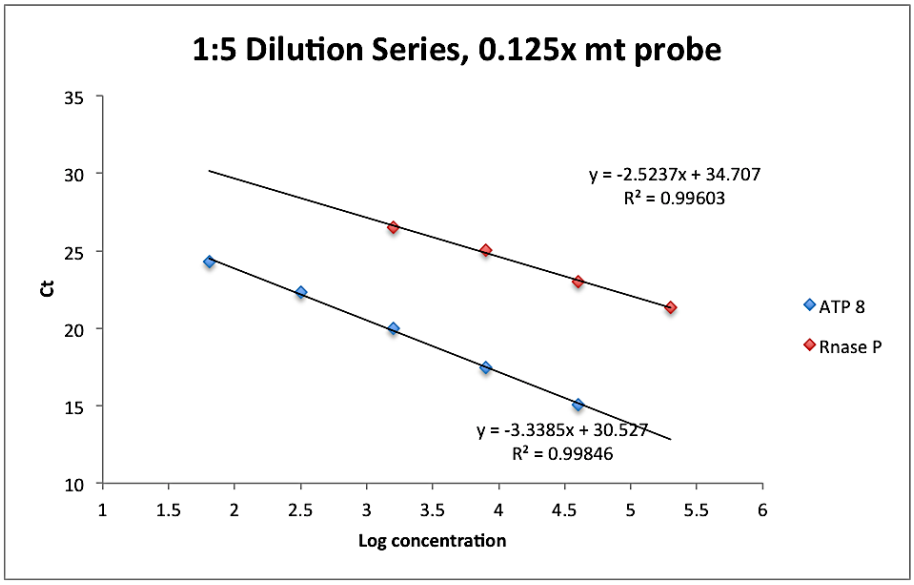

Figure 8.5: 1:5 dilution series standard curve with 0.125x mitochondrial probe, efficiency calculated for mtDNA probe 99%; efficiency of the RNase P probe 149%

Reducing the amount of mitochondrial probe used did initially look like it was improving the assay, and the calculated efficiency of the RNase P probe dropped closer to 100% (Figure 8.3, Figure 8.4), however, this effect dropped off as the mitochondrial probe was further decreased in amount (Figure 8.5).

The efficiency of the assays were then tested in single-plex to make sure that without added competition of another assay being run alongside, the probes were acting efficiently. For the

RNase P reference probe, this appeared to be the case, with the efficiency tested at 94% (Figure 8.6), however, the mitochondrial assay was less efficient with a calculated efficiency of 125% (Figure 8.7).

This means that the assays, contrary to assumption, were not necessarily pre-optimised which has consequences regarding the data and the way it can be analysed.

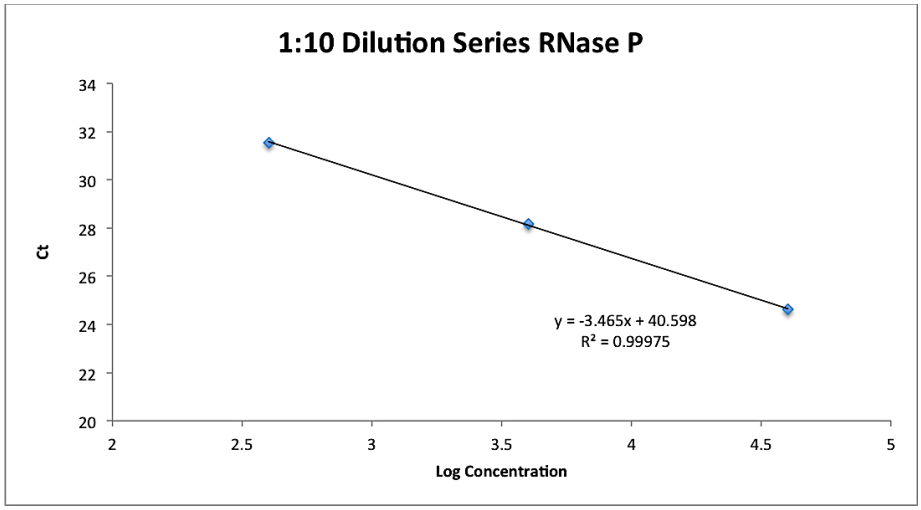

Figure 8.6: 1:10 dilution series standard curve with RNase P probe only (low concentrations of DNA failed to work here), efficiency calculated for probe was 94%

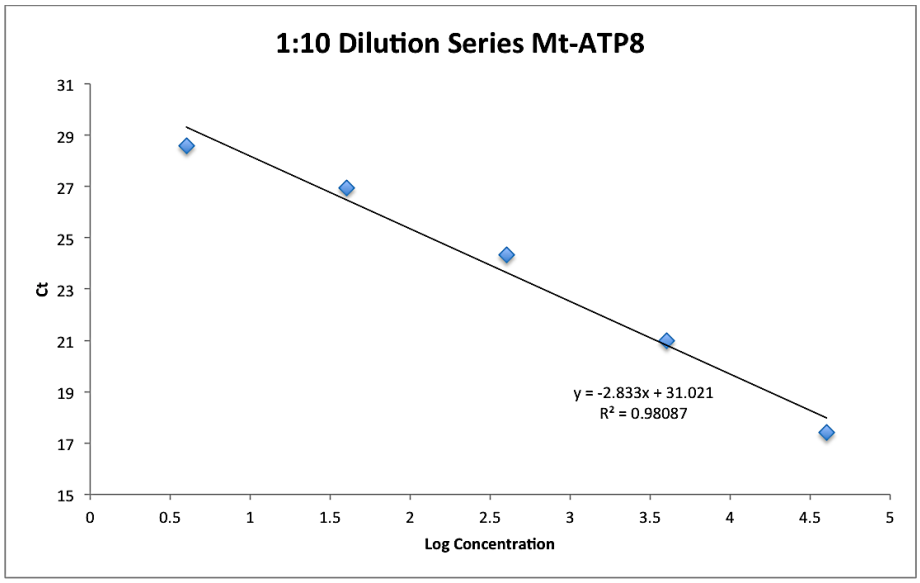

Figure 8.7: 1:10 dilution series standard curve with mitochondrial ATP8 probe only, efficiency calculated for probe was 125%

8.4.2 Supplementary statistics tables

Table 8.7: qPCR results with those individuals who were sequenced in the Whole Genome and Resequencing Cohorts excluded

Sample set	n	Gout cases/controls	OR (95% CI)	Pr (>|z|)
All Polynesians	379	191/188	1.07 (0.78-1.46)	0.68
East Polynesians	213	111/102	1.21 (0.82-1.79)	0.32
West Polynesians	166	80/86	0.89 (0.49-1.58)	0.70
Males	239	158/81	1.09 (0.75-1.61)	0.64
Females	140	33/107	0.91 (0.50-1.64)	0.76
Hyperuricaemics	103	85/18	1.03 (0.45-2.34)	0.95

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Figure 6.10**:** Electon micropscope images of mitochondrial morphology in carotid artherosclerosis patients compared with a healthy control. Originally published by Sobenin et al. (2013). Permission for image reuse not required as image was published in PLOSone, and thus open access.

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