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Molecular genetics of type 2 diabetes in New Zealand Polynesians

Nicola Renee Poa

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

November 2001

School of Biological Sciences
The University of Auckland
In memory of my grandfather

Leopold Pritchard
Abstract

The risk of developing type 2 diabetes is four fold higher in New Zealand (NZ) Polynesians compared to Caucasians. Hence diabetes is more prevalent in Maori (16.5% of the general population) and Pacific Island people (10.1%) compared to NZ Caucasians (9.3%). It is generally accepted that type 2 diabetes has major genetic determinants and heterozygous mutations in a number of genes have previously been identified in some subsets of type 2 diabetes and certain ethnic groups. The high prevalence of diabetes in NZ Polynesians, when compared with NZ Caucasians, after controlling for age, income and body mass index (BMI), suggest that genes may be important in this population. Therefore, the prevalence of allelic variations in the genes encoding amylin and insulin promoter factor-1 (IPF-1), and exon 2 of the hepatocyte nuclear factor-1α (HNF-1α) gene in NZ Polynesians with type 2 diabetes was determined. These genes are known to produce type 2 diabetes in other populations. The genes investigated were screened for mutations by PCR amplification and direct sequencing of promoter regions, exons and adjacent intronic sequences from genomic DNA. DNA was obtained from 146 NZ Polynesians (131 Maori and 15 Pacific Island) with type 2 diabetes and 387 NZ Polynesian non-diabetic control subjects (258 Maori and 129 Pacific Island). Sequences were compared to previously published sequences in the National Centre for Biotechnology Information database. Allelic variations in IPF-1 and exon 2 of the HNF-1α gene were not associated with type 2 diabetes in NZ Polynesians. However, in the amylin gene, two new and one previously described allele was identified in the Maori population including: two alleles in the promoter region (−132G>A and −215T>G), and a missense mutation in exon 3 (Q10R). The −215T>G allele was observed in 5.4% and 1% of type 2 diabetic and non-diabetic Maori respectively, and predisposed the carrier to diabetes with a relative risk of 7.23. The −215T>G allele was inherited with a previously described amylin promoter polymorphism (-230A>C) in 3% of Maori with type 2 diabetes, which suggests linkage equilibrium exists between these two alleles. Both Q10R and −132G>A were observed in 0.76% of type 2 diabetic patients and were absent in non-diabetic subjects. Together these allelic variations may account for approximately 7% of type 2 diabetes in Maori. These results suggest that the amylin gene maybe an important candidate marker gene for type 2 diabetes in Maori.
Preface

This work was carried out between January 1998 and January 2001 in the School of Biological Sciences, University of Auckland. This thesis is submitted for examination purposes only.

I would like to thank Dr. Shaoping Zhang for her advice and guidance during these three years of study, for her technical support and constructive criticism of this thesis. I would like to thank Dr. Gilgen for consultation with Tainui Iwi, for assisting with recruiting participants and collecting bloods; Dr. David Simmons for providing Pacific Island bloods; Tracy Ellison and the Hepatitis foundation for providing Maori non-diabetic bloods and measuring HbA$_{1c}$ levels; Dr. Tony Birch and Nan Neho for consultation with Ngapuhi Iwi, for assisting with recruiting participants and collecting bloods. I would also like to acknowledge Liam Williams and Kristine Boxen for their contribution to DNA Sequencing and technical support; Dr. Junxi Liu for her contribution to experiments and technical support. I would like to express my sincere thanks and appreciation to Dr. Paul Edgar for his constructive criticism and contribution to the preparation of this thesis, and for the tutelage and support he has provided throughout this study. This work was supported by grants from the Health Research Council of New Zealand.

I declare that this thesis is the result of my own work, includes nothing which is the outcome of this work done in collaboration and has not been submitted in whole or part to any other university.
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Abbreviations

Abbreviations used in the text are described below. Unit abbreviations are described in S.I. [System Internationale (d’Unites)] form, and standard notations are used for chemical formulae.

<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>A</td>
<td>adenine</td>
</tr>
<tr>
<td>Aβ</td>
<td>beta amyloid</td>
</tr>
<tr>
<td>ABI</td>
<td>Applied Biosystems Incorporated</td>
</tr>
<tr>
<td>AD</td>
<td>alzheimer’s disease</td>
</tr>
<tr>
<td>AP-1</td>
<td>activated protein –1</td>
</tr>
<tr>
<td>ApoE</td>
<td>apolipoprotein E</td>
</tr>
<tr>
<td>ATP</td>
<td>adenine triphosphate</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>β3AR</td>
<td>β-3-adrenergic receptor</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>C</td>
<td>cytosine</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CAT</td>
<td>chloroamphenicol acetyltransferase</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CAAT/enhancer binding protein</td>
</tr>
<tr>
<td>CPE</td>
<td>carboxypeptidase E</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP-response element</td>
</tr>
<tr>
<td>CREB</td>
<td>CRE-binding protein</td>
</tr>
<tr>
<td>dATP</td>
<td>deoxyadenosine triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxycytosine triphosphate</td>
</tr>
<tr>
<td>dDTP</td>
<td>deoxythymidine triphosphate</td>
</tr>
<tr>
<td>del</td>
<td>deletion</td>
</tr>
<tr>
<td>dGTP</td>
<td>deoxyguanosine triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>ESRF</td>
<td>end stage renal failure</td>
</tr>
<tr>
<td>FABP</td>
<td>fatty acid binding protein</td>
</tr>
<tr>
<td>fsdel</td>
<td>frame shift deletion</td>
</tr>
<tr>
<td>fsins</td>
<td>frame shift insertion</td>
</tr>
<tr>
<td>Grb2</td>
<td>growth factor receptor binding protein-2</td>
</tr>
<tr>
<td>GLUT</td>
<td>glucose transporter</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>HbA1c</td>
<td>glycosylated haemoglobin</td>
</tr>
<tr>
<td>HDL</td>
<td>high density lipoprotein</td>
</tr>
<tr>
<td>HLA</td>
<td>histocompatibility leucocyte antigen</td>
</tr>
<tr>
<td>HLH</td>
<td>helix loop helix</td>
</tr>
<tr>
<td>HNF</td>
<td>hepatocyte nuclear factor</td>
</tr>
<tr>
<td>HOMA</td>
<td>homeostasis model assessment</td>
</tr>
<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
</tr>
<tr>
<td>IAPP</td>
<td>islet amyloid polypeptide</td>
</tr>
<tr>
<td>IB-1</td>
<td>islet brain-1</td>
</tr>
<tr>
<td>IDDM</td>
<td>insulin dependent diabetes mellitus</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin growth factor</td>
</tr>
<tr>
<td>IGT</td>
<td>impaired glucose tolerance</td>
</tr>
<tr>
<td>IFG</td>
<td>impaired fasting glucose</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukin-6</td>
</tr>
<tr>
<td>ins</td>
<td>insertion</td>
</tr>
<tr>
<td>IPF-1</td>
<td>insulin promoter factor-1</td>
</tr>
<tr>
<td>IRS</td>
<td>insulin receptor substrate</td>
</tr>
<tr>
<td>Isl-1</td>
<td>Lim/homeodomain gene islet-1</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>Kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo daltons</td>
</tr>
<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
</tr>
</tbody>
</table>
PI3-K  phosphotidylinositol 3-kinase
WHO  World Health Organisation
Rab  ras related protein
Rad  Ras associated with diabetes
RFLP  restriction fragment length polymorphism
RIN  rat insulinoma
RNA  ribonucleic acid
rpm  revolutions per minute
RT  room temperature
SAPK  stress activated protein kinase pathway
SDS  sodium dodecyl sulphate
SH2  src homology domain 2
SNP  single nucleotide polymorphism
T  thymidine
TCA  tricarboxylic acid
TE  tris-EDTA buffer
TGFβ  transforming growth factor-β
thT  thioflavin T
TNF  tumour necrosis factor
tRNA  transfer ribonucleic acid
U  units
UTR  untranslated region
UV  ultra violet
V  volts
VLDL  very low density lipoprotein
VNTR  variable number of tandem repeats
vol  volume
w/v  weight per volume
Chapter 1: Introduction

1.1 The health of Maori & Pacific Island Polynesians

New Zealand is located in the South Pacific and a high proportion of its population is Polynesian. Maori, the tangata whenua or indigenous people of New Zealand, are descendants from Polynesians that arrived in New Zealand 800-1200 years ago (Figure1.1). Other South Pacific Island Polynesians include Western Samoans, Tongans, Cook Island Maori, Niueans and Tokelauans who migrated to New Zealand post European colonisation. In 1997 Maori and Pacific Island Polynesians comprised 15% and 5% of the total New Zealand population (3,715,700) respectively. The remaining ethnic populations include Caucasian (70%), Asian (5%) and other ethnic minority’s [1]. The majority of Maori live in Auckland City and the upper half of the North Island, which in contrast is largely a rural region. The Pacific Island community are predominantly urban based with approximately 66% also living in Auckland. Therefore, Auckland is the largest Polynesian populated city in the world [2].

Maori have suffered dramatic population losses through disease post-European settlement. Despite demographic recovery over the last century Maori men and women have a much lower life expectancy (67.2 and 71.6 years respectively) compared to the total New Zealand population (74.3 and 79.6 years) [1]. In New Zealand, both Maori and Pacific Island Polynesians have relatively poor health status, which can be related to living conditions, smoking, exercise and diet. However, the consensus is that it is mostly due to poor social-economic status.

In 1997, mortality rates in Maori were twice that of non-Maori. The leading cause of death for the combined New Zealand population was ischaemic heart disease followed by lung cancer, diabetes, cerebrovascular disease, chronic respiratory disease and breast cancer. Overall, Maori mortality rates from heart and lung disease were at least 60% higher than those for non-Maori [1]. Of particular concern are disparities between Maori and non-
Maori mortality rates for diabetes mellitus and hypertensive disease. In NZ Type 2 diabetes affects between 16.5% and 10.1% of the Maori and Pacific Island population, compared to only 9.3% of Caucasians, and is a major cause of mortality and morbidity in Polynesians (Table 1.1). Although the likelihood of death from these diseases are relatively low compared to that for cancer or heart disease, Maori are seven times more likely to die from either of these diseases compared to non-Maori [1]. A five-year survey reported that almost one-quarter of middle aged Maori known to have diabetes died within 5 years of diagnosis. Diabetes therefore contributes significantly to the relatively low survival rate of Maori into old age when compared to non-Maori [3]. In addition, mortality from diabetes is under-reported because it is often associated with other chronic diseases such as ischaemic heart disease, which is preferentially recorded as the underlying cause of death.

Figure 1.1 Map of the Pacific region illustrating the three major geographical and ethnic groups - Micronesian, Melanesian, and Polynesian [4].
Diabetes Mellitus in Polynesians

Diabetes mellitus is a group of metabolic disorders characterised by hyperglycaemia resulting from a combination of insulin resistance and defects in insulin secretion. There are two major forms of diabetes (Appendix 1.1): type 1 diabetes, also known as insulin-dependent diabetes mellitus (IDDM); and type 2 diabetes, also known as non-insulin-dependent diabetes mellitus (NIDDM). Type 1 diabetes results from insulin deficiency caused by cell-mediated autoimmune destruction of pancreatic β cells. The disease usually manifests at an early age and accounts for 10–15% of the diabetic population worldwide [5]. Type 2 diabetes is characterised by chronic hyperglycaemia resulting from a combination of insulin resistance and impaired insulin secretion. Type 2 diabetes generally develops at a later age and accounts for 85-90% of the diabetic population [5]. The remaining major forms of diabetes include “Other specific types”, such as maturity-onset diabetes of youth (MODY), and gestational diabetes (Appendix 1.1).

Like Polynesians elsewhere in the South Pacific, diabetes is almost exclusively type 2 accounting for 95% of diabetes in Maori and 85% in Pacific Island people. The prevalence of diabetes in Maori and Pacific Island people is 16.5% and 10.1% respectively compared to only 9.3% in NZ Caucasians (Table 1.1) [1]. For both Maori and Pacific Island populations, diabetes appears to be more common in men than woman. Women commonly have diabetes diagnosed during pregnancy [6]. The average age at onset in NZ Caucasians is 50-57 years, whereas Maori and Pacific Island people usually present 8-10 years earlier. Providing that life expectancy continues to improve for Maori and Pacific Island people over the next 50 years, type 2 diabetes will comprise 11.3% and 1.5% of the NZ population over 65 years of age. Since type 2 diabetes prevalence increases with age, and with an increasingly aging population, the incidence of diabetes in Maori and Pacific Island people can be expected to grow by 50% in the next 20 years compared to only 20% in Caucasians [1].
Table 1.1 Estimated prevalence (%) of type 2 diabetes (by glucose criteria) between the three major ethnic groups in New Zealand [1].

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Maori</th>
<th>Pacific Island</th>
<th>Caucasian</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 - 39</td>
<td>1.5</td>
<td>0.7</td>
<td>0.8</td>
</tr>
<tr>
<td>40 - 59</td>
<td>10.8</td>
<td>7.2</td>
<td>2.9</td>
</tr>
<tr>
<td>&gt; 60</td>
<td>16.5</td>
<td>10.1</td>
<td>9.3</td>
</tr>
</tbody>
</table>

1.1.2 Diabetes associated complications in NZ Polynesians

Maori and Pacific Island people experience high rates of diabetes related macrovascular and microvascular complications [7]. Macrovascular complications result from damage to the arterial wall, leading to cardiovascular disease. Of patients diagnosed with cardiovascular disease, 37% of Polynesians had type 2 diabetes compared to 15% of Caucasians [6]. Population studies in the U.S. and Europe report that cardiovascular disease is the principal cause of death among people with type 2 diabetes [8] and this also seems to be the case for Maori and Pacific Island people.

Microvascular complications arise from impaired blood supply to specific organs and can lead to a number of other clinical complications such as retinopathy, nephropathy, and neuropathy. Diabetic retinopathy, or eye disease, is a common cause of blindness in adults. The risk of blindness is 25 times greater in those with type 2 diabetes than in non-diabetics [9]. The overall prevalence of retinopathy in Auckland Polynesians was 16 – 44% [6]. Blindness could be attributed to diabetes in 8% of Pacific Island people and 6% of Maori but only 2% of Caucasians. Maori and Pacific Island people were also more likely to develop cataracts or receive laser treatment as a result of their diabetes [6].

Diabetes is the major cause of nephropathy and resulting end stage renal failure (ESRF) in New Zealand. The prevalence rates for ESRF in Maori, Pacific Island people and Europeans are 4.7%, 3.3% and 0.3% respectively [10]. Following cardiovascular disease, ESRF is the second major cause of mortality in Maori with type 2 diabetes accounting for
32.4%, 15% and 4% deaths in Maori compared to Pacific Island people and Caucasians respectively [11]. Maori and Pacific Island people with type 2 diabetes also experience elevated urinary albumin excretion (microalbuminuria) and proteinuria [12]. Microalbuminuria confers a higher risk of diabetic nephropathy and cardiovascular disease [11], while proteinuria is a predictor of ESRF in Pima Indians with type 2 diabetes [13]. The prevalence of microalbuminuria in Maori, Pacific Island people and Caucasians with diabetes is 72%, 76% and 39% respectively [14]. However, the role of microalbuminuria in Polynesian diabetic complications is unclear since there is a high incidence of this in non-diabetic Maori and Pacific Island people also.

Morbidity from neuropathy includes peripheral vascular disease, foot infection, ulceration and ultimately amputation. People with diabetes are 15 to 20 times more likely to require lower limb amputation than non-diabetics, making diabetes the major cause of lower extremity amputation in New Zealand [15]. Gangrene and ulcers of the feet are also more common in patients with diabetes. There have been no ethnic differences in diabetic amputation observed so far although foot care is poorer in Polynesians compared to Europeans.

Therefore diabetic nephropathy and cardiovascular disease together are the major causes of morbidity and premature death among Polynesians with type 2 diabetes. In addition, the apparent prevalence of nephropathy in diabetes is increasing rapidly.

1.1.3 Diabetes associated risk factors in NZ Polynesians

The main risk factors for type 2 diabetes are shown in Table 1.2. Family history is the strongest risk factor and has therefore become a focal point for intervention and modification. Individuals who are obese and have a positive family history for type 2 diabetes are ten times more likely to develop diabetes compared to individuals with obesity alone [16]. Thus, in genetically predisposed individuals, the presence of additional risk factors can exacerbate susceptibility to type 2 diabetes.
Obesity, especially android obesity, is associated with insulin resistance and hyperinsulinaemia [17]. In traditional Polynesian society obesity was regarded as a sign of high social status and beauty. Android obesity, reported to be associated with heart disease, hypertension and type 2 diabetes in Pima Indians [18], is particularly common in Maori and Pacific Island people [10]. This in part can be attributed to their larger intake of calories per day. Compared to NZ Caucasians, Maori and Pacific Island people consume larger amounts of protein, fat, saturated fat, and cholesterol, and considerably less amounts of carbohydrates, sucrose, fibre, and calcium. Standardisation for household income and education showed no significant difference in total energy intake between populations and is therefore independent of social economic status [19]. Instead, these ethnic differences in diet may reflect different cultural practices such as larger portion sizes and different cooking methods. For example, Maori and Pacific Island were reported to use butter or lard to fry or roast meats and vegetables, where as Caucasians used vegetable oils.

Individuax with android obesity and type 2 diabetes have an increased risk of multiple metabolic abnormalities collectively known as the Insulin Resistance Syndrome or Syndrome X [20]. These patients are characterised by insulin resistance, hyperinsulinaemia, hypertension, dyslipidaemia [increased very low density lipoprotein-triglyceride (VLDL-TG) and low density lipoprotein-cholesterol (LDL-C), and decreased high density lipoprotein-cholesterol (HDL-C)], all of which are established risk factors for coronary heart disease. Compared to NZ Caucasians, Maori and Pacific Island people have higher rates of coronary heart disease mortality [21]. The latter two populations also experience higher rates of blood pressure and higher levels of triglycerides, but lower levels

### Table 1.2 Main risk factors for type 2 diabetes mellitus

<table>
<thead>
<tr>
<th>Family history</th>
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<tr>
<td>Obesity (BMI ≥ 30 kg/m²)</td>
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<tr>
<td>Physical inactivity</td>
</tr>
<tr>
<td>Ethnic background</td>
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<tr>
<td>Age</td>
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<tr>
<td>Gestational diabetes</td>
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of total LDL and HDL cholesterol [22, 23]. Coronary heart disease in Maori and Pacific Island is inversely associated with serum LDL-cholesterol levels and therefore present a different clinical profile for predictive risk factors compared to NZ Caucasians.

Another risk factor for type 2 diabetes is reduced physical activity. An increase in energy expenditure of 500kcal/day reduces the risk of diabetes by 6%, even when not associated with a reduction in weight. This is evident in Aboriginal communities whereby implementation of exercise programs is associated with steady prevalence of diabetes despite significant increases in mean BMI [25]. No differences in physical activity between ethnic groups in NZ have been reported thus far. However, Polynesians living in western societies are generally less active than those living a more traditional lifestyle and are therefore less protected against the development of diabetes compared to their ancestors [4].

The incidence of insulin resistance and type 2 diabetes increases with age and is consistent between ethnic groups in NZ (Table 1.1). This may be explained in part by a more sedentary lifestyle, increased adiposity, and decreased body mass as the individual gets older. However, the earlier age at onset in Maori and Pacific Island people (>47 years) compared to NZ Caucasians (>57 years) is indicative of a strong genetic determinant in Polynesian diabetes that has yet to be identified.

Gestational diabetes, which is particularly prevalent in non-European groups, is also an important risk factor for type 2 diabetes [25]. Compared to NZ Caucasians, Maori and Pacific Island women are twice as likely to develop diabetes during pregnancy which increases the risk of diabetes in their offspring by a factor of two [26]. Gestational diabetes increases the risk of type 2 diabetes in the mother by a factor of two, 5-1 years following pregnancy.

Therefore Maori and Pacific Island people have a greater background prevalence of the necessary risk factors for type 2 diabetes compared to NZ Caucasians. These multiple
factors, in combination with genetic components, may contribute to the early development and high incidence of type 2 diabetes in NZ Polynesians.

1.2 Genetic & environmental factors in type 2 diabetes

Type 2 diabetes mellitus is a heterogeneous disease caused by interaction of genetic and environmental factors (Figure 1.2). A strong genetic component is suggested by its familial aggregation and transmission patterns, its high concordance rate in twins (60-100%), and its high prevalence in certain ethnic populations after controlling for lifestyle factors [27, 28]. The role of environmental factors is demonstrated in populations that are genetically similar but have different living conditions. For example, a study of rural Tokelauans who migrated to urban New Zealand showed that the prevalence of diabetes rose from 3.0% in non-migrants to 7.5% in migrants [29]. Similarly, the prevalence of diabetes in rural Melanesians in Fiji is 1.5% compared to 30% in urbanised Micronesians in Nauru [4]. This was attributed to a high calorie and protein diets increased alcohol consumption, greater weight gain and reduced levels of physical activity.

The propensity to type 2 diabetes following a lifestyle change may be explained by the "thrifty genotype" hypothesis [30]. According to this theory, factors in early humans that favoured fat deposition during periods of abundant food conferred survival advantage during subsequent periods of hardship and perhaps starvation, leading to the selection of genes for insulin resistance. Thus selective insulin resistance in the liver and increased efficiency for fat storage may have favoured survival in a traditional hunter-gatherer society. However, these factors may be detrimental to the individual post-urbanisation, resulting in the development of diabetes during the normal ageing process or with the onset of obesity. Relative resistance to glucose uptake in skeletal muscle would lead to increased adiposity thus contributing to the pathogenesis of diabetes and other metabolic abnormalities.
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In contrast, the "thrifty phenotype" theory proposes that the excesses of modern living are the major cause for insulin resistance. These excesses lead to reprogramming of carbohydrate metabolism and subsequent β cell dysfunction and insulin resistant tissues.

Disparities in type 2 diabetes and its complications between ethnic groups living in the same geographical location (ie. Polynesians and Caucasians living in NZ) suggests that type 2 diabetes may represent a selection of particular genotypes in response to different evolutionary pressures. The increased rate of diabetes in Maori and Pacific Island people following rapid transition to Westernised lifestyles suggest that Polynesians may represent a "thrifty genotype" population.

A role for genetic components in NZ Polynesian diabetes is indicated by the reported ethnic differences in the rates of type 2 diabetes in a multiracial workforce survey of 5677 subjects following standardisation for environmental factors [28]. Compared with NZ Caucasians, the prevalence of type 2 diabetes and impaired glucose tolerance was approximately four to six fold higher in Maori and Pacific Island people, after controlling for age, income and BMI. Although obesity was proposed to be a major reason for increased diabetes among NZ Polynesians, the relative risk and prevalence for type 2 diabetes remained significantly higher in Maori and Pacific Island people when adjusted for BMI. This suggests that other factors, such as genetic determinants, may also contribute to the increased rates of diabetes among NZ Polynesians.

The clustering of type 2 diabetes and nephropathy in Maori and Pacific Island families are also indicative of a genetic component [11]. Although there is insufficient Maori and Pacific Island parent/sibling and sibling/sibling information to provide direct evidence for a genetic origin for type 2 diabetes, familial clustering of nephropathy in Maori and Pacific Island are indicative of a founder effect, caused by an initial gene pool. Recently, the Diabetes Control and Complications Trial (DCCT) in Europe found a strong intra (familial) correlation (0.2) between parent/sibling pairs and the presence of nephropathy [31]. This report provides evidence for a possible contribution of genetic factors for this complication in Europeans and may also account for the high rates of nephropathy in NZ Polynesians.
Based on this evidence, that is the high prevalence of diabetes and its related complications when standardised for environmental factors, it has been postulated that Maori and Pacific Island people primarily have an increased genetic susceptibility to type 2 diabetes when compared to Caucasian populations.

1.3 Cellular basis for type 2 diabetes

1.3.1 General

The cellular basis for type 2 diabetes is insulin resistance and defects in insulin secretion. Insulin reduces blood glucose levels by stimulating glucose uptake, mainly in muscle and liver, thereby promoting glucose oxidation and glycogenesis. Insulin also controls hepatic glucose production and inhibits lipolysis, which is the breakdown of fat in adipose tissue (Table 1.3). Insulin action is initiated by its binding to the extracellular domain of the α-subunit in the insulin receptor, which leads to autophosphorylation of multiple tyrosine residues in the intracellular domain of the β subunit (Figure 1.3). Tyrosine kinase activation of the insulin receptor, by insulin, then leads to phosphorylation of tyrosine residues on the insulin receptor substrates 1 & 2 (IRS-1 & IRS-2), shc, and various other uncharacterised intracellular proteins. The phosphotyrosines of these proteins bind to SH2 domains on other signal kinases thus triggering the insulin-signalling cascade. IRS proteins preferentially signal different pathways for glucose uptake and glycogen synthesis. They also transfer the growth promoting and mitogenic signals of insulin to the nucleus, thereby stimulating protein synthesis [32].
1.3.2 Insulin resistance

The inability of insulin to maintain normoglycaemia in type 2 diabetes is caused by a combination of insulin resistance and defects in glucose stimulated insulin secretion [32]. Insulin resistance refers to an impaired biological response to insulin by one or more of its target tissues resulting in reduced glucose disposal. This resistance, as detected by hyperinsulinaemia, has been observed in patients 10 to 20 years prior to diagnosis of type 2 diabetes and is a strong predictor for type 2 diabetes.

Defects in glucose oxidation and glycogen synthesis in skeletal muscle are the major causes of insulin resistance in type 2 diabetes [32]. In adipocytes taken from type 2 diabetes patients, insulin stimulated glucose uptake is impaired and lipolysis is increased. In muscle, elevated circulating free fatty acids may induce insulin resistance by reducing glucose oxidation through the glucose fatty acid (Randle) cycle. Increased levels of free fatty acids can also induce insulin resistance by reducing hepatic clearance of insulin and enhancing gluconeogenesis [33]. Recent studies also suggest that long-chain fatty acids modulate transcription of the pancreatic β cell transcription factor, HNF-4α, or directly affect the activity of glycogen synthase [34].
Insulin resistance may also result from increased expression of cytokines. In obese people, tumour necrosis factor (TNF-α), which is over expressed in the adipose tissue, inhibits phosphorylation of the insulin receptor and IRS-1, thereby blocking the insulin-signalling cascade (Figure 1.4) [35]. Over expression of a low affinity insulin receptor isoform, and hybrid receptors in type 2 skeletal muscle and fat are also proposed to contribute to insulin resistance [36]. The latter results from fusion of the insulin and IGF receptors. The insulin receptor has greater affinity for IGF than does insulin and is negatively correlated with in vivo insulin sensitivity.

It has also been suggested that insulin resistance is caused by poor foetal and postnatal nutrition, as detected by low birth weight, leading to β cell dysfunction and insulin resistant tissues [37]. According to this hypothesis, termed the "thrifty phenotype", these individuals develop diabetes during adulthood or with the onset of obesity. In contrast, the "thrifty genotype" theory proposes that smaller birth weight is genetically determined and enables the foetus to store energy and fat more efficiently in an unfavourable intrauterine environment [38]. For example, the maternally inherited 16189 mitochondrial DNA variant is associated with restrained foetal growth and historically could have enhanced the likelihood of maternal survival. Although this genotype confers a survival advantage, it also increases the risk of developing diabetes at the onset of childhood obesity and in later life.

1.3.3 Insulin secretion

Insulin is secreted by pancreatic β cells in response to rising plasma glucose concentrations. Several abnormalities in β cell insulin secretion have been detected in subjects with type 2 diabetes. Insulin response to intravenous glucose is decreased in patients with type 2 diabetes, with loss of the first phase insulin secretion response, defective pulsatile patterns, and hyperproinsulinaemia [39].
Type 2 diabetes is characterised by a 20-50% reduction in β cell mass and may be a precipitating factor in defective insulin secretion [40]. Progressive loss of β cell function is associated with insulin resistance in skeletal muscle, and formation of islet amyloid deposits in the pancreas, which can induce pancreatic β cell death in vitro [41]. The pattern of loss is initial defect in early or first-phase insulin secretion followed by a reduction in glucose to potentiate non-glucose signals, and finally β cell failure that requires insulin treatment [32]. Development of islet amyloid in mice is associated with hyperglycaemia, and has been attributed to a high fat diet [42]. However, a 50% reduction in β cell mass alone is insufficient to explain the fasting hyperglycaemia observed in these animals, suggesting that the amyloidogenic process may impair islet cell function prior to cell death and reduction in islet mass.

Insulin action may also be affected by mitochondrial DNA (mtDNA). Mitochondria are the major site for Krebs cycle function, ATP synthesis and transfer of fatty acids. Elevated ATP in the cytoplasm is necessary for the membrane-dependent increase in cytosolic Ca$^{2+}$, which in turn triggers insulin exocytosis by the β cell. Patients with type 2 diabetes have approximately 35% less mtDNA in their peripheral blood leukocytes. This reduction has been observed to precede the onset of diabetes [43]. Low copy numbers of mtDNA are inversely correlated to fasting plasma glucose levels in patients with type 2 diabetes, and are associated with insulin resistance and defective insulin secretion in the offspring of diabetic mice [44]. Mothers with low mtDNA in their peripheral blood leukocytes tend to have offspring with low birthweight indicating that mtDNA content may be an important feature of the heritable "thrifty phenotype". The relationship between low mtDNA levels and diabetes is unknown. In vitro, β cell depletion of mitochondrial DNA results in altered mitochondrial morphology and inhibition of glucose-stimulated ATP production that is required for downstream insulin release by the islet β cell [45].

Another mechanism for defective insulin secretion may be glucose toxicity. Prolonged exposure of β cells to high glucose levels impairs insulin gene transcription resulting in decreased insulin secretion [46]. Chronic exposure to high levels of glucose can also cause
defective K\(^+\) channel function and a reduced expression of GLUT-2, responsible for glucose storage and transportation to the cell membrane.

Severe hyperglycaemia is correlated with increased insulin deficiency [47]. Once hyperglycaemia has developed, glucose toxicity can induce insulin resistance and decreased pancreatic \(\beta\) cell function. It has been predicted that a 50% decrease in \(\beta\) cell function (ie. decreased \(\beta\) cell function) in the presence of insulin resistance may induce hyperglycaemia. In addition, as \(\beta\) cell function decreases, glucose responsive tissue resistance to insulin will increase [48]. Hyperglycaemia can be significantly improved by treatment with diet, insulin therapy or sulfonylureas resulting in reduction of insulin resistance and glucose toxicity.

Insulin secretion and resistance are therefore interconnected at multiple levels, however it is unclear which defect is primary in the aetiology of diabetes. It has been predicted that a 50% decrease in \(\beta\) cell function in the presence of insulin resistance results in significant levels of hyperglycaemia [43]. Once hyperglycaemia develops, glucose itself leads directly to a loss of glucose induced insulin release and impairment in glucose disposal (glucose toxicity), resulting in exacerbation of the disease state.

1.4 Candidate genes for type 2 diabetes

Numerous investigators have sought to identify a genetic determinant that may be severe enough to substantially impair one or more of the major transduction pathways. The aetiology and pathogenesis of insulin resistance involve multiple signalling events and pathways, and therefore a number of target genes. Whole genome studies have demonstrated linkage of diabetes to different genetic loci in different populations [49]. Alternatively, the candidate gene approach, which tests for the association of a particular gene mutation and diabetes, has identified mutations in individual genes resulting in defective insulin signalling and secretion, and type 2 diabetes (Table 1.4). However, with
the exception of the monogenic form of diabetes, a single gene mutation that may explain the vast majority of type 2 diabetes patients has not been identified.

<table>
<thead>
<tr>
<th>Candidate genes</th>
<th>Chromosome</th>
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<tbody>
<tr>
<td><strong>Insulin resistance genes</strong></td>
<td></td>
</tr>
<tr>
<td>Insulin receptor</td>
<td>19p13</td>
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<tr>
<td>Insulin receptor substrate-1</td>
<td>2q36</td>
</tr>
<tr>
<td>Glucose transporter-4</td>
<td>17p13</td>
</tr>
<tr>
<td>Glycogen synthase</td>
<td>19q13</td>
</tr>
<tr>
<td>Amylin</td>
<td>12</td>
</tr>
<tr>
<td>β-3-adrenergic receptor</td>
<td>Not known</td>
</tr>
<tr>
<td>Fatty acid binding protein 2</td>
<td>4q26</td>
</tr>
<tr>
<td>Obesity</td>
<td>Not known</td>
</tr>
<tr>
<td>Prohormone convertase-2</td>
<td>20p11</td>
</tr>
<tr>
<td>Tumour necrosis factor-α</td>
<td>6p21</td>
</tr>
<tr>
<td>Ras associated with diabetes</td>
<td>16q22</td>
</tr>
<tr>
<td><strong>Insulin secretion defects</strong></td>
<td></td>
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<tr>
<td>Insulin</td>
<td>11p15</td>
</tr>
<tr>
<td>Glucose transporter-2</td>
<td>3q26</td>
</tr>
<tr>
<td>Maturity onset diabetes of the young</td>
<td>7q13</td>
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<tr>
<td></td>
<td>12q</td>
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<td></td>
<td>13q12</td>
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<tr>
<td></td>
<td>20p</td>
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<tr>
<td>NeuroD1/β 2</td>
<td>2q</td>
</tr>
<tr>
<td>Mitochondrial DNA</td>
<td>mtDNA</td>
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<tr>
<td>Glucagon receptor</td>
<td>17q25</td>
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</tbody>
</table>

Table 1.4 Candidate genes which have been investigated and found to predispose to type 2 diabetes, except for the OB, PC-3, and CPE genes. Adapted from [33, 107, 120, 121, 138, 140, 144].

1.4.1 **Candidate genes for insulin resistance**

There are many possible targets for natural mutations to disrupt insulin signalling (Figure 1.3 & 1.4). Some of the known targets are discussed below.

Genetic defects in the insulin receptor gene account for 1% of type 2 diabetes (Figure 1.3). They are associated with hyperglycaemia and type 2 diabetes in Polish, Dutch, Caucasian, and Japanese populations [50, 51], and are predictors of hypertension in Chinese populations [52]. Mutations in the insulin receptor gene have been identified in both the α subunit, which alters insulin binding or recycling of the receptor, and the β subunit,
resulting in decreased insulin receptor tyrosine kinase activity [51]. The prevalence of these mutations range from 0.4-7.8% depending on the type 2 diabetes affected populations studied.

Mutations in the IRS-1 gene account for approximately 15% of type 2 diabetes in Japanese, Danish and Italian populations (Figure 1.3) [53, 54]. The G972R variant, observed in both the Danish and Italian populations, leads to both reduced glucose and reduced sulphonylurea-stimulated insulin secretion through the PI3-kinase pathway [55, 56]. PI3-kinase downstream signals mediate protein synthesis and mitogenesis; plus activation of the ras related protein, Rab, which has been proposed to regulate translocation of glucose transporter (GLUT) storage vesicles to the cell membrane (Figures 1.3 and 1.4). Therefore, a defect in this pathway may induce several mechanisms for insulin resistance. Mutations in the IRS-1 gene are rare in Mexican Americans, Taiwanese and Pima Indians and are not a causative factor for diabetes in these populations [57, 58].

Genetic defects in the GLUT-4 gene are extremely rare and do not contribute significantly to type 2 diabetes prevalence (Figure 1.3) [59, 60, 61]. These studies observed GLUT-4 mutations in both type 2 diabetics and healthy controls indicating that the decreased expression of GLUT transporters in type 2 patients may be secondary to other genetic or environmental defects.
Figure 1.2 Genetic defects affecting insulin signalling. Defects in the insulin receptor gene and downstream targets such as the IRS-1, GLUT-2, and glycogen synthase genes that affect the insulin-signalling cascade. Mutations in these genes have been found to produce diabetes in certain subsets of type 2 diabetes and specific ethnic groups. Adapted from [49].
Figure 1.3 Potential inhibitors affecting insulin signalling. Defects in genes encoding PC-2, glycoprotein and TNF-α inhibit insulin action and subsequent insulin-signalling, while mutations in the rad gene inhibits insulin stimulated glucose uptake. Mutations in these genes are reported to produce type 2 diabetes in a number of ethnic groups. Adapted from [49].
Glycogen synthase catalyses non-oxidative glucose metabolism in skeletal muscle and liver. A defect in glycogen synthesis has been observed in type 2 diabetes indicating that the glycogen synthase gene may be an important susceptibility gene for diabetes [62]. The glycogen synthase gene has been mapped to chromosome 19q13, however there is no evidence of linkage for type 2 diabetes to this region [63]. A restriction fragment length polymorphism (RFLP) in the glycogen synthase gene is associated with familial type 2 diabetes, hypertension and insulin resistance in Finland [64]. However, no association between the RFLP and these problems were found in French, Japanese, Chinese or Russian populations [65, 66]. Carriers for this mutation have reduced glycogen synthase protein accumulation in skeletal muscle [67]. This mutation is not known to affect expression or normal function of glycogen synthase suggesting that this polymorphism may be linked to other functional polymorphisms on chromosome 19, therefore explaining type 2 diabetes in French, Japanese, Chinese or Russian populations.

The β3-adrenergic receptor (β3-AR) is expressed in the adipose tissue and regulates energy balance by increasing lipolysis and thermogenesis. Adipocytes from obese humans have increased 2-ARs, 2-/β3-AR ratios, and 2-AR-mediated responses, which, in mice, have been shown to promote high fat-diet induced obesity and adipocyte hyperplasia [68]. A mutation in β3-AR, W64R, is associated with early onset type 2 diabetes in Pima Indians and Japanese [69, 70]. Subsequently, this allele has been found in all populations studied including Caucasians, African Americans, Mexican Americans, Chinese, and Samoan, with the exception of Nauruans [71, 72]. In obese patients, this phenotype increased the capacity to gain weight and develop type 2 diabetes. Defects in the β3-AR gene appear to increase susceptibility to both insulin resistance and obesity and may therefore be a common marker to the typical forms of type 2 diabetes [73].

A missense mutation, A54T, in the gene encoding intestinal fatty acid binding protein (FABP2) is associated with hyperinsulinaemia and insulin sensitivity in Pima Indians and Mexican Americans [74, 75]. This mutated receptor is proposed to increase absorption and/or processing of dietary fatty acids by the intestine thereby increasing fat oxidation leading to increased insulin resistance. Genetic variability in the FABP2 locus is rare in
both Caucasians and Japanese, and does not contribute to the common form of type 2 diabetes [76, 77].

Mutations in the obesity (OB) gene result in profound obesity and type 2 diabetes in mice. The OB gene product, leptin, is over expressed in adipocytes of obese patients [78]. Mutations in the OB gene are rare in patients with type 2 diabetes or obesity and are not a common cause for these phenotypes [79, 80, 81]. Results of a Nauruan study indicated that OB alleles are low penetrant mutations that confer increased risk for decreased insulin resistance and type 2 diabetes, particularly when inherited with mutations in the leptin receptor gene, OB-R [82].

The discovery of elevated levels of proinsulin in subjects with type 2 diabetes has prompted the search for mutations in the proinsulin processing enzyme prohormone convertase 2 (PC-2) and carboxypeptidase E (CPE). The identification of a mutation in the CPE gene of the fat/fat mouse resulting in hyperinsulinaemia and late-onset obesity also suggests a role for these enzymes in the pathogenesis of diabetes. However, no mutations in the CPE gene were found in Japanese patients with type 2 diabetes when screened [83]. However, a microsatellite polymorphism in intron 2 of the PC-2 gene was found to co-segregate with type 2 diabetes in some Japanese pedigrees [84]. PC-2 catalyses cleavage of proinsulin and proamylin (a peptide hormone that is co-secreted with insulin) at their carboxyl and amino terminals respectively to produce the mature peptides [85]. Mice homozygous for the PC-2 null mutation have no circulating mature amylin peptide. Thus mutations in the PC-2 gene may increase susceptibility for insulin resistance through defective processing of the β cell hormones, insulin and amylin (Figure 1.4).

As discussed previously (Section 1.3) TNF-α is hypersecreted by adipocytes in obese individuals and impairs insulin action by inhibiting insulin receptor kinase (Figure 1.4). A microsatellite polymorphism in the TNF-α locus was linked to obesity in a cohort of Pima Indians, but not linked in a larger population study [86]. Similarly, several subsequent studies have failed to find an association between type 2 diabetes and polymorphisms in the TNF-α promoter region indicating that defects in TNF-α secretion may be secondary to
obesity rather than a primary genetic determinant [87, 88, 89]. Other studies indicate that the TNF-α microsatellite polymorphisms are in linkage disequilibrium with the HLA-DQ haplotypes characteristic of type 1 diabetes, and may contribute to increase relative risk of type 2 diabetes [90].

Ras-related protein associated with diabetes (rad) is a prototypic member of Ras-related GTPases and is involved in the translocation of glucose transporters to the cell membrane (Figure 1.3 & 1.4). Rad is over expressed in type 2 diabetes muscle and has been reported to inhibit insulin stimulated glucose uptake (Figure 1.4) [32]. A microsatellite polymorphism in the rad gene was reported to be preferentially associated with type 2 diabetes in white Americans [91] but was not associated with type 2 diabetes or insulin resistance in Pima Indians nor Finnish diabetic patients [92, 93].

A missense mutation in the human amylin gene, S20G, is associated with early-onset type 2 diabetes (10%) in a Japanese population, and identified patients with a strong family history of late-onset type 2 diabetes (4.1%) [94, 95]. Amylin is co-secreted with insulin from pancreatic β cells and is the major component of amyloid deposits [96]. Although its precise function is unknown, amylin has been implicated in the regulation of insulin and glucose metabolism. A prominent feature of amylin is its ability to form amyloid fibrils and has been detected in 70% to 90% of patients with type 2 diabetes at autopsy and is associated with 50% loss of β cell mass [97]. These fibrils represent highly ordered rod-shaped structures comprised of large amounts of β-pleated sheet structures. The region of the amylin peptide that is responsible for amyloid formation corresponds to amino acids 20 to 29 (Figure 3.2) and has been shown to form amyloid fibrils spontaneously in vitro [98]. Amino acid substitution within the 20 to 29 peptide region can slow down fibril formation [99]. Comparison of human with rodent amylin, which does not form the β-pleated conformation in vitro, substitutes proline at positions 25, 28, and/or 29.

Substitution of serine for glycine at amino acid 20 increases the hydrophobic properties of amylin thereby enhancing its ability to form fibrils. In vivo, S20G peptides form amyloid rapidly and more efficiently than wild type amylin, increasing amyloid deposit mass by a
factor of two. The S20G peptide, which accumulates in the endoplasmic reticulum and Golgi apparatus, has enhanced cytotoxicity, increasing apoptosis of pancreatic β cells three fold. Hence the S20G mutation may be a pathogenic factor for type 2 diabetes through enhanced amyloidogenecity and increased intracellular cytotoxicity thereby by promoting β cell dysfunction and death [100].

The amylin gene promoter allele, -132G>A, is significantly associated with type 2 diabetes in Spanish Caucasians (9.7% of type 2 diabetics vs 1.5% of non-diabetics) [101] but not British (5.3% of type 2 diabetics vs 3.2% of non-diabetics) [102] or Danish Caucasians (4.1% of type 2 diabetics vs 7.1% of non-diabetics) [103]. Affected individuals were carriers for the heterozygous (G/A) genotype except for two homozygous (A/A) individuals in the Spanish Caucasian population both of whom had disturbed glucose metabolism [101]. In vitro, the -132G>A allele demonstrates a two-fold increase in amylin promoter activity compared to the wild-type construct [104]. Hence the -132G>A allele may be a pathogenic factor for type 2 diabetes in Spanish Caucasians through increased amylin gene expression. Alternatively it may act in synergy with additional genetic markers or environmental risk factors that are specific to Spanish Caucasians but not British or Danish Caucasians.

The +79C>A allele in intron 2 has also been observed in Spanish Caucasians. It is highly prevalent in type 2 diabetics (6.8%), non-diabetics (7.7%), and women with gestational diabetes (11.2%) and is not significantly associated with type 2 diabetes in Spanish Caucasians [105]. The +79C>A allele, however, is significantly associated with lower levels of LDL cholesterol in both non-diabetic and type 2 diabetic patients.

Other alleles identified in the amylin gene include the -259C>T and -229C>A alleles in a Japanese diabetic patient with marked amyloid deposition [106] and the +75A>G allele in two Danish type 2 diabetic patients [103]. Neither of these alleles are significantly associated with type 2 diabetes in these populations.
1.4.2 Candidate genes for defects in insulin secretion

a) Non-MODY genes

Mutations in the insulin gene have been observed in very few families and are a rare cause of diabetes. However, two types of mutations in the insulin gene have been described. The first type affect the primary sequence of the A and B insulin chain resulting in reduced insulin-receptor binding activity. Affected individuals have elevated circulating levels of defective insulin that cannot be readily metabolised [107]. The second type of mutations affect the proinsulin processing site and inhibit cleavage of proinsulin to the mature peptide. Carriers for insulin gene mutations present clinically with mild type 2 diabetes characterised by hyperinsulinaemia or hyperproinsulinaemia and sometimes mild hyperglycaemia.

GLUT-2, which is expressed in the liver and pancreatic β cells, facilitates glucose uptake by the cell (Figure 1.3 & 1.4). Both association and linkage analysis indicate that the GLUT-2 gene is not a major determinant for type 2 diabetes. Two GLUT-2 mutations are reported in separate individuals, P68L and V197I, the latter has been shown to inhibit insulin-stimulated glucose uptake in Xenopus oocytes [108]. An RFLP in the GLUT-2 gene is associated with type 2 diabetes in a British cohort but has not been observed in other populations [109].

Several factors implicate mitochondrial DNA in the pathogenesis of type 2 diabetes. Firstly, mitochondrial oxidative phosphorylation is involved in peripheral glucose metabolism and glucose stimulation of β cell insulin secretion. Secondly mitochondrial DNA is maternally inherited as are some forms of diabetes. Finally, the risk of DNA mutation in the mitochondria is 10-20 times greater than nuclear DNA and increases with age. Maternally inherited diabetes and deafness (MIDD) is caused by an A to G substitution at position 3243 of the tRNA-Leu gene (3243tRNALeu) [110]. It occurs in 1-3% of patients with type 1 and type 2 diabetes, and is characterised by insulin deficiency, maternal inheritance, loss of neurosensory hearing and increased risk of renal dysfunction.
In vitro, the 3243tRNA\textsubscript{Leu} mutation is associated with lactate acidosis, poor respiration and defective respiratory chain activities [110]. A reduction in the amount of pyruvate entering the mitochondrion to feed the tricarboxylic acid cycle (TCA) may impair synthesis of ATP, glutamate, and other mitochondrially-derived factors that mediate insulin exocytosis by the pancreatic $\beta$ cell. Deletion and duplication of mtDNA has also been observed in patients with diabetes and deafness, however, these mutations are a rare cause for type 2 diabetes [112].

Glucagon is a counter-regulatory hormone of insulin that acts to stimulate hepatic glucose production and potentiate glucose-induced insulin secretion. Patients with type 2 diabetes often have high circulatory levels of glucagon. A missense mutation in the glucagon receptor gene (G40S) was found to be associated with type 2 diabetes in French and Sardinian populations with a prevalence of 4.6% and 8.3% respectively [113]. The G40S mutation affects the extracellular domain of the glucagon receptor and reduces its binding affinity for glucagon threefold. In vitro, glucagon-stimulated insulin secretion is markedly reduced in rat insulinoma (RIN) cells expressing this mutant receptor indicating that the G40S mutation decreases the sensitivity of target tissues to glucagon. Mutations in the glucagon receptor however, are a rare cause of diabetes in German, Taiwanese, Russian Japanese, Finnish populations [114, 115].

Candidate genes associated with high birth weight and adult onset diabetes have also been reported. The insulin gene variable number of tandem repeat (VNTR) microsatellite allele, class III, is associated with enhanced foetal growth. Historically this genotype may have acted to promote survival in infancy during nutritional adversity, however, it may be a risk for diabetes in adult life [38]. Although the mechanism by which the class III allele predisposes to diabetes is unclear, the VNTR region is proposed to regulate expression of the insulin and neighbouring IGF-2 genes, both of which are vital for normal foetal growth.
b) **MODY genes**

Genetic studies have also identified mutations in the genes encoding the glycolytic enzyme glucokinase; three liver-enriched transcription factors, HNF-1α, HNF-1β and HNF-4α, and the pancreatic transcription factor, IPF-1, all of which give rise to a subset of type 2 diabetes called maturity-onset diabetes of the young (MODY) (Figure 1.4). MODY, which accounts for 2-5% of type 2 diabetes, is a monogenic form of diabetes mellitus characterised by autosomal dominant inheritance, early age onset (commonly diagnosed < 25 years), and primary defects in β cell function and insulin secretion [116]. Heterozygous mutations in the MODY genes causes diabetes through haploinsufficiency (gene dosage effect) or by a dominant negative effect. Each MODY gene is associated with a distinct phenotype, therefore a definite diagnosis of MODY predicts the prognosis and clinical course.

Mutations in the glucokinase gene give rise to *MODY2* [117]. This gene is expressed in pancreatic β cells and the hepatocytes, catalysing the phosphorylation of glucose to glucose-6-phosphate. Defective glucokinase activity leads to a decreased rate of glycolysis and is proposed to increase the blood glucose threshold thus triggering insulin secretion. More than 80 glucokinase mutations have been reported to date accounting for 50% and 17% of French and British MODY families respectively. Patients with *MODY2* present in early childhood and undergo a mild clinical course and few diabetic complications.

Mutations in the genes encoding hepatic nuclear factors, HNF-4α, HNF-1β and HNF-1α result in *MODY1*, 3 and 5 respectively [116]. These genes are expressed in pancreatic β cells, hepatocytes, and the kidney where they regulate expression of genes involved in glucose, cholesterol, and fatty acid metabolism (Figure 1.4). Patients carrying hepatic nuclear factor mutations usually present with MODY in their early to mid twenties, and are characterised by reduced insulin secretion response of the islet β cell to glucose.

To date, eight mutations in the HNF-4α gene have been identified in *MODY1* pedigrees [118]. The prevalence of HNF-4α mutations range from 2%-6% in Caucasian and Asian
subjects with MODY and are a rare cause of type 2 diabetes [119]. Patients with a clinical diagnosis of \textit{MODY1} are characterised by progressive loss of β cell function and severe micro- and macro-vascular complications [120]. HNF-4α is a member of the nuclear receptor superfamily and is a key regulator of hepatic gene expression. HNF-4α is also a positive regulator of HNF-1α transcription (Figure 1.4), thus mutations in the HNF-4α gene have been proposed to cause MODY through impaired HNF-1α gene function. HNF-4α activity is directly modulated by long chain fatty acids and may therefore be a mechanism linking free fatty acids to insulin secretion (Figure 1.4) [121]. Mutations in the HNF-1β gene, resulting in \textit{MODY5}, are also rare causes of diabetes. The HNF-1β transcription factor functions as a homodimer or heterodimer (Figure 1.4), with structurally related HNF-1α, and plays a central role in kidney development and pancreatic β cell function [122]. \textit{MODY5} is characterised by high rates of renal dysfunction, which is often diagnosed before the onset of diabetes [123].
Figure 1.4  Network of MODY gene transcription factors (HNF-4α, HNF-1α, HNF-1β, HNF-3β and IPF-1) and their downstream target genes (boxed) in the pancreas.
Mutations in the HNF-1α gene give rise to MODY3 (Figure 1.5) [124]. HNF-1α is a homeodomain transcription factor that is expressed in the liver, pancreas, intestine, stomach and kidney. In the liver, HNF-1α may regulate more than 100 liver specific genes such as β-fibrinogen, α -antitrypsin, phosphoenolpyruvate carboxykinase (PEPCK) and L-type pyruvate kinase (LPK) [124]. In the pancreas, HNF-1α regulates expression of genes involved in glucose metabolism and transport such as insulin, amylin, glucokinase, GLUT-2, and LPK. A total of 65 different HNF-1α mutations (Figure 1.5) have been reported in 116 MODY pedigrees. These are the most common cause of MODY in British, German, French, Danish, Italian, Finnish, American, Spanish, Brazilian and Japanese populations [63, 123, 125, 126, 127, 128, 129]. HNF-1α mutations are highly penetrant with 63% of mutation carriers having diabetes by 25 years, 78.6% by 35 years, and 95.5% by 55 years. Patients with MODY3 are characterised by progressive β cell dysfunction with increasing risk for complications and requirement for treatment with age. The most common HNF-1α mutation, P291fsinsC, is generated by a mutational hotspot in exon 4 (Figure 1.5) [130]. The P291fsinsC mutation generates a truncated protein lacking most of the transactivation domain thereby inhibiting endogenous HNF-1α transcriptional activity by a dominant negative mechanism [131]. Loss of function mutations include those affecting the dimerization domain sequence, and the promoter region sequence (Figure 1.5), thus affecting HNF-1α expression, or result in synthesis of truncated proteins that are unstable and readily degraded [132, 133].

The HNF-1α mutations are heterozygous and the mechanism by which they lead to diabetes remains to be determined. One theory proposes that reduced HNF-1α activity during pancreatic islet development in the foetus may limit β cell function in later life [134]. Alternatively, abnormal HNF-1α activity may lead to impaired transactivation of pancreatic β cell genes such as insulin and therefore diabetes. However, defects in insulin reaction is likely to produce type 2 diabetes at an early age and the late onset presentation of this disease does not support this mechanism. Although HNF-1α is associated with MODY, a late onset presentation does not preclude the importance of its genetic variants and instead may reflect interaction between environmental and genetic factors. Thus it
could be that subjects with these genetic variants may be "protected" due to low BMI or less urbanised lifestyle.

Figure 1.5 Location of HNF-1α gene mutations within exons 1 through to 10, 5'UTR, promoter, and splice sites. The functional domains of the HNF-1α protein are shown. The numbers in brackets refer to the codons [123, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134].
Mutations in the IPF-I gene result in MODY4 (Figure 1.6). The IPF-1 transcription factor (also known as STF-1, IDX-1, and PDX-1) is expressed in the pancreas where it regulates early pancreatic development, and mediates glucose-responsive stimulation of insulin and amylin gene expression [135]. Mutations in the IPF-I gene are rare in both early onset (3.5%) and late onset (2.7%) type 2 diabetes [136]. A total of 11 IPF-1 mutations have been reported in 58 MODY families (Figure 1.6), 7 of which have been confirmed to predispose to or cause impaired insulin secretion in type 2 diabetes. Although these pedigrees fit the clinical criteria for MODY, several of the IPF-1 mutations were observed in families with typical late-onset type 2 diabetes. To date, only one IPF-1 mutation resulting from a single nucleotide deletion at codon 63 (Pro63fsdelC), has been observed to completely co-segregate with diabetes [137]. A homozygous deletion has been reported to cause pancreatic agenesis in one individual, while heterozygous carriers develop early-onset type 2 diabetes. The Pro63fsdelC mutation results in synthesis of two IPF-1 isoforms lacking either the amino-terminal or the carboxy-terminal transactivating domains thereby inhibiting IPF-1 transactivation activity by gene dosage and dominant-negative effects respectively [138].

Although the remaining IPF-1 gene mutations are more prevalent than Pro63fsdelC, they are low penetrant mutations and often are insufficient to cause diabetes on their own. Instead, these mutations increase the relative risk for diabetes by 3-12 fold depending on the severity of the mutation, and therefore, they are more likely to represent predisposing alleles in the more common forms of diabetes resulting in late-onset diabetes [139]. Furthermore, several of the IPF-1 mutations have been observed to be inherited with mutations in the HNF-1α and islet brain-1 (IB-1) genes [139]. Individuals who inherited both markers had a more severe form of diabetes compared with those carrying IPF-1, IB-1, or HNF-1α mutations alone. This suggests that a polymorphic locus, or nearby mutation which is in linkage disequilibrium with the IPF-1 gene, may contribute to the polygenic nature of type 2 diabetes. Thus when screening for mutations in the IPF-1 gene, it may be of particular interest to screen for other MODY genes as well.

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**Figure 1.6** Location of human IPF-1 gene mutations within the 2 exons and promoter region of the gene. The functional domains of the IPF-1 protein are shown, numbers in brackets refer to the codons [136, 137, 138, 139].
Mutations in the NeuroD1/β2 gene have recently been reported to be associated with type 2 diabetes in two families, one of which meets the criteria for MODY3 [140]. NeuroD1 regulates expression of the insulin gene by forming heterodimers with the ubiquitous HLH protein E47 which in turn binds to the insulin promoter E-box motif [141]. In mice, targeted disruption of NeuroD1 results in reduced β cell mass and failure of islets to cluster, severe diabetes and perinatal death [142]. The NeuroD1 mutations contribute to the development of type 2 diabetes in carriers by impairing NeuroD1 transcriptional activation of the insulin gene.

To date, association studies using genetic markers as putative aetiological risk factors has identified a number of mutations that give rise to only a small percentage of type 2 diabetes. Often, these predisposing mutations are familial, as in the MODY genes, or are only reproducible in select populations. Type 2 diabetes is therefore a polygenic disease and is more likely to be caused by multiple genetic markers rather than a single gene defect. It is possible that a significant founder effect underlies the incidence of type 2 diabetes in some of these populations. Alternatively type 2 diabetes may result from a selection of genotypes responding to environmental pressures that are specific to that population or racial group. Hence genetic heterogeneity and interaction with environmental factors complicate the identification of type 2 diabetes susceptibility genes.

1.5 Candidate genes for type 2 diabetes in Polynesians

1.5.1 Previous studies

In NZ, both Maori and Pacific Island people have relatively poor health status. Type 2 diabetes is a major health problem, accounting for 95% of diabetes in Maori and Pacific Island people, and 75% in NZ Caucasians. Compared to NZ Caucasians, the prevalence of type 2 diabetes in Maori and Pacific Island people is increased by a factor of 4. As a consequence, Maori and Pacific Island people face a much higher than normal risk of heart disease, kidney failure, leg amputation, blindness and nerve disease. Furthermore, Maori
and Pacific Island diabetes-associated admissions to hospitals are under-reported by at least 30% so that the actual level of acute episodes resulting from diabetes is higher than the statistics indicate. The total health costs for diabetes and diabetes-related complications in NZ was estimated to be in excess of $NZ247 million for 2001 [143].

It is generally accepted that type 2 diabetes has major genetic determinants. The clustering of type 2 diabetes in families, high concordance rate in twins, and high incidence in certain ethnic populations when controlled for environmental factors support this concept. Evidence for the role of genetic components in NZ Polynesian diabetes is indicated by the increased rates of type 2 diabetes and impaired glucose tolerance in Maori and Pacific Island people, compared to NZ Caucasian after controlling for age, income and BMI [28]. Familial clustering of nephropathy in Maori and Pacific Island people are also indicative of a genetic component [11, 31]. Together this evidence suggests that Maori and Pacific Island people primarily have an increased genetic susceptibility to type 2 diabetes when compared to Caucasian populations.

Substantial research in NZ and Polynesia have investigated the incidence of diabetes-associated risk factors and lifestyle risk factors such as hypertension, hyperlipidaemia, obesity, diet, and physical activity, in the epidemiology of disease in Polynesians. However, little research has focussed on determining a genetic basis for type 2 diabetes in these populations.

To date few studies have investigated the genetic determinants for type 2 diabetes in Maori and Pacific Island people. Genetic studies in Nauruans (Micronesia) have identified few genetic markers, HLA Bw22, OB and OB-R (see Section 1.4.1), that increase the relative risk of early onset diabetes [44, 82]. Nauruans are a particularly high risk group for type 2 diabetes with a prevalence rate of 30% in 1975 despite the fact that diabetes was relatively rare prior to the 1950's. This rapid increase of diabetes reflects the change in Nauruan lifestyle and increased prosperity from mining of phosphate deposits on the Island.
Linkage analysis of the MODY2 (See section 1.4.2) locus on chromosome 7p13-15, in a Maori cohort with a strong history of type 2 diabetes found no linkage between the disease and markers in this region [144]. Furthermore, direct sequencing of the glucokinase gene did not identify any mutations that co-segregated with type 2 diabetes in Pacific Island subjects of this study [145].

Shaw et al., 1998 [146] reported positive linkage (LOD score of 3.65) between markers at the MODY3 region of chromosome 12 and late onset diabetes in a Pacific Island pedigree whereby late-onset diabetes was seen to co-segregate in a pattern consistent with an autosomal disorder in this pedigree. However, sequencing of the 10 exons and promoter of the MODY3 gene HNF-1α did not identify any causative mutation in this gene. This is in agreement with Green [147] who screened the HNF-1α gene for mutations in a Pacific Island population. Eight previously described, and two novel polymorphisms, were detected but found not to co-segregate with diabetes in this Pacific Island population. This Pacific Island population along with a Maori population are the subjects of this thesis.

Despite these findings, positive linkage between late onset type 2 diabetes associated with severe insulin resistance and the MODY3 locus suggests that another gene or genes at this locus on chromosome 12q may be operative in Polynesians [144]. A major susceptibility locus near the HNF-1α gene, NIDDM2, was found to be associated with low insulin secretion and late onset diabetes in a Finnish population [49]. The NIDDM2 locus is proposed to be allelic with MODY3, with severe mutations resulting in MODY and mild mutations giving rise to late-onset diabetes. Thus it is possible that mutations at NIDDM2 may be responsible for the LOD scores observed by Shaw et al [146].

1.5.2 The present study

To date, no genetic determinants for type 2 diabetes have been identified in Maori and Pacific Island people. Therefore, the main objective of the present study was to assess the prevalence of polymorphisms in the genes encoding amylin and IPF-1, and exon 2 of the
HNF-1α gene, in the common form of late-onset type 2 diabetes in Maori and Pacific Island people. The HNF-1α gene was chosen as it is among the most common type 2 diabetes predisposing genes in other populations and therefore may also be important in Polynesians. An uncommon predisposing gene, IPF-1, was also chosen because it mimicked the phenotype or clinical picture of Polynesian type 2 diabetes. Amylin, also an uncommon predisposing gene, was chosen because racial differences in an amylin gene mutation indicate that this mutation may be present in Polynesians. The glucokinase gene, although an important predisposing gene in other populations was not studied because polymorphisms in this gene have previously been shown not to co-segregate with type 2 diabetes in Pacific Island Polynesians and therefore were not investigated in the present study. With the exception of the IRS-1 and glucagon receptor genes, the remaining type 2 diabetes susceptibility genes as discussed previously, were not considered for the present study due to their low prevalence (<2%) in type 2 diabetes. The high frequency of IRS-1 mutations (15%) [53] and glucagon receptor mutations (8%) [113] suggest that these genes may be worth screening in future gene association studies.

Mutations in HNF-1α gene are the most common cause of MODY (63%) and are associated with increased rates of diabetes related complications, especially proteinuria, a common complication in Polynesians. The human HNF-1α gene, located on chromosome 12q, comprises 10 exons spanning approximately 23 Kb and encodes a 628 amino acid protein that is highly conserved in vertebrates [124]. Previous screening of the HNF-1α gene have failed to identify any mutations that co-segregate with type 2 diabetes in Pacific Island people [146, 147]. However, screening of a Northern European population recently identified a polymorphism in intron 2 of the HNF-1α gene. The polymorphism, present in both diabetic and non-diabetic, was observed in 17% of the Caucasian population studied. This polymorphism, nt +66G>C, was located in the reverse primer, as used by both Shaw [146] and Green [147] for PCR amplification of exon 2, and was proposed to result in non-amplification of 3 mutations beginning approximately 20 base pairs upstream of the primer annealing site in the coding region of exon 2 [148]. Two of these mutations have been observed to completely co-segregate with diabetes in MODY.
Therefore HNF-1α may still have a role in the pathogenesis of type 2 diabetes in Polynesians. One objective of the present study was to screen for mutations in exon 2 of the HNF-1α gene in Maori and Pacific Island subjects with type 2 diabetes. Since exon 2 is a frequent site for HNF-1α mutations in British, German, French, Danish, Italian, Finnish, American, Spanish, Brazilian and Japanese populations, this exon may contribute to genetic susceptibility for type 2 diabetes in Maori and Pacific Island populations.

The second candidate gene examined in the present study is the MODY4 gene, IPF-1. The IPF-1 gene, located on chromosome 13q12, comprises 2 exons extending over a 5Kb region of the human genome [138]. Mutations in the IPF-1 gene are not a common cause for type 2 diabetes in Caucasians (3-4%), however it was chosen because it mimicked the phenotype or clinical picture of Polynesian type 2 diabetes [139]. The average age at onset for MODY4 (>35 years) is later than other MODY syndromes (<26 years) and similar to that in Polynesians (>40 years). Furthermore, IPF-1 mutations are proposed to increase the risk of type 2 diabetes in the presence of other factors such as BMI. This information, coupled with the high incidence of environmental determinants in Polynesians, indicated that the IPF-1 gene was worth screening for polymorphisms in diabetic Polynesians for efficacy as a diabetic marker.

The third susceptibility gene investigated in the present study is the amylin gene. The amylin gene, located on chromosome 12 comprises 3 exons extending over a 6Kb region of the human genome [149]. A missense mutation in the coding region of the amylin gene, S20G, has previously been shown to predispose to type 2 diabetes in Japanese (4.1%) and Chinese populations (0.4%) [91, 146], but not Caucasians [151, 152]. There is cumulative evidence pointing to a genetic origin for Polynesians in South East Asia. Y chromosome [155] and mitochondrial lineage analysis [156] indicate that the prehistoric settlement of Polynesia came from South East Asia, with NZ being the last major settlement in eastern Polynesia [157]. This coupled with the increased prevalence of type 2 diabetes mellitus in Maori, Pacific Island and Japanese compared with Caucasians when controlled for environmental influences, suggest that these ethnic groups are more likely to share similar polymorphisms for type 2 diabetes including S20G.
1.5.3 Significance of the present study

Type 2 diabetes occurs in 16.5% and 10.1% of Maori and Pacific Island people respectively and is therefore a major cause of mortality and morbidity in Polynesians. Genetic factors are important in the aetiology of type 2 diabetes, however, specific diabetes-susceptibility genes in Polynesians remain to be identified. It is possible that a significant founder effect underlies the high prevalence of type 2 diabetes in Polynesians with type 2 diabetes. Therefore, the aim of the present study was to assess the prevalence of and determine whether mutations in glucose homeostasis and β cell specific genes found in other populations are important causes of type 2 diabetes in Maori and Pacific Island Polynesians in NZ. Specifically genes encoding HNF-1α, IPF-1 and amylin were examined. If candidate marker genes are present in NZ Polynesians with type 2 diabetes they would also be worth screening for in non-diabetic Polynesians for efficacy as diagnostic markers. Determination of diabetes-associated genes may aid a definitive diagnosis of type 2 diabetes and help predict the likely prognosis and clinical course in susceptible individuals. Intervention programs pertaining to diet and exercise can then be applied to at risk individuals to minimise or prevent the onset of type 2 diabetes.
Chapter 2: Materials and Methods

2.1 Materials

Chemicals: all from BDH Chemicals, Dorset, UK or from Sigma, NSW, Australia.

Enzymes: enzymes for DNA manipulation were purchased from Gibco BRL, NY, USA, except:

1. Amplitaq Perkin-Elmer, Roche Molecular Systems, Inc., NJ, USA.
2. Exonuclease I USB Corporation, OH, USA.
3. Shrimp alkaline phosphatase USB Corporation, OH, USA.

Oligonucleotides: Sigma, NSW, Australia.

Kits:

1. QIAamp DNA Blood Mini Kit Qiagen, CA, USA.
2. Concert™ Rapid PCR purification system Gibco BRL, NY, USA.
3. ABI Prism BigDye™ Terminator Perkin-Elmer, PE Biosystems Division, CA, USA.

Cycle Sequencing Ready Reaction Kit

Molecular weight markers: Gibco BRL, NY, USA.
2.2 Methods

2.2.1 Blood Collection

Venous blood were collected from 146 Maori and Pacific Island patients with type 2 diabetes and 387 Maori and 129 Pacific Island non-diabetics. Prior to beginning this study, permission to use Maori genomic DNA for sequence analysis was approved by Iwi (Ngapuhi and Tainui) in accordance to Maori protocol. At blood sampling informed consent was obtained from each individual as approved by the regional Human Ethics Committee. Ethnicity was recorded according to the 1996 New Zealand census question [158]. Subjects were excluded if they self-identified as Indian or Asian. Due to the small sample population in the present study participants of Samoan, Cook Island Maori or Tongan descent were classified as Pacific Island. Maori cases and controls used in this study included those who identified first and second generation as either Maori or Maori and New Zealand Caucasian. The study group was randomly selected from clinics where they were being treated for diabetes as specified by the US National Diabetes Data Group (fasting plasma glucose ≥ 126mg/dL and a 2-h post glucose load > 200 mg/dL) [159], and ranged from 18-75 years of age. For each patient, date of birth, age at diabetes onset, family history for diabetes, complications, therapy, measurement of height, weight and body mass index (BMI) were recorded. Non-diabetic control subjects were randomly selected from clinics offering free testing for diabetes to the general public. Control subjects were classed as non-diabetic if they had an HbA\textsubscript{1c} levels between 3.0-6.0%. Date of birth and family history was collected for each control subject. The average age for non-diabetics was 50 years or over. To our knowledge all type 2 diabetic patients and control subjects were unrelated. Blood samples were collected in 5-10mL EDTA tubes (purple top tubes) and stored at -20 °C until ready for DNA extraction. HbA\textsubscript{1c} was measured using the Biorad Variant II HbA\textsubscript{1c} system.
2.2.2 Genomic DNA extraction

a) Micro hand genomic DNA extraction

Leukocyte nuclei were isolated from frozen 5-10 mL blood samples by thawing at 23 °C and mixing with an equal volume of PBS (2.5 mM KCl, 137 mM NaCl, 1.5 mM KH₂PO₄, 8mM Na₂HPO₄, pH 7.4) in a 50 mL polypropylene tube. The nuclei were collected by centrifugation at 3,300 g for 10 min. The supernatant was removed from the nuclei pellet by aspiration using a drawn out glass Pasteur pipette. Nuclei were resuspended in 250 µl CDTA buffer (10 mM CDTA, 50 mM Tris HCl, 100 mM NaCl, pH 7.5) and frozen at -70 °C until further extraction. Suspended nuclei were added to 250 µl of Lysis Buffer (200 mM NaCl, 20 mM Tris HCl, 50 mM EDTA, 1 % (w/v) SDS, pH 8.0) and 20 µl of proteinase K (20 mg/ml) and incubated overnight at 55 °C with gentle agitation (Thermomixer compact-Eppendorf). Two hundred and fifty microlitres of Tris HCl saturated phenol (pH 7.9) was added to the mixture before centrifuging at 15,000 g for 6 min. The upper aqueous phase was recovered and the phenol extraction step repeated. The final aqueous phase was extracted by adding 250 µl of chloroform/isoamyl alcohol (24:1) and centrifuged as above. The DNA was precipitated from the aqueous phase by the adding 5 µl of 5M NaCl and 650 µl of cold 95 % (v/v) ethanol. Precipitated DNA was centrifuged at 15,000 g for 5 min and washed in 1 mL of cold 70 % (v/v) ethanol. The DNA was dried for 20 min at 23 °C and resuspended in 200 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

b) "Mini Kit" genomic DNA extraction

The QIAamp DNA Blood Mini Kit was used according to the manufacturers instructions to prepare genomic DNA. Approximately 3.0 µg of genomic DNA (A₂₆₀/₂₈₀ ratio of 1.6-1.9) was prepared from 200 µl of leukocytes. Leukocytes were isolated from 5-10 mL of whole
blood (contains approximately $5 \times 10^6$ leukocytes/ mL of blood) by centrifugation at 3,300 g for 10 min. The upper layer containing plasma was removed by aspiration using a drawn out glass Pasteur pipette. The intermediate layer containing leukocytes was transferred to a fresh tube and resuspended in PBS to a final volume of 200 µl.

2.2.3 Agarose gel electrophoresis

DNA fragments were separated by electrophoresis in submerged TBE-buffered 0.7-1.5% (w/v) agarose gels. DNA loading dye (4 % (w/v) sucrose, 0.25 % (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol) was added to DNA to a final volume of 5 µl before loading onto the gel. Electrophoresis was carried out at 80 V in TBE buffer (90 mM Tris, 90 mM Boric acid, 1.25 EDTA, pH 8.0) containing ethidium bromide (0.5 µg/mL). DNA was visualised by illumination with UV light at 365 nm and photographed using a UVP gel documentation system (Watson and Victor Ltd). All agarose gels were electrophoresed with 1 kilobase ladder, 100 bp ladder or a Low Mass DNA ladder as appropriate.

2.2.4 Quantification of DNA

The concentration of DNA was determined by spectrophotometry using a GeneQuant RNA/DNA calculator (Pharmacia). DNA yield was determined by measuring the concentration of DNA diluted in H₂O (1:1000) by absorbance at 260 nm. The ratio between readings at 260 nm and 280 nm were used as quantification of purity, where pure preparations of DNA have an $A_{260/280}$ ratio > 1.6. Low concentrations of DNA or DNA segments such as PCR products were estimated by electrophoresis alongside a dilution of a known amount of DNA, ie. Low DNA Mass Ladder, in a 1.5 % (w/v) agarose TBE gel.
2.2.5 Polymerase chain reaction (PCR)

a) Oligonucleotides

Stock solutions of oligonucleotides were prepared by reconstituting the lyophilised material in H₂O or TE buffer to a concentration of 50 µM. Primers used are listed in Appendix 2.1.

b) Standard PCR parameters

The conditions for PCR amplification varied depending on the enzyme used and were optimised for gene sequence analysis. For the ELONGASE™ Enzyme Mix system standard reactions contained 0.1-0.2 µg of genomic DNA, 0.1-0.2 µM each of forward and reverse primers, 0.2 mM of each dNTP, 5 µl of Buffer A (60 mM Tris SO₄ (pH 9.1), 18 mM (NH₄)₂SO₄), 5 µl Buffer B (9 mM MgSO₄), 0.2-1.0 units of ELONGASE™ Enzyme Mix, and H₂O to a final volume of 50 µl.

For the AmpliTaq DNA Polymerase system standard reactions contained 0.1-0.2 ng of genomic DNA, 0.1-0.2 µM each of forward and reverse primers, 0.2 mM of each dNTP, 10 µl of Buffer II (10 mM Tris HCl, 50 mM KCl pH 8.3), 1.0-1.5 mM MgCl₂, 0.5-1.0 units of AmpliTaq DNA Polymerase and H₂O to a final volume of 50 µl.

For the Platinum TaqPCRx DNA Polymerase system standard reactions contained 0.1-0.2 ng of genomic DNA, 0.1-0.2 µM each of forward and reverse primers, 0.2 mM of each dNTP, 5 µl of PCR Amplification Buffer, 1.5 mM MgSO₄, 10 µl of PCR Enhancer solution, 1.0-1.5 units of Platinum Taq DNA Polymerase and H₂O to a final volume of 50 µl.

The reactions were carried out in a Master Gradient (Eppendorf) thermocycler using the following parameters: 94 °C for 5 min, 35 x (94 °C for 0.5 min, 55-68 °C for 1.5 min, 68-72 °C for 1 min), 68-72 °C for 5 min.
Details of primers and optimal conditions for specific PCR reactions are given in Appendices 2.1 and 2.2.

2.2.6 DNA purification

a) Ammonium acetate purification

Ten microlitre of PCR product was added to 1 μl of 4 M ammonium acetate and 25 μl of cold 95% (v/v) ethanol. The mixture was incubated on dry ice for 30 min then centrifuged at 15,000 g for 15 min at 4 °C. The supernatant was aspirated and the pellet washed with 1 mL of 70% (v/v) ethanol by inverting the tube several times and recentrifuged. The supernatant was aspirated and the pellet was dried in a speedvac evaporator. The DNA pellet was resuspended in 50 μl of TE buffer.

b) Column purification

PCR products were purified using the Concert™ Rapid PCR purification system according to the manufacturers instructions.

c) ExoI & SAP purification

This enzymatic purification of the PCR product was performed just prior to sequencing. PCR products (2 ng of PCR product per 100 bp) were mixed with 0.5-1.0 units of exonuclease I, 0.5-1.0 units of shrimp alkaline phosphatase and H2O to a total volume of 5 μl.

The reactions were carried out in a Master Gradient (Eppendorf) thermocycler using the following parameters: 37 °C for 15-30 min, 80 °C for 15 min, cool to 4 °C.
2.2.7 DNA sequencing

a) DNA sequencing reaction

The DNA sequencing reaction was carried out in a 96 well MicroAmp tray (Gibco BRL). The sequencing reaction contained 4-30 ng of template DNA (2 ng of PCR product per 100bp), 4 µl of ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction premix (Big dye terminators, dNTP, Amplitaq DNA polymerase, MgCl₂, Tris HCl, pH 9.0), 5 pmol of primer and H₂O to a final of 10 µl.

Thermocycling was carried out in a Master Gradient (Eppendorf) thermocycler using the following parameters: 25 x (96 °C for 10 sec, 50 °C for 5 sec, 60 °C for 4 min).

b) DNA sequencing reaction purification

The DNA sequencing reaction was precipitated in 1.0 µl of 3M sodium acetate (pH 4.6) and 25.0 µl of 95 % (v/v) ethanol. The tray was sealed and inverted to mix, then left to precipitate in the dark at 23 °C for 45 min. The tray was centrifuged at 2000 g for 45 min and the supernatant discarded by inverting the tray onto a paper towel and re-centrifuging at 700 g for 1 min. The pellet was washed in 200 µl of 70 % (v/v) ethanol and centrifuged at 2000 g for 10 min. The supernatant was discarded as above and the pellet left to air-dry in the dark.

c) DNA sequencing

DNA sequencing was carried out by Liam Williams (Centre for Gene Technology, University of Auckland) in an ABI Prism 377XL DNA sequencer. This system utilises the dideoxy chain termination method with dideoxy terminators [160]. Sequences were analysed by ABI Sequence Navigator and compared with nucleotide database sequences at
Sequences that were ambiguous were repeated and then determined on the antisense strand. Analysis of nucleotide and amino sequences was performed on the Expert Protein Analysis System (ExPASy) proteomics server of the Swiss Institute of Bioinformatics (SIB) at [http://www.expasy.ch/](http://www.expasy.ch/) using Translate for translation of DNA sequences.

### 2.2.8 Statistical analysis

For statistical analysis, results were presented as means ± SD. Statistical significance of the -215T>G amylin gene allele, the 185T IPF-1 gene allele, and -42G>A HNF-1α allele was determined by the Fishers Exact test. The 2 x 3 chi square table was used to compare frequencies of the -230A>C allele. Association of both mutations with diabetes was determined by the McNears test. A p value less than 0.05 was considered statistically significant. Relative risk was estimated using odds ratios, and 95 % confidence limits were calculated.

The frequency of alleles was tested by the Hardy-Weinberg equilibrium model \( p^2 + 2pq + q^2 = 1 \), where \( p \) is defined as the frequency of the dominant allele and \( q \) is the frequency of the recessive allele trait.
Chapter 3: Results

3.1 Patient sample characteristics

The aim of the present study was to screen for mutations in the genes encoding amylin and IPF-1, and exon 2 of the HNF-1α gene in Maori and Pacific Island people living in NZ, and to assess whether these mutations co-segregate with type 2 diabetes in these two populations. Most reports on the genetic similarities and/or differences between Maori and Pacific Island populations have focussed on the origins of Polynesians from South East Asia. Analysis of Y-chromosome variations reported three haplotypes common to Asian, Maori, and other Polynesian but not European populations [154]. Only one of these Y-chromosome haplotypes, h33, is unique to Maori and other Polynesians indicating that this lineage is indigenous to the Pacific. Mitochondrial DNA analysis identified four different haplotypes common to Maori and other Polynesians [157] plus a 9-bp deletion motif previously reported to predominate in Asian populations [154]. Finally, comparison of HLA DQA alleles between Maori, Pacific Island, NZ Caucasian and Northern Caucasian report that Maori and Pacific Island share HLA DQA allele frequency distributions more similar to each other compared to other populations studied [161].

Few gene association studies in both Maori and Pacific Island have been reported to date. Linkage analysis and gene association of the glucokinase gene in Pacific Island type 2 diabetics and a Maori cohort have failed to identify a role for this gene in Maori or Pacific Island diabetes [144, 145]. Similarly, gene association studies of the HNF-1α gene did not identify a role for this gene in Pacific Island diabetes, [146, 147]. This however, has not been investigated in Maori diabetes to date.

The first study population consisted of 131 Maori subjects with a clinical diagnosis of type 2 diabetes and blood samples were provided by Dr David Gilgen of Waahi Pa Medical Centre and Dr Tony Birch of Hauora Hokianga. Maori non-diabetic blood samples (n=258) were provided by the Hepatitis Foundation in Whakatane. Permission to use Maori DNA
for genetic analysis by DNA sequencing was approved by Iwi (Tainui and Ngapuhi) in hui in accordance with Maori protocol. At the time of blood sampling informed consent was obtained from each individual as approved by the Auckland Ethics Committee. The second study population consisted of 15 Pacific Island subjects with a clinical diagnosis of type 2 diabetes and 129 Pacific Island non-diabetics. Blood samples for these patients were provided by Dr David Simmons from the South Auckland Diabetes Clinic.

Maori type 2 diabetics, 59 women and 72 men, were aged 56.9 ± 12.4 years (mean ± SD), had a body mass index (BMI) of 31.7 ± 4.4 kg/m² and an HbA₁c of 9.5 ± 1.8 % (Table 3.1). Of those who responded to the “types of therapy” question during the interview (n=85), 31.8% (n=27) were treated with diet, 41.2% (n=35) with oral hypoglycaemic agents (OHA), and 27% (n=23) with insulin. The average age at diagnosis of diabetes for this group was 46.0 ± 9.2 years. Of this group, 39% reported a history of diabetes in a first-degree relative. The control Maori non-diabetic group, 138 women and 120 men, were aged 61.4 ± 7.9 years, had an HbA₁c of 5.9 ± 0.1%, and to their knowledge had no known familial history of diabetes (Table 3.1). BMI was not calculated for the control group due to inconsistent weight measurements when using a portable weighing machine.

Pacific Island type 2 diabetics, 10 women and 5 men, were aged 53.6 ± 18.4 years, had a BMI of 38.8 ± 5.3 kg/m² and fasting plasma glucose levels of 12.3 ± 0.5 mmol/l (Table 3.1). The average age at diagnosis of diabetes for this group was 49.0 ± 8.2 years. The control Pacific Island non-diabetic group, 65 women and 64 men, were aged 39.2 ± 15.1 years and had an HbA₁c of 5.5 ± 0.6%. Pacific Island subjects were of Samoan, Tongan or Cook Island descent. Information on HbA₁c, treatment for diabetes and family history for the disease was unavailable for this sample group.
### Table 3.1 Clinical characteristics for Maori and Pacific Island subjects.

Obesity occurs when body weight is ≥ 20% above the ideal body weight and was defined as BMI ≥ 30 kg/m². Treatment requirement was recorded for only 85 Maori diabetics and none of the Pacific Island diabetics. OHA, oral hypoglycaemic agents. Data are means ± SD.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Maori</th>
<th></th>
<th>Pacific Island</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patients</td>
<td>Controls</td>
<td>Patients</td>
<td>Controls</td>
</tr>
<tr>
<td>Subjects</td>
<td>131</td>
<td>258</td>
<td>15</td>
<td>129</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>72/59</td>
<td>138/120</td>
<td>5/10</td>
<td>64/65</td>
</tr>
<tr>
<td>Age (years) ± SD</td>
<td>57.0 ± 12.4</td>
<td>61.4 ± 7.9</td>
<td>53.6 ± 18.4</td>
<td>39.2 ± 15.1</td>
</tr>
<tr>
<td>BMI (kg/m²) ± SD</td>
<td>31.7 ± 4.4</td>
<td>—</td>
<td>38.8 ± 5.3</td>
<td>—</td>
</tr>
<tr>
<td>Age at diagnosis</td>
<td>46.0 ± 9.2</td>
<td>—</td>
<td>49.0 ± 8.2</td>
<td>—</td>
</tr>
<tr>
<td>Duration of diabetes (years)</td>
<td>12.0 ± 9.7</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>HbA₁c (%)</td>
<td>9.5 ± 1.8</td>
<td>5.9 ± 0.1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Fasting plasma glucose (mmol/l)</td>
<td>—</td>
<td>—</td>
<td>12.3 ± 0.5</td>
<td>5.5 ± 0.6</td>
</tr>
<tr>
<td>Treatment (diet/OHA/insulin)</td>
<td>27/35/23</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Prevalence of obesity</td>
<td>63%</td>
<td>—</td>
<td>80%</td>
<td>—</td>
</tr>
</tbody>
</table>
3.2 Identification of gene sequence variants in Maori and Pacific Island people with late onset type 2 diabetes

3.2.1 Amylin gene allelic variations

To determine whether diabetes-associated variants in the amylin gene were present in Maori and Pacific Island populations, the amylin gene was PCR amplified and directly sequenced to look for allelic variations in the following regions: 393 nucleotides in the 5' upstream region flanking exon 1, exon 1, exon 2, intron 1, the coding region of exon 3 (approximately 187 bp) and 130 nucleotides following the stop codon. The present study identified three new mutations in the amylin gene: a missense mutation in exon 3, Q10R, and two alleles in the proximal promoter -132G>A and -215T>G (Figure 3.5). A previously reported polymorphism -230A>C was also observed [174]. None of these alleles matched Single Nucleotide Polymorphism's for the amylin gene reported in the NCBI database (locus ID: 3375). The sequences for exons 1 and 2 of the amylin gene in all subjects screened were identical to the published sequence (Genbank accession #: X68830).

a) The Q10R mutation

Direct sequencing of exon 3 revealed a homozygous missense mutation, detected by a single nucleotide peak at +48bp, in one Maori diabetic out of the 131 subjects tested (0.76%) (Figure 3.3). No heterozygotes were observed and therefore did not agree with the Hardy-Weinberg model where the expected heterozygote frequency was 1.5%. The T/C allele, which creates a Kasl restriction site, is predicted to result in a glutamine to arginine substitution (Q10R) (Figure 3.2) at amino acid 10 of the mature amylin molecule. The carrier is a 60-year-old female, diagnosed at the age of 56 years and treated since then with insulin. The patient has a BMI of 28.4 kg/m² and reports a family history of diabetes (mother and siblings). The Q10R mutation was not found in the remaining Maori subjects with type 2 diabetes or the controls, nor was it detected in Pacific Island subjects.
<table>
<thead>
<tr>
<th>Animal</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human WT</td>
<td>KCNTATCATQRLANFLVHSSNNFGAILSSTNVGSNTY-NH$_2$</td>
</tr>
<tr>
<td>Human Q10R</td>
<td>KCNTATCATRRRLANFLVHSSNNFGAILSSTNVGSNTY-NH$_2$</td>
</tr>
<tr>
<td>Monkey</td>
<td>KCNTATCATQRLANFLVRSNNFGTILSSTNVGSNDY-NH$_2$</td>
</tr>
<tr>
<td>Rat</td>
<td>KCNTATCATQRLANFLVRSNNNLGPVLPPTNVGSNTY-NH$_2$</td>
</tr>
<tr>
<td>Mouse</td>
<td>KCNTATCATQRLANFLVRSNNNLGVPVLPPTNVGSNTY-NH$_2$</td>
</tr>
<tr>
<td>Dog</td>
<td>KCNTATCATQRLANFLVRTSNNLGAILSPTNVGSNTY-NH$_2$</td>
</tr>
<tr>
<td>Cat</td>
<td>KCNTATCATQRLANFLVRSNNLGAILSPTNVGSNTY-NH$_2$</td>
</tr>
<tr>
<td>Guinea Pig</td>
<td>KCNTATCATQRLANFLVRSNNLGAIALLPTDVGSNTY-NH$_2$</td>
</tr>
<tr>
<td>Hamster</td>
<td>KCNTATCATQRLANFLVHSSNNFGAILSPFTNVGSNTY-NH$_2$</td>
</tr>
<tr>
<td>Rabbit</td>
<td>KCNTATCATQRLANFLIHSSNNFGAIFSSPVGVSENTY-NH$_2$</td>
</tr>
<tr>
<td>Hare</td>
<td>KCNTATCATQRLANFLIHSSNNFGAIFSSPVGVSENTY-NH$_2$</td>
</tr>
</tbody>
</table>

**Figure 3.1** Amino acid sequence of human wild type amylin, the mutation Q10R, and amylin sequences from nine other animals. This comparison shows that an R residue does not occur at position 10 in any of the non-human sequences. Residues that differ from human amylin at equivalent positions are shown in bold type. The amyloidogenic region at amino acids 20 to 29 is underlined.
Figure 3.2 The Q10R mutation. Sequence electropherogram for the C/C homozygote (bottom) at +48 bp of exon 3 of the amylin gene, resulting in a glutamine to arginine substitution at amino acid 10 (Q10R) compared to wild type (top).
b) The -132G>A allele

The -132G>A allele was observed in one Maori diabetic subject out of the 131 tested (0.76%) but in none of the 258 control subjects. This heterozygote allele, as detected by two nucleotide peaks at -132bp in the electropherogram (Figure 3.3), results in deletion of an MwoI restriction site. This allele did not deviate from the Hardy-Weinberg equilibrium. The carrier was a 56-year-old female, who was diagnosed with type 2 diabetes at the age of 45 years and treated since then with insulin. She had a family history of diabetes. The patient had a BMI of 26.7 kg/m² and was therefore below the cut off range for obesity (BMI ≥ 30 kg/m²). Family members of this proband were not tested for -132G>A as the patient declined from the study shortly after the variant was identified. The -132G>A variant was not observed in any of the Pacific Island patients.

<table>
<thead>
<tr>
<th>Location in the amylin gene</th>
<th>Nucleotide sequence change</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Maori diabetics (n=131)</td>
<td>Maori controls (n=258)</td>
</tr>
<tr>
<td>5’upstream region</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-230</td>
<td>A → C</td>
<td>24.0%</td>
</tr>
<tr>
<td>-215</td>
<td>T → G</td>
<td>5.34%</td>
</tr>
<tr>
<td>-132</td>
<td>G → A</td>
<td>0.76%</td>
</tr>
<tr>
<td>Exon 3</td>
<td></td>
<td>—</td>
</tr>
<tr>
<td>Codon 10</td>
<td>CAG(Gln) → CGG(Arg)</td>
<td>0.76%</td>
</tr>
</tbody>
</table>

Table 3.2 Amylin gene sequence allelic variants identified in Maori and Pacific Island populations with late-onset type 2 diabetes. The frequency of each allelic variation was determined by genotyping 258 and 129 unrelated normal healthy Maori and Pacific Island populations respectively.
Figure 3.3 The -132G>A mutation. Sequence electropherogram for the G/G homozygote (top), and G/A heterozygote (bottom) at -132bp of the amylin gene. R = guanine or adenine
c) The $-230A>C$ polymorphism

The allelic variation at $-230$bp, resulting in substitution of adenine (A) for cytosine (C), was observed in both the heterozygous and homozygous state (Figure 3.4). The frequency of this allele in Maori with type 2 diabetes and the non-diabetic control group was 26.4% and 24% respectively (odds ratio = 1.02, [0.63-1.64], $p = 0.94$) and is therefore not associated with type 2 diabetes in Maori (Table 3.3). Similarly, the frequency of the $-230A>C$ allele in Pacific Island patients with type 2 diabetes and non-diabetic control group was 13.3% and 10% respectively (odds ratio = 1.0, [0.43-2.34], $p = 0.84$) is and therefore not associated with type 2 diabetes in this population. These genotype frequencies did not deviate from the Hardy-Weinberg equilibrium.

<table>
<thead>
<tr>
<th></th>
<th>A/A</th>
<th>A/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maori control</td>
<td>178 (69%)</td>
<td>68 (26.4%)</td>
</tr>
<tr>
<td>Maori type 2 diabetics</td>
<td>92 (70.23%)</td>
<td>31 (24%)</td>
</tr>
<tr>
<td>Pacific Island controls</td>
<td>113 (84.53%)</td>
<td>13 (10.08%)</td>
</tr>
<tr>
<td>Pacific Island type 2 diabetics</td>
<td>13 (86.67%)</td>
<td>2 (13.33%)</td>
</tr>
</tbody>
</table>

Table 3.3 Frequency of the adenine (A) and cytosine (C) alleles at position $-230$bp of the amylin gene in Maori and Pacific Island populations.

d) The $-215T>G$ allele

The $-215T>G$ allele was observed in 7 type 2 diabetic Maori patients (5.34%) and 2 non-diabetic Maori controls (0.76%) but not Pacific Island subjects (summarised in Table 3.4). All affected subjects were heterozygous for the $-215T>G$ allele, as detected by two nucleotide peaks at the same position in the electropherogram (Figure 3.4). The allelic variations at $-215$ bp did not deviate from the Hardy-Weinberg equilibrium. The $-215T>G$ allele was significantly associated with type 2 diabetes in Maori with an odds ratio of 7.23, [1.5-35.3], $p = 0.008$. Both the non-diabetic carriers had healthy BMI's of 24.38 and 25.29 kg/m$^2$, whereas the average BMI for diabetic carriers was 28 ± 2.5 and were therefore
classed as overweight. However, the statistical significance for differences in BMI was unable to be calculated due to lack of BMI data for the control group for comparative analysis. Similarly, the statistical differences in age were not calculated because age range was highly variable for this sample group.

Of the 7 diabetics carrying the -215T>G allele, 4 also presented with -230A>C (Table 3.4). Thus 3.1% of Maori diabetics were carriers for both the -215T>G and -230A>C mutations. The occurrence of these two alleles together was greater than that due to chance ($X^2 = 46.7; p > 0.01$) and the odds ratio of -230A>C carriers inheriting the -215T>G mutation was 2.27. There was no significant difference in BMI ($p=0.459$) and HbA1c levels ($p=0.356$) between subjects carrying both -215T>G and -230A>C compared to -215T>G only carriers.

<table>
<thead>
<tr>
<th>Subject number</th>
<th>Sex</th>
<th>Age at diagnosis (yrs)</th>
<th>Family history</th>
<th>BMI (kg/m²)</th>
<th>HbA1c (%)</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM069*</td>
<td>F</td>
<td>50</td>
<td>yes</td>
<td>28.95</td>
<td>9.55</td>
<td>OHA</td>
</tr>
<tr>
<td>DM081</td>
<td>M</td>
<td>48</td>
<td>yes</td>
<td>31.51</td>
<td>9.3</td>
<td>Diet</td>
</tr>
<tr>
<td>DM087*</td>
<td>F</td>
<td>42</td>
<td>yes</td>
<td>28.23</td>
<td>8.8</td>
<td>Diet</td>
</tr>
<tr>
<td>DM106</td>
<td>F</td>
<td>62</td>
<td>yes</td>
<td>27.99</td>
<td>12.87</td>
<td>OHA</td>
</tr>
<tr>
<td>DM120*</td>
<td>M</td>
<td>57</td>
<td>yes</td>
<td>28.60</td>
<td>8.5</td>
<td>Diet</td>
</tr>
<tr>
<td>DM093</td>
<td>F</td>
<td>66</td>
<td>yes</td>
<td>26.67</td>
<td>10.8</td>
<td>OHA</td>
</tr>
<tr>
<td>DM119*</td>
<td>M</td>
<td>41</td>
<td>yes</td>
<td>29.01</td>
<td>9.24</td>
<td>diet</td>
</tr>
<tr>
<td>NM8645</td>
<td>F</td>
<td>55</td>
<td>no</td>
<td>24.38</td>
<td>5.6</td>
<td>—</td>
</tr>
<tr>
<td>NM8716</td>
<td>M</td>
<td>59</td>
<td>no</td>
<td>25.29</td>
<td>5.8</td>
<td>—</td>
</tr>
</tbody>
</table>

Table 3.4 Clinical profile of carriers for the -215T>G allele. DM = Maori subjects with type 2 diabetes; NM = non-diabetic Maori subjects. *These patients presented with both -215T>G and -230A>C.
Figure 3.4 The -230A>C and -215T>G mutations. Sequence electropherograms for A/A (top), A/C (middle) & C/C (bottom) alleles at -230bp; and T/G (middle) allele at -215bp of the amylin gene. M = cytosine or adenine. K = guanine or thymine.
Figure 3.5 Location of human amylin gene mutations within exon 3 and promoter region of the amylin gene. The missense mutation, Q10R, and two mutations in the non-coding promoter region, -215T>G and -132G>A, account for approximately 7% of type 2 diabetes in Maori. The previously described mutations -230A>C and S20G do not contribute to type 2 diabetes in NZ Polynesians [98, 100, 150, 174, 175].
The allelic variants identified in this study lie within the minimal control region of the amylin gene (−390 to +450 bp). Comparisons with the rat and mouse amylin promoter region show that −230A>C and −132G>A occur at conservative positions, however −215T>G does not. The −132G>A allele results in a nucleotide change in the E-box consensus sequence (CANNCG/A) at −140/−130 bp [162] while the TAAT motifs A1 (−87/−82 bp), A2 (−149/−143 bp) and A3 (−249/−244 bp), and insulin enhancer-like sequence (−245/−238 bp) remain unaffected. To determine whether these alleles might possibly disrupt the binding site for transcription factors, potential binding sites in the vicinity of these variants were screened for using the MatInspector program on the TRANSFAC 4.0 database. Four potential binding sites for activator protein−1 (AP−1), two sites for AP−4, one for HNF−1, one for nuclear factor of activated T cells (NF−AT), and one for CAAT/enhancer binding protein β (C/EBPβ) were identified in the minimal control region. The −215T>G allele was found to lie in the binding site for the ubiquitous factor Activator Protein−1 (−217/−207 bp) changing a nucleotide in the core region, represented by four highly conserved consecutive nucleotides, of the AP−1 consensus sequence. Both −230A>C and −132G>A affected the flanking sequences for an AP−4 putative binding site in the reverse orientation. The −132G>A allele also lies within the flanking sequence of another AP−1 potential binding site. The selection criterion for the putative binding sites was a matrix similarity > 0.8 with core sequence equal to 1 (Table 3.5).

<table>
<thead>
<tr>
<th>Allelic variation</th>
<th>Conservative a position</th>
<th>Putative binding sites</th>
<th>Location b (orientation)</th>
<th>Ntd Seq</th>
<th>Core similarity</th>
<th>Matrix similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>−230A&gt;C</td>
<td>yes</td>
<td>AP−4 flanking; AP−1</td>
<td>−227/−218bp (-)</td>
<td>caCAG</td>
<td>1.0</td>
<td>0.914</td>
</tr>
<tr>
<td>−215T&gt;G</td>
<td>no</td>
<td>AP−1</td>
<td>−217/−207bp (+)</td>
<td>Caaga</td>
<td>1.0</td>
<td>0.921</td>
</tr>
<tr>
<td>−132G&gt;A</td>
<td>yes</td>
<td>E1; AP−4 flanking; AP−1</td>
<td>−140/−130bp (+); −130/−121bp (-);</td>
<td>Cacacc</td>
<td>1.0</td>
<td>0.905</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>−145/−136 (+)</td>
<td>Caga</td>
<td>1.0</td>
<td>0.921</td>
</tr>
</tbody>
</table>

Table 3.5 Location and characteristics of nucleotide amylin gene promoter allelic variants. *Comparison of the promoter region sequences of the human, rat and mouse amylin gene. *Location is given according to the start of exon 1. *Transcription factor binding site located in DNA strand in a 5’ to 3’ direction (+), or in the complementary DNA strand in a 5’ to 3’ direction (−).
3.3 **The G55G mutation in the IPF-1 gene**

The transcription factor IPF-1 plays a role in the development of the early pancreas and regulation of β cell genes including insulin, Glut 2, glucokinase, and amylin [135, 136]. The human IPF-1 gene comprises two exons extending over a 5 kb on chromosome 13q12 [138]. A number of allelic variations identified in the IPF-1 gene represent predisposing alleles and increase the relative risk of late-onset diabetes [136]. To determine whether diabetes-associated alleles in the IPF-1 gene were present in type 2 Maori and Pacific Island diabetes the IPF-1 gene was screened by PCR and direct sequencing of genomic DNA from 131 and 15 type 2 diabetic Maori and Pacific Island patients, plus 258 and 129 non-diabetic Maori and Pacific Island control subjects. The clinical characteristics of type 2 diabetic and control subjects are summarised in Table 3.1. The region of IPF-1 gene screened for mutations included the following: the upstream enhancer sequence (approximately 1.8 kb upstream of exon 1 start codon), the proximal promoter region (approximately 240 bp upstream of exon 1), exon 1 (approximately 410 bp) and 71 nucleotides of flanking intron 1, the coding region of exon 2 (approximately 470 bp) and 16 nucleotides following the stop codon. Because IPF-1 gene sequences are highly G-C rich, the PCRx Enhancer System (BRL) was used for PCR amplification. This system contains additional co-solvents for amplification of sequences that are 50% to 90% G-C rich.

In the exon 1 coding sequence of IPF-1, a novel nucleotide sequence variation GGC→GGT was identified in two unrelated Maori type 2 diabetic patients but not the Pacific Island type 2 diabetic patients or in the control groups. The 185T allele was not significantly associated with type 2 diabetes in Maori (p = 0.113). This allele is predicted not to affect the amino acid sequence at codon 55, G55G, and is therefore a silent mutation. Both affected patients were homozygous for the 185T allele, as detected by a single nucleotide peak at the same position in the electropherogram (Fig 3.6). One carrier was a 57-year-old male, diagnosed at the age of 49 years, had a BMI of 26.43 kg/m² and was treated by oral hypoglycaemic agents. The other carrier was a 51-year-old female, diagnosed at the age of 44 years, had a BMI of 23.7 kg/m² and was treated by diet alone. The coding sequence of
IPF-1 in the remaining subjects with type 2 diabetes and non-diabetics was the same as the published sequence (Genbank accession #: AH005051) and that determined from PAC80C14. Likewise, the upstream enhancer sequence and proximal promoter region in all subjects screened was the same as the published 5'flanking region (Genbank accession #: AF192496).

**Figure 3.6** The C185T allele. Sequence electropherogram for the C/C homozygote (top) and T/T homozygote (bottom) at 185 bp of exon 1 in the IPF-1 gene.
3.4 The -42G>A polymorphism in the HNF-1α gene

The transcription factor HNF-1α is expressed in the liver, pancreas, intestine, stomach and kidney where it regulates expression of many different genes [124]. The HNF-1α gene comprises 10 exons extending over a 23 kb region on chromosome 12q. A total of 65 mutations and 14 polymorphisms in the HNF-1α gene have been reported to date, a number of which represent a common cause of MODY in the majority of populations studied [134]. Previous screening for allelic variations in all 10 exons, flanking introns and promoter region of HNF-1α failed to identify any alleles that may be associated with Pacific Island type 2 diabetes [147]. The Pacific Island population used in this previous study are also the subjects for this thesis. Since then, a polymorphism, nt +66G>C, located in the reverse primer annealing site for PCR amplification of exon 2 has been reported [148]. The nt +66G>C polymorphism resulted in non-amplification of three missense mutations (K117E, H143Y and R171X) shown to co-segregate with diabetes in the pedigrees studied. To determine whether diabetes-associated alleles in exon 2 of the HNF-1α gene were present in Maori and Pacific Island diabetes, exon 2 was screened by PCR and direct sequencing of genomic DNA using an alternative reverse primer (Appendix 1.2) located 52 nucleotides downstream of the original primer annealing site used by Green [147]. The clinical characteristics of Maori and Pacific Island type 2 diabetic patients and non-diabetic controls are summarised in Table 3.1.

This study identified a previously reported intronic polymorphism resulting in substitution of guanine (G) for adenine (A) in intron 1, 42 nucleotides upstream of exon 2 [134]. The 327-42G>A polymorphism was observed in 51 (39%) and 5 (33%) Maori and Pacific Island type 2 diabetic patients, and 79 (31%) and 46 (36%) Maori and Pacific Island non-diabetic controls respectively (Table 3.6). The -42G>A polymorphism was not significantly associated with type 2 diabetes in Maori or Pacific Island people ($p = 0.118$ and 1.00 respectively). None of the missense mutations described by Ellard et al [148] were observed in this study and is therefore in agreement with Green’s report [147] that
HNF-1α alleles in exon 2 are not a common cause of Maori and Pacific Island type 2 diabetes.

<table>
<thead>
<tr>
<th></th>
<th>G/G</th>
<th>G/A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maori type 2 diabetics</td>
<td>80 (61%)</td>
<td>51 (39%)</td>
</tr>
<tr>
<td>Maori control subjects</td>
<td>179 (69%)</td>
<td>79 (31%)</td>
</tr>
<tr>
<td>Pacific Island type 2 diabetics</td>
<td>10 (67%)</td>
<td>5 (33%)</td>
</tr>
<tr>
<td>Pacific Island control subjects</td>
<td>83 (64%)</td>
<td>46 (36%)</td>
</tr>
</tbody>
</table>

*Table 3.6* Frequency of the guanine (G) and adenine (A) alleles at position -42bp of intron 1 of the HNF-1α gene in Maori and Pacific Island populations.
Chapter 4: Discussion

NZ Maori and Pacific Island people are high risk populations for diabetes. They experience a high incidence of diabetes-related complications and at an earlier age compared to Caucasians [1]. The aim of this project was to assess the role of mutations in the genes encoding amylin and IPF-1, and exon 2 of the HNF-1α gene in the pathogenesis of type 2 diabetes in the Maori and Pacific Island populations. Variations in these genes have previously been shown to be present in other populations with a high incidence of diabetes. These target genes were sequenced for variations in their proximal or minimal promoter segments, exons and adjacent intronic sequences. The results of this study suggest that mutations in the amylin gene contribute to approximately 7% of the genetic risk for type 2 diabetes in Maori and Pacific Island people.

4.1 Polymorphisms in the amylin gene

4.1.1 Background

Amylin is a 37 amino acid polypeptide that is co-secreted with insulin from the pancreatic β islet cell in response to nutrient intake [163]. It is the main constituent of extracellular amyloid deposits often found in human insulinomas and in pancreatic islets of patients with type 2 diabetes. Plasma concentrations of amylin are decreased in patients with type 1 diabetes and elevated in patients in the early stages of type 2 diabetes. Elevated plasma amylin levels have also been described in patients with impaired glucose tolerance, obese subjects and in pregnant women with normal glucose tolerance and women with gestational diabetes.
Although its precise function is unknown, amylin has been implicated in the regulation of insulin and glucose metabolism. In skeletal muscle, amylin promotes glycogenolysis and inhibits glycogenesis resulting in elevated serum lactate [164]. Pancreatic perfusion of amylin induces insulin resistance in rats by reducing glucose uptake and increasing hepatic glucose production, which in turn can be reversed by perfusion with the amylin antagonist, amylin-(8-37). Amylin is also a potent inhibitor of gastric emptying. This is mediated in part by the area postrema of the hindbrain, which contains the vagus nerve nuclei required for gastric motility. It has putative amylin receptors [165]. Hence amylin may act to regulate glucose flow to the circulation by influencing the rate of gastric emptying, and thus the rate at which meal-derived glucose enters the system. Amylin may also inhibit glucose release and hepatic glucose production in the postprandial period.

β-amyloid protein forms fibrils in Alzheimer’s disease and is toxic to neurons [166], therefore a similar mechanism has been proposed for amylin in pancreatic β cell loss. In vitro, human amylin inhibits β cell proliferation and induces islet cell apoptosis [167]. Toxicity may be mediated by the large fibrillar form of the mature amyloid deposit and require direct contact of the fibrils with the cell surface. Alternatively, over expression of amylin in pancreatic β cells results in accumulation of intracellular amyloid deposits localised to the ER and Golgi apparatus [168]. Intracellular amyloid deposits evoke specific apoptotic signalling pathways in these cells. This process requires less amylin to be cytotoxic than amylin added exogenously in cell culture studies.

Another proposal is that an amylin protofibrillar intermediate might be the cytotoxic factor. This is supported by the observation that transgenic mice, homozygous for the human amylin gene, spontaneously develop diabetes without amyloid deposition, however contain amorphous intra- and extracellular aggregates [169]. Furthermore, smaller aggregates derived from freshly prepared amylin solutions, but not larger mature amyloid deposits, evoke apoptosis of mouse and human islets in vitro [166]. Finally, in the high-fat induced diabetic strain db/db, β cell loss appeared to be insufficient to explain the severity of hyperglycaemia in these mice, suggesting that the amyloidogenic process may impair β islet function before cell death. Based on the association of amylin deposits with
hyperinsulinaemia, a predictor for impaired glucose tolerance in islet cell tumours, fibril formation has been proposed to begin during impaired glucose tolerance. This is after other factors cause the initial defects in early insulin secretion and insulin action.

Collectively this information suggests that over expression of amylin and the amyloid deposition process may be important contributors to the development of type 2 diabetes. Therefore, sequence variations in the 5' regulatory region of the amylin gene affecting transcriptional activity, such as those found in the present study, may be candidates for inheritable defects in the common late-onset form of type 2 diabetes. To address this question in Polynesian populations, the promoter sequences critical for regulation of amylin gene expression were screened for sequence variants. There are two regions of the 5' upstream sequences in the amylin gene known to be important for islet β cell function. These include the intronic region from +104 to +434 base pairs which appear to be important in post-transcriptional regulation of amylin expression [162]; and the promoter proximal region between −222 and −91 base pairs which contain the cis acting elements, E1 and A-box DNA-binding motifs, required for islet-specific transcript activity [170]. The amylin gene A-boxes, which occur at similar sites in the insulin promoter, have been shown to bind IPF-1 [171], and the LIM homeodomain transcription factor Isl1 [172], both of which play a critical role in the developing pancreas. The present study identified two novel and one previously described mutation in the proximal promoter of the amylin gene.

In addition to the 5' untranslated region, mutations in the coding sequences that affect the normal function of the mature protein may also be an important mechanism for inducing disease. The S20G amylin gene mutation reported in both Japanese and Chinese patients with early and late onset diabetes [94], results in a more hydrophobic amyloidogenic domain which is proposed to increase the fibrillogenic properties of the peptide [100]. Intracellular amyloid accumulation activates specific apoptotic signalling pathways [173] and may be a mechanism for β cell loss in type 2 diabetes. Therefore the enhanced ability for S20G to form amyloid fibrils in vitro may be a factor in β cell pathophysiology and the genesis of type 2 diabetes. This study identified a single a missense mutation, Q10R, in exon 3. None of the mutations in the promoter or coding region of the amylin gene,
matched the SNPs for the amylin gene reported in the NCBI database (locus ID: 3375). No mutations were observed in the post-transcriptional regulatory region downstream of the transcription start site, or in the remaining two exons.

4.1.2 The $-230A>C$ amylin polymorphism

The previously described polymorphism, $-230A>C$ [174], has been identified in a Japanese diabetic subject with marked islet amyloid. However, no association of this polymorphism and type 2 diabetes was found in a larger Japanese cohort [175].

Similarly, there was no significant difference in frequency of the $-230A>C$ polymorphism between patients with type 2 diabetes and the control group in both the Maori and Pacific Island populations (Table 3.3) indicating that on its own, the $-230A>C$ polymorphism is unlikely to be associated with type 2 diabetes in New Zealand Polynesians. The $-230A>C$ polymorphism was observed in 24% of Maori, 10% of Pacific Island, and 60% of Japanese patients with type 2 diabetes [175]. However, the differences in frequency between these three populations is unlikely to be significant due to the relatively small sample size tested in the Japanese study ($n=35$ diabetic patients & 49 control subjects) compared with the present study.

4.1.3 The $-215T>G$ amylin allele

The T/G amylin gene allele at position $-215$ bp, which was observed in Maori but not Pacific Island people, has not yet been described in other populations. There was a significant association between $-215T>G$ and diabetes in Maori, 5.34% in type 2 diabetics and 0.76% in controls ($p=0.008$). These findings suggest that $-215T>G$ may contribute to the genetic susceptibility for type 2 diabetes in Maori. However, its low penetrance in non-diabetics indicate that it may be insufficient to cause diabetes by itself. The $-215T>G$ allele predisposes the carrier to type 2 diabetes with a relative risk of 7.23, the severity of
which may increase in the presence of additional environmental or genetic factors. Both
the non-diabetic carriers had healthy BMI's of 24.38 and 25.29 kg/m², whereas the average
BMI for diabetic carriers was 28 ± 2.5 and were therefore classed as overweight. Thus a
low BMI may be an important factor for maintaining normoglycaemia in these subjects.
However, the statistical significance for differences in BMI was unable to be calculated due
to lack of BMI data for the control group for comparative analysis. The statistical
significance in differences in age between diabetic and non-diabetic carriers was not
calculated because age range was highly variable for this sample group. The present study
was therefore unable to determine the affect of BMI and age in propensity to diabetes in
-215T>G carriers. It is possible that the non-diabetic carriers may develop type 2 diabetes
later in life. Thus it would be of interest to test the non-diabetic carriers for signs of
deterioration of glycaemia and β cell function by fasting glucose and OGTT, and perhaps
homeostasis model assessment (HOMA). The former test measures plasma glucose levels
while fasting and two hours following a 75g-glucose load. The HOMA model is a measure
of insulin resistance and secretion from fasting glucose and insulin concentrations, and uses
a mathematical model of the body's glucose and insulin interaction as a frame of reference
(HOMA insulin resistance index = fasting glucose [mmol/L] x fasting insulin [μU/ml]).

Of the seven diabetics carrying -215T>G, four also presented with the -230A>C polymorphism. Thus 3.1% of patients with type 2 diabetes were carriers for both the
-215T>G and -230A>C alleles. This was not observed in any of the non-dietetics. The
occurrence of these two mutations together was greater than that due to chance (p < 0.001) and suggests linkage disequilibrium exists between these two alleles. This is likely since
the -215T>G and -230A>C alleles are separated by only 15 bp. Thus it is worth testing for
both the -215T>G and -230A>C alleles together in future population screenings. To prove
any synergistic effect of -215T>G and -230A>C, as well as the effect of -215T>G allele,
screening for co-segregation of these alleles in the seven families will need to be conducted. The inheritance of two or more diabetes susceptibility markers together, has
been reported in the genes encoding IPF-1, HNF-1α and islet brain-1 (IB-1) [139]. Subjects who inherited both markers had a more severe form of diabetes compared with
those carrying IPF-1, IB-1 or HNF-1α mutations alone. This suggests a nearby mutation
that is in linkage disequilibrium with a known diabetogenic gene, which could contribute to the polygenic nature of type 2 diabetes. Finally, intrafamilial analysis would determine whether the -215T>G allele co-segregates with type 2 diabetes in the family as well as determine the mode of transmission.

The absence of -215T>G in Pacific Island people may be due to the relatively small sample size of diabetics (n=15) compared to Maori diabetics (n=131). To completely rule out a role for -215T>G in Pacific Island diabetes, a larger cohort of Pacific Island diabetics would need to be screened. Similarly, it would be of interest to screen for the -215T>G in NZ Caucasians with type 2 diabetes to confirm that this amylin promoter variant is specifically associated with Polynesian diabetes only. Both these investigations will help determine the efficacy of amylin -215T>G as a diagnostic marker for type 2 diabetes in Maori. Alternatively, the absence of -215T>G in Pacific Island people may indicate that Maori may represent a different subset of type 2 diabetes in Polynesians as determined by the presence of -215T>G. The functional significance of this mutation in the pathogenesis of type 2 diabetes in Maori can be confirmed by further clinical studies, as described above, and subsequent functional analysis of the promoter variant. Collectively, these future investigations may help determine whether the -215T>G allele is a causative factor of type 2 diabetes in Maori.

All the promoter amylin gene alleles identified in this study lie within or near the proximal promoter region (-222 and -91 base pairs), which contains the cis-acting elements, required for islet-specific transcriptional activity [176]. Inspection of the amylin proximal promoter by MatInspector program identified four potential transcriptional factor binding sites for activator protein-1 (AP-1), two sites for activator protein-4 (AP-4), one for hepatocyte nuclear factor -1 (HNF-1), one for nuclear factor of activated T cells (NFAT), and one for CAAT/enhancer binding protein (C/EBPβ). HNF-1 is reported to bind the proximal promoter and negatively regulate transcription of the amylin gene [147], however, the functional significance of the remaining transcription factor sites remains to be demonstrated. A role for these putative cis-acting elements can be investigated by Electrophoretic Mobility Shift Assay (EMSA) and reporter gene assay utilising a series of
deletion or substitution mutant constructs spanning sequences at -174 to -168 base pairs (HNF-1), -142 to -457 base pairs (C/EBPβ), -85 to -97 base pairs (NFAT). These proposed functional and DNA binding assays may demonstrate novel functional cis-regulatory elements and their trans-active factors for regulating human amylin promoter activity.

The -215T>G allele, results in a single nucleotide change at a position that is not conserved between human and rat. This position lies in a potential binding site for the transcription factor AP-1 (Figure 3.6). AP-1 is a DNA binding complex comprised of members of the Fos and Jun family of proteins, which regulate the expression of many downstream genes involved in cell proliferation, differentiation and apoptosis. Several Fos related (c-Fos, FosB, Fra-1, Fra-2) and Jun related (c-Jun, JunB, JunD) proteins have been described. These leucine zipper proteins form the active AP-1 complex as Fos/Jun heterodimers or Jun/Jun homodimers [177]. AP-1 mediated transcriptional activity was first reported in LLC-PK1 cells treated with 12-O-tetradecanoylphorbol-13-acetate (TPA). Since then, AP-1 activity is reported to be induced by a range of stimuli including growth factors, cytokines, neurotransmitters and cellular stress [178].

Modified promoter activity resulting from a substitution in an AP-1 binding site has been described [180]. The -82A>G allele in the promoter region of the matrix metalloelastase (MMP-12) gene has decreased binding affinity for AP-1 in EMSA, and reduced MMP promoter activity in reporter assays. This allele is associated with coronary artery luminal dimensions in diabetic patients with coronary artery disease. The -215T>G mutation may therefore produce type 2 diabetes in a subset of Polynesians by way of interfering with AP-1 binding and regulation of the amylin promoter. The role of AP-1 in the regulation of the human amylin gene is unknown, however, in the pancreatic β cell, glucose regulates expression of immediate-early response genes c-Fos, c-Jun, and JunB [181]. c-Fos and JunB are proposed to facilitate glucose/cAMP induction of the insulin gene by sequestering c-Jun through dimerization, which in turn is thought to repress insulin promoter activity (Figure 4.1). Since the amylin promoter shares a range of features similar to that of the
insulin promoter [183], these transcription factors may also regulate expression of the amylin gene through its AP-1 binding domain.

The human amylin gene sequence from −217 to −207 base pairs, TCTGACACACCC, has high homology to the AP-1 consensus motif. A functional role for AP-1 elements in the amylin proximal promoter has not been reported to date. Characterisation of the binding protein to this region can be determined by EMSA using synthetic oligonucleotides containing the AP-1 consensus sequence at −217 to −207 base pair of the wild type amylin promoter. This would determine if AP-1-like factors bind to the amylin promoter. A shift in DNA-protein complex would indicate that proteins do interact with this sequence. Inhibition of this DNA-protein complex supershift with a competitor oligonucleotide containing the consensus AP-1 binding site would also confirm this. Supershift of DNA-protein complex by antibodies to the Jun and Fos family members would indicate whether this DNA binding protein is composed of Jun homodimers and/or heterodimers with Fos, as expected for AP-1 complexes.

The −215T>G allele changes a nucleotide in the putative AP-1 core region, represented by four highly conserved nucleotides (T/GGAC). Previous reports have shown that disruption of the AP-1 core region in the IL12 p40 promoter eliminates binding of the AP-1 complex in EMSA, indicating that this motif is critical for its function [179]. To investigate the functional significance of −215T>G, binding of AP-1-like elements to synthetic oligonucleotides (−217 to −207 base pairs) containing the −215T>G mutation can also be examined by EMSA as described above. If this sequence is proven to be a functional cis-acting element, then there is likely to be a change or supershift in AP-1 DNA binding activity compared to the wild type amylin promoter.

Luciferase reporter gene constructs containing the amylin promoter sequence, −217 to −207 base pairs, can also be used to investigate a functional role for the AP-1-like element in the regulation of amylin gene transcription. Comparison of luciferase reporter gene constructs containing the −215T>G promoter allele or the wild type amylin promoter would determine whether the −215T>G allele can negatively or positively regulate amylin gene promoter
activity. Co-expression with Jun and other Jun or Fos family members may help to identify the possible dimer combination for AP-1 and their potential transcriptional activity. In the human insulin gene c-Jun is reported to repress insulin gene expression [182]. It has been speculated that up regulation of Fos and Jun-B proteins might sequester c-Jun through dimerization and consequently facilitate induction of the insulin gene [181]. Thus the proposed reporter gene analysis of the promoter region -217 to -207 base pairs may indicate whether 1) the amylin gene is similarly regulated by c-Jun through this element and 2) whether this is affected by the -215T>G allele.

Reversed orientation of an AP-1 element [184] and increased spacing between an AP-1 site and its synergistic C/EBP site [179] has been shown to reduce reporter assay activity. This suggests that correct spacing and orientation of cis-acting elements may be necessary for the interaction of transcriptional activators with the basal transcriptional machinery. Alternatively it may be required for synergistic binding and interaction of transcription factors. These spacing restrictions may involve distance of alignment of sites on the DNA double helix. If the AP-1 element at -217 to -207 base pairs is proven to be an important cis-acting element, the functional effects of spacing between this AP-1 element and the putative cis-regulatory elements identified in this study (AP-1, AP-4, HNF-1, NFAT, and C/EBPβ) can be determined by reporter assay using insertion mutants constructed within the -222 to -91 base pair proximal promoter region that increase the distance between AP-1 and the putative transcription factor binding sites by five, ten and fifteen base pairs. These distances correspond to approximately half, one, and one and a half helical turns of DNA. Similar insertion constructs can be utilised to determine the spacing requirements between AP-1 element at -217 to -207 base pairs and other cis-regulatory elements, such as the E1 box and A-box DNA motifs, all of which have previously been reported to be critical for amylin promoter activity [176]. Collectively these assays will help delineate AP-1 interaction with the amylin promoter and determine whether this interaction requires other trans-acting factors. These investigations may give more insight on possible mechanisms for the -215T>G allele to affect promoter activity.
The proposed EMSA and reporter assays may help to identify novel functional cis- and trans-active factors important for amylin promoter activity. To date regulation of the amylin promoter activity through its AP-1-like elements has not been described. If the AP-1-like element at -217 to -207 base pairs is sufficient for promoter activity, subsequent EMSA and functional analysis of the -215T>G promoter will determine whether this allele can negatively or positively regulate amylin promoter activity. Based on the literature to date, it is reasonable to speculate that the -215T>G allele is likely to affect transcriptional activity through dysfunctional AP-1 binding to its mutated core sequence. Collectively these investigations may help determine whether the -215T>G amylin gene mutation is a causative factor of type 2 diabetes in Maori, by regulation of amylin gene expression through its AP-1-like element at -217 to -207 base pairs.

AP-1 family members are prototypic targets for the c-Jun N-terminal kinase (JNK) and p38 mitogen activated protein (MAP) kinase signal transduction pathways [183]. MAP kinase signalling pathways influence AP-1 activity by increasing expression of the AP-1 components c-Fos and c-Jun, and by stimulating their activity directly. The role of JNK or p38 pathways in amylin promoter activity has not been determined, however, description of an AP-1 site, and glucose responsive expression of c-Fos, c-Jun and Jun B in pancreatic β cells in islet β cells [181] suggests that their pathways may be involved. Regulation of AP-1 activity by JNK or p38kinase signalling pathways could be investigated by EMSA, or in vitro using JNK or p38 specific inhibitors, such as CEP-1347 and SB202190 respectively. Activation of the JNK signalling cascade has been implicated in the pathophysiology of diabetes and Alzheimer's disease (AD) [178]. Exposure of beta-amyloid (Aβ), which is a major component of senile plaques in AD, to cultured neuronal cells activates the JNK pathway evoking neuronal cell death. This in turn can be blocked by CEP-1357, preventing neuronal apoptosis [185]. Similarly, direct toxicity of human amylin may contribute to the pancreatic islet β cell apoptosis observed in type 2 diabetes [186]. Amylin exposure to pancreatic β cells result in increased expression and phosphorylation of c-Jun, thereby implicating a role for the JNK pathway in human amylin induced β cell apoptosis [187]. However, the ability of dominant negative mutant inhibitors to block upstream JNK signalling and subsequent β cell apoptosis due to amylin remains to be demonstrated.
Collectively, these data indicate that the JNK pathway may be a potential target for therapeutic intervention for both Alzheimer’s disease and type 2 diabetes.

**Figure 4.1** AP-1 activation in islet β cells. Glucose regulates expression of the immediate early-response genes, Jun and Fos, in islet β cells [181]. Members of the Fos and Jun family dimerise to form the AP-1 transcription factor [177]. A number of metabolic genes such as insulin, amylin, GLUT-2, and PCK contain AP-1 binding sequences in their regulatory elements [183]. AP-1 may therefore have a role in regulating expression of these genes.
The \(-132G\textgreater A\) amylin allele

A previously described allele, \(-132G\textgreater A\), was observed in one Maori diabetic subject indicating that this allele is rare (0.76%). The carrier was diagnosed at 45 years of age, earlier than the mean age of diagnosis, and had a family history of diabetes. To evaluate the possible contribution of this allele for type 2 diabetes, family members of the affected individual should be screened by DNA analysis, OGTT and HOMA. If the allele is found to co-segregate with diabetes in this family, it may provide a useful tool for identifying Maori at risk of developing diabetes.

The \(-132G\textgreater A\) allele is significantly associated with type 2 diabetes in Spanish Caucasians (9.7% of type 2 diabetics vs 1.5% of non-diabetics) [101]. Affected individuals were carriers for the heterozygous (G/A) genotype except for two homozygous (A/A) individuals whom had predicted diagnosis for diabetes mellitus and impaired glucose tolerance. The \(-132G\textgreater A\) allele is also prevalent in British (5.3% of type 2 diabetics vs 3.2% of non-diabetics) [102] and Danish Caucasians 4.1% of type 2 diabetics vs 7.1% of non-diabetics) [103], and is not associated with type 2 diabetes for either of these populations. Differences in the frequency of the \(-132G\textgreater A\) allele between Spanish Caucasians, British, and Danish Caucasians are probably due to differences in their genetic background. Similarly, the low prevalence of this allele in Maori may be due to Caucasian [188] or Spanish [189] admixture in the Maori gene pool. The significantly high frequency of \(-132G\textgreater A\) in Spanish Caucasian but not British or Danish Caucasian type 2 diabetics suggests that this allele may act in synergy with additional genetic markers or environmental risk factors that are specific to Spanish Caucasians. Thus it would be worth screening for nearby genetic markers on chromosome 12 that could be linked to the \(-132G\textgreater A\) allele in the Spanish population.

In vitro, the \(-132G\textgreater A\) construct demonstrates a 2-fold increase in amylin promoter activity compared to wild type amylin [104]. The \(-132G\textgreater A\) construct reproduces the same pattern of amylin promoter activity as the wild type in response to various stimuli such as 6-deoxy-glucose or mannoheptulose, but with higher levels of activity. Hence the \(-132G\textgreater A\) allele
may be a pathogenic factor for type 2 diabetes through elevated amylin expression and secretion in islet amyloidogenesis.

Transcriptional regulation of the amylin gene is dependent on the proximal promoter region between -222 and -91 base pairs. The -132G>A allele, which is conserved between human, rat and mouse, results in a single nucleotide change in the amylin E-box consensus sequence (CANNCG/A) located at -140 to -130 base pairs [170]. The E-box, also known as E1, is a cis-acting element and is proposed to negatively regulate amylin gene transcription [176]. In the human insulin gene, the E-box binds the helix-loop-helix (HLH) protein upstream stimulatory factor, USF [191] and is important for controlling tissue specific expression [192]. In the human amylin gene, USF does not bind to the E-box [176]. Certainly, identification of a transcriptional binding factor will help determine whether the -132G>A allele increases promoter activity through the E-box motif.

The amylin proximal promoter also contains a series of A-box motifs (CTAAG) located at positions -83 to -91 (A1), -142 to -154 (A2) and -172 to 163 base pairs (A3). Both A2 and A3 have been shown to bind IPF-I, which requires a functional E-box motif [170]. A mutation within any on these regulatory elements abolishes promoter activity. Glucose regulation of amylin gene transcription is dependent on IPF-I activity at these DNA motifs. In IPF-1 mutant mice, only 43% of all insulin and amylin producing cells co-express these two proteins, leading to loss of β cell phenotype and early onset of type 2 diabetes [192]. Collectively these studies indicate that regulation of the amylin gene is dependent on IPF-1 activity at the A2 and A3 sites both of which act synergistically with the E-box. To investigate this, co-expression of reporter gene constructs containing the wild type promoter region or -132G>A with IPF-1, would indicate whether this allele affects IPF-1 regulation of the amylin promoter. Since the binding of IPF-1 to the A2 and A3 sites requires a functional E-box [176] one can speculate that the -132G>A may affect IPF-1 binding to the amylin promoter thereby affecting amylin gene expression.

In addition, the -132G>A variant is located in the flanking sequences of putative binding sites for AP-4 in the reverse orientation, and AP-1. AP-4 is thought to stabilise the
transcriptional initiation complex and recruit additional factors through a number of its dimerization domains to enhance transcription [193]. Mutational analysis in SV40 DNA demonstrates that mutations in the flanking sequences of the AP-1 binding site restrict access of AP-1 to its target site by altering DNA folding. A functional role for the AP-1 and AP-4-like elements in the amylin promoter region has not been reported to date. EMSA and reporter gene assays can be utilised to identify binding proteins to the AP-1 (-145 to -136 base pairs) and AP-4 (-130 to -121 base pairs) like elements using synthetic oligonucleotides (-145 to -120 base pairs) containing the wild type promoter. Inclusion of a competitor oligonucleotide containing either the consensus AP-1 binding site or the AP-4 binding site may determine whether these AP-family members function alone or together. Supershift of DNA-protein complex by antibodies to Jun and Fos would also help characterise the AP-family members. Collectively these investigations may help to identify functional cis-acting elements in transcriptional regulation of the amylin gene. If the -132G>A allele is shown to act through the E-box or AP-family like elements these investigations may provide a mechanism for the -132G>A allele to produce type 2 diabetes.

### 4.1.5 The Q10R amylin mutation

The present study identified a single homozygous missense variation in amino acid 10 of the mature amylin molecule (CAG\text{Gln} to CGG\text{Arg}). The Q10R variant was observed in only one diabetic patient (0.76%) indicating that this variant is uncommon in Polynesians. The carrier was a 60-year-old-female, diagnosed at 56 years of age and treated with insulin. No heterozygotes were observed and therefore did not agree with the Hardy-Weinberg model where the expected heterozygote frequency is 1.5%. The homozygous variant was confirmed by sequencing in both directions and is therefore not an artefact of DNA sequencing. The patient reported a family history of diabetes in first-degree relatives (mother and siblings) whom should also be tested to determine whether the Q10R mutation co-segregates with type 2 diabetes in this family. Family analysis may also help to identify any heterozygote alleles. The Q10R mutation creates a KasI restriction site, thus
subsequent screening can be performed by a simple test such as RFLP analysis based on the KasI restriction site. Unfortunately, family members of this proband were not tested for Q10R as the patient declined from the study shortly after the variant was identified. Similarly this simple test could be used to ascertain the frequency of this mutation in different Polynesian populations.

The glutamine residue at position 10 of the amylin molecule is conserved, being common to at least 9 mammals [164]. Therefore the possibility arises that the Q10R mutant amylin has reduced biological activity. The Q10R variant does not occur in the region responsible for fibril formation (amino acids 20 to 29) [99], that has been proposed to contribute to the cytotoxic and amyloidogenic properties of amylin. However, while amylin is implicated in regulating insulin and glucose metabolism its precise function is unknown. Over expression of amylin has been proposed to contribute to the pathogenesis of diabetes through its inherent tendency to form amyloid fibrils, which may cause initial defects in early insulin secretion and insulin action and/or lead to apoptosis of affected cells.

Ability to form amyloid could be tested using wild type amylin and Q10R synthetic peptides by the thioflavin T (ThT) assay [194]. The ability of Q10R to induce apoptosis could be tested using RINm5F pancreatic islet β cells over expressing wild type or Q10R amylin and measuring DNA fragmentation upon agarose gel electrophoresis [190], or by Fluorescent-activated cell sorting (FACS) analysis.

The overall synthesis rate of Q10R in vivo, in comparison to wild type amylin could be determined by high pressure liquid chromatography (HPLC) analysis of amylin immunoreactivity in postprandial plasma extracted from affected patients. Unfortunately, this method requires more than 20mL of plasma and may be difficult in obtaining individual consent to draw enough blood (> 50 mL) from Polynesian patients.

The affect of Q10R on β cell function could be investigated using simple fasting and OGTT assessment. Patients with this mutation may have defective glucose sensing or β cell insulin secretion. β cell defects in type 2 diabetes mellitus are often characterised by
elevated proinsulin-to-insulin ratios, which can be tested for using homeostasis model assessment (HOMA). With these tests glycaemia and β cell function between diabetics and control subjects would be compared. These tests detect deterioration of glycaemia and β cell function due to increasing age (for age standardisation), and between non-diabetic and diabetic carriers. Clear differences in β cell function that can be attributed to a genetic marker may aid the determination of the clinical course for this subset of diabetes.

### 4.1.6 The S20G amylin mutation

The amylin gene coding region mutation, S20G, previously reported in Japanese and Chinese [94], was not observed in patients or controls from this study and is therefore unlikely to be a contributing factor for type 2 diabetes in Polynesians despite evidence for a common genetic origin for these populations. This suggests that while Polynesians may share a common heritage with South East Asians [155, 156] their genetic background may be sufficiently different to exclude the S20G mutation from Maori and Pacific Island populations.

Another reason for the discrepancy between the occurrence of S20G in Japanese, Chinese and Pacific Island may be due to the relatively small number of late onset diabetics tested in this study ($n=15$) compared to the Chinese and Japanese study ($n=86$ and 294 respectively). This mutation was observed in 0.4% and 4.1% of Chinese and Japanese patients respectively, with late onset diabetes. Recently, a large scale study ($n=1538$) observed the S20G mutation in just 40 (2.6%) Japanese patients with type 2 diabetes [195]. This study concluded that both large scale and strict criteria for non-diabetic control subjects were critical in a case-control study of genetic susceptibility for type 2 diabetes. Thus a small sample size in gene association studies may preclude any conclusions on the importance of the S20G mutation in type 2 diabetes. Based on these statistics the expected prevalence of S20G in Polynesians would range from 0-4%. Therefore, in the Pacific
Island population studied, the frequency of this mutation if it exists at all is likely to be below these rates.

4.1.7 **Summary of the amylin gene alleles**

In summary, we found that approximately 7% of Maori with type 2 diabetes have alleles in the promoter region of the amylin gene while only 0.8% of Maori without type 2 diabetes have these alleles (Figure 4.2). The -215T>G allele is associated with increased type 2 diabetes, with a relative risk of 7.23, and could be linked with the previously described amylin promoter polymorphism, -230A>C. These results suggest that amylin gene alleles maybe an important contributor to type 2 diabetes in Maori.

While the functional significance of these promoter mutations require additional investigation they may predispose to type 2 diabetes by altering basal transcriptional level of the amylin gene in pancreatic β cells. Based on the literature to date, one can speculate that the -132G>A allele may induce diabetes through a dysfunctional E-box although the AP-family elements also require investigation. Similarly, if the AP-1-like element at -207 to -217 base pairs is proven to be a functional cis-regulatory sequence, one can speculate that the -215T>G allele may affect transcriptional activity through dysfunctional AP-1 binding to its mutated core sequence. Thus a putative reduction in amylin promoter activity may be due to differences in affinity for nuclear proteins caused by these mutations. Modified transcriptional activities of mutant promoters predispose to other disease. They have previously been described in the lipoprotein lipase and the apolipoprotein E [195], whose genes are associated with the risk for hyperlipidaemia and Alzheimer’s respectively [196].

Finally, incomplete penetrance of -215T>G in the Maori population suggests that this allele is not sufficient enough to cause diabetes by itself and therefore may function in a polygenic and multifactorial context. The average BMI in non-diabetic carriers was lower than that for diabetic carriers, and may therefore be an important factor for maintaining
normoglycaemia in these subjects. However, the statistical significance of this remains to be determined. Thus the \(-215T>G\) allele may increase susceptibility to late-onset type 2 diabetes, particularly in the presence of other additional risk factors such as increased body mass and obesity. Approximately 60-80% of the diabetic subjects participating in this study were obese with a BMI ranging from 32-39 kg/m\(^2\). Therefore these patients, whom have a body weight 20-40% in excess of their desirable weight, have a 2-10 fold increased risk of developing type 2 diabetes. The \(-215T>G\) allele was inherited with a previously described amylin promoter polymorphism \((-230A>C)\) in 3% of Maori with type 2 diabetes but not the control group. These alleles are separated by 15 base pairs only and are likely to be linked. The occurrence of \(-215T>G\) and \(-230A>C\) together was greater than that due to chance \((p<0.001)\). Thus it is worth screening for both alleles in future investigations.

It should be noted that the non-coding region studied here lies in the "minimal control region" of the amylin gene spanning sequences from \(-390\) to \(+450\) base pairs relative to the transcriptional start site. It was chosen because it contains the proximal promoter region, the insulin-like NIR and FAR boxes, and the E2-like domain, and therefore probably contains the major sites controlling \(\beta\) cell specific expression of human amylin. However, enhancer elements can regulate transcription thousands of nucleotides upstream or downstream of the promoter. The 5' regulatory region of amylin gene that extends 2Kb upstream of the transcription start site [183] and the 3' regulatory sequences were not examined here. Therefore, these studies do not exclude the possibility of other promoter variants that may lead to abnormal expression of amylin thereby contributing to the development of diabetes.
Type 2 Maori diabetic patients (n=131)

<table>
<thead>
<tr>
<th>No mutation</th>
<th>91 (69.5%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q10R</td>
<td>1 (0.8%)</td>
</tr>
<tr>
<td>-132G&gt;A</td>
<td>1 (0.8%)</td>
</tr>
</tbody>
</table>

Non-diabetic Maori patients (n=258)

<table>
<thead>
<tr>
<th>No mutation</th>
<th>68 (26.4%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-230A&gt;C</td>
<td></td>
</tr>
<tr>
<td>&lt;0.4%</td>
<td></td>
</tr>
<tr>
<td>2 (0.8%)</td>
<td></td>
</tr>
<tr>
<td>-215T&gt;G</td>
<td></td>
</tr>
</tbody>
</table>

No mutation 188 (72.8%)

---

**Figure 4.2** The prevalence of amylin gene alleles among Maori patients. Allele \(x\%\) where \(x\) is the number of individuals with a mutation and \(y\%\) is \(x/n \times 100\), where \(n\) is the total number of type 2 diabetic and non-diabetic patients screened for mutations.
4.2 IPF-1 allelic variations

The homeodomain transcription factor IPF-1 is involved in the early development of the pancreas and in the transcriptional regulation of endocrine pancreas related genes such as insulin, GLUT2 and glucokinase [135, 136]. IPF-1 is normally expressed in all cells of the pancreatic embryonic bud, and its absence in mice arrests pancreas development of the bud stage leading to pancreatic agenesis.

Mutations in the IPF-1 gene account for less than 1% of diabetes, and therefore are not a common cause for diabetes [129]. To date, only one MODY family has been reported to carry an IPF-1 mutation (Pro63fsdel) that clearly segregates with diabetes [138]. In this family, a homozygous carrier has pancreatic agenesis and heterozygous carriers develop MODY4. IPF-1 deficiency in this MODY4 subtype results from expression of an internally translated dominant negative isoform that inhibits the wild type expressed IPF-1 isoform. An in-frame insertion of a proline (InsCCG243) was found to co-segregate with diabetes and an insulin secretion defects in two families with an autosomal dominant-like transmission [139]. The remaining missense mutations in the IPF-1 coding region identified in both British (C18R, D76N, R197H) and French (Q59L, D76N) type 2 diabetic patients are more prevalent but are considered to be low penetrant MODY mutations as non-diabetic carriers have also been identified [136, 139]. Functional analyses of these mutations demonstrated reduced basal insulin promoter activity in beta-cell lines. The relative risk for these carriers to develop diabetes ranges from 3.0–12.6. Therefore these mutations are more likely to represent predisposing alleles for the more common form of type 2 diabetes.

None of the previously described IPF-1 gene mutations were observed in this study of Maori and Pacific Island type 2 diabetes. The present study however identified a novel allelic variation, 185T, in two unrelated Maori type 2 diabetic patients. The 185T allele was not significantly associated with type 2 diabetes in this population (p = 0.113). This allele is predicted not to affect the amino acid sequence at codon 55, G55G, and therefore should not affect the normal function of IPF-1 in these subjects.
In summary, the prevalence of allelic variations in the IPF-1 gene is less than 0.76% in Maori and is not associated with type 2 diabetes. The absence of IPF-1 allelic variations in the remaining patients screened suggest defects in this gene is uncommon in Maori and Pacific Island type 2 diabetes.

4.3 Polymorphisms in the HNF-1α gene

Positive linkage of the MODY3 loci and late-onset diabetes in an extended Pacific Island pedigree have implicated the HNF-1α gene in the development of type 2 diabetes in Polynesians [146]. However, subsequent sequence analysis of the ten exons, flanking introns and promoter region of the HNF-1α gene in Pacific Island type 2 diabetic patients did not support this hypothesis [146, 147]. Since then, Ellard et al [148] reported that the failure to detect any mutations in exon 2 specifically may have been due to non-amplification of an allelic dropout, nt =66G>C located in the reverse primer.

In the present study resequencing of HNF-1α exon 2 using an alternative primer found no evidence of allelic variations to be associated with Maori and Pacific Island type 2 diabetes, although a previously described polymorphism, -42G>A [197], was found in both diabetic and non-diabetic individuals. The -42G>A polymorphism was not associated with type 2 diabetes in Maori of Pacific Island people (p=0.118 and p=1.00 respectively).

The absence of HNF-1α mutations that predispose to diabetes in Maori and Pacific Island people may reflect the genetic heterogeneity in diabetes susceptibility between different ethnic groups. Mutations in the HNF-1α gene are the most common cause of MODY in the UK, and are also present in German, Finnish, Danish, Italian, North American and Japanese MODY pedigrees [71, 123, 126, 127, 128, 129]. However, there is no evidence for linkage of type 2 diabetes with the MODY3 locus (HNF-1α) in Mexican-American [198], Ashkenazi Jews [199], or Pima Indians [200].
Association studies of the MODY genes indicate that they are only weakly associated with late-onset diabetes. Mutations in the HNF-1α gene were observed in 8% of Japanese subjects with early-onset diabetes, 15% with MODY, but only 1% with late-onset diabetes [201]. Similarly, Glucksman et al [128] observed no HNF-1α mutations in a study of late-onset diabetics with relative insulin deficiency.

Matahani et al [49] demonstrated linkage to a locus near the HNF-1α gene, NIDDM2, in Finnish families with low insulin secretion and late-onset of diabetes (mean age = 58 years). They proposed that NIDDM2 is allelic with MODY3 and that severe mutations in the MODY3 region cause MODY while mild mutations give rise to late-onset diabetes. It is possible that NIDDM2 is actually the linked locus rather than MODY3 identified by Shaw et al [146] in a Pacific Island pedigree at the region of chromosome 12q. Thus, it would be worth scanning chromosome 12q for linkage with type 2 diabetes Maori and Pacific Island cohorts in New Zealand.

In summary, the present study confirms Shaw et al [146] and Green’s [147] finding that HNF-1α mutations are unlikely to be a significant cause of type 2 diabetes in Pacific Island people. The present study also indicates that HNF-1α alleles in exon 2 are not associated with type 2 diabetes in Maori.

4.4 Challenges in the investigation of genetics in type 2 diabetes in Maori and Pacific Island populations

There is considerable interest in the genetic basis of type 2 diabetes. Whole genome studies have linked diabetes or glucose intolerance to many different genetic loci in certain ethnic populations. Single-gene mutations that impair insulin action or secretion (eg. IRS-1 and β3AR) have been identified in a few populations. However, the candidate susceptibility genes reported thus far are often only important in certain subsets of diabetes or specific ethnic populations. Furthermore, some of the more common mutations are often neither essential nor sufficient to produce type 2 diabetes on their own. Hence there is little
consensus for one specific locus that may confer genetic susceptibility to the common form of late-onset type 2 diabetes mellitus.

The present study demonstrates significant genetic association in Maori type 2 diabetes. This could have been advanced by confirmation in larger sample groups and in different populations as all newly described gene association studies would. However, the results described here are not only statistically significant but are of a sample size that indicates clinical significance. The search for candidate genes in a Pacific Island population has been complicated by the difference in numbers of Pacific Island patients with diabetes \((n=15)\) compared to the Maori diabetic group \((n=131)\). Obviously, small sample sizes run the risk of observing allele frequencies either lower or higher than the true frequency and may explain the lack of amylin promoter alleles \((-215T>G\text{ and }-132G>A)\) in the Pacific Island population.

Although the present study demonstrates genetic association in Maori type 2 diabetes, it did not address the affect of Caucasian admixture on the Maori gene pool. European colonisation in NZ began in the early 19th century, and by 1850 already outnumbered Maori, who today represent about 15% of the total NZ population. Since most subjects used in the present study are likely to have Caucasian ancestry this could complicate interpretation of the Maori gene pool. Indeed it would be very rare to find Maori cases in NZ that are genetically entirely Maori. It has been proposed that genetic admixture for a given population may reflect a difference in allele frequency in gene association studies [153]. For example, gene association studies in Pima Indians have been limited by the finding that Pima Indians with a high degree of Caucasian ancestry had lower susceptibility to type 2 diabetes. Population stratification in case-control studies, using unrelated control individuals, can be detected by including a second genotyping procedure for marker loci that are unlinked to the candidate locus [153]. It has been proposed that 15-20 microsatellite loci provide sufficient power to detect stratification. Failure to identify the \(-215T>G\) allele in the European populations to date, suggest that this allele may be unique to Maori. However, further testing for population stratification as described by above would support this hypothesis. Alternatively, screening for the Y-chromosome haplotype, h13, which is known to be common in Maori and other Polynesians, but not Caucasians or
Asians [154], would improve the power to detect stratification in this study. Thus population stratification may further support a role for the \(-215T>G\) allele in Maori diabetes.

One limitation of this study is the lack of co-segregation studies in affected family members. A common problem encountered in this study is the relatively late age at onset and premature mortality associated with type 2 diabetes. Unfortunately, for several probands in this study, the mode of transmission was not analysed because multigeneration families were either unavailable or family members had died. Another reason for the lack of co-segregation studies is the increased reluctance of Maori to participate in genetic studies. Maori worldview regards blood, tissue and genes as taonga (belonging to Maori only) and is not to be meddled with by scientists. This lead to a number of potential patients to decline from participating in the present study due to their reluctance to being identified with a “rogue” gene that may cause diabetes. Consequently, only control samples could be collected for the first two years of this study.

Another limitation of this study is the lack of functional analysis of the observed \(-215T>G\) and \(-132 G>A\) alleles. Based on the literature to date, one can speculate that the \(-132G>A\) allele may induce diabetes through a dysfunctional E-box although the AP-family elements also require investigation. Similarly, if the AP-1-like element at \(-207\) to \(-217\) base pairs is proven to be a functional cis-regulatory sequence, it is reasonable to speculate that the \(-215T>G\) allele is likely to affect transcriptional activity through dysfunctional AP-1 binding to its mutated core sequence.

The present study is also limited by the unavailability of diabetes-associated complications in such as dyslipidemia, hypertension, albuminuria, and obesity in both the controls and type 2 diabetic patients. Incomplete penetrance of the \(-215T>G\) allele in the Maori population suggests that this allele is not sufficient enough to cause diabetes by itself and therefore may function in a polygenic and multifactorial context. The statistical significance of this allele in the presence of other additional risk factors such as increased body mass and obesity therefore requires further investigation.
Finally, individual testing of multiple candidate genes by DNA sequencing is an expensive and labour intensive method for identifying disease-susceptibility genes. This is typical of the candidate gene approach particularly if the susceptibility genome is large. Using simulated data it has been calculated that it would be necessary to screen a single nucleotide polymorphism (SNP) every 10,000 to 30,000 bp to locate disease-susceptibility alleles [202]. Susceptibility genes have been identified in Japanese, Chinese and several Caucasian populations, these polymorphisms do not appear to be a common cause for type 2 diabetes in NZ Polynesians. This suggests that Maori and Pacific Island people represent a different subset of diabetes compared to the above populations and hence different genetic factors for disease. This emphasises the importance of research in this area as results from overseas may not apply to NZ Polynesians.

Subsequent screening for the -132G>A and Q10R alleles in affected family members and future population studies can be advanced by RFLP analysis. The -132G>A and Q10R alleles creates MwoI and KasI restriction enzyme sites respectively and therefore provide a simple test for rapid detection. Sequencing type 2 diabetic patients first and development of PCR-based assays for genotyping control subjects could have advanced the present study. Unfortunately such assays were not developed in the present study due to the unavailability of type 2 diabetic patients for the main duration of this study. Consequently all control subjects were sequenced prior to identification of type 2 diabetic patients and sample collection.

An alternative approach for identifying susceptibility genes in Polynesians would be to use random probes to "walk" along a chromosome. If a marker is identified, positional cloning identifies the gene in that region of the chromosome. However, while this technique is a powerful tool for detecting monogenic disorders, it is often not sensitive enough to detect low penetrant mutations and requires supplementation with gene association studies. This method is also labour and time intensive.
Identifying new SNP locations by such methods as those utilised in the present study can be extended to a much larger-scale effort, SNP microarray analysis, to determine SNP genotypes of many genomes from different populations. SNP's are single-base pair differences in the DNA sequence that can be observed between individuals in the population and are present throughout the human genome with a frequency of approximately 1 per 1000 base pairs [202]. High-throughput SNP analysis is increasingly used to identify genes that may contribute to complex diseases and other phenotypes, explore the evolutionary history of human populations, and to elucidate genetic factors underlying different drug efficacies. The steps for SNP genotyping involve DNA sample preparation, PCR amplification of specific locations, and microarray analysis. The latter step labels SNP locations of both alleles in the DNA sample, which is then hybridised to DNA arrays containing 20-80mer oligonucleotides specific for the sequence being investigated. A DNA array can contain between 64 and 6000 oligonucleotides, which may differ by only one or a few specific nucleotides. The amount of hybridisation is determined or scored by laser technology and stored into a computer database for construction of a SNP map. High-throughput SNP genotyping enables recording of SNP location base values of thousands of people from a given population. From this set of genotypes, one can also determine unique patterns of SNPs or haplotypes that may be specific to a population. This would be particularly useful for identifying haplotype patterns that may be unique to Maori or Pacific Island people which can be further used to determine whether there is any correlation with the prevalence of type 2 diabetes in these populations. Once a SNP pattern is established to be associated with the disease, SNP microarray technology can be used to test an individual for that disease expression pattern to determine whether they are susceptible to diabetes. When genomic DNA from an individual is hybridised to an array loaded with various SNPs, the sample DNA will hybridise with greater frequency only to specific SNPs associated with that person. Those spots on the microarray will fluoresce with greater intensity, demonstrating that the individual may have or be at risk for developing type 2 diabetes. Thus SNP mapping is a powerful tool for reducing the detection of multiple SNP's associated with the disease in a relatively small linkage disequilibrium region. This method has been used to investigate the ApoE locus in Alzheimer's disease and is currently being used to examine susceptibility loci for type 2
diabetes on chromosome 12 [203]. Thus SNP mapping has the advantage of rapidly reducing the size of DNA region containing disease-susceptibility genes from millions to thousands of base pairs thereby accelerating the identification process. However, the disadvantages of SNP mapping is high costs and that SNP maps from other ethnic populations may not be transferable to Polynesians due to the heterogeneous nature of type 2 diabetes.

4.5 Other possible candidate type 2 diabetes genes

The genes investigated in this study were chosen because substantial evidence supports a role for defects in genes encoding hormones and transcription factors expressed in pancreatic β cells in the pathogenesis of type 2 diabetes. NZ Polynesians share several features in common with the MODY phenotypes including the high incidence of familial disease and diabetes-related complications, such as proteinuria and ESRF, as well as earlier age at onset, indicating that these genes may be important in NZ Polynesians. Despite the absence of MODY alleles in NZ Polynesians, as determined by the present study, the high prevalence of type 2 diabetes in this population compared to Caucasians when controlled for environmental factors, still point to a role for susceptibility genes for diabetes. The amylin gene alleles discovered in the present study may account for approximately 7% of type 2 diabetes in Maori.

A common polymorphism, C825T, in exon 10 of the gene encoding the β3 subunit of heterotrimeric G protein (GNβ3) on chromosome 12p13, is frequently observed in the general population. Heterotrimeric G proteins mediate stimuli from heptahelical and tyrosine kinase receptors into the cell, and subsequent activation of numerous intracellular signalling pathways and effectors including the adenyl cyclase pathway, phospholipase C and the MAP kinase pathway. The 825T allele is associated with enhanced signal transduction via G proteins through the generation of a splice variant termed GNβ3 [204]. The 825T allele has also been reported to be associated with increased BMI in the presence
of other influences such as reduced physical activity and pregnancy. In hypertension the 825T allele is associated with low renin activity and is a strong predictor of ventricular hypertrophy. In type 2 diabetes the 825T allele predisposes type 2 diabetic patients to end-stage renal failure [204]. The 825T allele is more prevalent in black African and black American populations, pygmies, and Aborigines from Australia and Papua New Guinea (80-90%). It is also prevalent in Japanese, Koreans, Chinese and Native American Indians (40-60%); and least prevalent in Caucasians (30%). The geographic distribution of this polymorphism is consistent with the "out of Africa" hypothesis for modern humans.

In the presence of obesity, hypertension and diabetes-related phenotypes, the 825T allele may increase the risk for disease in Australian Aborigines indicating that this allele may represent a true "thrifty genotype". Like Polynesians, type 2 diabetes is more prevalent in Australian Aborigines (11%) compared to Australian Caucasians (2.3%) [24]. Since the epidemiology of diabetes in Maori and Pacific Island people follow a similar pattern to that in Australian Aborigines it would be of interest to determine whether the GNβ3 gene constitutes a thrifty genotype in Polynesians.

4.6 Conclusion

Type 2 diabetes mellitus affects 16.5% and 10.1% of Maori and Pacific Island people respectively, and is a major cause of morbidity and mortality in NZ Polynesians. It is generally accepted that type 2 diabetes has major genetic determinants. A high concordance rate in twins, familial transmission patterns, and high incidence in certain ethnic populations when adjusted for environmental factors support this concept. Heterozygous mutations in a number of genes have previously been identified in certain subsets of type 2 diabetes or specific ethnic groups. The high prevalence of diabetes in NZ Polynesians after controlling for age, income and BMI, suggest that genetic determinants may have a causative role for type 2 diabetes in these populations. The present study therefore investigated the role for the genes encoding amylin and IPF-1, and exon 2 of the HNF-1α gene in NZ Polynesians with type 2 diabetes. The HNF-1α gene was chosen as it
is among the most common type 2 diabetes predisposing genes in other populations and therefore may also be important in Polynesians. An uncommon predisposing gene, IPF-1, was also chosen because it mimicked the phenotype or clinical picture of Polynesian type 2 diabetes. Amylin, also an uncommon predisposing gene, was chosen because racial differences in an amylin gene mutation indicated that this mutation may be present in Polynesians.

In summary, the present study identified three new alleles in the amylin gene of Maori including a missense mutation in exon 3 (Q10R) and two alleles in the non-coding promoter region (−132G>A and −215T>G). Both Q10R and −132G>A were present at a rate of 0.76% (one patient each). The −215T>G allele was present at a rate of 5% and predisposed the carrier to type 2 diabetes with a relative risk of 7.23. These alleles were not detected in the Pacific Island diabetes group nor in the Maori and Pacific Island non-diabetic group. Together these alleles may account for approximately 7% of type 2 diabetes in Maori.

Incomplete penetrance of the −215T>G promoter allele in the Maori population suggests that this mutation is not sufficient enough to cause diabetes by itself and therefore may function in a multifactorial or polygenic context. A lower BMI may be a factor in maintaining normoglycaemia in non-diabetic carriers, however the present study was unable to test its statistical significance. The −215T>G allele was also inherited with a previously described amylin promoter polymorphism (−230A>C) in 3% of Maori with type 2 diabetes (p<0.001), which suggests that linkage disequilibrium exists between these two allelic variations. Thus it is worth screening for both alleles in future investigations.

A causative role for the amylin gene alleles in type 2 diabetes require further functional and clinical analysis. The amylin gene promoter alleles are predicted to affect putative transcriptional factor binding sites and cis-regulatory sequences and may therefore modify amylin promoter activity by affecting affinity for nuclear proteins to its AP-1-like element (−207 to −217 base pairs), the E-box motif (−138 to −131 base pairs), and other transcription factor binding and AP-family like elements. Intrafamilial analysis would determine
whether these alleles co-segregate with diabetes in the family as well as determine the mode of transmission. It would also be of interest to screen for the -215T>G allele in a larger Pacific Island sample population and NZ Caucasians with type 2 diabetes to confirm that this mutation is specifically associated with Maori diabetes only. Alternatively, population stratification by screening for markers unique to Maori and Pacific Island only, may control for Caucasian admixture. Collectively, these investigations will determine the efficacy of -215T>G as a diagnostic marker for type 2 diabetes in Maori.

The previously described amylin promoter polymorphism, -230A>C, was found not to be associated with type 2 diabetes on its own. The present study also confirmed that mutations in the MODY genes IPF-1 and exon 2 of HNF-1α are not associated with type 2 diabetes in Maori and Pacific Island Polynesians.

The candidate gene approach used in this study suggests that amylin gene alleles in the promoter region maybe an important contributor to type 2 diabetes in Maori. However, alternative environmental and genetic factors that may account for the majority of type 2 diabetes in Polynesians remain to be identified. Although the present and previous studies eliminate the role of HNF-1α, IPF-1 and glucokinase genes in the pathogenesis of diabetes in New Zealand Polynesians, the strong familial history and high incidence of diabetes and related metabolic abnormalities after adjusting for environmental factors still points to a strong genetic component in these populations. Although some of these metabolic abnormalities such as obesity have a genetic determinant, they are compounded by behavioural determinants such as high calorie, high fat diets, smoking and decreased energy expenditure. Hence the overall significance of diabetes-related genes and other gene-gene and gene-environmental interactions in New Zealand Polynesians still remain to be identified.
Appendix

Appendix 1: Etiological classification of diabetes mellitus

<table>
<thead>
<tr>
<th>Type 1 diabetes (β cell destruction, usually leading to absolute insulin deficiency)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autoimmune</td>
</tr>
<tr>
<td>Idiopathic</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Type 2 diabetes (predominantly insulin resistance with relative insulin deficiency or predominantly insulin secretory defect with or without insulin resistance)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Other specific types</td>
</tr>
<tr>
<td>Genetic defects of β cell function</td>
</tr>
<tr>
<td>Chromosome 20, HNF-4α (MODY1)</td>
</tr>
<tr>
<td>Chromosome 7, glucokinase (MODY2)</td>
</tr>
<tr>
<td>Chromosome 12, HNF-1α (MODY3)</td>
</tr>
<tr>
<td>Chromosome 13, IPF-1 (MODY4)</td>
</tr>
<tr>
<td>Chromosome 20, HNF-1β (MODY5)</td>
</tr>
<tr>
<td>Mitochondrial DNA</td>
</tr>
<tr>
<td>Others</td>
</tr>
</tbody>
</table>

| Genetic defects in insulin action                                             |
| Type A insulin resistance                                                     |
| Leprechaunism                                                                 |
| Rabson-Mendenhall syndrome                                                    |
| Lipoatrophic diabetes                                                         |
| Others                                                                         |

| Diseases of the exocrine pancreas                                           |
| Fibrocalculous pancreatopathy                                                |
| Pancreatitis                                                                 |
| Trauma/pancreatectomy                                                        |
| Neoplasia                                                                    |
| Cystic fibrosis                                                              |
| Haemochromatosis                                                             |
| Others                                                                        |

| Endocrinopathies                                                             |
| Cushing’s syndrome                                                           |
| Acromegaly                                                                   |
| Phaeochromocytoma                                                            |
| Glucagonoma                                                                  |
| Hyperthyroidism                                                              |
| Somatostatinoma                                                              |
| Aldosteronoma                                                                |
| Others                                                                        |

| Drug- or chemical-induced                                                   |
| Vacor                                                                        |
| Pentamidine                                                                  |
| Nicotinic acid                                                               |
### Appendix 1 Etiological classification of diabetes mellitus

<table>
<thead>
<tr>
<th>Other specific types</th>
<th></th>
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<tbody>
<tr>
<td>Drug- or chemical-induced</td>
<td></td>
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<tr>
<td>Glucocorticoids</td>
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<td>Thyroid hormone</td>
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<td>Diazoxide</td>
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<tr>
<td>β-adrenergic agonists</td>
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<td>Dilantin</td>
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<td>Others</td>
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<td>Congenital rubella</td>
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<tr>
<td>Cytomegalovirus</td>
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<tr>
<td>Others</td>
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<tr>
<td>Uncommon forms of immune-mediated diabetes</td>
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<tr>
<td>Insulin autoimmune syndrome (antibodies to insulin)</td>
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<tr>
<td>Anti-insulin receptor antibodies</td>
<td></td>
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<tr>
<td>‘Stiff man’ syndrome</td>
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</tr>
<tr>
<td>Others</td>
<td></td>
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<tr>
<td>Other genetic syndromes sometimes associated with diabetes</td>
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</tr>
<tr>
<td>Down’s syndrome</td>
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</tr>
<tr>
<td>Klinefelter’s syndrome</td>
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<tr>
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<td>Friedreich’s syndrome</td>
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<td>Huntington’s disease</td>
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<tr>
<td>Laurence-Moon-Beidl syndrome</td>
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<td>Polyphyria</td>
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<tr>
<td>Prader-Willi syndrome</td>
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<td>Others</td>
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<td>Gestational diabetes</td>
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**Appendix 2: PCR parameters**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Region</th>
<th>Forward primer (5'-3')</th>
<th>Reverse primer (5'-3')</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>amylin</td>
<td>R</td>
<td>atgcgaccccttgactccag</td>
<td>atgccccatgtctctcaaat</td>
<td>1702</td>
</tr>
<tr>
<td>amylin</td>
<td>exon 1</td>
<td>tgctcatgtcagagctgag</td>
<td>acaacaggtgtcattctct</td>
<td>228</td>
</tr>
<tr>
<td>amylin</td>
<td>exon 2</td>
<td>ctcttgattcagtgtgga</td>
<td>ggcaagtattatttgacagt</td>
<td>212</td>
</tr>
<tr>
<td>amylin</td>
<td>exon 3</td>
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<td>aggtatatgtctctagcac</td>
<td>238</td>
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<td>IPF-1</td>
<td>UES</td>
<td>gcgcagacaatggaactc</td>
<td>agatgccttgcttcacc</td>
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</tr>
<tr>
<td>IPF-1</td>
<td>PP</td>
<td>gctactgctcttgtagcgt</td>
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<td>315</td>
</tr>
<tr>
<td>IPF-1</td>
<td>exon 1</td>
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</tr>
<tr>
<td>IPF-1</td>
<td>exon 2</td>
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<td>tgaagccctcagccag</td>
<td>499</td>
</tr>
<tr>
<td>HNF-1</td>
<td>exon 2</td>
<td>caagttctctgtctcatgacc</td>
<td>tccacagctctcttcccc</td>
<td>516</td>
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**Appendix 2.1** Primers used to sequence exons, intron 1, and 5' regulatory region (R) of the human amylin gene (Genbank accession # GI32589); the upstream enhancer segment (UES), proximal promoter (PP) and two exons of the human IPF-1 gene (Genbank accession # GI6911059); and exon 2 of the HNF-1 gene (Genbank accession # U72612).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Region</th>
<th>DNA concentration (ng)</th>
<th>Primer concentration (μM)</th>
<th>Annealing temperature (°C)</th>
<th>Annealing time (minutes)</th>
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<td>0.5</td>
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<td>exon 1</td>
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<td>0.2</td>
<td>55</td>
<td>1.0</td>
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<tr>
<td>amylin</td>
<td>exon 2</td>
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<td>0.2</td>
<td>52</td>
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<td>0.2</td>
<td>56</td>
<td>1.0</td>
</tr>
<tr>
<td>IPF-1</td>
<td>exon 1</td>
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<td>0.2</td>
<td>59</td>
<td>0.5</td>
</tr>
<tr>
<td>IPF-1</td>
<td>UES</td>
<td>200</td>
<td>0.2</td>
<td>54</td>
<td>0.5</td>
</tr>
<tr>
<td>IPF-1</td>
<td>PP</td>
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<td>0.2</td>
<td>53</td>
<td>0.5</td>
</tr>
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<td>IPF-1</td>
<td>exon 2</td>
<td>400</td>
<td>0.2</td>
<td>60</td>
<td>0.5</td>
</tr>
<tr>
<td>HNF-1</td>
<td>exon 2</td>
<td>400</td>
<td>0.2</td>
<td>56</td>
<td>0.5</td>
</tr>
</tbody>
</table>

**Appendix 2.2** Optimised PCR conditions for the amplification exons, intron 1, and 5' regulatory region (R) of the human amylin gene (Genbank accession # GI32589); the upstream enhancer segment (UES), proximal promoter (PP); and two exons of the human IPF-1 gene (Genbank accession # GI6911059); and exon 2 of the HNF-1 gene (Genbank accession # U72612).
Appendix 3: Publications arising from this research

Poa, NR, Cooper, GJS, Edgar, PF. Amylin gene promoter mutations predispose to Type 2 diabetes in New Zealand Maori. Diabetologia 2003, 46, 574-578.
Amylin gene promoter mutations predispose to Type 2 diabetes in New Zealand Maori

N. R. Poa1,2, G. J. S. Cooper1, P. F. Edgar2

1 The Biochemistry and Molecular Biology Group, School of Biological Sciences, University of Auckland, New Zealand
2 Molecular Psychiatry Research Group, Christchurch School of Medicine, University of Otago, New Zealand

Abstract

Aims/hypothesis. Amylin gene mutations are known to predispose Chinese and Japanese subjects, but not Caucasian subjects, to Type 2 diabetes. New Zealand Maori, who have a high prevalence of Type 2 diabetes, have genetic origins in South East Asia. Amylin gene mutations could therefore predispose New Zealand Maori to Type 2 diabetes.

Methods. The amylin gene was screened for mutations in the proximal promoter region, exons 1 and 2, intron 1, and coding region of exon 3 by polymerase chain reaction amplification and direct sequencing of 131 Type 2 diabetic Maori patients and 258 non-diabetic Maori control subjects.

Results. We identified three new amylin gene mutations: two mutations in the promoter region (-215T>G and -132G>A) and a missense mutation in exon 3 (Q10R). The -215T>G mutation was observed in 5.4% of Type 2 Maori diabetic patients and predisposed the carrier to diabetes with a relative risk of 7.23. The -215T>G mutation was inherited with a previously described amylin promoter polymorphism (-230A>C) in 3% of the Maori with Type 2 diabetes, which suggests linkage disequilibrium exists between these two mutations. The -230A>C polymorphism on its own, however, was not associated with Type 2 diabetes in Maori subjects. The -132G>A and Q10R mutations were both observed in 0.76% of Type 2 diabetic patients and were absent in non-diabetic subjects.

Conclusion/Interpretation. The amylin gene mutations identified in this study are associated with Type 2 diabetes in 7% of Maori. Amylin is likely to be an important susceptibility gene for Type 2 diabetes in Maori people. [Diabetologia (2003) 46:574–578]

Keywords Type 2 diabetes, amylin gene, New Zealand, Maori, Caucasian, mutation, polymorphism.

New Zealand Maori, the indigenous people of New Zealand, are descendants from Polynesians that arrived in New Zealand 800 to 1200 years ago and now comprise approximately 15% (557,700) of the total New Zealand population [1]. The prevalence of Type 2 diabetes in Maori and Caucasian subjects is 10.8% and 2.9% respectively, and is, therefore, a major cause of morbidity and mortality in Maori people [2]. It is generally accepted that Type 2 diabetes has major genetic determinants and mutations in a number of genes that have been identified in clinical subsets of Type 2 diabetes and certain ethnic populations. The prevalence of these mutations range from 1 to 15% in late onset Type 2 diabetes [3]. That disturbed gene function could cause diabetes in Maori people is indicated by the increased rates of Type 2 diabetes, when compared with New Zealand Caucasian subjects, after controlling for age, income and BMI [4]. The clustering of diabetes-related complications, such as nephropathy, in Maori pedigrees also indicates an inherent genetic component [5]. Together this evidence sug-
gests that Maori have an increased genetic susceptibility to Type 2 diabetes. Despite these findings, screening of the MODY genes, glucokinase and hepatocyte nuclear factor-1α (HNF-1α), in Maori and Pacific Island populations [6], so far have not identified Type 2 diabetes susceptibility genes for Polynesian people.

There is cumulative evidence of Y chromosome [7] and mitochondrial lineage analysis [8] pointing to a genetic origin for Polynesian people in South East Asia. A missense mutation in the amylin gene, S20G, has been shown to predispose Japanese and Chinese [9, 10], but not Caucasian populations [11, 12] to Type 2 diabetes. This mutation was prevalent in 0.4% of Chinese and 4.1% of Japanese patients with late-onset Type 2 diabetes. Amylin is a 37 amino acid polypeptide that is co-secreted with insulin from the pancreatic beta-islet cell in response to nutrient intake [13]. Although its precise function is not known, amylin has been implicated in the regulation of insulin and glucose metabolism. A prominent feature of amylin is its ability to form amyloid fibrils. Extracellular plaques formed from these fibrils have been detected in 70 to 90% of patients with Type 2 diabetes at autopsy [14]. The ability of intracellular amyloid deposits to evoke specific apoptotic signalling pathways in beta cells is a feature of beta-cell loss in Type 2 diabetes [15]. The S20G mutation occurs in the region necessary for amyloid formation, amino acids 20 to 29, and increases the fibrillogenic properties of the peptide [16]. Therefore, this enhanced ability for S20G to form amyloid fibrils in vitro, could be a factor in the genesis of Type 2 diabetes in Japanese and Chinese populations.

The genetic origins of New Zealand Maori being in South East Asia along with amylin gene mutations predisposing East Asian populations to Type 2 diabetes, lead us to hypothesise that amylin gene mutations could also be important in Type 2 diabetes in Maori populations. Therefore, we screened for mutations in the protein encoding and 5' regulatory sequences of the amylin gene in Maori subjects with late-onset Type 2 diabetes.

Subjects and methods

Subjects. The study population consisted of 131 Maori patients with a clinical diagnosis of Type 2 diabetes and 258 Maori non-diabetic subjects as the control group. The study group was recruited from clinics where they were being treated for diabetes which was diagnosed by the US National Diabetes Data Group criteria (fasting plasma glucose >126 mg/dL and a 2-h post glucose load of >200 mg/dL). Non-diabetic control subjects were enrolled from the general public by offering free testing for diabetes and were included if they had HbA1c values of 3.0-6.0%. The clinical data for subjects are summarised in Table 1. Before carrying out this study informed consent was obtained from each individual as approved by the Auckland ethics committee and local tribes. Ethnicity was recorded according to the 1996 New Zealand census question [17].

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Type 2 diabetic patients</th>
<th>Non-diabetic controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects</td>
<td>131</td>
<td>258</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>72/59</td>
<td>138/120</td>
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<tr>
<td>Age (years) ± SD</td>
<td>57.0±12.4</td>
<td>61.4±7.9</td>
</tr>
<tr>
<td>BMI (kg/m²) ± SD</td>
<td>31.7±4.4</td>
<td>5.7±0.3</td>
</tr>
<tr>
<td>Age at diagnosis ± SD</td>
<td>46.0±9.2</td>
<td>9.5±1.8</td>
</tr>
<tr>
<td>Duration of diabetes (years) ± SD</td>
<td>12.0±9.7</td>
<td>27/35/23</td>
</tr>
<tr>
<td>Hba1c (%) ± SD</td>
<td>5.7±0.3</td>
<td>63%</td>
</tr>
</tbody>
</table>

Data on patients were included if they identified themselves as Pacific Islanders, Indian or Asian. The Maori subjects who participated in this study were first and second generation Maori or Maori and New Zealand Caucasian.

Methods. Genomic DNA was extracted from peripheral blood leukocytes using the QIAamp DNA Blood Mini kit according to the manufacturers instructions (Qiagen, Valencia, Calif., USA). The proximal promoter region, exon 1, exon 2, intron 1, coding region of exon 3 (approximately 187 bp) and 130 nucleotides following the stop codon, of the amylin gene were amplified by PCR using the following forward and reverse primers: 5'-atgcaccctgtactcag-3', 5'-cgttcgctgtctacatgtc-3' for the proximal promoter region, exon 1 and intron 1: 5'-tcgttcgctgtctacatgtc-3', 5'-tcgttcgctgtctacatgtc-3' for exon 2; and 5'-tcgttcgctgtctacatgtc-3', 5'-tcgttcgctgtctacatgtc-3' for exon 3. Each PCR amplification reaction contained 100 ng of genomic DNA, 0.1 to 0.2 µmol/l each of forward and reverse primers, 0.2 mmol/l of each dNTP, 0.5 to 1.0 units of AmpliTaq DNA Polymerase (Roche Molecular Systems, Nutley, N.J., USA), 10 µl of Buffer II (10 mmol/l Tris HCl, 50 mmol/l KCl pH 8.3), 1.0-1.5 mmol/l MgCl₂, and H₂O to a final volume of 50 µl. The reactions were carried out in a Master Gradient (Eppendorf, Hamburg, Germany) thermocycler at 94°C for 5 min, 35x (94°C for 0.5 min, 55-68°C for 1.5 min, 68-72°C for 1 min), 68 to 72°C for 5 min. PCR products were enzymatically purified in 0.5 units of shrimp alkaline phosphatase and 0.2 units of Exonuclease I at 37°C for 15 min followed by 80°C for 15 min. The PCR products were sequenced on both strands using a BigDye terminator cycle sequencing ready reaction kit (Perkin-Elmer, PE Biosystems Division, Foster City, Calif., USA). Mutations were identified using Sequence Navigator software (Perkin-Elmer).

Analytical methods. Haemoglobin A1c was measured using the Biorad Variant II Hba1c system with a normal range of 4.0 to 6.0%.

Statistics. For statistical analysis, results were presented as means ± SD. The Fishers Exact test was used to compare frequencies of the -215T>G mutation. The 2×3 chi square table was used to compare frequencies of the -230A>C mutation. Association of both mutations with diabetes was calculated by the McNears test. A p value of less than 0.05 was considered statistically significant. Relative risk was estimated using odds ratios, and 95% confidence limits were calculated.
Results

We identified three new mutations in the amylin gene: two mutations in the proximal promoter region, (-215T>G and -132G>A), and a CTG→CCG nucleotide change in exon 3 predicted to result in a glutamine to arginine substitution in codon 10 (Q10R) (Fig. 1). A previously reported mutation, -230A>C, in the non-coding promoter region was also identified in this population [18]. The genotype frequencies of these mutations are summarised (Fig. 2). The sequences for exons 1 and 2, and intron 1 of the amylin gene in all subjects screened were identical to the published sequence (Genbank accession #: X68830).

The -215T>G promoter mutation was observed in seven Type 2 diabetic patients (5.4%) and two control subjects (0.76%) (Fig. 2). All affected subjects were heterozygous for the -215T>G mutation. The -215T>G mutation was associated with Type 2 diabetes in this population with an odds ratio of 7.23, [1.5–35.3], p=0.008. Both non-diabetic carriers had BMI’s of 24.38 and 25.29 kg/m², whereas the average BMI for diabetic carriers was 28 kg/m²±2.5 (mean±SD). Of the seven Type 2 diabetic patients carrying the -215T>G mutation, four also had the -230A>C promoter mutation (Fig. 2). This combination of mutations was not observed together in any of the non-diabetic control subjects. The occurrence of these two mutations together was significant (χ²=46.7; p<0.001) and the odds ratio of -230A>C carriers inheriting the -215T>G mutation was 2.27. There was no difference in BMI (p=0.459) and HbA₁c values (p=0.356) between subjects carrying both -215T>G and -230A>C when compared with -215T>G only carriers.

The -132G>A mutation was observed in one patient with Type 2 diabetes (0.76%) and was absent in the non-diabetic control subjects (Fig. 2). The -132G>A mutation is a heterozygous mutation and results in the deletion of a MwoI restriction enzyme site. The carrier was a 56-year-old woman, diagnosed with Type 2 diabetes at the age of 45 years and treated with insulin. This patient has a BMI of 26.7 kg/m² and reported a family history of diabetes. The Q10R mutation was observed in one patient with Type 2 diabetes (0.76%) and was absent in the non-diabetic control subjects (Fig. 2). The Q10R missense mutation is a homozygous mutation in exon 3, and creates a Kasi restriction enzyme site. The carrier was a 60-year-old woman, diagnosed with Type 2 diabetes at the age of 56 years and treated with insulin. This patient had a BMI of 28.4 kg/m² and reported a family history of diabetes. The association of -132G>A and Q10R mutations with Type 2 diabetes was not statistically significant.

The -230A>C mutation was observed in both the heterozygous and homozygous state. The frequency of this mutation in patients with Type 2 diabetes and the control group was 26.4% and 24% respectively (odds ratio=1.02, [0.63–1.64], p=0.94) and is therefore not associated with Type 2 diabetes in this population (Table 1).

To determine whether the mutations identified in this study disrupt the binding site for transcription factors, potential binding sites in the vicinity of these mutations were screened for using the MatInspector program on the TRANSFAC 4.0 database [19]. The -215T>G mutation was found in the binding site for the activator protein-1, AP-1, transcription factor (−217–207 bp). Both the -230A>C and -132G>A mutations affected the flanking sequences for an AP-4 putative binding site in the reverse orientation (Fig. 1). The -132G>A mutation also lies within the flanking sequence of another AP-1 potential binding site.

Discussion

The amylin gene mutations identified in this study are associated with 7% of Type 2 diabetes in Maori people. The -215T>G mutation was present at a frequency of 5.4% and 0.76% in Type 2 diabetic and non-diabetic Maori subjects respectively. This mutation was therefore associated with Type 2 diabetes in this population (p=0.008) predisposing the carriers to Type 2 diabetes with a relative risk of 7.23. The non-diabetic carriers for the -215T>G mutation had healthy BMI’s, whereas the average BMI for diabetic carriers at
28 kg/m²±2.5 classed them as overweight. Therefore a low BMI could be an important factor for maintaining normoglycaemia in these subjects. However, the statistical significance for differences in BMI was unable to be calculated due to the lack of BMI data for the control group in this study.

The –215T>G mutation was observed in combination with the –230A>C mutation in 3.1% of patients with Type 2 diabetes. The occurrence of –215T>G and –230A>C mutations together was greater than that due to chance (p<0.001) and suggests linkage disequilibrium exists between these two mutations. Thus it is necessary to test for both the –215T>G and –230A>C amylin gene mutations together in future population screenings. The inheritance of two or more diabetes susceptibility markers together, has been reported in the genes encoding IPF-1, HNF-1α and islet brain-1 (IB-1) [21, 22]. Subjects who inherited both markers had a more severe form of diabetes compared with those carrying IPF-1, IB-1 or HNF-1α mutations alone. This suggests a nearby mutation that is in linkage disequilibrium with a known diabetogenic gene which could contribute to the polygenic nature of Type 2 diabetes.

The –230A>C amylin gene polymorphism has been identified in a Japanese diabetic subject with marked islet amyloid deposition [18]. However, no association of this mutation and Type 2 diabetes was found in a larger Japanese cohort [20]. We also found that there was no difference in frequency of the –230A>C allele between patients with Type 2 diabetes and the control Maori group, indicating that on its own, the –230A>C mutation is not likely to be associated with Type 2 diabetes in Maori people. This allele was present in 24% of Maori and 60% of Japanese subjects with Type 2 diabetes. However, the differences in frequency between these two populations is not likely to be statistically significant due to the relatively small sample size tested in the Japanese study (n=35 diabetic patients and 49 control subjects).

The –132G>A mutation is rare in this population (0.76%). The carrier reported a family history of Type 2 diabetes in first-degree relatives, therefore co-segregation of the –132G>A mutation with diabetes should be tested in family members of this carrier. Alternatively, functional studies of this mutation could validate its association with Type 2 diabetes.

All the promoter amylin gene mutations identified were found in putative transcription factor binding sites within or near the proximal promoter region (–222 and –91 base pairs), which is required for islet-specific transcriptional activity [23]. Thus it would be of interest to investigate whether these mutations alter basal transcriptional level of the amylin gene in pancreatic beta cells.

The Q10R mutation was observed in one patient with Type 2 diabetes indicating that this mutation is rare in Maori people (0.76%). The carrier reported a family history of Type 2 diabetes in first-degree relatives, therefore it has yet to be determined whether Q10R co-segregates with diabetes in this family. The Q10R mutation does not occur in the region responsible for fibril formation (amino acids 20 to 29) [24], that has been proposed to contribute to the cytotoxic and amyloidogenic properties of amylin. Therefore it is not likely to contribute to the pathogenesis of Type 2 diabetes through enhanced fibril formation.

The amylin gene coding region mutation, S20G, reported in Japanese and Chinese populations [9, 10], was not observed in Maori patients or control subjects and is therefore not likely to be a major contributing factor for Type 2 diabetes in Maori people despite evidence for a common genetic origin for these populations [7, 8].

In summary, we found that approximately 7% of Maori with Type 2 diabetes have mutations in the promoter region of the amylin gene while only 0.8% Maori without Type 2 diabetes have these mutations. The –215T>G is associated with increased Type 2 diabetes susceptibility, with a relative risk of 7.23, and could be linked with the previously described amylin promoter polymorphism, –230A>C. These results suggest that amylin gene mutations may be an important contributor to the polygenic nature of Type 2 diabetes in Maori people.

Acknowledgements. We wish to thank Nga Puhi and Tainui iwi, hapu and whanau (tribes, sub-tribes and families) for their willingness to participate and support this study; Dr. T. Birch, N. Neho, from Hokianga Health, and Dr. D. Gilgen for assisting with recruiting patients and collecting blood samples; T. Ellison and The Hepatitis Foundation for providing non-diabetic blood samples and measuring HbA1c values; and Dr C. Frampton for statistical analysis. This study was supported by grants from the Health Research Council of New Zealand.

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factor-1beta gene are associated with familial hypoplastic glomerulocystic kidney disease.  


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