A CROSS-SECTIONAL STUDY TO CHARACTERISE YOUNG-ONSET DIABETES IN UK ETHNIC GROUPS

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Abstract

Diabetes subtypes are challenging to differentiate, particularly in people who present at young age. Assigning the correct subtype of diabetes is critical as it informs treatment and misclassification can lead to erroneous management. The impact of ethnicity on diabetes phenotypes is poorly studied but recognition of diabetes subtypes within non-white ethnic groups, is increasingly problematic. Maturity onset diabetes of the young (MODY) is a monogenic form of diabetes that is frequently misdiagnosed in white populations and not widely studied in other ethnicities.

The work in this thesis aims to investigate the phenotypes of young-onset diabetes across white, south Asian and African-Caribbean ethnic groups, with the aim of improving recognition of these phenotypes.

Results from a retrospective analysis of referrals for MODY testing in the UK, showed a significantly lower detection rate of finding a positive MODY mutation in people referred from south Asian ethnicity compared to white European ethnicity. It was not clear if this significant disparity represented a lower prevalence of MODY in south Asian individuals or if there was a bias in testing referrals based on the traditional clinical criteria for MODY.

To fully investigate this finding, the MY DIABETES study was initiated; a multi-centre cross-sectional study that systematically phenotypes young-onset diabetes across south Asian, African-Caribbean and white European ethnic groups. Fasting C-peptide and pancreatic auto-antibodies are used to stratify individuals for genetic testing of all known MODY genes.

Results of the initial 728 participants recruited, showed an overall detection rate of 9.5% that was not significantly different by ethnicity and confirmed pathogenic MODY mutations were detected across all ethnic groups. These results suggest that a systematic approach to stratification in south Asian and African-Caribbean individuals may be warranted if clinical criteria are less sensitive due to the overlapping phenotypes of other types of diabetes in these groups.

Declaration of Originality

The majority of the work outlined in this thesis was undertaken by the author (Dr Shivani Misra). Additional information used from third parties has been referenced accordingly. Contributions from collaborators and where assistance has been provided, is outlined below:

Chapter 4 & 5

C-peptide analyses on serum samples from sites were undertaken with assistance from Mr Ludwig Lupak, Senior Biomedical Scientist, Imperial College Healthcare NHS Trust.

Chapter 6

Preliminary investigations into pathogenicity were conducted in collaboration with Dr Kevin Colclough, clinical scientist at Exeter Molecular Genetics laboratory

Detailed structural analysis of the effect of the A251T variant on HNF1A protein structure was undertaken by Mr Richard Caswell (Clinical Scientist, Exeter Molecular genetics laboratory).

Functional assays were undertaken by the author whilst hosted in Professor Anna Gloyn's laboratory, Oxford. Mutant HNF1A constructs were generated by Dr Amanda Bennett and Dr Neelam Hassanali, scientists in Professor Gloyn's laboratory.

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Abbreviations

AC	African-Caribbean
BMI	Body Mass Index
CCG	Clinical commissioning group
CRN	Clinical Research Network
DNA	Deoxyribonucleic acid
EMSA	Electromobility shift assay
ExAC	Exome aggregation consortium
FCP	Fasting C-peptide
GAD	Glutamate acid decarboxylase
GCK	Glucokinase
HDL	High density lipoprotein
HGMD	Human genetic mutation database
HLA	Human Leukocyte Antigen
HNF1A	Hepatic Nuclear Factor 1-Alpha
HNF1B	Hepatic Nuclear Factor 1-Beta
HNF4A	Hepatic Nuclear Factor 4-Alpha
HOMA	Homeostasis assessment model
hsCRP	High sensitivity C-Reactive Protein
IA-2	Islet antigen 2
LDL	Low density lipoprotein
MMTT	Mixed meal tolerance test
MODY	Maturity Onset Diabetes of the Young
PCR	Polymerase chain reaction
SA	South Asian
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
tNGS	Targeted next generation sequencing
UCPCR	Urine C-peptide to creatinine ratio
VUS	Variant of unknown significance
WE	White European
WT	Wild-type
Znt8	Zinc transport 8

Publications and Outputs

The following list outlines peer-reviewed publications, abstracts and other outputs published in relation to the work undertaken in this thesis.

Original Manuscripts

Misra S, Shields B, Colclough K, Johnston DG, Oliver NS, Ellard S, et al. South Asian individuals with diabetes who are referred for MODY testing in the UK have a lower mutation pick-up rate than white European people. Diabetologia. 2016;59(10).

Reviews

Misra S, Oliver NS & Dornhorst A. Diabetic ketoacidosis, not always due to type 1 diabetes. BMJ.2013.346;f5301

Abstracts

Oral Presentations

Misra S, Colclough K, Lupak L, Walker E, Johnston DG, Hattersley AT & Oliver NS. Clinical and biochemical definitions of type 1 diabetes do not agree in a multi-ethnic young-onset diabetes cohort: results from the MY DIABETES study. Accepted at DUK 2017, Manchester

Poster presentations

Misra S, Colclough K, Halleron K, Williams E, Hatterlsey AT, Johnston DJ & Oliver NS. Type 1 diabetes phenotype in white and South Asian people with young onset diabetes in the UK: results from the MY DIABETES study. EASD, 2016, Munich

Misra S, Hassanali N, Smith C, Colclough K, Ellard S, Hattersley A, et al. Homozygous loss of function HNF1A variant resulting in sulfonylurea-responsive early-onset diabetes. Diabetes. 2016;65 (Supp 1::A360-A431) ADA 2016, New Orleans

Misra S, Kaur A, Walkey H, Godsland I, Dayan C, Peakman M, Bingley P, Dunger D, Johnston DJ & Oliver NS. Reclassification of diabetes subtype following a diagnosis of type 1 diabetes: data from the After Diabetes Diagnosis Research Support System (ADDRESS-2) cohort. Diabetes UK, 2016, Glasgow

Misra S, Jairam C, Smith C & Oliver NS. The use of continuous glucose monitoring in the safe-cessation of insulin in people with monogenic forms of diabetes. Poster presentation, ATTD, 2015, Paris

Book Chapters

Misra S & Hattersley AT. Monogenic forms of Diabetes, Chapter 18. Textbook of Diabetes 5th Edition (Ed R Holt). 2016 Wiley Blackwell. ISBN: 978-1-118-91202-7

Chapter 1 : General Introduction

1.1 Diabetes

Diabetes mellitus is a condition characterised by hyperglycaemia, resulting from defects in insulin secretion and/or insulin sensitivity (1–3). The prevalence and incidence of diabetes continues to rise worldwide and the morbidity and mortality from diabetes impacts significantly on healthcare systems globally posing a significant burden to all healthcare budgets (4). Reduction of hyperglycaemia through glucose-lowering therapies is the fundamental approach to management of all diabetes cases; the specific treatment approach is primarily determined by the type of diabetes. Diabetes has been broadly categorised into type 1, an autoimmune-mediated beta-cell failure and type 2, insulin-resistance driven beta-cell failure. Though an aetiological separation, the distinction primarily influences treatment choice. Type 1 cases require life-long insulin from diagnosis and type 2 diabetes cases initially, at least, is managed with non-insulin based therapies. However, a variety of paths, influenced by an interplay of genetic and environmental factors lead to hyperglycaemia and the rudimentary distinction of type 1 from type 2 diabetes is likely to be a simplified approach (5).

Over time, knowledge of other types of diabetes and understanding of distinct phenotypes within one type of diabetes, has expanded significantly. The recognition that these alternative types of diabetes may be treated differently to the standard care provided for both type 1 and type 2 diabetes, has meant that differentiating these other and more unusual forms from type 1 and type 2 diabetes, is worthwhile.

The impact of ethnicity on the classification and recognition of diabetes types is a poorly understood area. In recent years there has been increasing recognition of the heterogeneity with which diabetes can occur, present and progress in people from different ethnic groups (6). However, studies systematically investigating these differences are scarce and effecting changes in clinical practice may be even harder.

The work presented in this thesis aims to define, investigate and describe the variable phenotypes of young-onset diabetes in UK south Asian (SA) and African or Caribbean (AC) ethnic groups, with an aim of identifying similarities and differences that can

impact clinical practice and improve the identification of distinct subtypes that influence treatment choice in future studies.

1.2 The Challenge of Diabetes Classification

The American Diabetes Association and the World Health organisation are two of the most-widely cited classifications for diabetes, in use. They broadly categorise on the basis of aetiology, however they do not address precise criteria for the differentiation of one subtype from another (see table 1.1) (7,8). This primarily reflects the challenge of accurately defining types of diabetes by clinical parameters. The traditional clinical criteria such as age at diagnosis, body mass index or presence of ketoacidosis, now poorly discriminate the two main types of diabetes from one another. For example, type 1 diabetes though more common in childhood, may present at any age (9) and the presence of obesity should not preclude the diagnosis, as rates of obesity in the population as a whole are now much higher and obesity is frequently encountered in youth with type 1 diabetes (10). Meanwhile ketoacidosis, a complication thought to exclusively occur in people with type 1 diabetes can also occur in type 2 diabetes (11).

The Royal College of General Practitioners, provided some practical criteria for the differentiation of diabetes type, which as well as age at diagnosis, incorporated a clinical definition relating to the continuous use of insulin from diagnosis (12). However, this approach is also not without limitations, as continuous use of insulin from diagnosis may signify beta-cell failure, but it also may occur in people inappropriately treated with insulin.

Although other forms of diabetes may be proportionally less common, identifying the correct subtype in a newly presenting person is crucial as it ensures correct treatment modalities are provided, impacts on the type of diabetes education delivered and influences overall control. At its most serious, misclassification of diabetes subtype could mean that a person with type 1 diabetes requiring life-sustaining insulin therapy, does not receive it if they are misclassified as having type 2 diabetes. Conversely a person with type 2 diabetes may be unnecessarily treated with insulin. This may not seem problematic, but it is important to consider the adverse effects associated with insulin use such as hypoglycaemia, impact on quality of life and potentially education

and employment, which may be needlessly experienced in the case of misdiagnosis. At the other end of the spectrum, more uncommon forms of diabetes, for example maturity onset diabetes of the young (MODY) can often be managed without insulin, or in some cases requires no treatment at all.

The differentiation of type 1 from type 2 diabetes has proven to be a useful therapeutic distinction, but it has not helped to explain the wide variety of diabetes manifestations; for example the lean non-ketotic individuals from specific ethnic groups; the differential response to treatment in type 2 diabetes; the variable rates of beta-cell failure in both type 1 and type 2 diabetes or the rapid progression to complications in some individuals (13). These differences in turn have led to the recognition of new clinical entities, for example latent autoimmune diabetes of adulthood (LADA) or ketosis-prone type 2 diabetes (14).

The one size fits all approach to treatment is also to be challenged with increasing recognition of heterogeneity in treatment responses in type 2 diabetes and also in understanding that genotype may influence treatment choice in people with genetic forms of diabetes(15–17). The intersection of genotype, phenotype, ethnicity and lifestyle factors has given momentum to the concept of precision medicine in diabetes, to ensure people with diabetes are correctly classified and treated (13).

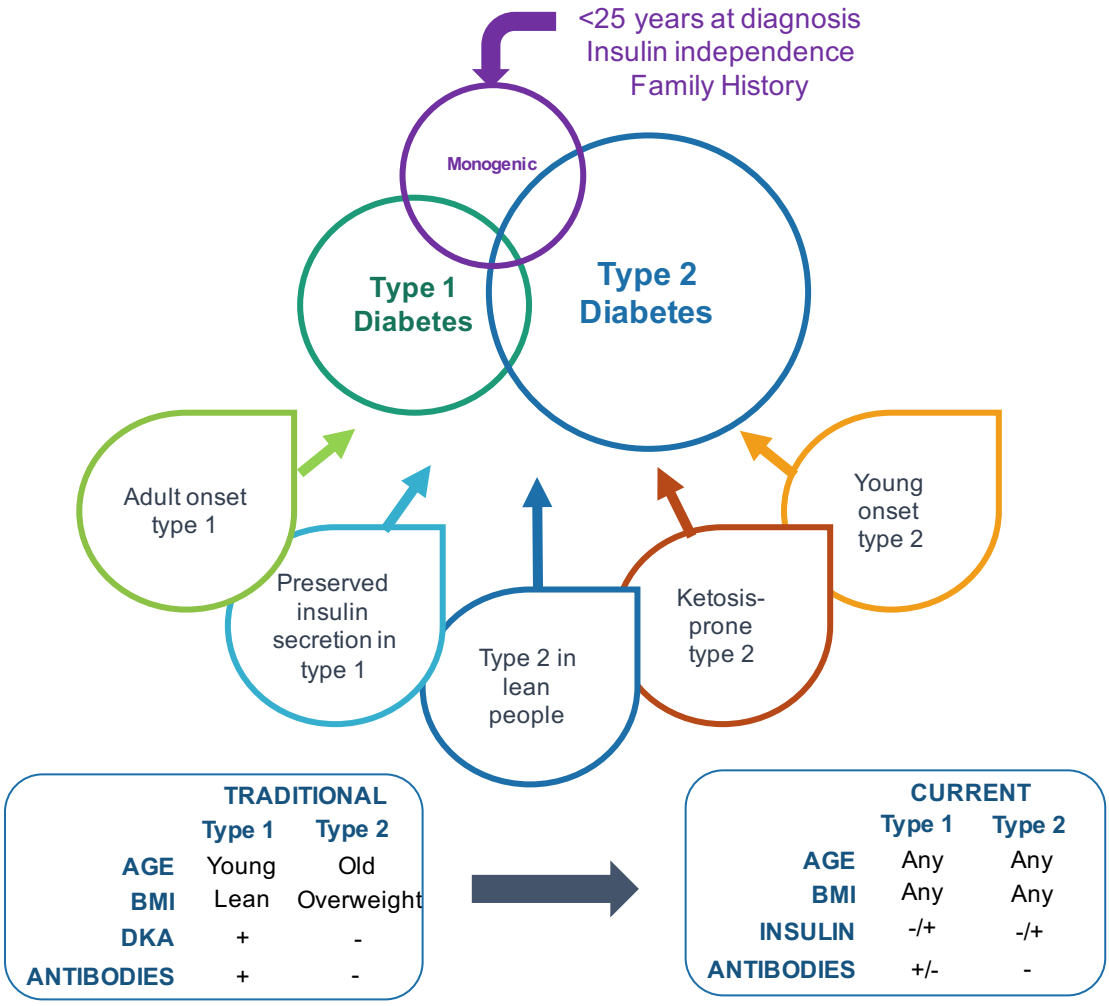
Arguably the challenge of classification is even greater in people with young onset diabetes, where the relative prevalence and incidence of all types of diabetes overlap (5). People from different ethnic minority groups also pose a challenge as the prevalence of young onset type 2 diabetes is reportedly higher and the incidence of type 1 diabetes variable (5), and possibly dependent on migration status (18–20).

In the next section (section 1.3), each of the main types of diabetes are described and the challenges in their recognition, explored.

1.3 Subtypes of Diabetes and their Recognition

The major forms of diabetes and the challenges around classification are summarised in figure 1.1 and table 1.1.

Figure 1.1: Schematic of the Overlapping Types of Diabetes and Phenotypes that Obscure Clinical Separation



DKA, Diabetic ketoacidosis; BMI, Body mass index; antibodies denotes the present or absence of pancreatic autoantibodies.

Table 1.1: Summary of Diabetes Subtypes Based on Underlying Pathology

Underlying pathology	Subtype
Destruction of beta-cells	Type 1 diabetes, latent autoimmune diabetes of adulthood, CEL (carboxylesterlipase) MODY, drug-induced, primary pancreatic pathology
Low beta-cell mass from birth	HNF1-beta MODY, PDX1-MODY
Defective response to glucose	Type 2 diabetes, glucokinase (GCK) MODY, HNF1/4 alpha MODY, mitochondrial diabetes, endocrinopathies, genetic insulin resistance syndromes, drug-induced
Defective processing of insulin	WFS1 (Wolframin gene) diabetes
Unknown	Ketosis-prone type 2 diabetes

Modified from (21).

1.3.1 Type 1 Diabetes

Type 1 diabetes is characterised by autoimmune beta-cell destruction and can occur at any age (8). Young people with type 1 diabetes are typically thought to be slim, although this, with rising obesity levels, is less likely to be the case. The condition is typified by an acute presentation, with associated low beta-cell reserves at onset (22). Affected individuals are expected to be relatively insulin sensitive and almost invariably possess human leukocyte antigen (HLA) susceptibility haplotypes (23). However, these features do not reliably differentiate between type 1 and type 2 diabetes. Additionally, it is recognised that a proportion of individuals with phenotypic type 1 diabetes, do not have pancreatic autoantibodies, so-called type 1b (22). Older individuals with type 1 diabetes, may have a slower onset of symptoms with a more insidious presentation and the coexistence of insulin resistance may unmask beta-cell failure earlier (24).

The autoimmune nature of the condition, means that pancreatic autoantibodies can be detected in the serum of newly presenting patients, however current guidance does

not support the routine measurement of these in adults (25), although paediatric guidelines do support this (26). This is primarily to avoid insulin cessation in adults who are antibody negative, recognising the proportion of individuals who are antibody negative, even at diagnosis. The adult guidelines from the National Institute for Health and Care Excellence, recommend testing pancreatic autoantibodies if type 1 diabetes is suspected and there are atypical features of type 1 diabetes, for example having a body mass index (BMI) above 25kg/m^2, or in people aged over 50 years (25). Pancreatic autoantibodies are not without limitations and these are discussed in section 1.4.2.

C-peptide, a marker of beta-cell function, is also not routinely recommended in the diagnosis of type 1 diabetes. Levels around diagnosis are difficult to interpret and unlike other hormonal axes, C-peptide levels are also influenced by tissue insulin resistance (27). The identification of type 1 diabetes is therefore predominantly based on clinical features. Several studies have shown that these features, for example age-at-diagnosis, BMI or continuous insulin treatment, have variable sensitivity and specificity (28,29). In one study, age at diagnosis and the time to insulin treatment were the most sensitive predictors of type 1. However, the criterion against which these parameters were compared (continuous insulin treatment within 3 years of diagnosis) was also open to incorrect diagnoses and the population studied was predominantly white European (29). Studies validating the utility of different phenotypic features in identifying type 1 diabetes, are additionally thwarted by the absence of a gold-standard definition for type 1 diabetes, particularly at the time of diagnosis.

The presentation of type 1 diabetes may be different in certain ethnic groups, where there is proportionally more young-onset type 2 diabetes, variability in insulin sensitivity and body habitus. These differences also add to the challenges around diagnosis.

1.3.1.1. Type 1 Diabetes in Ethnic Groups

Very few studies have evaluated the phenotype of type 1 diabetes in SA and AC individuals. One small paediatric study has shown similar phenotypes of SA and white

individuals in the UK (30), but reports from the Indian subcontinent and in African populations are limited. There is a recognition from studies in native countries that people of non-white ethnicity with type 1 diabetes are less likely to be pancreatic autoantibody positive (31–34).

However, the situation may be more complex than just phenotypic differences alone, as risk of type 1 diabetes (and possibly phenotype) may vary according to whether the ethnic group studied are in their native country or have migrated to a country with a different incidence of type 1 diabetes. For example, second generation children of south Asian migrants in Leicestershire, Somali migrants in Finland and East African migrants in Sweden, appear to have higher rates of type 1 diabetes than local white European children (18–20).

1.3.2 Type 2 Diabetes

Type 2 diabetes is characterised by insulin resistance and beta-cell dysfunction. Traditionally, it has been associated with obesity but increasingly and particularly in certain ethnic groups, type 2 diabetes has been shown to occur in relatively lean individuals. This recognition has led to the development of ethnic-specific BMI cut-offs (35). That some ethnic groups and indeed some individuals in any ethnic group, develop type 2 diabetes without being overweight, whilst some obese individuals do not develop type 2 diabetes at all, suggests that the underlying mechanisms influencing the developing of type 2 diabetes vary across individuals (36).

Later age at diagnosis was thought to discriminate type 2 diabetes, but now type 2 diabetes has been reported in children and increasingly in young-adults (37). In some respects, it is the changing demographics of type 2 diabetes that have impacted on the recognition of other types; the increasing frequency of type 2 diabetes in people relatively lean, and at young age, overlaps with the peak time period when type 1 diabetes, monogenic diabetes and other types present, obfuscating the detection of these types.

1.3.2.1 Type 2 Diabetes in Ethnic Groups

In ethnic groups with a high prevalence of type 2 diabetes the challenge is harder to overcome. Differentiating young-onset type 2 diabetes from type 1 diabetes is difficult as type 1 diabetes can occur at any age and in any body weight category. Meanwhile the absence of obesity accompanying young SA people with diabetes coupled with an inadequate way to assess insulin resistance and the fact that insulin resistance may accompany any form of diabetes, makes the delineation of diabetes type a considerable problem in SA people. Additionally, in AC individuals, type 2 diabetes is noted to have more of an insulin secretory deficit; again suggesting different underlying pathophysiological pathways to hyperglycaemia(38,39).
The pathophysiological differences that may underlie the variability in type 2 diabetes phenotype are discussed below.

South Asian Diabetes

Diabetes in SA individuals is an increasing problem globally. Most data relates to type 2 diabetes in SA populations (from the Indian subcontinent) as very little is known about type 1 diabetes or other types of diabetes in these groups. Both within the Indian subcontinent and in the SA diaspora increasing type 2 diabetes poses a considerable health burden. Migrant SA populations have a much higher risk of developing type 2 diabetes when compared to the population prevalence of the country they move to (40).
The risk of diabetes is also variable within the SA ethnic group, a heterogenous mix of different genetic groups, with the highest in people of Bangladeshi origin, approximately 4 times the risk of white European (WE) ethnicity and lowest in Indian and Pakistani people at approximately 2 times the risk (40,41).
The underlying issues that contribute to the excess risk observed in SA individuals are not fully understood, but are likely to be multifactorial.

There is some evidence to suggest that differences in beta-cell capacity may account for the higher risk of type 2 diabetes in SA individuals, but this is not conclusively proven. Data from prospective studies has demonstrated that beta-cell function as assessed by HOMA-B% was higher in SA individuals, but did not increase with age,

as observed in WE individuals (42,43). Consequently, it appears that although SA individuals produce more insulin at a younger age to compensate for insulin resistance, there is an earlier reduction in beta-cell function which underlies the transition to diabetes (40). Sattar et al hypothesise that these differences may represent early beta-cell exhaustion secondary to higher insulin production throughout life, a lower beta-cell capacity from birth, or perhaps ectopic pancreatic fat but this requires further investigation to fully elucidate mechanisms (40).

Underlying genetic predisposition to type 2 diabetes has been studied in SA individuals without revealing a mechanism. Genome-wide association studies revealed 24 single nucleotide polymorphisms (SNPs) that were associated with type 2 diabetes in SA individuals, however no differences were observed between the risk associated with these SNPs or their frequency when compared to WE individuals (44). Other studies have shown higher DNA methylation scores in SA individuals compared to WE, which accounted for approximately 32% of the higher risk observed in SA individuals (45).
The risk of developing type 2 diabetes itself appears to be present throughout early life in people of SA ethnicity. Higher cord insulin levels were noted in SA neonates compared to WE and data has also demonstrated that UK SA children have higher fasting insulin and triglyceride concentrations throughout childhood (46,47). These findings are consistent with a degree of insulin resistance in SA individuals, not observed in matched WE groups.

Differences in adiposity may in part account for the higher risk observed in SA individuals. Several studies have demonstrated higher visceral adiposity for any given body mass index in SA individuals. This has led to the fat overflow hypothesis, which suggests an innate inability to store fat safely, with preferential deep subcutaneous fat or visceral fat deposition (48,49). Further prospective work is needed to corroborate this along with assessment of ectopic fat stores in the liver and pancreas (40).

Regardless of the underlying process, the excess risk observed cannot be overlooked. The importance of this higher risk has led to the development of ethnic-specific BMI cut-offs, which recognise that the risk of diabetes occurs at a lower BMI in SA individuals than WE (50,36). This in turn has led to the adoption of a BMI of 23 kg/m^2 or more to define ‘at risk of diabetes’ in SA individuals (51).

People of SA ethnicity also appear to transition from pre-diabetes to diabetes more rapidly. A study in India showed that the annual progression rate of people with impaired glucose tolerance of 12-18% for SA people, compared with the significantly lower rate observed in WE individuals of 5-11% (52).

African-Caribbean Diabetes

AC people also have an increased risk of type 2 diabetes when adjusted for other confounders (2 times risk compared to WE), but there appears again, to be differential risk according to gender and origin, for example Black African men (1.7 times) conferred the highest risk and Caribbean women (0.7 times) had lower risk compared to WE (41).

Similar to SA populations, this risk is likely to be multifactorial, but has been less well studied than SA populations.
Genome wide association studies have been undertaken in African-American populations. Studies have identified most of the SNPs encountered in WE populations, which also have an effect size and direction that is similar in African-American populations, however novel loci have also been identified that have not been encountered in WE groups, suggesting a different underlying genetic architecture (53). Trans-ethnic genome-wide association studies have identified more novel loci, but it seems unlikely that these loci alone will account for the excess risk observed in African-Caribbean groups (54).

Most of the excess diabetes risk is likely to be related to differences in underlying pathophysiology. African-Caribbean ethnicity has been shown to be associated with insulin secretory defects before, most notably in the NHANES III population survey (55), which assessed fasting C-peptide in people without diabetes and showed low fasting C-peptides but high fasting insulin. Studies in people with diabetes have also shown lower C-peptide levels in African-American and Ghanaian populations (38,39,56). However, these studies need to be replicated in other cohorts.

African-Caribbean ethnicity is also associated with lower triglyceride and higher high density lipoprotein (HDL) cholesterol levels, which suggests at baseline they are more insulin sensitive, however this appears to be coupled with higher fasting insulin levels (57,58).

East Asian Diabetes

There is also evidence of rising type 2 diabetes in East Asian populations (59) and similar to south Asian populations, east Asian diabetes seems to develop at a lower body mass index than that observed in WE groups (60).
In east Asian populations there also appears to be more visceral adiposity for any given body mass index, which is associated with insulin resistance (59).

A key features of east Asian diabetes appears to be significant beta-cell dysfunction. This has been observed in a variety of studies that demonstrate failure to compensate for insulin resistance adequately, as assessed in Japanese individuals with and without diabetes undergoing frequently samples intravenous glucose tolerance tests showed a reduced disposition index, but relatively little difference in insulin sensitivity (61,62).

Genome-wide association studies have revealed a few novel loci in east Asian individuals that contribute to type 2 diabetes risk, but the effect size of all the SNPs are not thought to account for the excess risk of diabetes encountered and the effect sizes of shared SNPs between east Asian and WE people are similar (54).

1.3.3 Latent Autoimmune Diabetes of Adulthood

Latent autoimmune diabetes of adulthood (LADA), is the term given to a slowly progressing, relatively late onset form of diabetes(14). As a clinical entity it gained momentum, following the identification of glutamic acid decarboxylase (GAD) positivity in people with type 2 diabetes (63) who were noted to progress to insulin treatment much faster than those who were negative. These individuals become insulin dependent sooner than antibody negative type 2 diabetes, but it is unclear if they represent a distinct group from people with type 1 diabetes. Controversy exists around its exact definition and a study comparing genotypes of individuals has

demonstrated conflicting results, possibly thwarted by the lack of an agreed definition of what constitutes LADA (64–66).

1.3.4 Ketosis-Prone Type 2 Diabetes

Ketosis prone type 2 diabetes (KPDM) is particularly challenging to identify as patients present with diabetic ketoacidosis and require insulin treatment, but can subsequently maintain euglycaemic remission off insulin, making the diagnosis critical to on-going management, as misclassification as type 1 diabetes may mean long-term insulin treatment (11). Flatbush diabetes, as it is also known, was first described in African-American adolescents in New York, but has now been described in almost every ethnicity and age group (67).

The concept of KPDM is an interesting one, as it challenges our understanding of pathophysiology (68,69) – why should a person with type 2 diabetes, acutely deteriorate beta-cell function to such an extent that they develop ketoacidosis, only to recover function subsequently? Classically, people present in ketoacidosis, usually without a precipitant (70). The hallmarks of KPDM are the absence of pancreatic autoantibodies at diagnosis and recovery of beta-cell function by 3-6 months, with euglycaemia off all treatment (69,71). Similar to LADA, there is a lack of an agreed definition about what constitutes ketosis-prone type 2 diabetes, especially as any person with type 2 diabetes can theoretically develop ketoacidosis if catabolic or acutely unwell. Unlike LADA, the clinical benefits of recognising this distinct phenotype are very clear; affected individuals can maintain euglycaemic remission off insulin and although they are at risk of future episodes of ketoacidosis, many maintain normoglycaemia for decades; so clearly distinguishing type 1 diabetes from KPDM is of value (11).

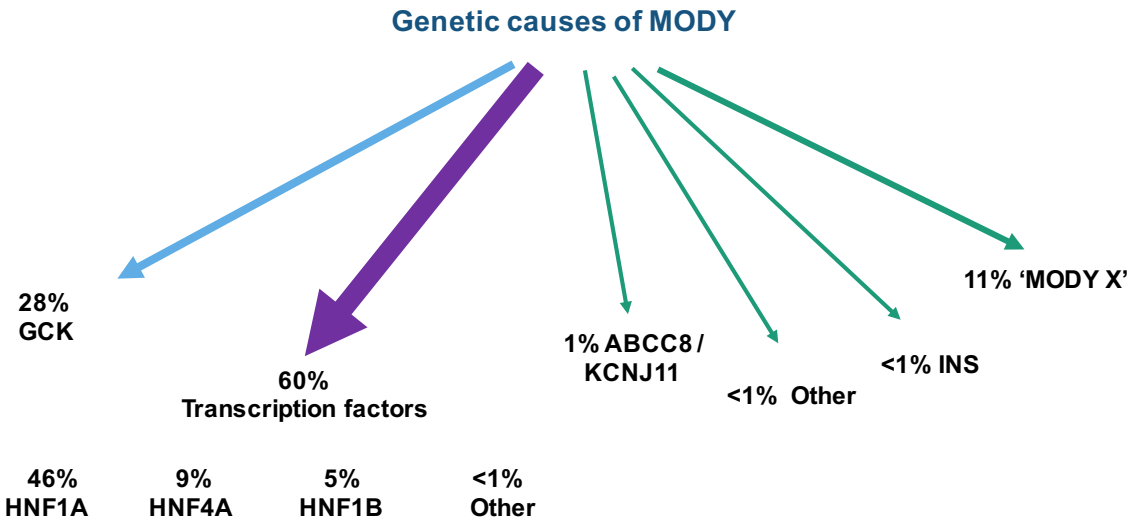
Most cases of KPDM have been described in people of African or Caribbean heritage, but reports in nearly all ethnic groups, including non-Hispanic white, exist. There is no clear advance on the pathophysiological mechanism behind the condition and detailed metabolic, genetic and physiological studies are yet to provide a clear answer. One study demonstrated an increase in frequency of the PAX4 transcription factor variant, but this was likely to be a benign variant (72).

Further understanding of this phenotype in the UK population would be valuable as seminal studies to date have been undertaken in African-American and French migrant populations. There is every possibility that within the African diaspora, variability exists in this intriguing phenotype, but these are yet to be explored in detail in the UK.

1.3.5 Maturity-Onset Diabetes of the Young

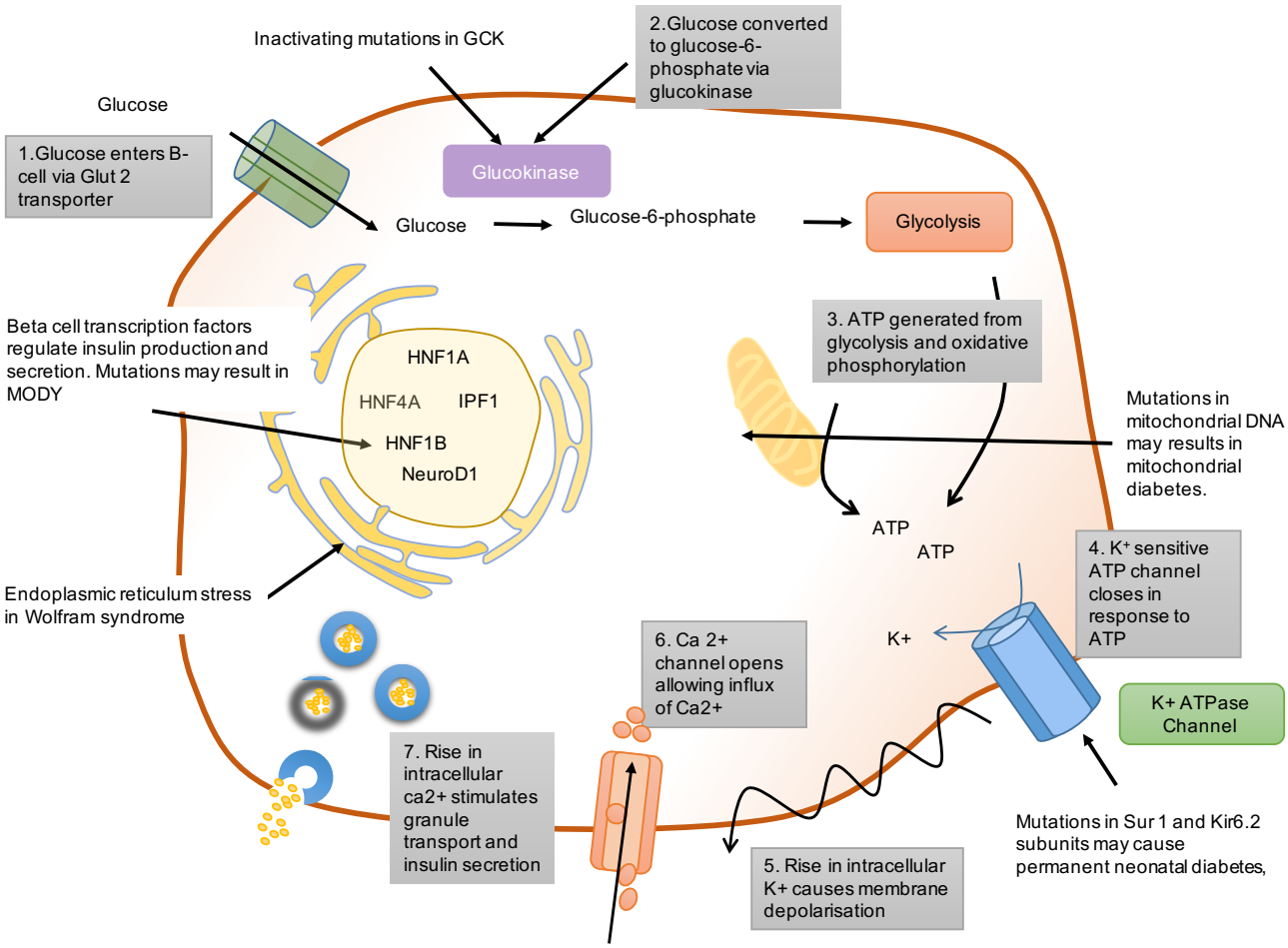
Maturity-onset diabetes of the young (MODY) is a collective term given to monogenic cause of diabetes. Classically, it is autosomal dominantly inherited with a 3-generational history of diabetes, does not require insulin immediately and is diagnosed before the age of 25 years (73). These criteria were described by Tattersall in 1974 (73,74) and now there is a recognition that these clinical criteria alone are insensitive at detecting all MODY cases (75). A variety of genes have been implicated in causing MODY, and most affect beta-cell function rather than insulin sensitivity (73,74). MODY can be classified according to the gene involved (7,8). Mutations in the enzyme glucokinase (*GCK*) and several transcription factors that affect beta-cell development and function, account for most cases the frequencies of MODY, which are summarised in figure 1.2 and their role in beta-cell physiology in figure 1.3.

Figure 1.2: The Different Genes Implicated in the Cause of MODY



MODY X denotes dominantly inherited young-onset non-insulin dependent diabetes fitting clinical criteria for maturity-onset diabetes of the young where mutations in known MODY genes have not been identified. Adapted from McCarthy & Hattersley (76)*, Shields et al* (77)*, reproduced with permission from* (78)*.*

Figure 1.3: Schematic of the Beta-Cell



Key Steps in glucose sensing and insulin secretion outlined in shaded boxes and other proteins and organelles that can cause monogenic diabetes, highlighted. Adapted from Fajans et al(79) *and reproduced with permission from* (78).

The way in which MODY presents is largely influenced by the gene affected (see below); for example people with *GCK* mutations have life-long, mild fasting hyperglycaemia, which is non-progressive and does not require treatment (80). There is real benefit to making the diagnosis in an individual as studies have shown that the hyperglycaemia encountered is non-progressive and does not result in microvascular or macrovascular complications (81). If someone is identified with *GCK* MODY they can usually be advised to stop any diabetes treatment.

Hepatic nuclear factor- 1 alpha (*HNF1A*) and -4 alpha (*HNF4A*), the two most common transcription factor MODY types are characterised by a progressive beta-cell failure that can be managed effectively with low-dose sulphonylurea therapy (82–84). This was a seminal finding and enables most people with the mutations in both genes to come off insulin. However, recognising cases is a challenge as the peak age at presentation overlaps with type 1 diabetes age brackets and the lack of insulin requirement mimics type 2 diabetes (85).

These management strategies contrast with the usual care offered for both type 1 diabetes and type 2 diabetes (83). Therefore, correct identification of MODY is crucial for diabetes education and informs lifelong treatment. Misclassification can result in inappropriate insulin therapy, risking hypoglycaemia, and impacting on complications, lifestyle, employment and quality-of-life. In addition, a confirmed genetic diagnosis identifies risk in family members of affected individuals, facilitating further improved treatment and identification of cases.

1.3.5.1 Glucokinase (GCK)

In the first, rate-limiting step of glycolysis, glucose is converted to glucose-6-phosphate and this is catalysed by the enzyme glucokinase (see figure 1.3) (78). GCK therefore acts as a glucose sensor, ensuring insulin release is initiated at an appropriate level of glycaemia (86). The dose-response curve is right-shifted in people with heterozygous loss-of-function mutations (87) and blood glucose levels are therefore regulated at a higher set point but, critically, in contrast to other forms of diabetes, remains under physiological control with homeostatic measures intact. Affected individuals have a mild fasting hyperglycaemia from birth, usually between 5.4–8.3 mmol/L and HbA1c results range between 40-60 mmol/moL (88,89). Since *GCK* MODY is asymptomatic there may be no known family history of diabetes, despite its autosomal dominant inheritance.

In contrast to type 1 diabetes, the hyperglycaemia encountered in *GCK* MODY is mild, pancreatic autoantibodies are usually negative and (if tested) a parent is likely to also be affected. Fasting C-peptide will remain detectable and the post-prandial rise in glucose levels will be smaller than those encountered in in type 1 or type 2 diabetes,

as homeostasis is maintained. Differentiating people with *GCK* mutations from type 2 diabetes can be challenging as both conditions may cause mild fasting hyperglycaemia with a strong family history. The absence of obesity or features of insulin resistance, a small 2-hour increment on oral glucose tolerance testing and non-progressive all suggest mutations in *GCK*. However, as discussed background obesity in the population should not preclude the diagnosis.

With the exception of during pregnancy, treatment of *GCK* is not warranted or recommended as it is very challenging to lower glucose levels past the set point and when achieved this usually triggers a counter-regulatory response with associated symptoms of hypoglycaemia (80,90). Additionally, studies to date suggest that people with *GCK* MODY do not develop microvascular of macrovascular complications(81,89).

1.3.5.2 Hepatic Nuclear Factor 1 Alpha & 4 Alpha

Hepatic nuclear factors 1A and 4A (*HNF1A* and *HNF4A) are transcription factors that control expression of a variety of genes involved in insulin secretion.* Most individuals with MODY in the UK have heterozygous mutations in one of these transcription factor genes.

Transcription factor mutations are thought to alter insulin secretion and levels of other proteins that are involved in metabolism, including the GLUT-2 glucose transporter along with enzymes involved in the mitochondrial metabolism of glucose (91–93). The progressive deterioration in beta-cell function that is observed with *HNF1A* and *HNF4A* mutations, may reflect reduced beta-cell proliferation and increased apoptosis (93–96), but is not fully explained. Mutations in *HNF1A* account for up to 70% of cases of MODY with nearly 200 different mutations reported. *HNF4A*, the next most common, accounts for approximately 3% of cases (97).

Affected individuals usually have normal glucose tolerance (in contrast to *GCK* mutations) at birth but demonstrate progressive beta-cell dysfunction, presenting before the age of 25 years and 79% present by the age of 35 years; it has been demonstrated that the age of diagnosis reflects the nature and location of the

underlying mutation and gene (98–100). As with other forms of diabetes, but in contrast to *GCK* mutations, microvascular complications can occur if hyperglycaemia is inadequately treated (101). However, in contrast to people with type 2 diabetes, individuals tend to be insulin sensitive not resistant. This latter point, in part, accounts for the marked response to sulphonylurea therapy, which often results in significant hypoglycaemia(83).

The sensitivity to sulphonylurea therapy encountered, makes it this drug an excellent first line treatment (102,103) and blood glucose control is often better than with insulin treatment (82,103). Transfer to sulphonylurea treatment is successful in the majority of patients although insulin therapy may be required as diabetes progresses (104). GLP-1 agonists have also been shown to be effective at reducing glycaemia (105).

The differences between *GCK* and transcription factor MODY mutations are summarised in table 1.2.

Table 1.2: Key Differences Between GCK and HNF1A/HNF4A mutations

	Glucokinase MODY	HNF1A/ HNF4A MODY
Onset of hyperglycaemia	Birth	Adolescence/early adulthood
Presentation	Usually asymptomatic, detected by screening or on routine testing	Usually symptomatic
Progress	Minimal increase in glycaemia with age Mild (fasting glucose usually 5.4–8.3 mmol/L) HbA1c 40-60 mmol/mol	Progressive deterioration of glycaemia with age; HbA1c variable depending on age and treatment, may be high
Microvascular complications	Rare	Frequent
Treatment	Pharmacologic treatment rarely needed	Sensitive to sulphonylurea treatment May progress to require insulin

Adapted and reproduced with permission, from (78)

1.3.5.3 Hepatocyte Nuclear Factor 1 Beta

Mutations in the hepatocyte nuclear factor 1 beta (*HNF1B*) transcription factor account for between 5-10% of all MODY cases (78,106). *HNF1B* is a member of the homeodomain superfamily of transcription factors that regulates gene expression in a number of tissues including the pancreas, kidneys, liver genital tract and gut (107). *HNF1B* mutations arise spontaneously in 50% of cases. Thus evidence of autosomal inheritance may not always be present.

There is considerable variation in the clinical presentation of *HNF1B* mutations, even in individuals with the same mutation and within the same kindred. This heterogeneity is not fully explained and may suggest additional genetic or environmental factors that influence phenotype. It has also been suggested that random variation in the temporal expression of HNF1B during early development may also contribute to the variability in clinical presentation (106,108–112).

The recognition of *HNF1B* mutations giving rise to MODY was first described in a Japanese family with renal disease and subsequently confirmed in other kindreds (113). HNF1B is expressed in a number of different tissues including the pancreas and kidney, the gut, genital tract and liver (114). Heterozygous mutations may result in developmental abnormalities in any of these tissues, for example, genital tract malformations, hypomagnesaemia, abnormal liver enzymes, hyperuricaemia and exocrine pancreatic failure (106). However, the most common manifestations are diabetes and renal disorders, in which a variety of manifestations have been described (108). Up to 50% of mutations arise spontaneously, so there may be no history of autosomal dominant inheritance. Unlike *HNF1A* and *HNF4A* mutations, most people with *HNF1B* mutations require insulin to manage glycaemia.

The extra-pancreatic manifestations may not always segregate with diabetes, making the identification of *HNF1B* mutations based on clinical features alone problematic, particularly in people with spontaneous mutations. Early insulin therapy is usually required for management of diabetes. The sulphonylurea sensitivity seen in other transcription factor diabetes is not seen in *HNF1B*, but the need for renal surveillance

and the variety of extra-pancreatic manifestations, make the diagnosis worthwhile in enabling effective management(78).

1.3.5.4 Mitochondrial Diabetes

Mitochondrial mutations can also result in diabetes such as maternally inherited diabetes and deafness (MIDD), which is characterised by maternal inheritance (115,116). MIDD classically presents with sensorineural deafness but a wide range of other features are reported too. It affects up to 1% of all diabetes cases, but is frequently misdiagnosed (116).

Nearly all mitochondrial diabetes results from the m.3243A>G point mutation in mitochondrial DNA (117). The mutation affects the mitochondrial respiratory chain and results in cellular energy deficiency. Organs with high metabolic activity such as the pancreas and cochlea are most affected and therefore diabetes and hearing loss are typical common features (118–122). Diabetes is progressive but may present acutely and is usually insulin requiring with ketoacidosis occurring in approximately 8% of cases (118,119,123). Age at diagnosis of diabetes is highly variable (119,123,124).

Mitochondrial diabetes is challenging to differentiate from type 1 or type 2 diabetes and additionally, heteroplasmy may result in significant heterogeneity of presentation within affected families (85). Pancreatic autoantibodies are usually, but not always negative (125–127). The presence of deafness in the patient or clustering of diabetes and deafness in maternal relatives should raise clinical suspicion. The diagnosis can be confirmed by testing for the m.3243A>G mutation, in blood or urine (78). Maternal relatives of affected patients and children of female patients should be assumed to carry the m.3243A>G mutation and screening for complications of MIDD may be advisable (85).

The major phenotypic differences between the types of diabetes are summarised in table 1.3.

Table 1.3: Summary of phenotypic differences in major types of diabetes presenting at young-age (modified from (78))

Features	Type 1 diabetes	Young onset type 2	Ketosis-prone type 2 diabetes	HNF1A MODY	MIDD	GCK MODY
Typical clinical presentation	Acute presentation with or without ketoacidosis, weight loss, short duration	Slower onset, incidental pick-up or mild symptoms of hyperglycaemia	Present with ketoacidosis, but remain off insulin in the long-term	Acute of sub-acute presentation with symptomatic hyperglycaemia	Later onset 20-40 years, mild symptoms	Usually incidental finding, without symptoms.
Insulin dependent	Yes from diagnosis, but people with slow-onset may not be initially.	Not initially	At presentation, but then can stop	Not initially, but may require with time	Likely to be insulin-requiring, but not always	No
Parent affected	2–4%	Common	Common	Yes	Mother	Yes
Typical age of onset	6 months – young adult	Adolescent and young adult	Variable	Teens – young adult	Young adult	Birth (may be diagnosed at any age)
Obesity	Population frequency	Yes, but not in all ethnic groups	Population frequency	Population frequency	Population frequency	Population frequency
Acanthosis nigricans	Not usual, but insulin resistance may co-exist	Yes, signs of insulin resistance may be present	Unclear	Not usual, but insulin resistance may co-exist	Not usual, but insulin resistance may co-exist	Not usual, but insulin resistance may co-exist
Glycaemia	High	Variable	High initially, euglycaemia after	High	Variable	Mild
Pancreatic autoantibodies	Usually present, but not in all	No	No	No	No	No
C-peptide levels	Should be low or undetectable with long-duration, but not necessarily at diagnosis and insulin resistance may increase levels	Usually high	Low at presentation with ketoacidosis, but then recovers	In the absence of insulin resistance and off sulphonylurea treatment, should be low but not undetectable	Seem to be much lower than with MODY at risk of ketoacidosis	In the absence of insulin resistance and off sulphonylurea treatment, should be low but not undetectable

1.3.6 Prevalence of MODY

Only a few studies have systematically screened large populations to estimate the prevalence of MODY in people with diabetes. These include; the UNITED study in the UK, which demonstrated 3% of all diabetes diagnosed <30 years (128) and 2.5% of diabetes in paediatric populations, were accounted for by MODY (129); the SEARCH study in the United States, which identified a minimum prevalence of MODY in 1.2% of children and adolescents with diabetes (130); a Norwegian nationwide study also identified a minimum prevalence of monogenic diabetes in children with diabetes of 1.1% (131); and finally a study from Poland that identified a minimum prevalence of 3.1–4.2% (129,132) in children with diabetes.

MODY has a minimum estimated population prevalence of 108 cases per million, but this is likely to be an underestimate (77). Prevalence estimates from large systematic surveys, as above,, are between 1-3% of young-onset diabetes. A population study based on screening pregnant women estimated the glucokinase prevalence to be 1.1 in 1000 population prevalence (or 1100 cases / million population) (133), suggesting most glucokinase patients are not coming to medical attention or are not being diagnosed. Based on current estimates of MODY prevalence 80-90% of cases are likely to be misdiagnosed or unrecognised, highlighting the need for improved case-finding.

Data on MODY prevalence in non-white populations are limited. Mutations in the *GCK*, *HNF1A* and *HNF4A* genes have been detected in patients from a variety of ethnic groups (134–138) and a large paediatric population based study in the US identified cases of MODY from several ethnic groups (130). There is, however, a paucity of epidemiological data relating to the population prevalence of MODY in non-white ethnic groups, though some studies have suggested that this may be lower (139). The prevalence and characteristics of MODY in populations other than northern European, where it has been extensively studied, is important to establish as it underpins improvements in the diagnosis of young-onset diabetes in multi-ethnic regions.

In the absence of large population prevalence studies, research has focussed on the detection rate of finding MODY mutations in people selected for testing. A study by Thanabalsingam et al observed a detection rate of 11% in those with type 2 diabetes diagnosed before 45 years or 10% of those with type 1 diabetes, from individuals selected on the basis of antibody negativity and preserved C-peptide, but this was predominantly in a white British population (140). In the SEARCH study, 8% (47/586) of children screened for MODY were found to have a mutation. This group comprised 20% black African-American and 31% Hispanic (130).

In the UK, MODY was first reported in the south Asian ethnic group in a systematic survey of childhood diabetes(141). A 2006 study revealed a lower than expected prevalence of referrals for MODY testing in the south Asian ethnic group(142). However, it was unclear if this reflected a genuinely lower MODY prevalence in this group or under-diagnosis due to referral bias and numbers were too small to explore the clinical characteristics of patients. Meanwhile *HNF1A*, *HNF4A* and *GCK* mutations have been detected in various reported studies from India, however the assessment of what constitutes a pathogenic mutation versus a benign nucleotide change, remains challenging(136,137,139,143).

1.3.7 Strategies to Improve Case-Finding

In recent years, efforts to improve the diagnosis of MODY have been studied and involve the use of biomarkers or algorithms to predict MODY (78). The latter approach recognises that the major barrier to identifying MODY is actually to consider whether there are clinical features that are unusual for type 1 and type 2 diabetes and to undertake genetic testing in these individuals to confirm mutations. This issue of bias in referrals was demonstrated clearly in a publication examining referrals and confirmed mutations by county in England and demonstrating a significant disparity by geographical region that can only be explained by differences in referral rate (77).

MODY probability calculator

The MODY probability calculator (144) offers an objective way of establishing if a diagnosis of MODY is likely: it is a mathematical prediction model that uses clinical information to determine if MODY testing is worthwhile. In the published evaluation, it

significantly improved the specificity and sensitivity of identifying MODY compared to standard criteria for diagnosis (144,145). This could certainly be a useful additive tool, considering that one of the major barriers to genetic testing, even when it is clinically considered, has been cost. However, it has been developed and validated in white European individuals only and therefore is currently invalid for use in people of other ethnicities.

The model uses information on the following parameters to calculate a positive predictive value for undertaking genetic testing; age at diagnosis, current age, current treatment, HbA1c, time to insulin treatment, body mass index and parental history of diabetes. As it is primarily clinical data that the model is based on, it is likely that other ethnic groups will require different algorithms that weight data in an ethnicity-specific way. The model also works to predict likelihood of MODY, on the basis of established prevalence and presentation of type 1 and type 2 diabetes and it is likely that models in other ethnic groups will require different prevalence's. Whilst the tool may be useful for practitioners who may lack clinical experience in the features of MODY, it is arguable whether a low probability in an individual where clinical suspicion is high, should preclude testing.

Another limitation of the model, is that it was validated within the pool of cases from which it was developed (using jackknife statistical validation) and has yet to be evaluated in unselected populations. Therefore, the high sensitivity and specificity observed in the initial evaluation may prove to be different in an unselected cohort. Studies are therefore needed to provide further validation of this tool.

Biomarkers such as C-peptide, pancreatic autoantibodies, lipid profiles and high-sensitivity C-reactive protein (CRP), have been used to assist with identification of MODY and these are discussed in the section 1.4.

The diagnosis of MODY is confirmed on genetic testing and this is discussed in section 1.5.

1.4 Diabetes Classification - Biomarkers and their Limitations

1.4.1 C-peptide

Pro-insulin is cleaved to form insulin and C-peptide, which are produced in equimolar amounts. C-peptide is an established marker of endogenous beta-cell function and is preferable to measure over insulin as it has a longer half-life, avoids hepatic first pass metabolism and can differentiate whether measured insulin is endogenously produced or exogenously administered (27). Levels are considered to be low in established type 1 diabetes (146,147), but in practice many patients continue to produce moderate amounts of C-peptide for decades (148,149). This means that, unless undetectable, C-peptide levels are not independently useful in discriminating type 1 diabetes from other types, particularly immediately post-diagnosis. Though attempts have been made to establish cut-offs for C-peptide levels that differentiate one type of diabetes from another, these cut-offs are not robustly evaluated in a variety of populations, use different assays and therefore, lack a firm evidence base (27,150,151).

Unlike other hormonal axes, C-peptide concentrations are not only influenced by beta-cell function, but levels are affected by insulin sensitivity. Levels in isolation, are therefore difficult to interpret. Whilst it is recognised that routine C-peptide measurement has not been at the forefront of clinical diabetes practice, it is perhaps some of the difficulties around interpretation and previously, measurement, that have led to its lack of routine measurement in clinical practice.

However, that situation is now changing and it is recognised that knowledge of C-peptide levels can provide additional support in subtype differentiation. For example, persistent C-peptide production in a person labelled with T1DM may indicate an alternative diagnosis such as MODY(152). Routine serum measurement has been hindered by the need for a timed sample (fasting or stimulated) and rapid separation, although in one study random serum C-peptide was equally effective in differentiating subtype as stimulated and fasting measures (151,153). Recently C-peptide has been measured in spot urine samples and expressed as a ratio to creatinine. Urine C-peptide to creatinine ratio (UCPCR) has been shown to be a practical alternative to serum C-peptide in both the fasting and post-meal states (153–155). The non-invasive nature and comparative stability (72 hours with boric acid preservation) have

made it an attractive alternative to an invasive serum measurement, particularly as these assays are not routinely available. UCPCR measurement is offered by one laboratory in the UK, validated using a single assay (Roche Elecsys) (153), in a predominantly white European population, but its performance on other analytical platforms and in a multi-ethnic population has not been assessed.

Whilst UCPCR is clearly an attractive modality for measuring C-peptide, it requires further investigation before being used widely across all ethnic groups. It has long been established that people of south Asian, African-Caribbean and white Europeans with and without diabetes, differ markedly in their insulin secretion and sensitivity characteristics (156,157). It cannot, therefore, be assumed that beta-cell function assessed by UCPCR that has been predominantly evaluated in white European individuals with diabetes will apply equally to people of African origin or south Asian individuals.

There is insufficient knowledge about how C-peptide levels vary with diabetes type across ethnic groups and whether, in those with undetectable C-peptide, phenotype of type 1 diabetes is similar by ethnicity. The relationship between serum C-peptide and UCPCR in people of different ethnicities had also not been established; use in people from non-white ethnic groups, is not recommended.

1.4.2 Pancreatic Autoantibodies

Islet cell antibodies (ICA) were first detected in the 1970's using immunofluorescence (IF) techniques(158). These assays provided the basis for the IF assays in use today, which incubate patient sera with primate pancreas to provide a qualitative or semi-quantitative result for 'islet cell positivity'(159). Since this initial discovery, the individual islet antigens contributing to heterogeneous islet cell autoantibody immunofluorescence staining have been identified and most can now be specifically tested for(159). Measuring individual antibodies offers several advantages over IF techniques. The latter is technically demanding, non-standardised, operator dependent and potentially non-specific as it detects inter-species antigen-antibody binding. The international antibody standardisation programme has also provided data

showing the IF technique lacks sensitivity in the detection of antibody positivity (160,161).

Islet antigens include the GAD65 isoform of glutamate decarboxylase (162), tyrosine phosphatase-related protein islet antigen 2 (IA-2), insulin, zinc transporter 8 (ZnT8) and, more recently, tetraspanin-7. GAD antibodies were isolated in 1990 and shown to contribute to ICA fluorescence (162). The IA-2 protein, also termed ICA-512, A-2 is a member of protein-tyrosine-phosphatase protein family and antibodies were isolated from serum of people with type 1 diabetes (163). It is not clear if the antigen is the target of the immune process in type 1 diabetes or if antibodies develop to islet antigens as a consequence of exposure following beta-cell destruction, which provokes an immune response. ZnT8 is a cation efflux transporter in the beta- cell and has been shown to be a major autoantigen in type 1 diabetes (164). The most recent auto-antigen to be discovered is tetraspanin 7, which is a transmembrane protein in beta-cells (165).

Most people with type 1 diabetes will have multiple islet cell autoantibodies detectable in their blood at diagnosis (159,166). Adults with type 1 diabetes are most likely to be GAD 65 antibody positive, however around 20% of individuals who test negative to GAD antibodies, are IA-2 positive (159,167).

Limitations of antibody testing:

Whilst positive diabetes antibodies are a useful test for detecting islet autoimmunity and guiding insulin treatment, the test has many limitations that mean a negative test does not exclude a diagnosis of type 1 diabetes, these include:

- Incomplete testing of autoantibodies in clinical practice, such that testing of usually one, or at most two, antibodies is undertaken.
- Diabetes antibody positivity is lower for individuals with type 1 diabetes when diagnosed in adulthood rather than childhood
- Diabetes antibody positivity is also lower in individuals with type 1 diabetes if tested after diagnosis
- A proportion of individuals in the population have background antibody positivity, for example GAD positivity has been reported between 0.4-4.7% of people who persistently did not have diabetes over time assessed, but had an

affected relative (168–170).

These limitations mean that a positive diabetes antibody result has a high positive predictive value for type 1 diabetes and can be helpful in confirming a diagnosis of type 1 diabetes, however negative results in someone with suspected type 1 does not exclude the condition, particularly as it is recognised that a proportion of individuals with type 1 are antibody negative (5,22,171).

Negative antibody testing may not exclude a diagnosis of type 1 diabetes, but it can be useful in increasing clinical suspicion of non-type 1 diabetes, for example MODY (172,173). Although a proportion of people in the population of have background antibody positivity, so a positive result cannot conclusively rule out MODY.

1.4.3 Other Biomarkers

Lipid profiles have been shown to vary with diabetes type. People with type 1 diabetes have lipid profiles similar to unaffected individuals (174). Patients with *HNF1A* mutations have elevated levels of high density lipoprotein cholesterol (HDL) which contrasts with the reduced HDL levels seen in type 2 diabetes (175).

From genome-wide association studies, it was noted that HNF1A also binds to the C-Reactive Protein (CRP) promoter to initiate CRP transcription and it was therefore discovered that people with HNF1A heterozygous mutations, characteristically had undetectable CRP levels, especially when compared to people with type 2 diabetes, which is a pro-inflammatory state (176). CRP was best measured in the low range of the assay, to ensure this difference was detected, giving rise to the high sensitivity assay (hs-CRP). This approach has shown in studies to be an effective way to pick up people with *HNF1A* mutations (176,177). However, its widespread use has been limited by the lack of availability of the hs-CRP assay (usually used to stratify people for statin treatment and not available at all biochemistry laboratories) and the fact that it only discriminated *HNF1A* MODY cases, such that other MODY types could still be present.

Plasma glycan proteins are also influenced by the HNF1A transcription factor and in a similar narrative to hs-CRP, have been shown to differentiate *HNF1A* mutations from those without (178). Fucosylation, a specific type of glycosylation, comprises the addition of fucose residues to glycans and appears to be highly regulated by *HNF1A*. However, measurement requires a manual high-performance liquid chromatography assay and this is currently not available routinely or even in specialist centres. Whilst this discovery has provided novel insights into *HNF1A* biology and may have value in functionally characterising novel variants in *HNF1A*, its clinical utility is currently limited and any future studies must address the impact of ethnicity on reference ranges or the definition of normality.

1.5 Approaches to Genetic Testing for MODY

The facility to sequence known MODY-causing genes using targeted next generation sequencing technologies has enabled the rapid recognition of mutations in people presenting with MODY phenotypes (179). However, the recognition of MODY still primarily relies on clinical suspicion and the initiation of genetic testing from specialist services.

Molecular genetic testing has traditionally been guided by the clinical phenotype and also the relative prevalence of mutations within any given population (78). In people with a very specific clinical phenotype, it might be pragmatic to request Sanger sequencing (a method that sequences a single gene in a single reaction) (180) of the selected gene alone; for example *GCK* testing in a patient with isolated fasting hyperglycaemia, or *HNF1B* testing in a patient with renal cysts and diabetes (78) (see table 1.4 for clinical information that may guide testing).

In other types of monogenic diabetes, however, it may be difficult to predict the affected gene on the basis of clinical features alone. Previously, this situation would result in sequential testing of multiple genes, using Sanger sequencing. This could often result in long delays before a diagnosis was obtained. However, advances in DNA sequencing technologies have meant that now panels of genes can be tested simultaneously using next generation sequencing platforms without the considerable costs and time associated with earlier sequencing approaches (179,181). This approach mitigates somewhat against the circular paradigm of defining specific

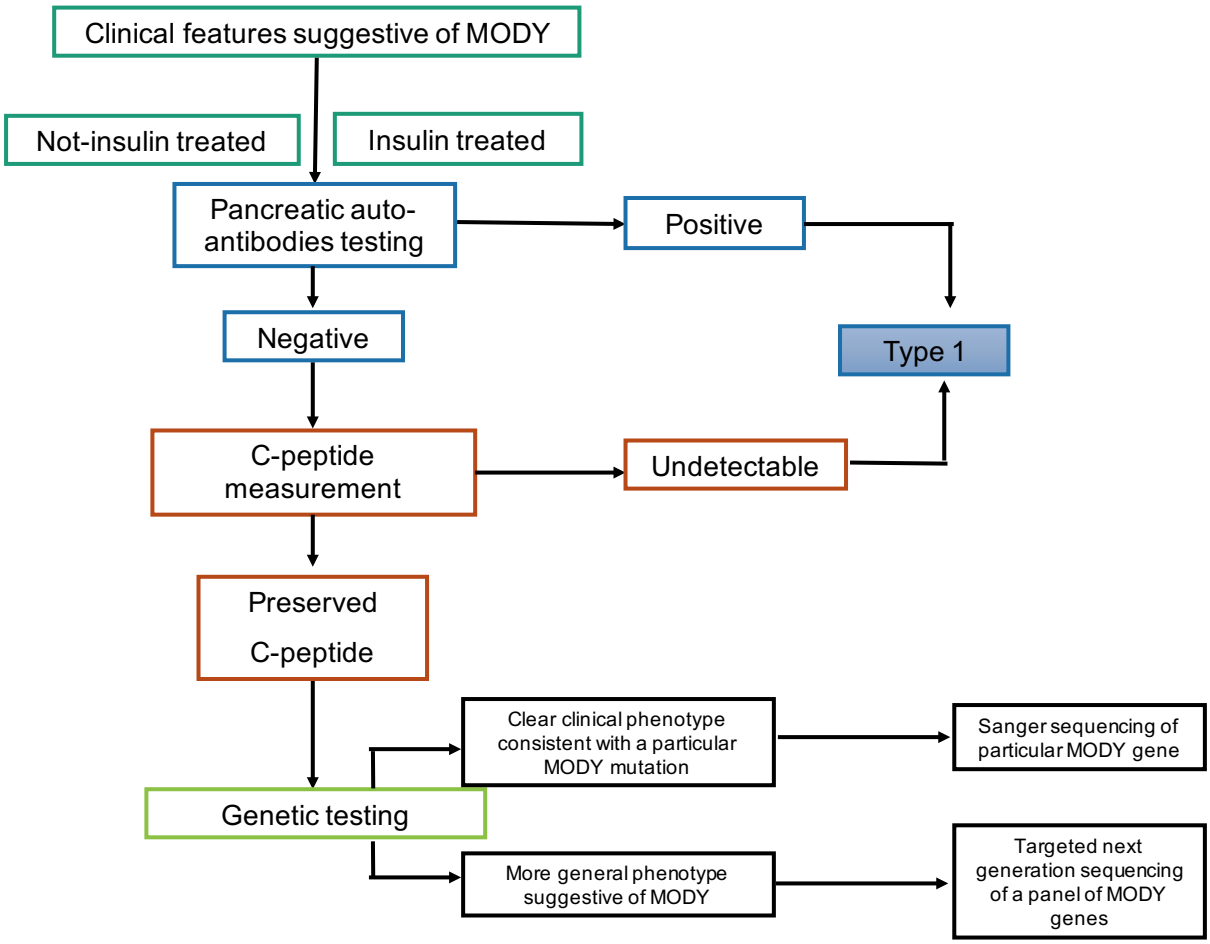
phenotype in individuals with associated genotypes, but only testing those individuals with the phenotype in the first place. Thus, it may be the case that individuals with the same gene affected, could present differently, but because only those with the 'defined' phenotype are being tested, those other individuals are not detected.

Table 1.4: Current information provided to guide genetic testing requests

	Clinical history	Biochemical / immunological test results
In the proband	Age at diagnosis Current age Body mass index Initial treatment and duration Current treatment Complication Birth weight Neonatal hypoglycaemia, Deafness Lipodystrophy Renal cysts or structural abnormality	C-peptide Oral glucose tolerance test results Pancreatic autoantibody HbA1c
Family members	Presence of diabetes in parents, grandparents, siblings or children. If present BMI, age at diagnosis and current treatment. Family history of deafness or renal disease	As above, if known

The integration of biomarkers with clinical information may be the best approach to not only identify MODY, but also define and classify other forms of diabetes. This is summarised in figure 1.4. However, two key challenges remain in the aftermath of undertaking genetic testing. The first is dealing with interpretation of variants in which pathogenicity cannot be confirmed and the second is how people who test negative for known MODY mutations should be classified.

Figure 1.4: Schematic Illustrating an Approach to Undertaking Genetic Testing for MODY



Adapted from (78).

1.6 Variant Interpretation

Single nucleotide changes are commonly encountered across coding and non-coding regions of the genome. Following the implementation of next generation sequencing, where multiple genes can be sequenced in one go, these variants are being frequently encountered. This issue is discussed in detail in chapter 6.

Determining pathogenicity of a variant is extremely important in the clinical context, as incorrect interpretation can have erroneous consequences to patient management (182). This is a particular issue in genes associated with monogenic diseases such as MODY, where distinguishing between variants that are clearly disease-causing versus those that impair protein function or are neutral, is problematic (183).

A variety of guidelines, have attempted to standardise the approach to investigating variant pathogenicity and to also standardise notation (182,184,185). However, these techniques are not without issue and further work is needed to shed light on variant functionality. These may be clinical studies in the individual or family members or by use of reliable *in vitro* functional and biochemical assays that assess the effect of the variant in a cellular system. Even then, confident assignment of pathogenicity may not always be possible due to the limitations of these techniques.

1.7 Absence of a Mutation on MODY Testing

People who are screened for MODY mutations using panels of genes and who are found to be negative, are likely to either have type 2 diabetes, presenting at young age, or a mutation in an as yet unidentified MODY causing gene (MODY X). This poses a difficulty in assigning classification, particularly if the associated biomarkers and features in the individual do not fit with type 2 diabetes. There are now studies using whole genome and whole exome sequencing technologies to identify new MODY genes and these have had some success (186,187). However, success rests on the correct approach, which involves sequencing trios (affected proband, unaffected parents) or multiple affected individuals with an extreme phenotype.

This issue may be more apparent in people from non-white ethnic groups, where the phenotype of young-onset type 2 diabetes overlaps considerably with MODY. In these individuals it is possible that novel MODY genes will be found. In the meantime, additional genetic approaches are available, for example, characterisation of type 2 diabetes risk polymorphisms and HLA single nucleotide polymorphisms, in so-called genetic risk scores (188,189). The type 1 risk score has been shown to differentiate type 1 diabetes from MODY. However, the type 2 score was less specific. The advantage of this approach is that it can be used around the time of diagnosis, when C-peptide levels are challenging to interpret. It remains to be seen if this score can be used effectively in all ethnic groups and whether those with a low type 1 risk score can still develop type 1 diabetes.

1.8 Summary

Diabetes is a heterogeneous condition with multiple phenotypes, treatments and presentations. There have been considerable advances in our understanding of various diabetes aetiologies and how to differentiate them, which is critical to ensure on-going appropriate and effective treatment. However, the impact of ethnicity on these developments has been largely understudied. There is a need and opportunity to explore these knowledge gaps in diabetes phenotypes in a multi-ethnic population. As the most significant overlap in diabetes types occurs in those diagnosed at young age, efforts should be directed to this cohort. In trying to understand the impact of ethnicity on young-onset diabetes phenotypes, data from three separate studies are presented in this thesis;

Study 1) Retrospective analysis of the clinical features of referrals in the Exeter MODY database, by ethnicity.

Study 2) Cross-sectional analysis of young-onset diabetes phenotypes, across UK south Asian and African-Caribbean people with diabetes.

Study 3) *In vivo* and *in vitro* functional assessment of a novel *HNF1A* variant to determine pathogenicity in a Turkish kindred with young-onset diabetes.

1.9 Hypotheses & Aims

To explore the features of young-onset diabetes across UK south Asian and African-Caribbean ethnic groups with young-onset diabetes, through a variety of systematic clinical and functional studies.

1.9.1 Study 1

Aim:

In chapter 2, the results of a retrospective analysis of the UK MODY database, by ethnicity, are presented. The aims of this study were:

- to determine if there had been an increase in referrals of south Asian people since the previous study in 2006

- to determine and compare the detection rate of a positive mutation by ethnicity
- and to investigate the phenotype of people of south Asian origin compared to white European, in those with and without MODY mutations.

Hypothesis:

1. The phenotype of people without mutations will be different between south Asian and white people referred for MODY testing.
2. The detection rate of finding a mutation will be lower in the SA group compared to white European.
3. The phenotype of people with MODY mutations will be similar between south Asian and white referrals.

1.9.2 Study 2

Aim:

In chapters 3-5, the methods and results of the MY DIABETES study are presented. The aim of the MY DIABETES study was to systematically characterise young-onset diabetes phenotypes in three matched ethnic groups, using fasting serum C-peptide and pancreatic autoantibody assessment to determine people eligible for MODY testing and to determine;

- the prevalence of MODY mutations in people of white, south Asian and African-Caribbean ethnicity, systematically studied
- the detection rate of finding a mutation in people tested from each ethnic group
- the phenotype of type 1 diabetes by ethnic group
- the phenotype of type 2 diabetes by ethnic group
- the effectiveness of the MODY probability calculator in each ethnic group once fully recruited, at identifying MODY.

Hypotheses

1. The prevalence of MODY will be lower in south Asian and African-Caribbean participants compared to white individuals
2. The detection rate of finding a mutation will be lower in the south Asian and African-Caribbean group compared to white individuals.

3. The phenotype of type 1 diabetes will be different in south Asian and African-Caribbean individuals compared to white individuals, specifically;
    a. Lower rates of antibody positivity will be observed
4. The phenotype of type 2 diabetes will be different between south Asian and African-Caribbean individuals versus white European.

1.9.3 Study 3

In chapter 6, the detailed results of *in vivo* and *in vitro* studies in an individual with a novel homozygous *HNF1A* variant (A251T) found during the course of the MY DIABETES study, are presented. This was of interest as homozygous mutations have not been described in *HNF1A* are thought to be embryonic lethal.
The aims of these studies were to determine:

- whether the A251T variant was indeed was pathogenic or benign
- whether the variant was hypomorphic, accounting for the fact that a homozygous change was possible
- whether the individual with the variant could transition off insulin onto sulphonylurea therapy, as would be expected for a pathogenic mutation.

Hypotheses:

1. The A251T variant is a hypomorphic variant that in a homozygous state produces a phenotype similar to heterozygous *HNF1A* mutations
2. *In vitro* studies in cellular systems will demonstrate a mild effect of this variant in comparison to wild-type *HNF1A*
3. The affected individual will be able to stop insulin treatment and transition to sulphonylurea therapy

Chapter 2 : A retrospective analysis of the MODY database by ethnicity

The contents of this chapter have been published in Diabetologia (190) *and an expanded version of this paper is presented here.*

Misra S, Shields B, Colclough K, Johnston DG, Oliver NS, Ellard S, et al. South Asian individuals with diabetes who are referred for MODY testing in the UK have a lower mutation pick-up rate than white European people. Diabetologia. 2016;59(10):2262–5 (see appendix 1)

2.1 Introduction and Aim

Mutations in the glucokinase (*GCK*), hepatic nuclear factor 1-alpha (*HNF1A*) and hepatic nuclear factor 4-alpha (*HNF4A*) genes have been detected in patients from a variety of ethnic groups (134–138) and a large paediatric population based study in the US identified cases of maturity onset diabetes of the young (MODY) from several ethnic groups (130). There is, however, a paucity of epidemiological data relating to the population prevalence of MODY in non-white ethnic groups, though some studies have suggested that this may be lower (139). The prevalence and characteristics of MODY in populations other than northern European, where it has been extensively studied, is important to establish as it underpins improvements in the diagnosis of young-onset diabetes in multi-ethnic regions.

In the United Kingdom (UK), MODY was first reported in the south Asian ethnic group in a systematic survey of childhood diabetes (141). A 2006 study revealed a lower than expected prevalence of referrals for MODY testing in the south Asian ethnic group(142). However, it was unclear if this reflected a genuinely lower MODY prevalence in this group or under-diagnosis due to referral bias and numbers were too small to explore the clinical characteristics of patients. Meanwhile *HNF1A*, *HNF4A* and *GCK* mutations have been detected in various reported studies from India (136,137,139).

The recognition that many cases of MODY are being missed (77) has led to the development of new strategies that utilise biomarkers and probability calculators to improve detection (144,172,173,177). The MODY probability calculator uses clinical features to predict the chances of finding a MODY mutation but its effectiveness in south Asian and other ethnic groups is unknown because it was developed and validated in a Caucasian population(144).

Aims:

1) To determine if referrals for genetic testing in people from minority ethnic groups had increased since the 2006 study,

2) To investigate whether the detection rate for a confirmed mutation is the same across ethnic groups and if the clinical features are different in people with and without MODY mutations across ethnic groups.
3) To examine the effectiveness of the MODY probability calculator at discriminating south Asian people with confirmed MODY mutations from those without mutations.

2.2 Methods

2.2.1 Study Design and Dataset

Clinical data and results of all referrals for MODY testing in the UK are held centrally in the molecular genetics laboratory at the Royal Devon and Exeter NHS Foundation Trust. The database is populated prospectively with demographics and relevant clinical information as recorded from a standardised genetic testing request proforma that accompanies all DNA samples (191). The proforma contains a free text box in which ethnicity can be reported by requestors. In the current study a retrospective analysis of this database was undertaken.

2.2.2 Study Population

Referrals originating from within the UK from 1996 to November 2015 were extracted. Probands with diabetes in whom initial Sanger sequencing for mutations in the most common and clinically important MODY genes (HNF1A, HNF4A, HNF1B or GCK) was requested, were selected. This group included people in whom testing of additional MODY genes was subsequently undertaken. From 2013 onward, in addition to the Sanger sequencing service, a targeted capture next generation sequencing (tNGS) panel of MODY genes was offered as a diagnostic test (179) and probands referred with diabetes for tNGS were also included in the analysis. Referrals diagnosed with diabetes in the first 12 months of life were not incorporated to exclude cases of neonatal diabetes.

Clinician reported ethnic origin of individual referrals were categorised into broader groups as being of white European (WE), south Asian (SA), African-Caribbean, Middle

Eastern, south east Asian or other/mixed, based on UK Census classification sub-categories (192). The WE group included people categorised as 'white', 'British' or from any European country. The SA group included people from India, Pakistan and Bangladesh or in whom ethnic origin was stated to be 'Asian' and excluded people with mixed ethnicity or from south East Asia. Cases in which no ethnicity data were recorded, were excluded from the main analysis.

Referrals were grouped according to outcome of genetic testing and ethnic origin. The detection rate of finding a MODY mutation after testing was compared by ethnic group and defined as the percentage of MODY mutations found from the total number of referrals for testing in that ethnic group.

To determine if there were differences in the phenotype of referrals by ethnicity, clinical parameters were compared in people found to have no mutation and those in whom MODY mutations were found (*GCK* and *HNF1A*/*4A* were analysed separately) in WE versus SA (the largest non-white ethnic group). Parameters assessed included the traditional clinical criteria for MODY diagnosis (young age at onset, insulin independence and familial history) and additional variables as assessed with the following criteria; age at diagnosis, current age, duration of diabetes, body mass index (BMI) at referral, percentage insulin-treated at diagnosis, percentage currently insulin-treated, percentage with a parental history of diabetes (one or both parents affected), fasting glucose and HbA1c. BMI was analysed in referrals currently aged above 16 years of age.

To assess the effectiveness of the MODY probability calculator in the SA population the prediction model was applied to SA people in the database with a confirmed mutation and those in whom no MODY mutation was found and probabilities obtained were compared between each group and to the WE data.

The probability is calculated differentially based on whether insulin treatment was started in the first 6 months and results were additionally sub-analysed according to this categorisation. The total number of predicted cases were compared to the actual number of MODY cases diagnosed in each ethnic group.

2.2.3 Statistical Analyses

Analysis was undertaken using STATA (version 13, StataCorp College Station, Tx). Data was nonparametric and median and interquartile ranges are given. Continuous variables were statistically compared using the Mann-Whitney U test and categorical variables using Chi-Square analysis.

2.3 Results

A total of 4688 proband referrals were identified from requests originating within the UK. Ethnicity was not recorded in 678 of the index cases resulting in a total of 4010 proband referrals with confirmed ethnicity.

2.3.1 Ethnicity of Patients Referred

White European referrals represented the largest group, accounting for 3472 of 4010 (86.6%). 293 cases referred were in people of SA ethnicity (7.3%) and the third largest group with 81 referrals (2.0%) were those from African or Caribbean backgrounds (table 1). See table 2.1 for full ethnicity breakdown.

Table 2.1: Breakdown of proband referrals for MODY genetic testing and confirmed MODY mutations by ethnic group.

Ethnic Group	*n*	%	MODY mutation (*n*)	Pick-up rate (%)
All groups	4688		1180	25.2
Individuals with known ethnicity	4010		1083	27.0
White	3472	86.6	1011	29.1
South Asian	293	7.3	37	12.6***
African-Caribbean	81	2	8	9.9
Middle Eastern	47	1.2	5	10.6
South East Asian	21	0.5	6	28.6
Mixed /Other	96	2.4	16	16.7
All Non-white ethnic groups	538	13.4	72	13.4

****p value<0.001 when compared to the white European group.*

2.3.2 Mutation Pick-Up Rate

In total, 1180 probands were found to have a monogenic cause of their diabetes, resulting in an all-ethnic group mutation detection rate of 25.2% (1180/4688), (table

2.1). The detection rate of finding a mutation in referrals differed significantly between ethnic groups with 29.1% (1011/3472) in the WE group, compared to a 12.6% (37/293) detection rate of finding a MODY mutation in the SA group (p<0.001). The detection rate in all non-white ethnic groups together was also lower (72/538 13.4%) than the WE group (p<0.001). The detection rate in the 678 people in whom no ethnicity had been recorded was similar to the non-white detection rate at 14.0% (95/678).

The detection rate of finding a positive MODY mutation in pre-pubertal children (defined as <12 years) referred for testing did not differ between ethnic groups and was higher than in all referrals; 32.6% in WE vs 26.7 % in SA children, p=0.356. (32.6% WE versus 26.7% SA, p=0.356).

14% of SA referrals had a targeted next generation sequencing gene panel test versus 7.6% of WE referrals (p<0.0001). However, the pickup rate of a genetic diagnosis by tNGS in SA people was lower than WE (12.2% vs 26%, p=0.05).

2.3.3 Proportion of MODY types by ethnic group

In the WE ethnic group the MODY mutations were most commonly detected in the HNF1A gene accounting for 43% of all mutations (429 / 1011), followed by GCK mutations in 35.5% (358 / 1011). In the SA group GCK mutations were the most common 45.9% (17/37), followed by HNF1A 32.4% (12/37), however the overall distribution of different mutations between the two groups was non-significant (p=0.482) (table 2.2).

Table 2.2: Proportions of Different MODY Mutations by Ethnic Group

Mutation type	White	South Asian	p value
GCK	358 (35.5%)	17 (45.9%)	
HNF1A	429 (43.0%)	12 (32.4%)	
HNF4A	120 (11.9%)	3 (8.1%)	
Other gene	104 (10.3%)	5 (13.5%)	0.482
Total	1011	37	

2.3.4 Phenotypes of Those with confirmed MODY mutations (table 2.3)

2.3.4.1 GCK MODY phenotype by ethnic group

SA GCK MODY patients were diagnosed earlier (6.5 vs 20 years, p<0.0001) than WE patients (table 3) but there was no difference in age at diagnosis in pre-pubertal children (5 SA vs 8 years WE, p=0.079). Referrals from both ethnic groups had similar proportions of parental history of diabetes (83.5%WE vs 93.8% SA, p=0.274) and were equally likely to not require insulin treatment (89.7% WE and 92.3% SA, p=0.762).

2.3.4.2 HNF1A /4A MODY phenotype by ethnic group

The HNF1A/4A MODY phenotypes in SA and WE patients were similar across all parameters except for HbA1c, which was higher in the SA group (8.6% (70 mmol/mol) versus 7.2% (55 mmol/mol), p=0.004), a phenomenon observed throughout all sub-analyses (table 2.3).

2.3.4.3 Phenotypes of those with no MODY mutations

SA people in whom no MODY mutation was found were younger at diagnosis (19 years vs 25 years, p<0.0001, were younger at referral and had a shorter duration of diabetes. BMI was also similar across both ethnic groups (WE 25.3 vs SA 25 kg/m^2 p=0.217). However, SA people were more likely to have a parent affected with diabetes (83.6% vs 68.2% p<0.0001 and more likely not to be treated with insulin at the time of referral (60.2% vs 50.6%, p=0.006).

Table 2.3: Clinical Characteristics of Referrals with GCK or HNF1A/ HNF4A MODY Mutations and Those Without MODY Mutations, Anaylsed by Ethnic Group.

MUTATION STATUS	No Mutation			GCK			HNF1A / HNF4A		
Variable	WE	SA	*p* value	WE	SA	*p* value	WE	SA	*p* value
Total (*n*)	2387	256		358	17		545	15	
Age at diagnosis (years)	25 (16–35)	19 (14–30)	**<0.0001**	20 (14–28)	6.5 (4.5–15)	**<0.0001**	18.5 (15–25)	16 (14–20)	0.054
Age at diagnosis in children <12 years (years)	9 (6–11)	11 (7–11.5)	**0.012**	8 (5–10)	5 (3–7)	0.079	11 (9–12)	11 (8–12)	1.000
Current Age (years)	35 (25–47)	28 (19–36)	**<0.0001**	29 (19–39)	13 (6–17)	**0.0044**	37 (26–48)	25 (20–48)	0.108
Duration of diabetes (years)	7 (2–14)	4 (2–9)	**<0.0001**	6 (2–11)	2 (0–5)	**0.0045**	14 (6–27)	11 (6–28)	0.658
BMI (kg/m^2) in patients aged≥16 years	25.3 (22.4–29.6)	25.0 (23.0–28.2)	0.217	22.1 (20.1–25.0)	19.7 (18.0–20.3)	**0.02**	24.1 (22.0–27.0)	24.0 (21.1–24.6)	0.246
Fasting glucose (mmol/l)	7.4 (6.1–10.1)	7.9 (6.5–10.7)	0.223	6.7 (6.3–7.3)	6.9 (6.5–7.1)	0.918	7.8 (6.3–9.9)	7.3 (6.7–8.2)	0.638
HbA_{1c} (mmol/mol)	57 (45–76)	62 (50–83)	**0.0065**	46 (43–50)	49 (48–52)	**0.04**	55 (45–68)	70 (65–73)	**0.004**
Meets all three clinical criteria (%)[a]	14.8	31.3	**<0.001**	43.5	85.7	**0.002**	44.7	50	0.736
Diagnosed <25 years (%)	43.4	58.6	**<0.001**	61.5	94.1	**0.006**	72.1	93.3	0.069
Currently non-insulin treated (%)	50.6	60.2	**0.006**	89.7	92.3	0.762	65.7	69.2	0.794
History of parental diabetes (%)	68.2	83.6	**<0.001**	83.5	93.8	0.274	89.9	92.9	0.720

p values in bold indicate a significant value. Data are presented as medians and interquartile ranges or %. SA, south Asian; WE white European. [a]Calculated using cases where the data set for all parameters is complete

2.3.4.4 Predictors of MODY in SA referrals

SA referrals with GCK or HNF1/4A MODY were leaner, diagnosed younger and less likely to be insulin treated compared to SA people without MODY (table 2.4). However similar proportions of cases with a parental history of diabetes were observed.

Table 2.4: Clinical differences in south Asian referrals with and without MODY mutations

	South Asian Referrals		
Parameter	Without MODY	With MODY	p-value
n	256	37	
Age at diagnosis (years)	19 (14-30)	14 (6-16)	**<0.0001**
Current Age years)	28 (19-36)	18 (12.5 -30.5)	**0.003**
Duration of diabetes (years)	4 (2-9)	5 (2-11)	0.453
BMI (kg/m^2) in patients aged≥16 yrs	25 (23 -28.2)	21.4 (20-24)	**0.001**
Fasting glucose (mmol/L)	7.9 (6.5 -10.7)	6.95 (6.5 -7.2)	**0.029**
HbA1c (%)	7.8 (6.7 -9.7)	7.2 (6.6 -8.6)	0.235
% Initially non-insulin treated	63.4	84	**0.04**
% Currently non-insulin treated	60.2	80.8	**0.04**
% with history of parental diabetes	83.6	93.3	0.162

p values in bold indicate a significant value.

2.3.5 Effectiveness of MODY probability calculator

Complete data to enable calculation of the probability score was available on 140 SA and 1666 WE referrals. The probability score in SA people with a genetic diagnosis of MODY was higher than those without MODY (76% with MODY v 46 no MODY, p=0.0007) (table 2.5). The probability score in SA people without MODY was significantly higher than that observed in WE people without MODY (46% vs 8.2%, p<0.0001). When sub-analysed according to time to insulin-treatment, scores in SA people who were not insulin-treated within 6 months were significantly different (6% no MODY vs 49% MODY p=0.044) and in those without a mutation SA scores were significantly higher than WE (62% vs 33%, p<0.0001). On comparison of actual versus predicted number of MODY cases found (see figure 2.0), the MODY calculator

overestimated cases significantly in the SA group, accounted for by patients who were not insulin-treated in the first 6 months (59 predicted versus 15 actual cases).

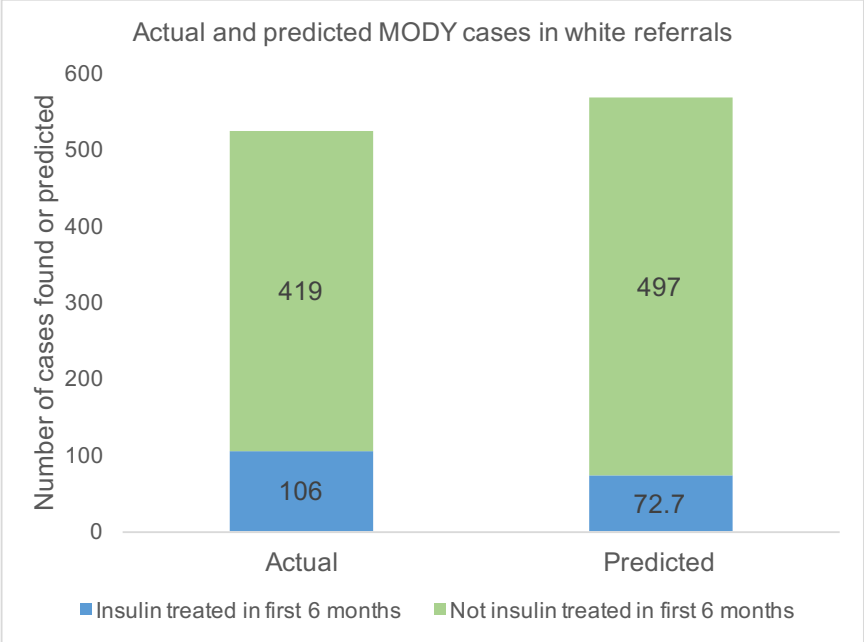
Table 2.5: Probability Scores Obtained from the MODY Probability Calculator

	South Asian		White European	
	No MODY	MODY	No MODY	MODY
n	125	15	1141	525
Median % Probability Score (IQR)	46 (6-76)	76 (62-76)	8 (5-49)	76 (21-76)
p value	**0.0007**		**<0.0001**	

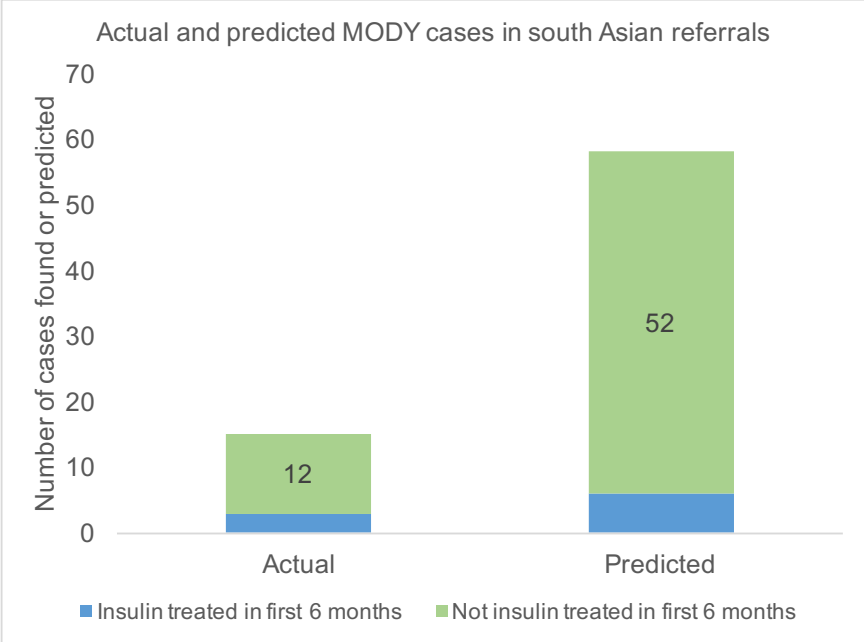
p values in bold indicate a significant value.

Figure 2.1: Actual and Predicted MODY Cases Based on the MODY Probability Calculator

a)



b)



Bars are split by whether insulin-treated within 6 months of diagnosis or no. Graph a) white European referrals and b) south Asian referrals

2.4 Discussion

In this analysis, SA people accounted for markedly more referrals than the previously reported 0.5%(142). Whilst this broadly reflects UK population demographics, the higher prevalence of type 2 diabetes in SA people may mean this is still lower than expected (193). The detection rate for finding a MODY mutation was lower in the SA group than for WE referrals. However, SA referrals without a mutation were more likely to meet the clinical criteria for MODY than white referrals. This may demonstrate a referral bias favouring SA cases that more stringently meet the clinical criteria. However, the results show that these criteria are insensitive in separating MODY from young-onset type 2 diabetes in this group.

SA people without mutations had higher BMIs than those with confirmed MODY mutations, suggesting that the low MODY detection rate in the SA group is due to a higher prevalence of young-onset, familial diabetes. This likely represents younger-onset type 2 diabetes.

The best predictors for finding a MODY mutation in the SA group were a lower BMI and age at onset and these factors may need to have a higher weighting when considering genetic referrals. BMI was lower than WE cases in the SA GCK group and HNF1A/4A groups, but higher and non-significantly different in the no mutation group at 25kg/m^2. This is in the overweight category for SA people and supports the possible higher prevalence of type 2 cases in this group.

BMI was lower than WE cases in the SA *GCK* group and *HNF1A/4A* groups, but higher and non-significantly different in the no mutation group. The lower BMI and age at diagnosis in SA referrals was primarily observed in the *GCK* mutation group and not seen in the HNF1A/4A or no mutation group. It is likely that this reflects a lower referral rate in adults, as in children alone the detection rate was similar in both ethnicities.
Evidence to support an underlying lower population prevalence of MODY in SA people is limited, with one exploratory study suggesting fewer *GCK* cases in Indian children (139), however, this may be an underpowered study as the estimated minimum prevalence from a recent (predominantly Caucasian) study is 1 in 1000 cases (133). It

is more likely that the lower detection rate reflects more younger-onset type 2 diabetes, which is supported in our analysis showing similar detection rates in children.

The MODY probability calculator assessed in the study was validated in a Caucasian population (144), and although it discriminated SA referrals with and without MODY statistically, the actual probability scores overlapped, and particularly overestimated probability in SA people who were not insulin-treated.
Our data highlight the need to consider the best way of identifying cases of MODY in people of non-white European ethnicity.

The phenotype of people with *HNF1A* and *HNF4A* mutations were broadly similar between ethnic groups. The lower BMI and age at diagnosis in SA referrals was primarily observed in the *GCK* mutation group and not seen in the *HNF1A/4A* or no mutation group. It is likely that this trend reflects a lower referral rate in adults, as in pre-pubertal children alone there was no significant difference in age at diagnosis and the detection rate was similar in both ethnic groups.

Consideration needs to be given to the best way of identifying cases of MODY in people of non-white European ethnicity. The clinical criteria alone are known to be ineffective in identifying all MODY cases in white European people and attention has turned to strategies that supplement the traditional criteria with additional parameters. These include the MODY probability calculator and the use of C-peptide, diabetes antibodies, highly sensitive CRP, and lipid profiles to help stratify cases (144,173,177,178,194). Additionally novel biomarkers have been shown to differentiate *HNF1A* MODY from other types of diabetes, for example plasma glycan profiles, though its applicability is limited by the availability of routine methodology(178). These approaches may well prove useful in people of SA ethnicity but are currently challenged by the paucity of data outlining the typical characteristics of type 1 and type 2 diabetes phenotypes in people of non-white ethnicity. For example, the presence of diabetes antibodies at diagnosis may be lower in some ethnic groups with type 1 diabetes (195,196) and slightly lower than white populations in Indian studies (196,197), whilst lipid profiles may be less useful due to concurrent insulin resistance in some populations (58,198). Furthermore as obesity levels rise and the presence of insulin resistance in relatively lean south Asians, the overlapping

phenotype of MODY with insulin resistance should also be considered (36). Changes to existing strategies, may also need to take into account the variation in prevalence of type 1 diabetes between native and migrant populations as children of migrants born in high incidence countries may adopt the local risk of type 1 diabetes (18–20,199).

The MODY probability calculator was highly effective at discriminating MODY cases in the WE group, but significantly overestimated the MODY pre-test probability in the SA group, particularly in those non-insulin treated.

The data in this study suggests that MODY cases may be more sensitively identified by using lower age at diagnoses and BMI cut-offs, as none of the confirmed MODY cases in the SA group had a BMI above 25kg/m^2 and only 1 case out of 12 was aged >20 years at diagnosis. In contrast to the no MODY group, where 48% had a BMI>25 mg/m^2 and 57% were aged >20 years. It is likely that changes to the BMI and age thresholds in the probability model may help better discrimination and this needs to be investigated in a non-selected cohort of SA individuals, diagnosed at young age and systematically investigated to ensure appropriate validation.

2.5 Limitations

This study is the first to explore the characteristics of referrals for MODY testing by ethnicity and highlights, for the first time, the lower detection rate of MODY mutations in people of non-white ethnicity. Its strengths lie in these novel findings and its large sample size ascertained through assessment of a central MODY genetic testing database, which holds data on referrals from a wide geographic area within the UK. The study is limited by retrospective analysis, including the inability to classify ethnicity in a number of individuals, who were excluded. Ethnicity was also not collected in a standardised format and it may include a mixture of self-reported and assumed ethnic origin. Finally, as not all MODY genes have been sequenced in each case it is possible that some of the cases categorised as without MODY have mutations in other genes that were not tested. However, when those with next generation sequencing were sub-anlaysed, the detection rate was still significantly lower in south Asians compared to white Europeans, making this explanation less probable.

2.6 Conclusion

Referrals for MODY testing in SA people have increased considerably, but may still be under-representative. Meanwhile the lower detection rate despite meeting referral criteria more stringently, suggest these criteria are insensitive when separating MODY from younger-onset type 2 diabetes.

Further work, in unselected cohorts, is needed to develop ethnic-specific criteria that facilitate the identification of MODY from type 2 diabetes in south Asian people with young-onset diabetes.

Chapter 3 : Methods and protocol of the MY DIABETES study

3.1 Introduction

There is a paucity of data exploring the phenotypes of young-onset diabetes in different UK ethnic groups. In particular, the prevalence and characteristics of MODY remain unexplored in non-white ethnic groups (190).
There is evidence to suggest that MODY is frequently misclassified in the white ethnic group and strategies to improve identification have been developed, but not validated in other ethnic groups (77,172).
In order to improve the identification of MODY in non-white ethnic groups, the characteristics of other diabetes types must first be investigated and the features of type 1 and type 2 diabetes in UK south Asian and African-Caribbean people with young-onset diabetes, is also largely unexplored.

To address these knowledge gaps and to gain insights into the variable phenotypes of young-onset diabetes across ethnicities, a cross-sectional study was designed.

The protocol of this study, which forms the core work of this thesis, is described in detail in this chapter.

3.2 Hypothesis

- The prevalence of MODY is lower in people of south Asian and African-Caribbean ethnicity as compared to a white control group, in people with young-onset diabetes across England.
- There are differences in diabetes phenotypes by ethnicity.

3.3 Aims

1) Establish the prevalence of MODY mutations in white, south Asian and African-Caribbean ethnic groups across the UK.
2) Explore the phenotypes of type 1 and type 2 diabetes by ethnicity
3) Make recommendations for strategies to improve case-finding and accurate diagnoses in south Asian and African-Caribbean people with young-onset diabetes.

Study Name: MODY in **y**oung-onset **diab**etes from different **et**hnicit**ies**

Study Acronym: The MY DIABETES study

Study Approvals: Approved by Chelsea LREC (13/LO/0944) 20th September 2013

Study Design: The MY DIABETES study is a non-interventional, cross-sectional study comparing UK-based individuals of white, African-Caribbean and south Asian ancestry with diabetes diagnosed before the age of 30 years. Recruited participants to date have undergone clinical, biochemical and genetic characterisation, to assess diabetes phenotype in a single visit study.

3.4 Population and Power Calculation:

Existing data on the prevalence of MODY in non-white ethnic groups, is limited (see introduction, section 1.3.6).
Population prevalence studies are limited in number and unfeasible to undertake, but a minimum prevalence of 108 cases per million has been suggested in the UK, however the true figure is likely to be considerably higher (77).
In predominantly white European populations, a variety of systematic surveys have found prevalence rates between ~1-3 % of people with young-onset diabetes (130). However, these studies are difficult to interpret with variable testing strategies, cohorts studied and genes sequenced. A previous study investigating MODY systematically in south west England and Tayside found a 3% prevalence of MODY in a predominantly white European diabetes population (128), who were aged <30 years at diagnosis and 2.5% when examining people aged less that 20 years at diagnosis (129).

Data from the retrospective study outlined in chapter 2 demonstrated the detection rate of MODY mutations in the south Asian group to be significantly lower than that of the white European group (190). Examination of the features of people without MODY, suggested that this was due to higher prevalence of young-onset type 2 diabetes. Although there are clear limitations to extrapolating the data from that initial pilot study,

which looked at detection rates for MODY, not prevalence, there is reasonable evidence to suggest that the prevalence of MODY mutations in south Asian individuals with young-onset diabetes is likely to be lower than that established in white populations.

With 80% power, alpha=0.025, and using a one-sample t-test design, it would be possible to detect as significant a difference from the predicted prevalence of 3% in the white European group, if the prevalence in the SA and AC groups was 0.6% or lower in each group. This provided a sample size of 561 participants in each ethnic group.
The effect size of this power calculation was difficult to estimate given the limited data available in this area. Based on pilot work and the feasibility of large-scale recruitment a one-tail design was selected and comparisons would be limited to south Asian versus white and African Caribbean versus white.

The primary objective was to design a study that could provide some information on the characteristics of diabetes types across ethnic groups and enable feasible recruitment to minority ethnic groups. It is accepted that this study was not designed to provide a definitive MODY prevalence across different ethnic groups.

3.4.1 Protocol Amendment

At the study outset only the south Asian and white European groups were selected for study, with a sample size of 458 people per group. However, in 2014, following an ethics amendment, the African-Caribbean group was also included as this was an ideal opportunity to dually recruit to both minority ethnic groups using the recruitment strategies employed and to provide novel data in this area, for both ethnic groups.

3.5 Subject Inclusion & Exclusion Criteria with Justification

Subjects were recruited if they were:

- Diagnosed with any type of diabetes on the basis of fasting glucose and/or oral glucose tolerance test and/or HbA1c as per World Health Organisation criteria (200,201).
- Aged below 30 years at diagnosis
- Of south Asian ancestry - defined as self-reported ancestry for entry into the study and at least 2 grandparents from the Indian Subcontinent (India, Pakistan, Bangladesh or Sri Lanka) for analysis.
- Of white European ancestry - defined as self-reported ancestry for entry into the study and at least 2 grandparents from within Europe for analysis.
- Of African and Caribbean ancestry - defined as self-reported ancestry for entry into the study and at least 2 grandparents from the African continent or the Caribbean for analysis.

Subjects were excluded if:

- An established secondary cause of diabetes existed (for example recurrent pancreatitis, pancreatectomy, new onset diabetes after transplantation, Cushing's syndrome, acromegaly, steroid induced or a condition that the principal investigator felt represented a secondary cause).
- They were enrolled in a clinical trial of an investigational medicinal product that was felt by the research team to potentially impact results from the study.
- They had an active malignancy or were under investigation for malignancy.
- They were pregnant beyond the first trimester
- They had a current diagnosis of gestational diabetes.

Subjects would be withdrawn from the study in the case of:

1. Loss of capacity to give informed consent
2. Terminal illness

Withdrawal was immediate and subjects were free to withdraw for any reason provided at any time at their discretion.

3.5.1 Selected Age Cut-Off

To limit the number of type 2 diabetes cases in the cohort, an age at diagnosis cut-off of 30 years was selected to represent a young-onset cohort. This was comparable to the previous UNITED study in Tayside and south-west England, allowing for direct comparison of the cohort (128). It is accepted that with this age cut-off some MODY cases will be missed, as age-at-diagnosis has been above 30 years in a variety of reported cases (78). However, as this study was not designed to be a population prevalence study and as the numbers of cases diagnosed beyond 30 years of age are likely to be small, this cut-off seemed appropriate for the intended purpose of the present study.

3.5.2 Ethnicity Definition

Ethnicity definitions were based on UK census categories (193). The definition of ethnicity is complex and much work has been undertaken to facilitate classification. Entry into the study was based on self-reported ethnic origin, in keeping with guidance from the Office of National Statistics (192).

However, to facilitate a more robust categorisation for analysis, data was also collected on parental and grandparental ancestral origin. During analysis, people with all four grandparents from a region of interest were included in either the white, south Asian or African-Caribbean groups respectively.

People with mixed ethnicity were also recruited into the study, but their analyses were kept separate in a fourth ‘mixed ethnicity’ group. Mixed ethnicity included participants with at least one set of grand-parents from a region of interest and the other set from either another region of interest or a different ethnic group to the core groups studied in this protocol. The mixed group is therefore ethnically diverse, however as many genetic studies exclude these individuals and as anecdotally, they may have unusual or unexplored phenotypes of diabetes, a decision was made to include them in the study but to analyse their data separately.

It is accepted that the core groups are also genetically heterogenous as, for example, the genetic ancestry of people from the Indian subcontinent is hugely variable and in one study allele prevalence was more variable within Indian sub-populations than in

Europe (202). Equally grouping the whole African continent together is also a simplification as it includes northern-African populations, that are more genetically aligned to middle-eastern populations rather than sub-Saharan African populations.
This genetic heterogeneity is likely to be a feature of any genetic study in an ethnic group conducted outside of native countries.
However, although a limitation, this is in equal measure a strength as using the study design outlined, there is an opportunity to sample a wide variety of sub-populations which will facilitated the assessment of multiple genetic groups for MODY prevalence within this. In addition, this approach provided an easier recruitment criteria as there are difficulties in defining which countries should be included / excluded within a continent or subcontinent or within a region of interest.
As this is not intended to be a true prevalence study and as data from this study will be used to study interesting populations in more detail, this approach offers considerable advantages. This broad approach for the identification of MODY in non-white ethnic groups also underlies the decision to recruit people from mixed ethnic backgrounds, who are frequently excluded from genetic studies.
In future work to further characterise ancestry, DNA samples may be analysed (anonymised) to determine genetic ancestry and better delineate ethnic groups. This strategy has been used effectively and utilises analysis of Y chromosome, mitochondrial DNA or single nucleotide polymorphisms. Of these three approaches analysis of single nucleotide polymorphisms may be preferable to enable analysis of both female and male participants. This strategy analyses differences in allele frequency between two continental populations and has been used effectively to determine population admixture in a variety of ethnicity studies (203,204).
Using this approach in the MY DIABETES study may help to genetically segregate the likely heterogenous ethnic groups based on grandparental origin. However, testing ancestral markers may require additional ethical approval and therefore decision-making around this will be determined at the end of the study.

3.5.3 C-peptide

In this study fasting C-peptide was chosen to assess endogenous beta-cell function.
Fasting C-peptide shows good correlation with stimulated C-peptide measured after a mixed meal tolerance test. Though the latter is considered to be a gold-standard, a

fasting measure was more convenient to undertake in the large sample size to be studied (150). A fasting measure was preferred to a random measurement to ensure a standardised approach that would allow for meaningful ethnic group comparisons. C-peptide analyses were undertaken on the Abbott Architect platform (see 3.11.1). Fasting C-peptide was used to establish if there was evidence of endogenous beta-cell function, which in turn assisted with stratification for genetic testing (see 3.11.4). Cut-offs chosen are discussed further in section 3.11.1.

3.5.4 Antibody measurement

The three main diabetes antibodies detected in people with type 1 diabetes were selected for measurement in this study; GAD-65, IA-2 and ZnT8. Standardisation of antibody assays are poor across laboratories. Antibody measurement was therefore centralised to one laboratory to ensure inter-assay imprecision was kept to a minimum. Insulin antibodies were not chosen for measurement as many people treated with insulin form antibodies to insulin that could potentially result in false positivity. Cut-offs for antibody positivity (see section 3.11.3) were based on 99th centile from reference range work undertaken in the Exeter laboratory following analysis of 500 non-diabetic samples (173).

3.6 Recruitment

Following ethical approval the study was adopted by the national NIHR Clinical Research Network (CRN), with the aim of recruiting individuals across England and focusing on areas with high ethnic minority populations across the country.

A variety of recruitment strategies were planned and the following approaches all received ethical approval:

1) Examination of hospital diabetes registries / out-patient clinics in adult and paediatric secondary care services, nationally.
2) Examination of general practice databases in local clinical commissioning groups (CCG) for people meeting recruitment criteria, either as participant identification centres or as study sites.

3) Examination of existing diabetes databases of patients who have consented to be contacted for future studies.
4) Direct advertising in national press, or local media focusing particularly on TV channels and radios for specific ethnic groups.

3.6.1 Recruitment from Secondary Care

Recruitment predominantly took place at secondary care sites from diabetes clinics. Once the study was adopted to the CRN portfolio, various research teams at NHS Trusts around the country made contact, following a call for expressions of interest in late 2014. In the initial round of interest any site, regardless of local community demographics, was screened and initiated, provided minimum infrastructure criteria were in place to support the study. Later on in recruitment, new sites were limited to those that could exclusively recruit to south Asian and /or African-Caribbean sites. All sites adopted different approaches, but recruitment occurred from all clinic types, including general diabetes, type 1 diabetes, type 2 diabetes and other specialist clinics e.g. transitional / young-adult clinics. A full list of sites is depicted in table 3.1.

3.6.2 Recruitment from Primary Care

Initial strategies deployed included the use of general practices as participation identification centres. This was tested as a pilot scheme in North West London where approval was sought to use all CCG's to identify potential participants. Primary care recruitment was felt to be a good strategy to employ as many people with young-onset type 2 diabetes may not be managed in secondary care.
However, in the present study, the call for expressions of interest yielded interest from only 6 practices. The participants meeting the criteria at each of these practices following System One / EMIS searches ranged from two to fifteen patients and of these, only two people responded to participate. As each individual practice had so few participants meeting the criteria, this approach was felt to be less feasible. However, it was left to individual sites to decide, based on their local diabetes community set up to determine if this approach was viable. A more fruitful approach has been the use of community diabetes clinics as a source of identifying participants.

In 2016, the first general practice site was set up for study visits as a hub for all practices in North West London. This approach had not been a priority due to the limited number of patients identified per site and the difficulty in procuring equipment (freezer and centrifuge), however with close links to the community diabetes clinic and a professional network of diabetes contacts, the site was approved and will recruit its first participant in January 2017.

Table 3.1: List of NHS sites participating in the MY DIABETES study

Site	Principal Investigator	Date first participant recruited	Description
Imperial College Healthcare NHS Trust	Dr Shivani Misra	28/11/ 2013	Adults
Warwick University Hospital	Dr Rajni Mahto	17/11/2014	Adults
University Hospitals Coventry	Professor Tom Barber	12/12/2014	Adults
Ealing Hospital, London	Dr Kevin Baynes	10/3/3015	Adults
Birmingham Children's Hospital	Professor Tim Barrett	16/6/2015	Paediatrics
University Hospital Lewisham, London	Dr Ruvan Kottegoda	18/6/2015	Adults
Kings College Hospital, London	Dr Martin Whyte	6/7/2015	Adults
Northwick Park Hospital, London	Dr Mushtaqur Rahman	28/7/2015	Adults
Northern General Hospital, Sheffield	Professor Simon Heller	5/10/15	Adults
St Thomas's Hospital London	Dr Anna Brackenridge	4/1/2016	Adults
West Middlesex university hospital	Dr Jayanti Rangasami	4/1/2016	Paediatrics
Bristol Royal Infirmary	Dr Natasha Thoroughgood	5/1/2016	Adults
Barth Health NHS Trust	Dr Bobby Huda	19/2/2016	Adults
Central Middlesex Hospital	Dr Daniel Darko	5/4/2015	Adults
Hillingdon Hospital, London	Dr Mark Edwards	25/4/2016	Adults
Newham General Hospital	Dr Shanti Vijayaragavan	28/4/2016	Adults
St Georges University Hospitals	Dr Ken Earle	28/4/2016	Adults
Royal Preston Hospital	Dr Satyan Rajbhandari	16/6/2016	Adults

Chorley and South Ribble Hospital	Dr Satyan Rajbhandari	21/6/2016	Adults
West Middlesex University Hospital	Dr Rashmi Kaushal	20/7/2016	Adults
Coventry University Hospital	Dr Heather Stirling	3/8/2016	Paediatrics
Birmingham Heartlands Hospital	Dr Sri Bellary	Site initiated	Adults
University College Hospital, London	Dr Sarita Naik	Site initiated	Adults
Park View Centre for Health and Wellbeing	Dr Samia Hassan	Site initiated	Adults, general practice hub
Pinderfield Hospital, Dewsbury	Dr Olivia Pereira	Site Initiated	Adults
Bradford University Hospital	Dr Donald Whitelaw	In set up	Adults
Royal Wolverhampton NHS Trust	Dr Nisha Pargass	In set up	Paediatrics
Royal Wolverhampton NHS Trust	Dr Ananth Viswanath	In set up	Adults
Sandwell and West Birmingham Hospital	Dr Chizo Agwu	In set up	Paediatrics

3.6.3 Recruitment Timeline

Recruitment to the study started in November 2013 and will continue until October 2018.

New sites are still being added if they can contribute significantly to south Asian and African-Caribbean recruitment.

The white European ethnic group is likely to reach target numbers in early 2017 and will be closed.

Initial recruitment projections at the beginning of the study were based on 13 sites recruiting an average of 6 participants per month. It rapidly became apparent that this approach was inconsistent as there was wide variability in the numbers recruited per month, with some sites exceeding expectations, but other sites recruiting none. This strategy was therefore amended and the study was opened more widely in 2015. Sites are now still being added on and by January 2017, there will be a total of 29 sites. With each site recruiting 1 or 2 participants a month, the target is projected to be reached before the end date.

3.7 Identification of Participants and Initial Approach

Participants were approached from a variety of sources depending on strategies employed, as above.

Two common approaches were as follows:

- Contact in diabetes clinics

  Members of the diabetes clinical teams at approved sites approached potential participants directly and gave them participant information sheets (see appendix 2). If potential participants were happy to be contacted, members of the research team initiated contact after a suitable interval (at least 24 hours) to discuss the study further, assess suitability and book study visits.

- Telephone contact following mail out

  Suitable participants were also identified from hospital registries of diabetes out-patient attendees or other clinical / research databases. These potential recruits were contacted with site-specific participant information sheets and were either contacted after a suitable interval, or approached if they responded themselves.

Following initial contact, a member of the research team member clarified that the participant was diagnosed with diabetes *before* the age of 30 years and checked the participants self-reported ethnicity to ensure they were from one of the eligible groups. The aim of the study was also briefly explained at this point as well as the instructions for the study visit.

If they were happy to participate an appointment for the visit was scheduled and instructions provided.

3.8 Instructions Prior to Study Visit

Day before:

- Adult participants (>16 years) were advised to fast from the night before. A fast of 10 hours was advised.
- Adult participants who preferred not to fast were still allowed to participate.
- Child participants were recruited non-fasted.

Day of study visit:

- Water was allowed on the morning of the visit and any non-diabetes medications were permitted.

Omission of medications until after the study visit in those fasting.

- Participants taking oral hypoglycaemic agents or non-insulin injectable agents, were advised to defer taking these until after the study visit.
- Long acting insulin injections were not stopped on the basis that little effect on C-peptide levels have been noted on and off basal insulin and the risks of stopping treatment were not acceptable(205).
- Short acting insulin injections taken with breakfast were omitted as participants were to be fasting.
- Participants on subcutaneous insulin infusion pumps were advised to continue basal insulin, but not bolus, as breakfast was to be omitted.
- For people on other insulin regimes, omission of specific treatment was advised by the local principal investigator.
- Participants who experienced hypoglycaemia overnight during their fast, were still allowed to participate non-fasting if they felt well enough. The information about the hypoglycaemic episode was recorded by the research team, on the case report form.

3.9 Study Visit

The study visit SOP can be found in appendix 3. A brief summary is provided here and summarised in figure 3.1.

Participants attended fasting, if agreeable. Before consenting, a capillary blood glucose was checked to ensure it was not in the hypoglycaemic range.

Written consent was then sought from individual participants, or in the case of children, assent with parental or guardian consent (see appendix 4 for age specific forms).

Following informed consent, a standardised paper-based case report (see appendix 5) form was filled by a member of the research team. This collected information on the variables depicted in table 3.2.

Figure 3.1: Schematic of Typical Study Visit

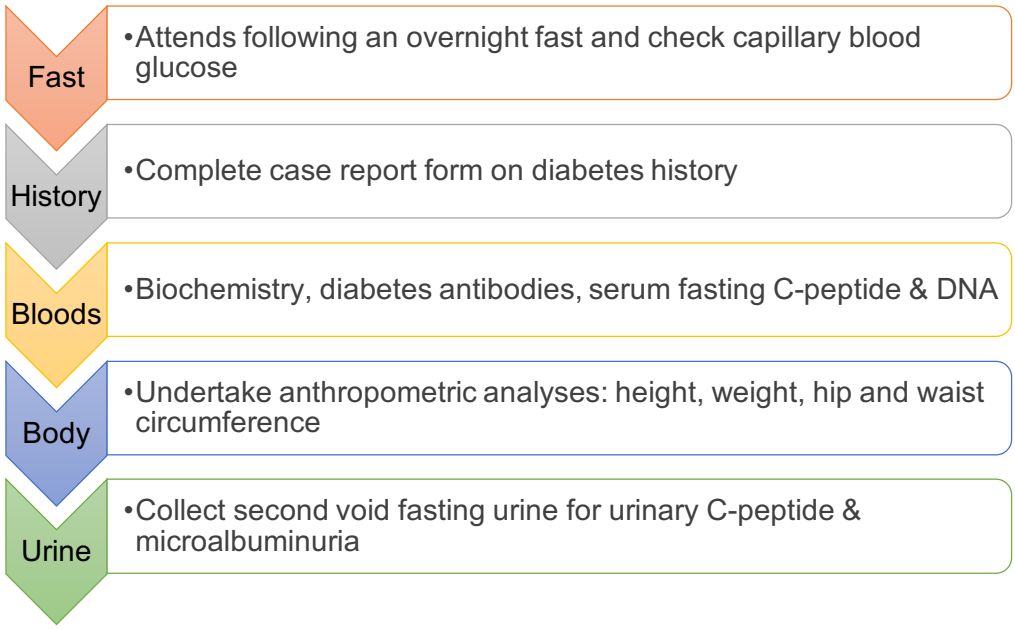


Table 3.2: Summary of Information Recorded on the Case Report Form

Section	Questions
Ethnicity	• Self reported ethnicity • Country of birth • Parental ancestral origin • Grandparental ancestral origin
Diabetes Diagnosis	• Age at diagnosis • Date of diagnosis • Diagnosed in pregnancy? • Known MODY? • Symptoms at presentation? • Weight loss? • Duration of symptoms • Diabetic ketoacidosis at diagnosis or subsequently • Birth weight

Treatment history	• Initial treatment; diet, tablet or insulin
	• Duration of initial treatment
	• Current treatment:
	  ○ Diet only
	  ○ Tablet type
	  ○ Insulin regime, dose and total units/24 hours

Medical History	• Young-onset deafness? • Learning difficulties? • Known renal cysts? • Diabetic nephropathy, neuropathy or retinopathy • Macrovascular complications?
Family History	• Presence or absence of diabetes in mother, father, maternal grandparents, paternal grandparents, children and siblings. • Where diabetes was present in a family member, approximate age at onset and treatment type was further queried.

Following completion of the CRF, a member of the research team undertook basic anthropometric measurement: height, weight, hip and waist circumference. That latter two were omitted in paediatric recruits. The presence or absence of *acanthosis nigricans* was also determined by examination of both axillae.

3.10 Blood Sample Collection

Table 3.3 shows the blood volumes, tubes and tests that were collected for each participant. There were some variations depending on local arrangements for pathology laboratory testing. As a minimum requirement a DNA sample and serum save needed to be taken to ensure that C-peptide and pancreatic autoantibodies could be measured. This enabled the participant to be eligible for inclusion in the primary outcome analysis, i.e. to determine whether or not genetic testing would be undertaken.

Table 3.3: Approximate Blood and urine Sample Requirements

Tube Number	Preservative	Volume	Tests
1 x yellow	SST / gel separator	2 ml	Renal, lipid profile, Liver functions, C-reactive protein
1 x small purple	EDTA	2 ml	HbA1c
1 x grey top	Fluoride oxalate	1 ml	Glucose
1 x large purple	EDTA	7-10ml	DNA extraction and antibodies
4 x large red	Plain tube	6 ml each	Serum to save for C-peptide
1x universal container	None / boric acid	1 ml at least	Micoalbuminuria and stored for urine C-peptide
Paediatric			
1 x small purple	EDTA	5 ml	DNA extraction and antibodies
2 x small red	Plain tube	4 ml each	Serum to save for C-peptide

Other biochemical blood results were usually available and accepted within 2 months of the study visit, if there was no funding to support the tests to be undertaken on the day of the study visit. This was a pragmatic decision reflecting the limited local funding arrangements and the limitation of the 2-month window period helped assure there would be less variability in results. A full description of how blood samples were processed is available in the sample processing SOP version 1 in appendix 6.

Volunteers were then provided with a urine pot to collect a sample for microalbuminuria analysis. As part of a sub-study (not reported in this thesis), an aliquot of the urine was decanted into a boric acid container for later urine C-peptide assessment. At this point the study visit was complete and participants were free to go.

3.11 Sample Analyses

3.11.1 C-peptide

Anonymised, coded, saved serum was collected in batches from sites and analysed for C-peptide using a centralised research assay at Imperial College Healthcare NHS Trust. This was the Abbott Architect chemiluminesent assay and its characteristics are summarised in table 3.4. The assay was internally quality controlled and calibrated. Although the kit stated a lower detection limit of 3 pmol/L, internal validation work revealed imprecision in this area and so for the purposes of the MY DIABETES analysis a lower detection limit of 27pmol/L was set, in keeping with the clinical assay. Therefore, a participant was said to have a detectable C-peptide if their value was >27 pmol/L.

Table 3.4: Characteristics of the C-peptide assay used to analyse samples

Parameter	Assay Characteristic
Imprecision	≤10%
Recovery	98.9% - 102.1%
Dilution Linearity	Mean recovery of 100 ± 15% of expected result
Lower detection limit	3 pmol/L
Upper detection limit	10,000 pmol/L
Dilution	Samples >10,000 undergo an on board 1 in 10 dilution using multiassay diluent (phosphate buffer)
Sample type	Serum, plasma, non-acidified 24-hour urine collections

3.11.2 Pancreatic Autoantibodies

GAD-65, IA-2 and ZnT8 auto-antibodies were measured centrally in the Royal Devon and Exeter NHS Trust immunology laboratories. The immunology laboratory has set up a procedure to enable measurement on samples extracted from plasma EDTA samples, meaning that a single sample can have antibodies measured on it and DNA extracted.

The process has been internally validated and involves pre-treatment with thrombin and calcium chloride.

Kits used and their characteristics are depicted in table 3.5.

Table 3.5: Pancreatic Autoantibody Assay Characteristics

Parameter	GAD-65	IA-2	ZnT8
Kit manufacturer	RSR	RSR	RSR
Sensitivity /specificity	98% specificity 92% sensitivity (DASP 2005)	100% specificity 64% sensitivity (DASP Study 2010)	99% specificity 72% sensitivity (IASP 2012 study)
Lower detection limit U/mL	5	7.5	15
Upper detection limit U/mL	2000	4000	2000
Reference range based on 99th centile in healthy volunteers	Negative <11 IU/ml Positive ≥11 IU/ml	Negative <15 IU/ml Positive ≥15 IU/ml	In those <30 years Negative <65 U/ml Positive ≥65 U/ml In those ≥30 years Negative <9.1 U/ml Positive ≥9.1 U/ml

The assay for ZnT8 antibodies became available in 2015. Prior to this only GAD-65 and IA-2 antibodies were available.

Participants enrolled into the study before ZnT8 assays became available were classed as antibody negative if both GAD-65 and IA-2 antibodies were negative and antibody positive if one or both was positive.

After the ZnT8 assay became available, antibody negativity was defined as all three antibodies negative and positivity as one or more.

This discrepancy will be addressed in coming months as the old samples (n= 330) without ZnT8 measurement are to be analysed for ZnT8. The current analysis does incorporate those in whom ZnT8 was not tested initially and it is accepted that there may be some ZnT8 positive participants who have been genetically tested. As the rates of ZnT8 antibody positivity are known to be low and since samples will be retrospectively analysed for ZnT8, this approach has been continued for now.

3.11.3 Genetic Testing

Genetic testing for MODY genes was undertaken in the Royal Devon and Exeter Molecular genetics laboratory in collaboration with Professor Sian Ellard and her team. EDTA samples were posted first class on the day of the study visit to the molecular genetic laboratory. DNA samples are stable at room temperature for 72 hours.

MODY testing was activated if criteria for testing were met (see below).
The automated technologies used to enable sequencing, are briefly outlined here. The team had previously designed a custom Agilent SureSelect exon-capture assay (Agilent Technologies, Santa Clara, CA, USA) with baits for 29 genes. The steps in this procedure are outlined:

1. DNA was isolated and purified following extraction from peripheral blood leukocytes using automated platforms that lysed the nuclear and cellular membrane.
2. Genomic DNA was sheared using sonication and fragments were adenylated and ligated to a 6 base pair unique tag, enabling each fragment to be uniquely identified back to the participant. This ensured multiplexing (mixing samples of multiple participants, a more efficient way to sequence) was possible.
3. Following ligation with the adaptor, each sample underwent 7 cycles of PCR amplification to enrich individual DNA libraries.
4. Adaptor-ligated genomic DNA fragments were then hybridised with biotinylated RNA-baits, which are 100 base pair RNA fragments specifically designed against regions of interest and appropriately balanced to enable capture of harder regions. Bait capture and density had been previously optimised for genes of interest (179).
5. Magnetic beads coated in streptavidin were then used to bind to the RNA baits which in turns captured the relevant fragments of genomic DNA. Captured libraries were then extensively washed and purified.
6. Finally captured and purified libraries underwent a 16 cycle PCR amplification.
7. Samples were then sent for parallel sequencing using the HiSeq Illumina
8. The next generation sequencing technology enabling massively parallel sequencing have been published previously here (206,207).

The Illumina HiSeq 2000 (Illumina, San Diego, CA, USA) was used to sequence genomic DNA in pools of 12. Sequencing generated multiple reads per base of genomic DNA (read depth) Sequencing was optimised to enable a minimum read-depth of 30 base pairs and targeted to enable 50 base pair upstream and 10 base

pairs down stream of each exon to be sequenced. The assay could also identify deletions and duplications >30 base pairs using changes in read depth.
Any mutations identified were confirmed using Sanger sequencing.

Table 3.6: Genes Sequenced in the Targeted Capture Array

Gene	Full name	Transcript	Phenotype
ABCC8	(ATP Binding Cassette Subfamily C Member 8	U63421 and L78208	M/ ND
BLK	B-lymphocyte specific tyrosine kinase	NM_001715.2	M
CEL	Carboxy-ester lipase	NM_001807.3	M
EIF2AK3	Eukaryotic Translation Initiation Factor 2 Alpha Kinase 3	AF110146.1	ND
FOXP3	Forkhead box P3	NM_014009.2	ND
GATA4	Zinc finger transcription factor 4	NM_002052.3	ND
GATA6	Zinc finger transcription factor 6	NM_005257.3	ND
GCK	Glucokinase enzyme	NM_000162.2	M/ND
GLIS3	GLI-similar zinc finger protein	NM_001042413.1	ND
HNF1A	Hepatocyte Nuclear Factor 1-Alpha	NM_000545.3	M
HNF1B	Hepatocyte Nuclear Factor 1-beta	NM_000458.1	M/ND
HNF4A	Hepatocyte Nuclear Factor 4-Alpha	LRG_483	M
IER3IP1	Immediate early response 3 interacting protein 1	NM_016097.3	ND
INS	Insulin	NM_000207.2	M/ND
IPF1 (PDX1)	Insulin promoter factor 1 (pancreatic and duodenal homeobox 1)	NM_000209.1	M/ND
KCNJ11	potassium voltage-gated channel subfamily J member 11	NM_000525.3	M/ND
KLF11	Kruppel like factor 11	NM_003597.4	M
LMNA	Lamin A/C	NM_005572.2	L
m.3243 region	Mitochondrial	NC_012920.1	Mt
NEUROD1	Neuronal differentiation 1	NM_002500.2	M/ND
NEUROG3	Neurogenin 3	NM_020999.2	ND
PAX4	Paired box 4	NM_006193.2	M
PPARG	Peroxisome Proliferator Activated Receptor Gamma	NM_005169.3	L
PTF1A	Pancreas transcription factor 1	NM_178161.2	ND
RFX6	Regulatory Factor X6	NM_173560.3	ND

SLC19A2	Solute Carrier Family 19 Member 2	NM_006996.2	ND
SLC2A2 (GLUT2)	Solute Carrier Family 2 Member 2	NM_000340.1	ND
WFS1	Wolframin	NM_006005.3	ND
ZFP57	Zinc finger protein 57	NM_001109809.2	ND

The transcript and disease phenotype are described; MODY (M), neonatal diabetes (ND), lipodystrophy (L) or mitochondrial diabetes (Mt).

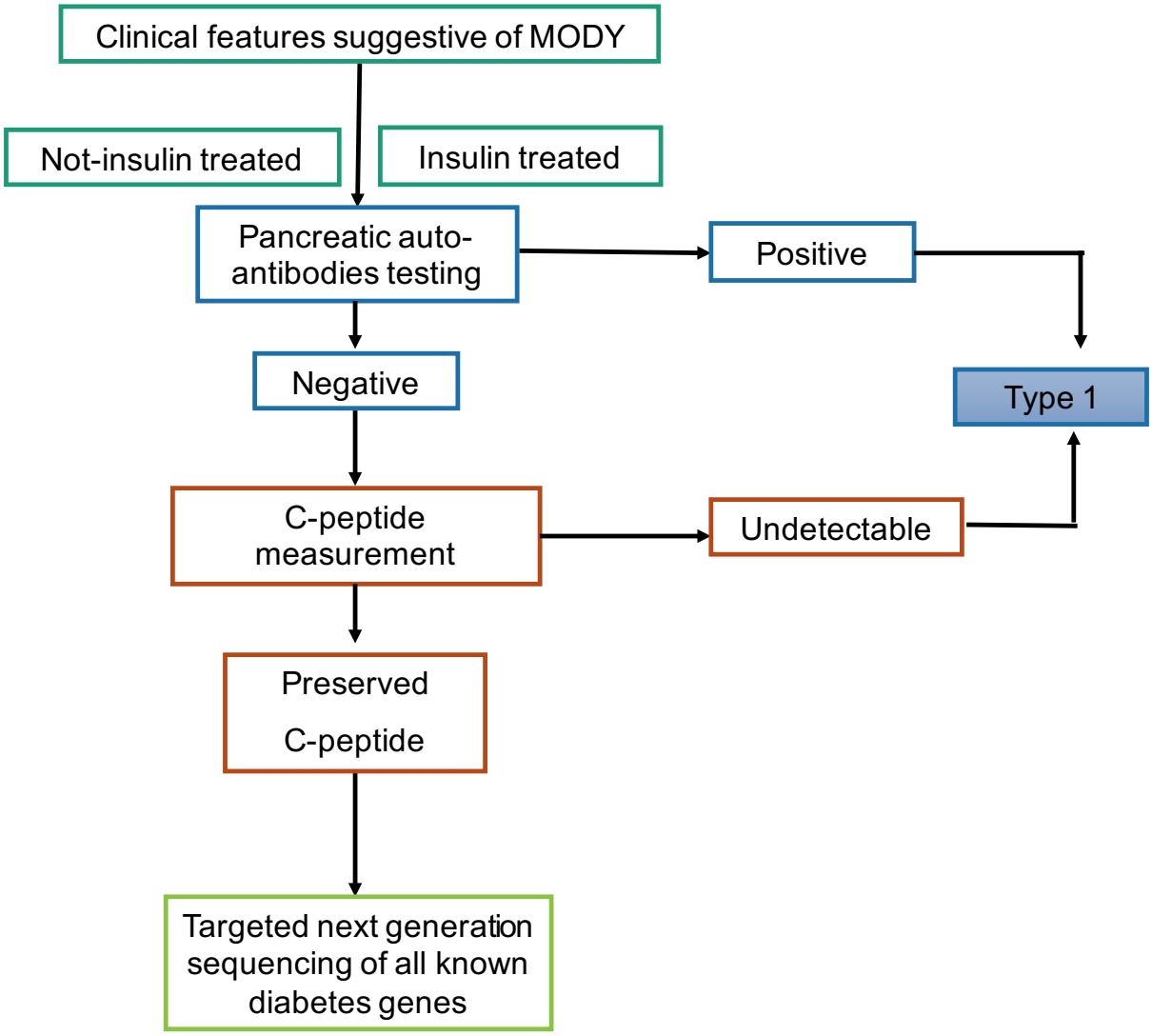
Using an established bioinformatics pipeline, a list of sequence variants (single nucleotide variants and insertions / deletions) was generated and variant interpretation was undertaken by clinical scientists.

Variant pathogenicity was determined according to national guidelines (184). The methodologies used are described in detail in chapter 6.
Briefly, a variety of *in silico* prediction models, database searches and information about species conservation along with effects on physiochemical properties were used to determine variant pathogenicity. Pathogenicity was defined as benign, likely benign, variant of uncertain significance, likely pathogenic and pathogenic.

3.11.4 Strategy for genetic testing

The strategy for genetic testing is depicted in figure 3.2. To summarise, people who tested negative for GAD-65 and IA-2 antibodies (and later ZnT8, see above) and in whom C-peptide was >27 pmol/L regardless of fasting status, were eligible for genetic testing of all known MODY genes. It is clear that this strategy could potentially include a subset of people with long-duration type 1 diabetes. However, as there is still debate as to what qualifies as a C-peptide level consistent with type 1 diabetes and as many people with MODY may eventually require insulin, a decision to use a strict low cut-off for ‘detectable C-peptide’ was made.

Figure 3.2: Strategy for genetic testing in recruited participants



GAD, glutamic acid decarboxylase; IA-2 insulin-antigen 2; T1DM, type 1 diabetes

People in whom genetic testing was not undertaken still consented for their DNA sample to be stored for future studies.

3.12 Informing Participants of Results

Standardised results letters were produced and ethically approved (see appendix 7) to inform participants of results. Once C-peptide and antibody results were available, these were collated at Imperial College and conveyed to individual sites.

- If participants had one or more antibody positive, regardless of C-peptide levels, they were sent the ‘Antibody positive’ letter, which explained the presence of pancreatic autoantibodies, its indication of a diagnosis of type 1 diabetes and informed them that MODY testing would not be undertaken.
- If antibodies were negative and C-peptide was$P \leq 27$ pmol/L, participants were sent the C-peptide negative letter, which explained that this was most consistent with type 1 diabetes and that MODY testing would not be undertaken.
- For those participants who were antibody negative and C-peptide >27pmol/L, no letter was sent initially and genetic testing was scheduled.
- If MODY testing was negative a standardised results letter informing participant of this was sent.
- If MODY testing identified a mutation, a fully accredited NHS report was issued and conveyed to the local principal investigator. Participants were then invited in to discuss their results and any implications in a face-to-face consultation.

3.13 Outcomes

3.13.1 Primary Outcome

- Determine the prevalence of MODY in white European, AC and SA individuals in the UK with young-onset diabetes.

3.13.2 Secondary Outcomes

- Compare the proportions of people meeting criteria for genetic testing by ethnicity.
- Assess the phenotypic characteristics of MODY and other diabetes subtypes in this population.
- Statistical comparison of antibody positivity, C-peptide levels and other biochemical parameters between ethnic groups.

3.14 Governance, Data and Biological Sample Management

Data During the course of the study visits all data was stored on encrypted laptop computers, not connected to the internet, for later statistical analysis. These data will be coded and non identifiable. Participant case report forms were stored in a locked filing cabinet in a secure room in Imperial College Healthcare NHS Trust. Only the research team (clinical research fellow and principal investigators) had access to the filing cabinet.
Missing, unused, and spurious data was assessed on an individual basis and usually included in the analysis.

Samples All blood samples received at Imperial College London were anonymised and identifiable only by study codes. These samples were stored on HTA premises in locked freezers.
Samples sent to Exeter for genetic testing were treated as NHS samples and were ethically approved to contain patient identifiable information, such that full genetic reports could be issued without the need for a repeat non-anoymised sample.
All sample transfer was covered by material transfer agreements between Imperial College and participating sites.

3.15 Analysis Plan

All data were analysed using Stata/SE version 13.1. Data were non-parametric, medians and interquartile ranges were therefore used.

Primary analysis:
As recruitment has not been completed at the time of the analysis, a surrogate primary outcome of numbers eligible for genetic testing in each ethnicity was undertaken and proportions compared using Chi-Square analyses.

Secondary analyses
The following secondary analyses were undertaken:

1) Exploring the phenotype of the group eligible for genetic testing

a. Description and statistical comparison of the group eligible for genetic testing (C-peptide>27 pmol/L and antibody negative), by ethnicity (WE vs SA and WE vs AC), examining the following variables:
    i. Age at diagnosis, duration, current age, BMI, waist circumference, waist-to-hip ratio, % currently not insulin treated, % with a parental history of diabetes and & diagnosed before 25 years of age.
    ii. HbA1c, total cholesterol, triglycerides, HDL cholesterol, LDL cholesterol, fasting C-peptide and fasting glucose.
b. Description and statistical comparison of the group eligible for genetic testing by ethnicity, as above, specifically examining individuals with C-peptide ≥200 pmol/L
c. Description and statistical comparison of the group in whom genetic testing has been undertaken, as above, but in whom no MODY mutation has been found.
d. Linear regression to determine predictors of C-peptide in the group who are eligible for genetic testing.

2) Exploring the phenotype of people classified as having type 1 diabetes
    a. Description and statistical comparison of the group with undetectable C-peptide (C-peptide ≤27 pmol/L), by ethnicity (WE vs SA and WE vs AC), examining the following variables:
        i. Age at diagnosis, duration, current age, BMI, waist circumference, waist-to-hip ratio, % currently not insulin treated, % with a parental history of diabetes and & diagnosed before 25 years of age.
        ii. HbA1c, total cholesterol, triglycerides, HDL cholesterol, LDL cholesterol, fasting C-peptide, fasting glucose and pancreatic auto-antibody status
    b. Description and statistical comparison of the group with C-peptide >27 pmol/L who were antibody positive, as above.
    c. Description and statistical comparison of the entire type 1 diabetes group, group with C-peptide either ≤27 pmol/L or who were antibody positive regardless of C-peptide level.

d. Linear regression to determine predictors of C-peptide in the antibody positive group, with C-peptide >27 pmol/L
e. Exploration of antibody positivity proportion and type of the whole cohort and by ethnicity

Analyses examining BMI were undertaken in participants over the age of 16 years at the study date and where noted, BMI cut-offs were ethnic specific, based on World Health Organisation recommendations.

Where clinical and biochemical parameters were compared by ethnicity, Mann-Whitney U tests were used to compare SA and AC to WE.

Significance was set at $p<0.05$ and post-hoc Bonferroni correction was not applied. Although multiple comparisons were undertaken, variables analysed were restricted to those with *a priori* hypotheses based on previous published data. However, this study analysed data from a novel cohort in which many published studies cannot be readily applied. Data were therefore be used to generate hypotheses for subsequent studies, in which Bonferroni correction will be undertaken.

Cases with missing data were included as long as C-peptide and antibody results were available.

Chapter 4 : Demographics, Classification and Analysis of the Genetic Testing Cohort in the MY DIABETES study

4.1 Description of the MY DIABETES Cohort

At the time of the presented analysis a total of 728 participants had been recruited to the MY DIABETES study from multiple centres around England.
The characteristics of the entire cohort are presented in table 4.1, representing the total number of recruits at the end of October 2016.

Table 4.1: Characteristics of All Participants Recruited up to October 2016

	% (n) / median (IQR)
Total Number	728
White	65 (469)
South Asian	18 (133)
African-Caribbean	12 (85)
Mixed / Other	5 (37)
Male	46 (334)
Children (<18yrs)	8 (59)
Clinician-assigned diagnosis	
"Type 1 diabetes"	82 (570)
"Type 2 diabetes"	9 (82)
"Don't' Know"	4 (24)
"Other"	2 (17)
Currently insulin-treated	88 (643)
Median age at diagnosis (years)	17 (11-24)
Median duration (years)	18 (7-33)
Median age at recruitment (years)	35 (26-49)
Antibody positive	56 (394)
C-peptide detectable (>27pmol/L)	39 (261)
C-peptide >201pmol/L	25 (168)
Glucose at study visit (mmol/L)	9.5 (6.5-12.6)
% Fasting at the study visit	79 (575)

Figures given are % (n) or median (inter-quartile range).

The white European (WE) group accounted for the largest group at 65%, followed by south Asian (SA) and African-Caribbean ethnic groups (18% and 12% respectively).

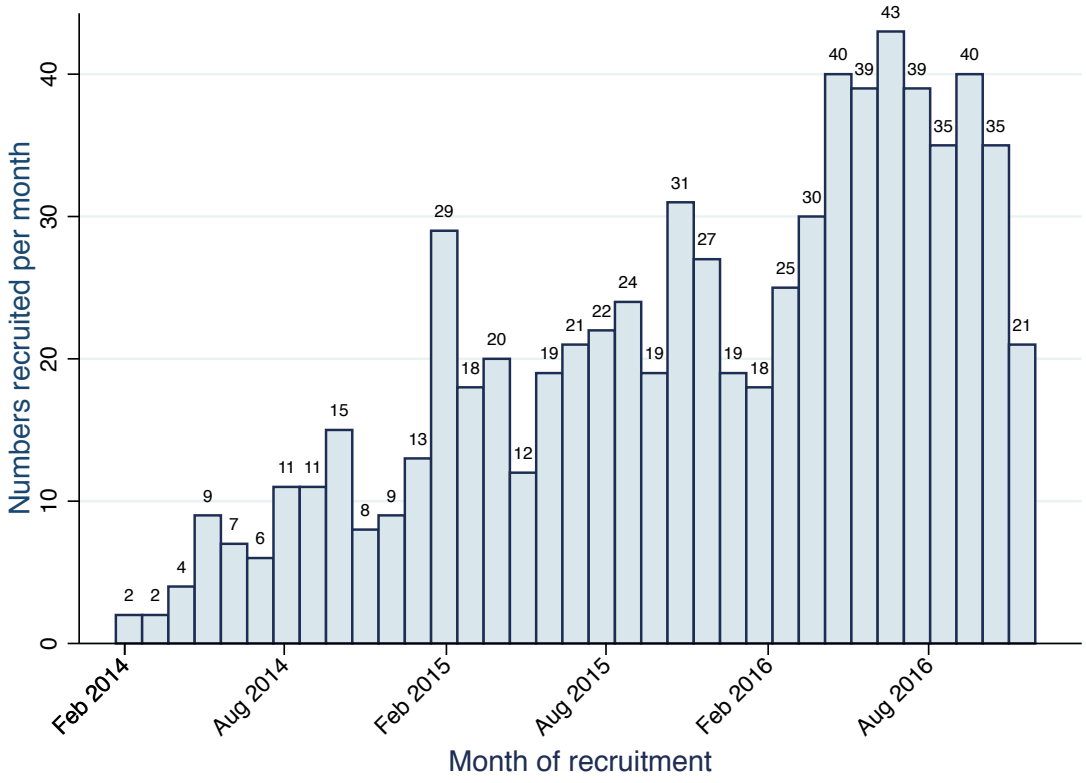
The majority of participants had a clinician-assigned diagnosis of type 1 diabetes (82%), with type 2 diabetes accounting for 11.8% of cases.

Antibody positivity to GAD-65, IA-2 or ZnT8 was observed in 56.2% of the whole cohort. The majority of individuals in the cohort had an undetectable C-peptide (<27 pmol/L) with 38% above the detection limit and 24.7% with a level above 200pmol/L (the DCCT study fasting cut-off, (208)).

4.2 Recruitment by Month

Figure 4.1 depicts the numbers of participants recruited per month throughout the duration of the study. The study is now averaging 35-40 recruits per month and continues to recruit until October 2018.

Figure 4.1: Recruitment figures per month



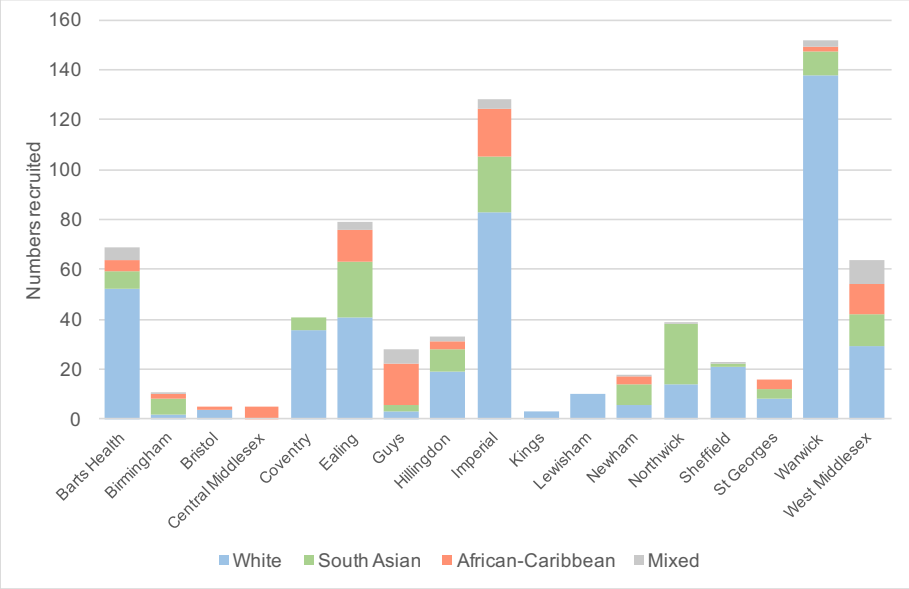
From February 2014 to November 2016. Numbers at the end of each bar are number of recruits per month.

4.3 Recruitment by Site

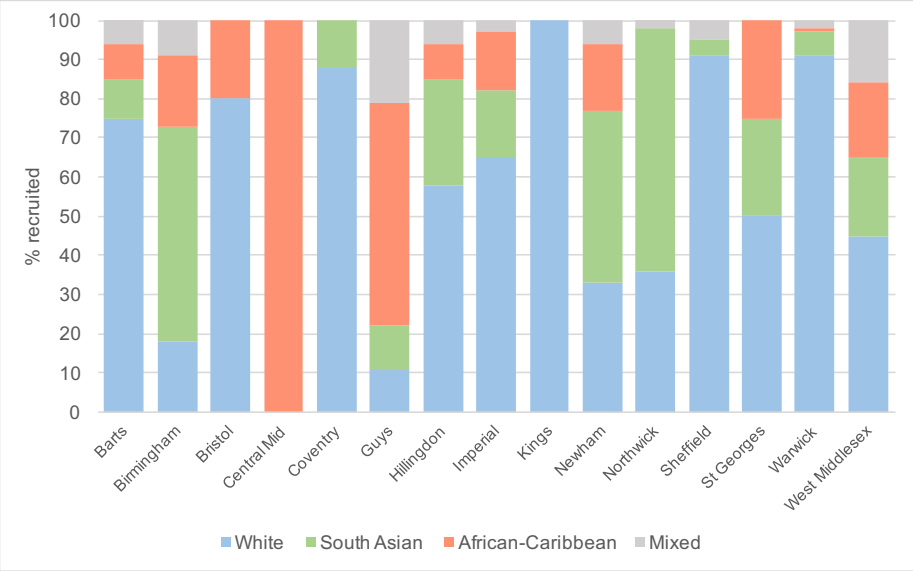
The ethnicity split of recruitment by site is depicted in figure 4.2 a) and b). At most sites, WE participants formed the largest proportion of recruits. Ethnicity also varied significantly by location, $p<0.0001$. However, the variable proportions of ethnic groups recruited mainly reflects the local population demographics. As recruitment occurred via the clinical research network, sites also had accrual targets to achieve and this was often accomplished early on, by recruiting known research participants from other research studies.

Figure 4.2: Bar Charts Depicting a) Numbers of Recruits by Site and b) Ethnicity or Recruits at Each Site

a)



b)



4.4 Stratification of the Cohort for Genetic Testing

Due to the lag in measurement of C-peptide and antibody results, antibody and C-peptide data were available on 698 participants to enable a decision to be made about genetic testing (see table 4.2). These data were used to stratify the cohort for genetic testing and to assign, where appropriate, a biochemical or immunological diagnosis of type 1 diabetes.

The results of this process are summarised in figure 4.3.

Table 4.2: Number of participants who were stratified for genetic testing according to available C-peptide and antibody results

n	Antibody negative	Antibody positive	Antibody currently unavailable	
C-peptide <27 pmol//L	152	251	14	
C-peptide >27 pmol/L	138	119	4	
C-peptide unavailable	17	24	9	**Total= 698**

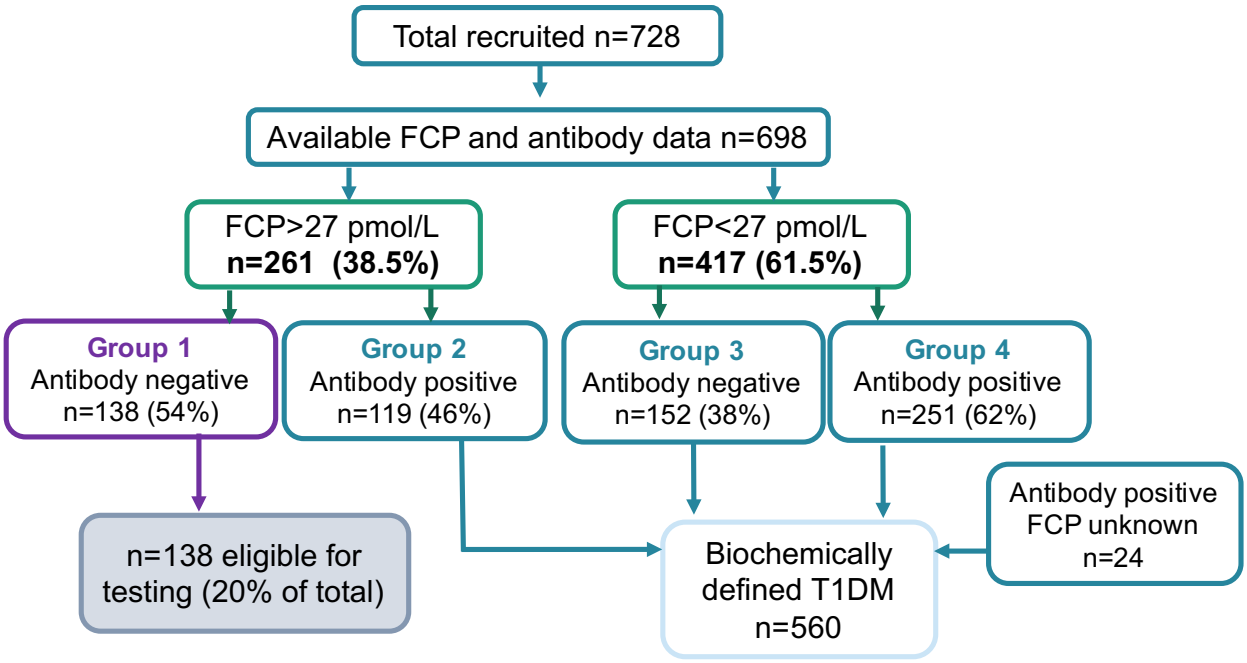
Numbers in green (bold) were included, those in black had insufficient information to make a decision about genetic testing.

A total of n=261 (38.5%) of the cohort had detectable fasting C-peptide of these, 138 individuals were antibody negative (group 1). A further n= 119 (46%) had detectable C-peptide but were antibody positive (group 2). In the n=417 with undetectable C-peptide, 38% of individuals were antibody negative (group 3). A further n=261 individuals were antibody positive, with undetectable C-peptide (group 4). Finally, 24 individuals were antibody positive, but C-peptide levels were unavailable, making a total n=560 (417 + 119 +24) individuals, classified as type 1 diabetes.

4.5 Stratification for Genetic Testing, by Ethnic Group

Participants were selected for genetic testing if they were antibody negative and had a detectable C-peptide (C-peptide>27 pmol/L).

Figure 4.3: Flowchart depicting stratification of cohort to determine eligible group for genetic testing



C-peptide, FCP; T1DM, type 1 diabetes.

Significant differences were observed in the proportions of people eligible for genetic testing using these criteria, by ethnic group (see table 4.3).

In the WE group, the majority of individuals (89%) did not meet the criteria. In contrast the south Asian and African-Caribbean groups had significantly more participants that met the criteria, 38% and 42% respectively (p<0.0001 vs WE).

Table 4.3: Percentage of participants meeting the criteria for genetic testing by ethnic group

	WE	SA	AC	Mixed
Does not meet criteria	408 (89%)	78 (62%)	43 (58%)	27 (77%)
Meets criteria	51 (11%)	48 (38%)	31 (42%)	78 (23%)
p - value versus WE		p<0.0001	p<0.0001	

Criteria for testing = C-peptide>27 pmol/L & antibody negative. WE, white European; SA, south Asian; AC, African-Caribbean. p value on Fisher's exact test

4.6 Characteristics of Group1: C-peptide positive, antibody negative

The criteria for genetic testing were met by 20% of individuals and as discussed above, proportions varied significantly by ethnic group. Biochemical and clinical characteristics were then compared by ethnic group in this cohort and are presented in table 4.4 and 4.6.

4.6.1 Clinical Characteristics

Although age at diagnosis did not vary significantly across ethnic groups, age at recruitment was significantly lower the AC group, median 23.9 years (inter-quartile range, IQR 19.1-42.6 years) than the WE ethnic group, 35.6 years (28.6 -47.7). Due to the younger age at diagnosis, duration was also significantly shorter in the AC group (see table 4.4).

BMI did not differ between the three ethnic groups, although SA participants had the lowest median BMI within the cohort at 27 kg/m^2. The SA BMI observed, according to updated WHO BMI cut-offs was in the 'overweight' category, whereas the WE BMI in the obese category. No significant differences were observed in waist circumference and waist-to-hip ratio across ethnicities in male or female participants. However, by established cut-offs all median waist circumferences and wait-to-hip ratios measured were in the high risk categories(209). BMI distribution is further examined in the next section (4.6.2).

Table 4.4: Clinical characteristics of individuals eligible for genetic testing

Parameter	WE	SA	p-value *	AC	p-value **	p-value ***
n	52	48		31		
Age at diagnosis (years)	20 (18-27)	22 (17-27)	0.724	18 (13.9 – 24)	0.136	0.196
Duration (years)	14.1 (6.2-24.6)	10.9 (2.5 – 21)	0.262	6.6 (1.1 -15.2)	**0.011**	**0.045**
Current age (years)	35.6 (28.6 – 47.7)	33.7 (22.8 -46.4)	0.240	23.9 (19.1 – 42.6)	**0.002**	**0.009**
BMI[#] (kg/m^2)	30.1 (23.8-35.8)	27 (22.7 -31.8)	0.055	28.3 (25.4-36.8)	0.832	0.098
Female waist circumference(cm)	106 (85-118)	93.9 (85-98)	0.088	92 (83-116)	0.663	0.273
Female waist:hip	0.88 (0.84-0.94)	0.9 (0.85-0.96)	0.493	0.9 (0.82-0.97)	0.824	0.813
Male waist circumference(cm)	99 (83-120)	101 (91-108)	0.737	90 (83.5-98)	0.443	0.421
Male waist:hip	0.97 (0.88-1.0)	0.97 (0.93-1.0)	0.308	0.9 (0.87-1.01)	0.737	0.278
% Parent affected	60%	89%	**0.001**	79%	0.078	0.004
% Currently not on insulin	33%	29%	0.703	39%	0.578	0.678
% Diagnosed <25 years	59%	55%	0.790	77%	0.090	0.147

*WE, white European; SA, south Asian; and AC, African-Caribbean. [#]BMI in people currently aged ≥16 years. Significant p-values in bold. *p-value WE vs SA; ** p-value WE vs AC; *** p-value WE vs SA vs AC.*

4.6.2 BMI Analysis by Cut-Offs

To further examine the impact of ethnicity on BMI, the group 1 cohort was split according to established BMI cut-offs. No significant differences were observed by World Health Organisation BMI category (see table 4.5), with equal proportions across lean, overweight and obese categories.

Table 4.5: BMI categories in people eligible for genetic testing

BMI category (kg/m^2)	WE	SA	AC	
18.5 -24.9	27.5%	36.4%	19.2%	p=0.451
25.0-29.9	19.6%	29.6%	34.6%	
>30	51.0%	31.8%	42.3%	

WE, white European; SA, south Asian; AC, African-Caribbean

When cut-offs were adjusted for Asian ethnicity, categories advised by the WHO in 2005, no additional differences were observed by ethnicity (see table 4.6).

Table 4.6.3: Ethnic-specific BMI categories in people eligible for genetic testing

Weight Category	WE*	SA**	AC*	
Lean	28%	23.3%	20.0%	
Overweight	20%	16.3%	36.0%	
Obese	52%	60.5%	44.0%	p=0.371

*Categories used correspond to standard WHO cut-offs (210) and south Asian**, specific cut-offs: lean 18.5-22.9, overweight 23-27.5 and obese >27 kg/m^2(35). WE, white European; SA, south Asian; AC, African-Caribbean*

4.6.4 Traditional Criteria to Diagnose MODY

Similar proportions of participants who were currently not insulin treated, were observed across the 3 ethnic groups. The proportions of individuals diagnosed before the age of 25 years was also not significantly different, however the AC group had a higher proportion, 77% vs 58% in the WE group.

Of note, the percentage with a parental history of diabetes was significantly higher in the SA group (89% versus 59% WE, p=0.001) and non-significantly higher in the AC group (79.3 vs 59% WE, p=0.068).

4.6.5 Biochemical Characteristics

Biochemical parameters were also compared by ethnicity in the group eligible for genetic testing (group 1) and are depicted in table 4.7.

Table 4.7: Biochemical results in people eligible for genetic testing

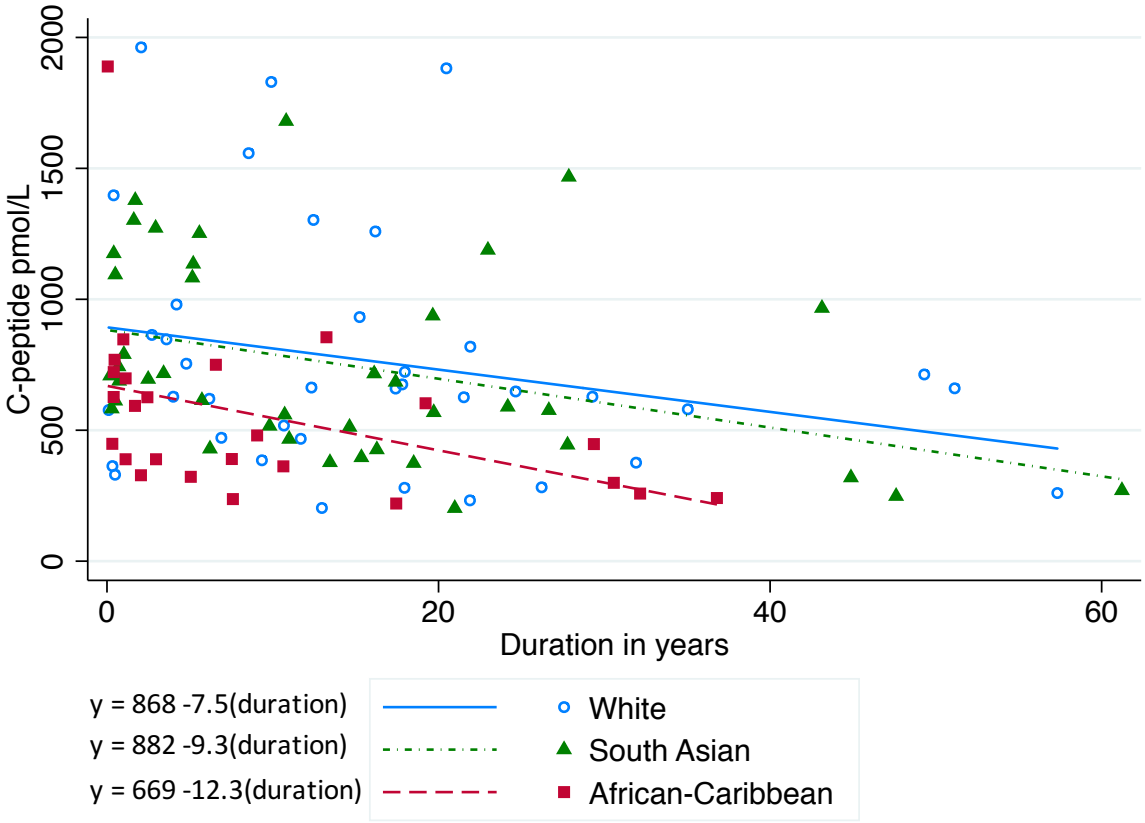
Parameter	WE	SA	p-value *	AC	p-value **	p-value ***
n	51	48		31		
HbA1c (mmol/mol)	58 (46-76)	58 (51-72)	0.883	73 (53-103)	0.066	0.126
Total cholesterol mmol/L	4.1 (3.5-5)	4.6 (3.5 -5.3)	0.375	4.3 (3.7 -5.1)	0.550	0.638
HDL cholesterol mmol/L	1.2 (0.99-1.4)	1.1 (1.0-1.3)	0.288	1.34 (1.1-1.6)	0.236	0.138
Triglycerides mmol/L	1.5 (0.93-2.2)	1.5 (1.0-1.9)	0.863	0.83 (0.6-1.2)	**0.0004**	**0.0002**
LDL cholesterol mmol/L	2.3 (1.6-2.9)	2.5 (1.7-3.2)	0.466	2.5 (2.2-3.4)	0.118	0.284
C-peptide (pmol/L)	591 (218-787)	600 (392-1024)	0.276	389 (237 -627)	0.275	0.082
C-peptide if >200 pmol/L	653 (469 -898)	684 (444-1094)	0.978	448 (322-698)	**0.022**	**0.032**
% with C peptide between 27-200	22	10.4		16		
Glucose mmol/L	9.6 (6.8-12.4)	7.9 (6.4-10.6)	0.141	8.7 (6-13.2)	0.615	0.376

*WE white European, SA south Asian and AC African-Caribbean, Significant p-values in bold. *p-value WE vs SA; ** p-value WE vs AC; *** p-value WE vs SA vs AC.*

HbA1c was similar across ethnic groups, but absolute values were higher in the AC group, though did not reach statistical significance. With the exception of lower triglyceride levels in the AC group compared to WE (0.83 vs 1.5 mmol/L), no other significant differences in lipid profiles were observed. When examining C-peptide in the sufficient range (>200 pmol/L), the median AC C-peptide was found to be significantly lower than the WE group (448 vs 653 pmol/L, p-value 0.022).

C-peptide levels were further examined in a scatter plot, by duration. As shown in figure 4.4, AC C-peptide levels appeared lower for any given duration than WE and SA ethnic groups.

Figure 4.4: Scatter plot of C-peptide (where C-peptide is ≥200pmol/L), by ethnicity with regression lines

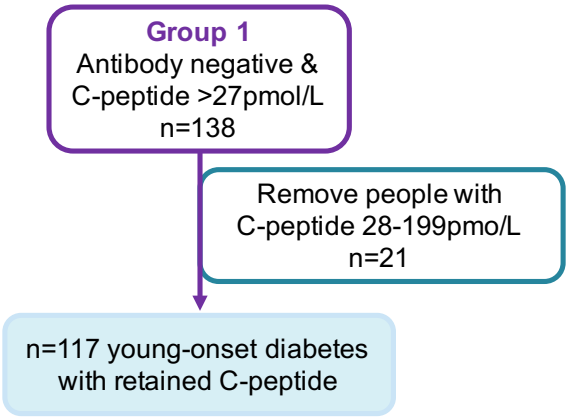


4.7 Predictors of C-peptide in Antibody Negative People with Preserved C-peptide

To further assess the impact of ethnicity on C-peptide levels, multiple variable regression was undertaken to ascertain independent predictors of the C-peptide levels observed in this cross-sectional dataset.

The people eligible for genetic testing, thus far described, were sub-categorised to exclude people with C-peptide levels between 27-199 pmol/L. This cut-off was applied in an effort to exclude people with long-duration type 1, such that a group of individuals with retained C-peptide production and in whom antibodies were negative, was created (see figure 4.5).

Figure 4.5: Schematic illustrating features of the group labelled antibody negative young-onset diabetes with retained C-peptide



The 21 individuals who were removed from the analysis group, had longer durations of diabetes - 16.8 years (IQR, 7.6-30.8 years) - than the 117 individuals with higher C-peptides, 10.7 years (IQR, 2.5 -20.1 years), but this was not statically significant (p=0.08). BMI was also not statically significantly different between the low C-peptide group, 26.2 (22.2 – 31.6 kg/m^2) and higher C-peptide group, 28.1 (23.4-33.9 kg/m^2) (p=0.59). Although neither reached significance, the finding of the longer durations and lower BMI's in the 21 individuals who were removed, is supportive of them being people with long-duration type 1 diabetes. However, it cannot be conclusively proven that this approach has completed mitigated against the inclusion of people with long-duration type 1 diabetes.

The clinical and biochemical features of this revised group with C-peptides ≥200 pmol/L are displayed in tables 4.8 and 4.9.

Table 4.8: Clinical characteristics of individuals eligible for genetic testing with C-peptide ≥ 200 pmol/L

Parameter	WE	SA	p-value *	AC	p-value **	p-value ***
n	41	43		26		
Age at diagnosis (years)	20.5 (18-27)	22.5 (17-27)	0.922	20 (14.8-24)	0.279	0.443
Duration (years)	12.5 (4.8-21.9)	10.8 (2.5 -19.7)	0.427	5.1 (19.7-43.0)	0.026	0.091
Current age (years)	34.8 (28.6 - 44.7)	31.8 (22.7-46.3)	0.403	24.3 (19.7-42.9)	0.012	0.059
BMI[#] (kg/m^2)	31.5 (23.4-35.4)	27.2 (23.7-32.1)	0.120	28.3 (25.4-35.1)	0.897	0.239
Female waist circumference(cm)	106 (86-114)	94 (85-98)	0.097	91 (81-111)	0.4096	0.277
Female waist:hip	0.89 (0.85-0.94)	0.9 (0.85-0.96)	0.672	0.91 (0.80-0.98)	0.8852	0.889
Male waist circumference(cm)	97 (85-119)	103 (91-114)	0.843	90 (85.8-107.3)	0.7170	0.640
Male waist:hip	0.98 (0.88-1.0)	0.98 (0.92-1.1)	0.331	0.92 (0.88-1.1)	0.8280	0.480
% Parent affected	69	90	0.016	83	0.212	0.049
% Currently not on insulin	41	33	0.398	44	0.857	0.706
% Diagnosed <25 years	55	55	0.983	76	0.088	0.169

*WE white European, SA south Asian and AC African-Caribbean. [#]BMI in people currently aged >16 years. Significant p-values in bold. *p-value WE vs SA; ** p-value WE vs AC; *** p-value WE vs SA vs AC.*

Table 4.9: Biochemical characteristics of individuals eligible for genetic testing with C-peptide ≥ 200 pmol/L

Parameter	WE	SA	p-value *	AC	p-value **	p-value ***
n	41	43		26		
HbA1c (mmol/mol)	55.5 (45-77)	58 (47-71)	0.849	74 (56-104)	0.031	0.068
Total cholesterol mmol/L	4.2 (3.5-5.2)	4.6 (3.8-5.4)	0.521	4.4 (3.8-5.1)	0.827	0.787
HDL cholesterol mmol/L	1.2 (0.9-1.4)	1.1 (1.0-1.3)	0.934	1.3 (1.1-1.6)	0.093	0.168
Triglycerides mmol/L	1.7 (1.1-2.3)	1.5 (1.1-1.9)	0.427	0.9 (0.6-1.2)	0.0001	**0.0001**
LDL cholesterol mmol/L	2.4 (1.8-3.0)	2.6 (1.8-3.2)	0.745	2.5 (2.2-3.4)	0.348	0.6092
C-peptide (pmol/L)	648 (467-864)	684 (444-1094)	0.826	448 (322-698)	0.038	**0.042**
Glucose mmol/L	8.3 (6.2-12.8)	7.8 (6.4-10.7)	0.568	8.4 (5.8-14.5)	0.812	0.871

*WE white European, SA south Asian and AC African-Caribbean. #BMI in people currently aged >16 years. Significant p-values in bold. *p-value WE vs SA; ** p-value WE vs AC; *** p-value WE vs SA vs AC.*

4.7.1 Linear Regression Analysis

The effect of ethnicity on C-peptide levels was first assessed using univariate linear regression, assessing the interaction between C-peptide levels and ethnic group, in people with antibody negative young-onset diabetes with retained C-peptide.

On linear regression analysis, African-Caribbean ethnicity was significantly associated with a 225 pmol/L reduction in C-peptide levels as compared to the white European group (p=0.026). South Asian ethnicity did not significantly associate with C-peptide levels as compared to white ethnicity. In total, ethnicity accounted for 3.5% of the variance observed in C-peptide levels (adjusted r^2 0.035, p-value= 0.05).

Further univariate analyses were undertaken to determine the other significant predictors of C-peptide. The following variables showed significant associations with C-peptide levels; current age, duration, BMI, ethnic-specific BMI weight category,

waist:hip and whether currently insulin-treated. Glucose, HbA1c, parental history of diabetes and age at diagnosis were not significant predictors.

Duration, ethnic-specific weight category and insulin-treatment status were incorporated into a multi-variable linear regression model, to determine whether the effect on C-peptide seen in African-Caribbean ethnicity was explained by these other independent predictors. Current age was left out of this model, as it demonstrated co-linearity with duration. Results are shown in table 4.10.

Table 4.10: Multivariable linear regression of independent predictors of C-peptide levels

Independent predictor of C-peptide (n=105 observations, breakdown where states))	Coefficient	t-statistic	p-value
Ethnic group compared to white (n=39)			
South Asian (n=41)	-44	-0.53	0.597
African-Caribbean (n=25)	-263	-2.71	0.008
Weight category compared to lean (n=27) * ethnicity adjusted			
Overweight* (n=22)	+187	1.65	0.102
Obese* (n=56)	+271	2.79	0.006
Waist:hip (≥0.9 male, ≥0.85 female)	+144	1.49	0.140
Duration (years)	-9	-3.33	0.001
On insulin treatment vs no insulin	-211	-2.83	0.006
Multivariable model: adjusted r^2 = 0.27, p-value <0.0001			

Coefficient represents magnitude and direction of change in C-peptide level. The lean, overweight and obese categories outlined, corresponded to ethnic specific cut-offs. P values in bold, significant.

As C-peptide was not normally distributed, multiple variable regression was repeated on log transformed data and the same significant results were ascertained. To aid interpretation, non-log transformed data are presented.

With incorporation of other independent predictors, African-Caribbean ethnicity was still associated with a reduction in C-peptide levels, as compared to white European ethnicity. The magnitude of this effect was, however, increased compared to the

univariate model (271 pmol/L reduction, p-value = 0.006). Significant negative predictors also included duration of diabetes; a 9 pmol/L reduction in C-peptide per year of diabetes duration (p=0.001) and insulin treatment; a 211 pmol/L reduction in C-peptide in people who were insulin treated versus those who were not. With the incorporation of BMI, the significance observed in waist:hip ratio using univariate models, was lost. In total this model accounted for 27% of the variance observed in C-peptide levels in the cohort.

4.8 Genetic Testing Results in People Eligible for Testing

Of the 138 participants eligible for genetic testing, results were available at the time of writing on n=82. This lag represents the 4-month turnaround time of next generation sequencing and the cumulative delays in collating C-peptide and antibody results, to be able to schedule genetic testing.

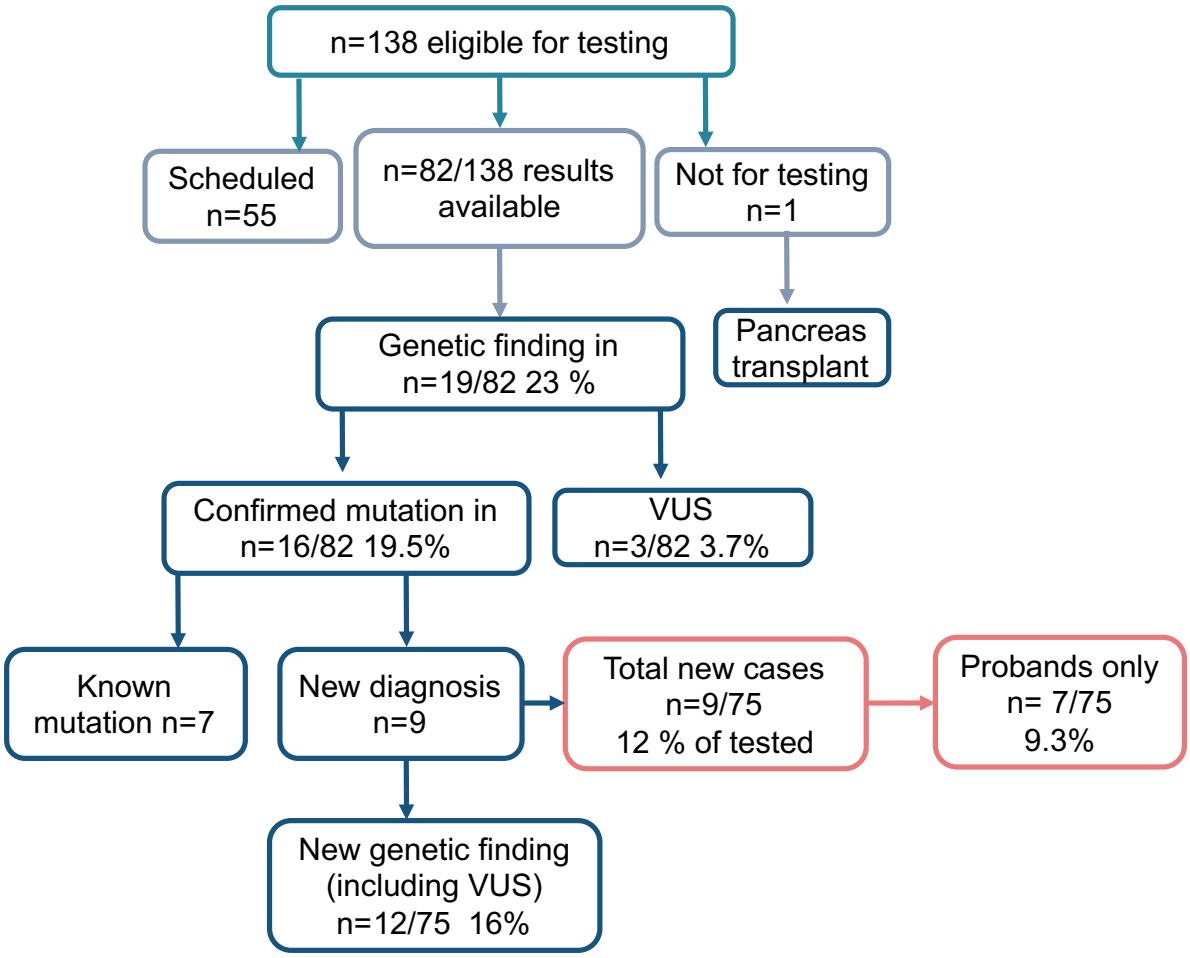
4.8.1 Frequency of Genetic Findings by Ethnic Group

The overall prevalence of a genetic finding in the 82 individuals tested was 19/82, 23%. Of these, 7 people had known MODY mutations and 3 variants of unknown significance. Therefore, the number of new cases of MODY found in the cohort was n=9/75, accounting for 12% of those tested. See figure 4.6.

Three of the individuals were members of the same kindred, therefore on examination of probands only, the MODY detection rate was 7/75 cases tested, 9.3%. One individual was eligible for testing based on C-peptide and antibody status, but was not scheduled for testing as they had undergone a simultaneous pancreas and kidney transplant for type 1 diabetes and renal failure, making their C-peptide value potentially uninterpretable.

Although the cohort hasn't been completely sequenced, Chi square analysis was undertaken to examine if any of the differences across ethnic groups were statistically significant. The mixed group was not included in this analysis due to the small numbers and as the study was not powered to detect changes in this group. The frequencies of MODY in each ethnic group are depicted in table 4.11.

Figure 4.6: Flowchart illustrating numbers of individuals tested for MODY and numbers with mutations detected



VUS, variant of unknown significance

Using the entire cohort recruited as the denominator, the overall prevalence of MODY mutations was 16/698 (2.3%) in those with data enabling classification. With the known cases removed (n=7) and focussing only on probands, the overall prevalence was 1.0% (7/689).

Table 4.11: Breakdown of genetic testing results by ethnic group

Result	WE	SA	AC	p-value
Eligible for testing	51	48	31	
Scheduled	19	20	10	
Results available	32	28	21	
No mutation found	24	20	15	
Confirmed mutation	7	4	4	
VUS	1	2	0	0.497
New cases only (known MODY removed)				
Result	WE	SA	AC	
No mutation found	24	22	17	
New mutation found	1	3	4	
VUS	1	2	0	0.363
Total	26	27	21	
New proband cases only				
No mutation found	24	22	17	
New mutation found	1	3	2	
VUS	1	2	0	0.617
Total	26	27	19	
Pick-up rate (new cases)	4%	12%	10.5%	0.572

Numbers scheduled for testing provided for information. Chi square analysis relates to the group that has reported genetic testing results only. VUS, variant of unknown significance. WE, white European SA, south Asian & AC African-Caribbean.

In people of mixed ethnicity, 7 people identified were eligible for genetic testing. Thus far 1 has been tested for MODY and a confirmed mutation was found.

4.8.2 Confirmed MODY Mutations Identified

The individuals with genetic findings are summarised in table 4.12 known MODY cases and 4.13 newly identified MODY during the study and the clinical details of the

new MODY cases are outlined in more detail in table 4.14. Newly identified MODY mutations were found in all of the ethnic groups studied. None of the identified mutations were novel and all had been reported previously.

Table 4.12: Known MODY Cases Recruited; Respective Nucleotide, Protein Effects and Predicted Pathogenicity

Case	Gene	Exon	Genotype	Nucleotide position	Amino acid change	Effect	Predicted pathogenicity	Previously published
IC-005	HNF1A	2	Heterozygous	c.811C>T	Ala116Val	Missense	Pathogenic	Klupa et al. 2002 (211)
IC-006	HNF4A	7	Heterozygous	c.802C>T	Gln268Ter (p.Q268*)	Nonsense	Pathogenic	Yamagata et al. 1996 (135,212)
IC-060	HNF4A	8	Heterozygous	c.869G>A	Arg290His	Missense	Pathogenic	Pearson et al. 2007 (213)
IC-066	HNF1A	1	Heterozygous	c.42_51delins TG	Ala15 frameshift	10 nucleotide deletion and insertion resulting in premature stop codon	Pathogenic	Colclough et al. 2013 (214)
IC-070	GCK	2	Heterozygous	c.128_133del	Arg43_Gly44del	In frame deletion of 2 amino acids	Likely pathogenic	Novel
IC-107	GCK	2	Heterozygous	c.446C>T	Thr149Ile	Missense	Pathogenic	Unpublished
WH-051	HNF1A	2	Heterozygous	c.427C>T	His143Tyr	Missense	Pathogenic	Colclough et al. 2013 (214)

Table 4.13: New MODY Cases Identified; Respective Nucleotide, Protein Effects and Predicted Pathogenicity

Case	Gene	Exon	Genotype	Nucleotide position	Amino acid change	Effect	Predicted pathogenicity	Previously published
BL-002	GCK	5	Heterozygous	c.491T>C	Leu164Pro	Missense	Pathogenic	Nam et al 2000 Diabetes Res Clin Pract 50: 169-176(215)
IC-033	Mitochondrial	N/A	N/A	Mt.3243A>G	N/A	N/A	Pathogenic	Goto et al 1990 Nature 348: 651-3(216)
IC-118	Mitochondrial	N/A	N/A	Mt.3243A>G	N/A	N/A	Pathogenic	Goto et al 1990 Nature 348: 651-3(216)
IC-088	HNF1B	N/A	Heterozygous	N/A	N/A	Whole gene deletion	Pathogenic	Bellane-Chantelot et al. 2005 Diabetes 54: 3126-32(217)
GH-007	HNF1A	2	Heterozygous	c.431T>C	Leu144Pro	Missense	Pathogenic	Colclough et al 2013 Hum Mutat 34: 669-685(214)
EH-037	ABCC8	38	Heterozygous	c.4610C>T	Ala1537Val	Missense	Pathogenic	Kapoor et al 2011 Diabetologia 54: 2575-2583(218)
EH,004, 015, 016	ABCC8	37	Heterozygous	c.4547C>T	Thr1516Met	Missense	Pathogenic	Banerjee et al 2011 Eur J Endocrinol 164: 737-740(219)

Table 4.14: Clinical Description of New MODY Cases Identified

AAR, Age at diagnosis; AAR age at recruitment; Rx , treatment; [#]proband

Reported Ethnicity	Sex	Gene / mutation	Initial Diagnosis	AAD	AAR	Initial Rx	Rx at visit	BMI	C-pep pmol/L	HbA1c mmol/moL	Family members with diabetes
Polish[#]	F	GCK, Leu164Pro	Type 2	10	31	Insulin	Diet	22	232	47	Mother
British[#] Pakistani	F	Mitochondrial, A3243G	Type 1	17	23	Insulin	Insulin	21	614	72	Mother and maternal grandparents
Caribbean[#]	F	Mitochondrial, A3243G	Type 1	22	24	Insulin	Insulin	26	593	51	Paternal grandfather
British Indian[#]	F	HNF1B, Whole gene del	Type 2	25	36	Metformin	Metformin	19	466	61	Nil
Mixed white /black[#]	F	HNF1A, Leu144Pro	Type 1	19	32	Tablets 9 months	Insulin	32	48	64	Mother borderline, maternal grandfather T2DM
Indian[#]	M	ABCC8, Ala1537Val	Type 1	26	70	Tablets 2 months	Insulin	23	319	70	Mother, 2 brothers
Black[#] British	F	ABCC8, Thr1516Met	Type 2	14	21	Insulin alone 3 months	Insulin, metformin gliclazide	35	390	84	Mother and brother
Ghanaian	F	ABCC8, Thr1516Met	Type 1	24	56	Insulin	Insulin	38	258	74	Mother, maternal grand other and 2 children
Black African	M	ABCC8, Thr1516Met	Don't Know	18	29	Tablets	Metformin gliclazide	24	362	110	Mother and sister

Mutations were identified in the three most common MODY genes; *HNF1A*, *GCK* and *HNF1B*, but also in the less common genes, with two probands identified with mutations in the *ABCC8* gene.

Probands had clinician-assigned diagnoses of type 1 and type 2 diabetes and in one case, unknown. Ages at diagnosis across the new MODY cases ranged between 10 and 26 years, with a median of 18.5 years (IQR, 16-25). The median duration was 12 years but ranged between 5.7 and 45 years. The oldest participant at recruitment was 70 years of age.

BMI at recruitment ranged between 19.5 and 38 kg/m^2, with a median of 23.4 kg/m^2. Fasting C-peptide levels were between 48 and 614 pmol/L. HbA1c results were highly variable ranging between 47 and 110 mmol/moL with a median of 71mmol/L. In all cases, except the HNF1B mutation, there was an immediate family member affected.

4.9 Variants of Unknown Significance

In total, three variants of unknown significance were identified during the study. These variants are now described here in more detail.

4.9.1 VUS: *HNF1A* Ala251Thr

MD-IC-004, an identical twin was identified as having a homozygous variant in exon 4 of the *HNF1A* gene. No other changes were identified on next generation sequencing of other MODY genes. The missense change substituted an alanine for threonine at position 251.
The proband was diagnosed with diabetes at the age of 12 years and was insulin-treated since diagnosis.
His self-reported ethnicity was white British, however on further questioning, his family was originally from Turkey.

The finding of a homozygous change in the HNF1A gene was of significant interest as, prior to this finding, homozygous HNF1A mutations were thought to be embryonic

lethal. In view of this unusual finding, detailed investigations into pathogenicity were undertaken. These are presented in full in Chapter 6.

4.9.2 VUS: HNF1B Pro60Leu

The Pro60Leu variant was identified in MD-IC-025, a male south-Asian participant born in the UK, of Indian ethnicity. He was diagnosed with diabetes aged 27 years and went on to have a renal transplant aged 30 years. The proband has a clinician-assigned diagnosis of type 2 diabetes, but is lean, insulin-treated and has a fasting C-peptide level in the detectable but deficient range (165 pol/L). At diagnosis he was treated with tablets for under a year, before switching to insulin. There is a generational family history of diabetes (see figure 4.7). Next generation sequencing of all known MODY genes revealed only the p.Pro60Leu variant in *HNF1B.*

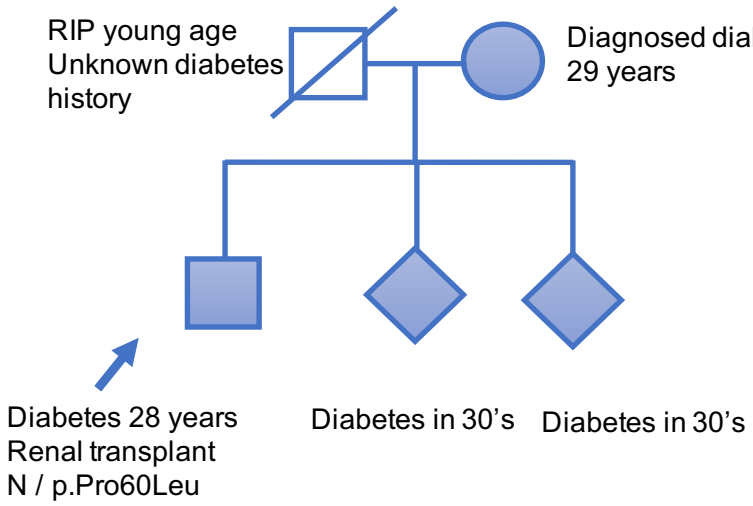
The variant p.Pro60Leu (c.179C>T) is a heterozygous variant of uncertain significance, resulting in a leucine for proline substitution in exon 1 of the *HNF1B* gene (see figure 4.7). Though listed in the Exome Aggregation Consortium (ExAC) database (220) and found in 1/8251 south Asian individuals, the mean allele frequency (MAF) was 0.006%, demonstrating its low population frequency. Two further variants at this amino acid position are listed in the ExAC database with low frequency; p.Pro60Ser identified as heterozygous in 1/8251 south Asian individuals (MAF 0.006%) and p.Pro60Arg identified as heterozygous in 6/ 32774 European individuals (MAF 0.001%). *In silico* predictions of pathogenicity of the *p.Pro60Leu* variant were inconclusive and revealed a variant of uncertain significance (see table 4.15). The Pro60Leu variant has not been previously published.

A different variant at the same amino acid residue, p.Pro60Ser, was identified in another south Asian individual diagnosed with diabetes aged 11 years, insulin treated from diagnosis with a strong generational family history but without renal complications. It is possible that this is therefore a south-Asian specific variant / phenotype. In addition, variants at amino acid residues in a similar position have been shown to associate with an *HNF1B* renal and diabetes phenotype, e.g. p.Val61Gly(221).

There is a strong clinical case to be made for the pathogenicity of the p.Pro60Leu variant and further functional assays are required to determine if the variant is pathogenic.

Figure 4.7: a) Pedigree of the proband with the HNF1B Pro60Leu variant and b) position of the variant in the HNF1B gene

a)



b)

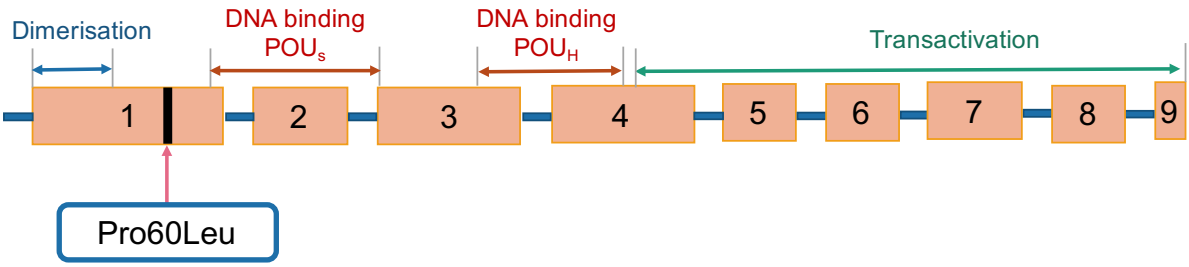


Table 4.15: Summary of In Silico Investigations into the Pathogenicity of the HNF1B VUS Pro60Leu

P60L (p. Pro60Leu)	Overall: Variant of unknown significance
Mutation Database searches (HGMD professional)	Not previously reported
Sequence Variant Database	Listed in ExAc in south Asian (MAF 0.006%). Not listed in Exome Variant Server or 1000 genomes
Amino Acid Change	Proline (non-polar) to Leucine (non-polar)
Species conservation	Conserved in 18 out of 19 orthologs, including 10 mammalian orthologs
In silico prediction	Likely pathogenic: SIFT, AlignGVGD & Grantham Distance Uncertain: PolyPhen2
Cryptic Splice Site	Not predicted to create splice site

ExAc Exome Aggregation Consortium

4.9.3 VUS: HNF1A Pro379Ser

MD-EH-008 is a 42-year-old female participant of Indian ethnicity, born in Malaysia. She was diagnosed with diabetes during her second pregnancy at the age of 27 years. She was initially treated with metformin, but subsequently switched to insulin. Both parents have been diagnosed with type 2 diabetes and her brother died from complications associated with type 1 diabetes at the age of 35 years, having been diagnosed at 25 years.

She has a BMI of 26.8 kg/m^2 and her C-peptide was 396 pmol/L with an HbA1c of 80 mmol/mol.

The variant Pro379Ser (c.1135C>T) results in a serine for proline substitution in exon 6 of the *HNF1A* gene, which corresponds to the transactivation domain (see table 4.15 for *in silico* predictions). Though listed in ExAC and found in 7/8205 south Asian individuals, the mean allele frequency (MAF) was 0.043% in south Asian people and 0.0059% in all ethnicities studied (see table 4.16 for summary of investigations).

The variant had been published previously as a MODY causing mutation(100), however one of the key issues questioning its pathogenicity was it's finding in another south Asian individual sequenced by the Exeter molecular genetics team. This individual had a heterozygous ABCC8 mutation causing congenital hyperinsulinism

but neither the individual nor family members had diabetes, suggesting the variant to be benign (unpublished).

Table 4.16: Summary of In Silico Predictions into Pathogenicity of the Pro379Ser Variant

P379S (p. Pro379Ser)	Overall: Variant of unknown significance
Mutation Database searches (HGMD professional)	Previously published
Sequence Variant Database	Listed in ExAc: Heterozygous in 7/8205 South Asian individuals. MAF 0.043% in south Asian, 0.0059% in all. Not listed in Exome Variant Server, 1000 genomes or dbSNP.
Amino Acid Change	Proline nonpolar, neutral to Serine polar, neutral
Species conservation	Conserved in 20 out of 20 orthologs
In silico prediction	Overall: Uncertain Align=GVGD: Likely benign SIFT: Likely pathogenic PolyPhen2: Likely pathogenic Grantham Distance: Likely benign
Cryptic Splice Site	Not predicted to cause aberrant splicing.

ExAc, Exome Aggregation Consortium; dbSNP, database of single nucleotide polymorphisms

Other variants at the same amino acid position have been identified, however they are also present in the ExAc database at higher frequencies than would be expected for a MODY causing mutation. This includes Pro379Arg, which has been published as a MODY causing mutation (222,223). Additionally, Pro 379Ala (c.1135C>G), previously published (100)has a mean allele frequency (15/118870) much higher than expected and functional studies published that showed the variant has no significant effect on transactivation of several promoters(224).

Further work is planned to help establish pathogenicity of this variant, however at present it has been classified as a variant of unknown significance and this result has been conveyed to the clinician and participant.

4.10 Polymorphisms Identified

In addition to the mutations and variants of unknown significance outlined, a number of benign variants were also identified in sequenced participants. These variants were classified as benign using UK based guidance(185). These are summarised in table 4.17 and provided for information.

Table 4.17: Benign variants identified, coding effects and locations in individuals sequenced as part of the study

cDNA, complementary DNA; 'p.=' no change in protein; 'p.?' unknown effect on protein

Gene	cDNA nomenclature	Protein nomenclature	Coding effect	Variant type	Frequency	Location
ABCC8	NM_001287174.1:c.2394G>C	p.=	synonymous	substitution	1	Exon 20
CEL	NM_000545.5:c.519G>A	p.=	synonymous	substitution	1	Exon 2
	NM_001807.4:c.1510G>C	p.Asp504His	missense	substitution	1	Exon 11
	NM_001807.4:c.1742A>G	p.Glu581Gly	missense	substitution	1	Exon 11
	NM_001807.4:c.1744A>G	p.Thr582Ala	missense	substitution	1	Exon 11
	NM_001807.4:c.1747G>C	p.Ala583Pro	missense	substitution	1	Exon 11
	NM_001807.4:c.1810G>C	p.Ala604Pro	missense	substitution	2	Exon 11
	NM_001807.4:c.1822C>A	p.Pro608Thr	missense	substitution	1	Exon 11
	NM_001807.4:c.1828A>C	p.Thr610Pro	missense	substitution	1	Exon 11
	NM_001807.4:c.1855C>G	p.Pro619Ala	missense	substitution	1	Exon 11
	NM_001807.4:c.1975G>C	p.Ala659Pro	missense	substitution	2	Exon 11
	NM_001807.4:c.2143G>A	p.Ala715Thr	missense	substitution	1	Exon 11
	NM_001807.4:c.2212C>A	p.Pro738Thr	missense	substitution	1	Exon 11
	NM_000545.5:c.519G>A	p.=	synonymous	substitution	1	Exon 2
	NM_000545.5:c.1015G>A	p.Gly339Ser	missense	substitution	1	Exon 5
HNF1A	NM_000545.5:c.1310-12C>T	p.?	unknown	substitution	3	Intron 7
	NM_000545.5:c.1502-29_1502-21del	p.?	unknown	deletion	1	Intron 8
	NM_175914.4:c.-129T>C	p.=	synonymous	substitution	1	Upstream 1
	NM_000458.3:c.244G>A	p.Asp82Asn	missense	substitution	1	Exon 1

	NM_000208.2:c.653-7_653-4del	p.?	unknown	deletion	1	Intron 3
	NM_000208.2:c.653-30_653-26del	p.?	unknown	deletion	1	Intron 3
HNF4A	NM_000208.3:c.653-7del	p.?	unknown	deletion	1	Intron 3
HNF1B	NM_000208.3:c.653-9del	p.?	unknown	deletion	1	Intron 3
INSR	NM_000208.2:c.2243C>T	p.Ser748Leu	missense	substitution	1	Exon 11
	NM_000208.2:c.2575C>T	p.His859Tyr	missense	substitution	1	Exon 13
	NM_000208.2:c.2946-14T>C	p.?	unknown	substitution	1	Intron 16
	NC_012920.1:n.MT-TL1:-20C>T	p.TL1:=		substitution	1	Upstream 1
	NM_000209.3:c.296C>A	p.Pro99His	missense	substitution	1	Exon 1
	NM_000209.3:c.670G>A	p.Glu224Lys	missense	substitution	1	Exon 2
	NM_000209.3:c.726_728dup	p.Pro244dup	In-frame	duplication	1	Exon 2
MT-TL1	NM_002666.4:c.1442G>A	p.Arg481His	missense	substitution	1	Exon 9
PDX1	NM_002691.3:c.13C>T	p.Arg5Trp	missense	substitution	1	Exon 2
	NM_002691.3:c.208G>A	p.Val70Ile	missense	substitution	1	Exon 3
	NM_002691.3:c.1276G>A	p.Gly426Ser	missense	substitution	1	Exon 11
PLIN1	NM_002691.3:c.1489C>T	p.=	synonymous	substitution	1	Exon 12
POLD1	NM_002691.3:c.1694A>C	p.His565Pro	missense	substitution	1	Exon 14
	NM_002691.3:c.2718-5C>T	p.?	unknown	substitution	1	Intron 22
	NM_002691.3:c.3219-25_3219-20dup	p.?	unknown	duplication	1	Intron 27
	NM_001134665.2:c.349-17dup	p.?	unknown	duplication	1	Intron 4
	NM_006005.3:c.505G>A	p.Glu169Lys	missense	substitution	1	Exon 5
	NM_006005.3:c.599T>C	p.Leu200Pro	missense	substitution	1	Exon 5
	NM_006005.3:c.1675G>A	p.Ala559Thr	missense	substitution	1	Exon 8

TRMT10A	NM_006006.3:c.2185G>A	p.Asp729Asn	missense	substitution	1	Exon 8
WFS1	NM_001109809.2:c.1118C>G	p.Ser373Cys	missense	substitution	1	Exon 4
ZFP57	NM_001109809.2:c.1118C>G	p.Ser373Cys	missense	substitution	exon	Exon 4

4.11 Clinical and Biochemical Characteristics of People Without Mutations

Finally, the characteristics of the cohort was studied with known and new MODY cases removed, which resulted in a group of individuals in whom the differential diagnoses were; young-onset type 2 diabetes, MODY in a novel gene as yet unidentified or antibody negative type1 diabetes with residual beta-cell function. See table 4.18 and 4.19. Similar findings were observed to the analysis where the whole of group 1 was included, with few significant differences by ethnicity. However, with the MODY cases removed the SA BMI became significantly lower than the WE group.

Further biochemical differences also became apparent, for example higher LDL and total cholesterol in the AC group compared to WE. C-peptide and HbA1c levels were non-significantly different.

Table 4.18: Clinical characteristics of individuals in whom no MODY mutation was found

Parameter	WE	SA	*p-value* *	AC	*p-value* **	*p-value* ***
n	21	20		15		
Age at diagnosis (years)	26 (22-27.5)	21.5 (15.5-26.5)	0.071	23.5 (18-28)	0.538	0.248
Duration (years)	13.8 (3.4-21.7)	18.0 (5.9-27.8)	0.203	11.1 (1.1-19.2)	0.551	0.207
Current age (years)	38.4 (31.6-47.3)	44.3 (27.3-56.4)	0.566	32.9 (22.9-44.8)	0.216	0.376
BMI[#] (kg/m^2)	32 (27.7-37.7)	28.6 (24.6-32.1)	**0.022**	30.9 (28.1-36.8)	0.736	0.066
Female waist circumference(cm)	110 (100-126)	97.8 (90.5-107)	0.114	99.5 (86-113.5)	0.277	0.259
Female waist:hip	0.92 (0.89-0.94)	0.94 (0.91-0.98)	0.219	0.91 (0.81-0.99)	0.884	0.506
Male waist circumference(cm)	116 (92-119)	103 (97-105)	0.404	91 (83.5-116.5)	0.405	0.556
Male waist: hip	0.99 (0.88-1.0)	0.99 (0.96-1.1)	0.284	0.94 (0.89-1.1)	0.654	0.457
% Parent affected	65	100	**0.005**	92	0.074	**0.008**
% Currently not on insulin	33	20	0.335	47	0.418	0.245
% Diagnosed <25 years	30	65	**0.027**	57	0.113	0.071

*WE white European, SA south Asian and AC African-Caribbean. [#]BMI in people currently aged >16 years. Significant p-values in bold. *p-value WE vs SA; ** p-value WE vs AC; *** p-value WE vs SA vs AC.*

Table 4.19: Biochemical characteristics of individuals without MODY mutations

Parameter	WE	SA	*p-value* *	AC	*p-value* **	*p-value* ***
n	21	20		15		
HbA1c (mmol/mol)	69 (47-79)	58 (45.5-76)	0.616	60 (39-103)	0.958	0.909
Total cholesterol mmol/L	3.85 (3.4-4.7)	4.5 (3.5-5.2)	0.379	5.0 (4.1-6.0)	**0.026**	0.076
HDL cholesterol mmol/L	1.2 (0.94-1.35)	1.1 (1.0-1.6)	0.607	1.3 (1.1-1.6)	0.114	0.391
Triglycerides mmol/L	1.7 (1.1-2.4)	1,5 (1.1-1.9)	0.636	1.1 (0.6-1.6)	0.054	0.090
LDL cholesterol mmol/L	1.9 (1.5-2.5)	2.2 (1.6-2.7)	0.949	3.3 (2.5-3.9)	**0.012**	**0.017**
C-peptide (pmol/L)	626 (518-864)	510 (259-1109)	0.433	447 (220-627)	0.095	0.262
Glucose mmol/L	11.4 (6.5-12.8)	7.9 (6.4-10.7)	0.053	7.4 (5.9-10.6)	0.192	0.149

*WE white European, SA south Asian and AC African-Caribbean. #BMI in people currently aged >16 years. Significant p-values in bold. *p-value WE vs SA; ** p-value WE vs AC; *** p-value WE vs SA vs AC.*

4.12 Discussion

In this chapter a focused analysis has been undertaken on the group eligible for genetic testing and a detailed description of genetic findings so far, has been provided.

4.12.1 Summary of key findings

The key findings from this chapter are as follows;

- The criteria for genetic testing were met by significantly more SA (38%) and AC (42%) participants than WE (11%) participants studied, p<0.001.
- Within the group who underwent genetic testing
    - 9.3% (7/75) had a new MODY mutation identified
    - The detection rate of finding new MODY case were 4% in WE, 12% in SA and 10.5% in AC and were non-significantly different.
    - All mutations found had been previously published
    - The overall MODY prevalence was 2.3% (16/698)
    - The overall frequency of new proband cases was 1% (7/689)

- Within the group eligible for testing;
    - Age at diagnosis, BMI, waist: hip ratio, % not on insulin and % diagnosed under 25 years did not vary between ethnic groups.
    - Duration and current age were significantly lower in the AC group compared to WE.
    - % With an affected parent was significantly higher in the SA group.
    - Triglyceride levels and C-peptide were significantly lower in AC individuals than WE.
- Within the group eligible for testing, examining only those with an C-peptide≥ 200pmol/L, multivariable linear regression revealed;
    - C-peptide was significantly lower in AC individuals and independent of potential confounders.
    - In addition to AC ethnicity, other predictors of C-peptide included BMI (favours higher C-peptide), insulin-treatment (favours lower C-peptide) and duration (favours lower C-peptide).
- Examining the sub-population in the group eligible for testing that had no mutation identified, the following was observed;
    - SA people without mutations had significantly lower BMI's than WE individuals.
    - SA and AC individuals were more likely to have a parent affected with diabetes than WE.
    - AC individuals had higher total cholesterol and LDL cholesterol levels than WE individuals.

In this first systematic assessment phenotyping young-onset diabetes in a multi-ethnic UK cohort, the detection rate of finding a MODY mutation was 9.3% in those tested and was not significantly different by ethnic group. The overall MODY frequency in the cohort analysed was 2.3%.

This study has identified undetected cases of MODY across all ethnic groups. It is noteworthy that all of the mutations identified have been previously published, suggesting that to date, the fewer observed cases of MODY in SA and AC people with diabetes may not entirely be due to novel mutations or mutations in novel MODY-

causing genes. Using a systematic approach with strict biochemical and immunological criteria has therefore proven to be an effective strategy across all ethnic groups at identifying MODY. Although numbers are currently small, these findings are impactful as they suggest that the lower number of MODY cases observed in people from minority ethnic groups reflects a bias is selection for referrals.

4.12.2 Pick-Up Rate of MODY Mutations and Impact of Findings

In the current analysis, no significant differences were observed in the detection rate across ethnic groups. This contrasts the analysis presented in chapter 2 (190) where the detection rate in SA people referred for testing was almost half that of the WE group (12.6% vs 29.1%). The overall detection rate observed in this analysis at 9.3% is lower than the overall 25% genetic detection rate observed in the retrospective analysis of the Exeter MODY database. This finding probably reflects differences in the screening criteria applied; the analysis in chapter 2 was based on clinician referral (likely a composite of biomarkers and clinical features stratifying for testing), whereas the current study is based on a strict cascade of biochemical criteria alone. The latter strategy will inevitably result in a broader number of individuals screened. If the hypothesis is correct, however, and there is a lower frequency of mutations in SA and AC individuals, then this may also account for the lower detection rate, but it should be noted that the lower detection rate was also observed in the WE group.

It is worth noting that the detection rate of finding a mutation was similar even though more people met the criteria for testing in the SA and AC groups. This needs to be reassessed when larger numbers have been recruited, but if this signal persists it would suggest that more MODY cases may be subsequently identified when SA and AC groups are fully recruited. It is unclear if this strategy is simply picking out the large numbers of SA and AC cases previously undetected early on, when people with atypical diabetes are referred, or whether this signal will reduce with larger numbers.

The observed detection rate is also lower than one of the key UK systematic surveys published looking at MODY detection in paediatric clinics, which found a MODY detection rate of 24% in those tested (129). However, the current study has predominantly recruited adults, where the prevalence of young-onset type 2 diabetes

is likely to be higher, thus diluting the pool of people eligible for testing, though the cut-off of 30 years in the inclusion criteria went some way to mitigate against this. Furthermore, compared to the study by Shepherd et al the criteria for testing applied in this study are more relaxed, with inclusion of a lower c-peptide cut-off (>27 pmol/L versus 0.2 nmol/mmol UCPCR, which is equivalent to a fasting serum C-peptide 80 pmol/L or stimulated C-peptide of 200 pmol/L(27)). This approach will likely have included some people with long-duration type 1 diabetes and residual beta-cell function, further diluting the detection rate. However, one of the new MODY cases detected was found to have a fasting C-peptide of 40 pmol/L, suggesting that lower levels than previously reported, may be possible(154). Of interest, this participant was also African-Caribbean and the analysis presented in this chapter has shown an independent association African-Caribbean ethnicity with lower C-peptide concentrations.

Comparing the detection rate with one of the few studies systematically studying adults with young-onset diabetes (Thanabalasingam et al (140)), the 9.3% detection rate was similar to that observed, with 11% of those with type 2 diabetes diagnosed before 45 years or 10% of those with type 1 diabetes. The lower detection rate in the current study, may reflect the mixed ethnicity population recruited. The detection rate of 9.3% is however similar to the only other study that has systematically screened for MODY in different ethnicities. In the SEARCH study, 8% (47/586) of children screened for MODY were found to have a mutation. This group comprised 20% black African-American and 31% Hispanic (130).

If this finding persists as more genetic results become available, it suggests that the reduced detection rate observed in the retrospective study outlined in chapter 2 is a result of a selection bias in referrals, as new cases of MODY were identified in all ethnic groups. It also suggests that the clinical criteria and strategies used to identify cases are not effective at picking up all cases, although this is not surprising as systematic screening for MODY is not routinely undertaken.

The current hypothesis suggests that the detection rate should be lower, based on the higher number of young-onset type 2 diabetes cases in SA and AC ethnic groups, but this is not borne out in the current data. It is possible that as more cases are recruited

the detection rate will indeed be lower, however an alternative possibility is that the hypothesis will be disproved. It may be possible that the structured screening approach to testing in this study is identifying more cases than usual in the non-white ethnic group where the clinical criteria for testing are insensitive and this is discussed in the next section or it may represent that most MODY cases have already been identified in the WE group.

The overall pickup rate including those with known MODY and those newly diagnosed in the cohort was 2.3%, but 1% with just new diagnoses in probands included. It is difficult to compare this prevalence to other studies, which have all used slightly different selection criteria. Reassuringly, this frequency is comparable to those reported from other large surveys, in the UK, US, Norway and Poland (129–132).

Based on the strict biochemical and immunological criteria used, more people in the SA and AC groups were eligible for testing than WE. This is not unexpected, as SA and AC people are reported to have more cases of young-onset diabetes, which by definition, meet the criteria for testing. However, systematically assessing a young onset diabetes cohort to determine proportions of type 1 diabetes vs non-type 1 is of interest and a novel finding as this has not been undertaken before in a UK multi-ethnic cohort. So far, this analysis has identified that SAs have a 3-fold higher and AC 4-fold higher number of participants meeting the criteria for genetic testing, compared to WE individuals diagnosed before 30 years. It remains to be seen what proportion of MODY versus young-onset type 2 diabetes, accounts for this disparity.

Though the incidence of type 1 diabetes is reportedly similar in the children of migrants to the UK, the proportion of type 2 diabetes may still be higher. This is explored further in chapter 5.

4.12.3 Clinical Characteristics of the Group Eligible for Testing and Impact on Identifying MODY

The characteristics of the group referred for genetic testing were broadly similar across ethnic groups with no differences in age at diagnosis or BMI / waist:hip ratio. The AC group were slightly younger at recruitment and had shorter durations because of this, but with the smallest number of individuals recruited to this group, there are limited

conclusions to draw at present. If we compare the significant differences observed in this cohort to those studied in chapter 2 (the Exeter MODY database) it is of interest, that most of the significant differences in that cohort are not observed here. For example, in the retrospective analysis, SA referrals without mutations were diagnosed younger with shorter durations, were more likely to not be on insulin and have a parent affected. In the current study, with the exception of higher proportions of parental history of diabetes in SA people and a lower BMI, no other significant differences were observed. It is likely that the systematic assessment in this study on the basis of antibody and C-peptide status, and the unselected referral criteria, have addressed the referral bias observed in the retrospective study.

The best practice guidelines for MODY testing include a parental history of diabetes, non-insulin requiring diabetes and being diagnosed before 25 years(75). The data outlined in tables 4.18 and 4.19, show that in people with no detectable mutations, the percentage of SA and AC people with a parental history is much higher. Therefore, the presence of a parental history of diabetes is an insensitive discriminator of MODY in people of SA and AC in comparison to WE individuals. Meanwhile age at diagnosis was not significantly different between ethnic groups and in the people identified with mutations, the highest age at diagnosis was 26 years, suggesting this may still be a valid criterion. BMI also ranged up to 38 kg/m^2. It is difficult to draw conclusions about the MODY phenotype in ethnic groups, given low numbers, but it appears there may be a broad spread of clinical features in people with MODY from non-white ethnic groups, perhaps due to population differences in insulin resistance and body habitus(225). This will require reassessment when recruitment is complete. However, if signals persist then clinical referral criteria for MODY may need to be revised, for example, not weighting a parental history of diabetes too high. Arguably the use of boad biomarker cut-offs with clinical features related to age-at-diagnosis may be the best approach.

4.12.4 Potential Selection Bias

Recruitment has primarily focussed on secondary care sites and early on it was felt that this may introduce a potential bias favouring type 1 diabetes cases that are usually preferentially managed in secondary care. This may be a particular issue in the SA

and AC ethnic groups where there are more cases of young-onset type 2 diabetes that are more likely to be managed in primary care or community diabetes clinics. This was not an issue for paediatric sites where usually all types of diabetes are managed in secondary care.

To address this issue, a pilot was undertaken to recruit from North West London CCG's however this yielded very few recruits and in fact the number of participants per practice meeting eligibility criteria was usually below 10/practice. It was therefor concluded that this would not be a feasible avenue to recruit to a large scale study. Instead an individualised approach to recruitment with each site deciding on best avenues, was opted for. That this was the best approach became apparent after it emerged that different secondary care sites managed their young-onset type 2 diabetes in different places depending on local agreements. This meant some secondary care sites had full access to type 2 cases, whilst others managed them in community diabetes clinics, which could act as PIC sites. For the vast majority of sites, secondary care recruitment remained the best approach and sites with community run clinics used these to identify more participants. However, it is accepted that the type 2 diabetes group may be under-represented in this study.

4.12.5 Predictors of C-peptide

Multivariable regression analysis has revealed interesting predictors of C-peptide in the cohort eligible for testing. The diagnoses in individuals categorised for testing (Group 1) are likely to be a mixture of MODY and presumed young-onset type 2 diabetes cases. As numbers increase the analysis can be repeated with MODY cases excluded. In this analysis AC ethnicity, duration and being on insulin treatment were all negative independent predictors of C-peptide. These are useful data, as for the first time, they have provided a cumulative assessment of all predictors and indicated the magnitude of the effect with the coefficients outlined. In total this model accounted for 27% of the variation observed in C-peptide levels, which is considerable.

In this analysis there was a 9pmol/L reduction in C-peptide with each year of duration of diabetes, which represented the strongest association with C-peptide levels (t statistic -3.33, p=0.001). We know from some studies that beta-cell function, as

assessed using HOMA declines with duration of type 2 diabetes (226) and others have shown a decline measuring C-peptide, but shown variability (227). However the same data for a multi-ethnic population is limited and subsequent studies have predominantly focused on C-peptide decline in type 1 diabetes (228). Of course the model assessed in this analysis is limited by lack of inclusion of current treatment apart from insulin, including sulphonylureas and when numbers are higher the analysis will be repeated to incorporate the additional measures.

Similarly, being on insulin treatment was associated with a lower C-peptide by a magnitude of 211 pmol/L as compared to not being on insulin treatment. This at first glance is not unexpected as we would expect people with lower circulating C-peptide to receive insulin treatment following clinical recognition of this. However, as participants in the study did not stop their basal insulin prior to attending their study visits, an alternative explanation may be suppression of endogenous insulin by exogenous insulin. Published studies have shown that continuation of basal insulin does not grossly affect fasting C-peptide and it was on this basis that the current approach was selected (205). One way to conclusively determine how this signal arises would be to measure insulin levels and compare the ratio of insulin and C-peptide. This is beyond the scope of the current body of work, but a variety of further biochemical analyses are planned on existing samples and insulin will be included in this to enable analyses such as this one to be undertaken.

The biggest effect size was seen for AC ethnicity, which was associated with a 267pmol/L reduction in C-peptide as compared to WE ethnicity. AC ethnicity has been shown to be associated with insulin secretory defects before, most notably in the NHANES III population survey (55), which assessed fasting C-peptide in people without diabetes and showed low fasting C-peptides but high fasting insulin. Studies in people with diabetes have also shown lower C-peptide levels in African-American and Ghanaian populations (38,39,56).

4.12.2 Conclusion

The MY DIABETES study has been successfully set up across multiple NHS sites and will continue to recruit to the SA and AC ethnic groups with CRN support. In this

analysis of the MY DIABETES cohort, systematic phenotyping has led to the identification of MODY mutations in 9.3% of those selected for testing and 1% of the whole cohort. Critically, these mutations were found across all three ethnic groups and none were novel mutations. The characteristics of the group eligible for testing were also broadly similar across ethnic groups. This contrasts the findings from the retrospective study in chapter 2, suggesting that a systematic approach to selection for genetic testing may mitigate against potential referral bias. A number of variants of unknown significance were also identified and further work in planned to help assign pathogenicity in these cases. As the study continues to recruit, repeat analyses can be undertaken to determine if the signals seen in this interim analysis persist with larger numbers.

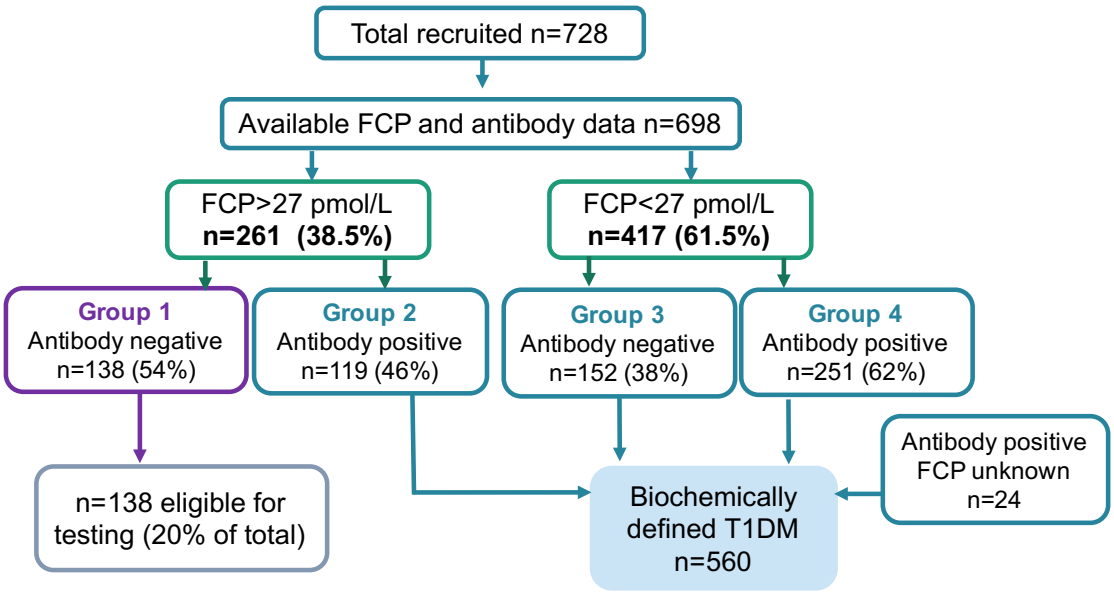
Chapter 5 : The Characteristics of Individuals Defined as Having Type 1 Diabetes using C-peptide and Pancreatic Autoantibodies.

5. 1 Introduction

People from ethnic minority groups are often misclassified as having type 2 diabetes instead of type 1, on the assumption that young-onset type 2 diabetes is more common. However, studies suggest that second generation migrants adopt the local type 1 diabetes risk prevalence of the country they live in (18,20,199,229). Few studies have systematically explored the relative proportions of type 1, type 2 and other types of diabetes in young-adult onset groups. Understanding the phenotype of type 1 diabetes in people from different ethnic groups is important as it may highlight phenotypic differences that should be taken into consideration when making the diagnosis of type 1 diabetes. Furthermore, although studies of type 1 diabetes phenotype may have taken place in native countries, it is possible that migrant populations present differently, for example lower IA-2 antibody positivity in one study (30). Ethnic specific differences in type 2 diabetes have been explored, but with the exception of a few studies, the differences in the phenotype of type 1 diabetes remains a largely unexplored area (30).

In chapter 4, features of the entire cohort and of the 138 individuals who met criteria for genetic testing, were presented. In this chapter the focus moves to the majority of the individuals in the MY DIABETES cohort who did not meet the criteria for genetic testing because they were categorised as having type 1 diabetes.
This, as outlined in figure 5.1, is a mixed group of individuals who either have undetectable C-peptide (≤27 pmol/L) and/or positive pancreatic antibodies. These 560 individuals have been classified as having type 1 diabetes on the basis of the MY DIABETES phenotyping criteria, outlined in chapter 3. If complete data was not available, for example C-peptide or clinical data, numbers included were less than 560 and are indicated in individual tables.

Figure 5.1: Classification of the MY DIABETES Cohort on the Basis of Antibody and Fasting C-peptide results



Fasting C-peptide (FCP)

5.2 Description of the Entire Type 1 Cohort and Impact of Ethnicity on Phenotype

Of the 728 people recruited, 560 people met the biochemical criteria for type 1 diabetes. As previously described in chapter 4, significant differences were observed in the proportions of people being defined as type 1 or those eligible for genetic testing, by ethnicity (presented again in table 5.1). In 4 of the individuals defined as having type 1 diabetes, ethnicity was unknown and these are therefore removed from the analyses incorporating ethnicity.

Table 5.1: Percentage of Participants Meeting the MY DIABETES criteria for Type 1 Diabetes, by Ethnic Group. Individuals with unknown ethnicity removed, therefore n=556 individuals

	WE	SA	AC	Mixed	Total
%Defined as type 1 diabetes (n)	89 (408)	62 (78)	58 (43)	77 (27)	556
% Not type 1 (n)	11 (51)	38 (48)	42 (31)	23 (7)	138
p value vs. WE		p<0.0001	p<0.0001		

WE, white European; SA south Asian; and AC African-Caribbean; Criteria for type 1 diabetes: C-peptide ≤27 pmol/L or any antibody positive

The type 1 group was predominantly formed of people of WE ethnicity, see table 5.2 for the overall features of the type 1 group. Most participants were adults. The vast majority of individuals had a clinician-assigned diagnosis of type 1 diabetes, but there were a proportion (4.1%) who had a diagnosis of type 2 diabetes and furthermore, approximately 4% of individuals were not insulin-treated at the time of recruitment. Antibody positivity was observed in 72% of the whole group. Approximately 10% of individuals had C-peptide levels above 200 pmol/L.

Table 5.2: Features of the Group Classified with Type 1 Diabetes Based on a Fasting C-peptide ≤27pmol/l and / or Positive pancreatic Antibodies

	n / median (IQR)	%
Meets criteria for 'type 1 diabetes'	556	
White	408	73
South Asian	78	14
African-Caribbean	43	8
Mixed / Other	27	4.9
Born in the UK	407	80
Male	264	47
Children (<18yrs)	46	8.2
Clinician-assigned diagnosis (if provided)		
"Type 1"	512	91
"Type 2"	22	4.1
"Don't' Know"	2	0.4
"Other"	3	0.5
Currently insulin-treated	541	96.6%
Median age at diagnosis (years)	15 (9.7-24)	
Median duration (years)	19.6 (8.6-34.6)	
Median age at recruitment (years)	36.4 (26.6 -49.8)	
Antibody positive	394	72.2
C-peptide undetectable (≤27pmol/L)	417	77.8
C-peptide ≤200pmol/L	485	90.5%
Glucose at study visit (mmol/L)	9.7 (6.5-12.9)	
% Fasting at the study visit		76.3

IQR, interquartile range

5.2.1 Features of Individuals Not Classified as Type 1 at Recruitment

Features of the 27 people who did not have a clinician-assigned diagnosis of type 1 at recruitment are shown in table 5.3 and compared to the cohort of individuals both referred as type 1 by their clinician and classified as type 1 (using MY DIABETES criteria).

Table 5.3: Comparison of Characteristics of Individuals Referred as Type 1 versus those Not Referred asType1

	Referred as type 1	Referred as non-type 1	*p-value*
Meets MY DIABETES criteria for type 1 diabetes	512	27	
White	76.9% (n=393)	33.3 (n=9)	
South Asian	11.7% % (n=60)	40.7% (n=11)	**<0.001**
African-Caribbean	6.7% (n=34)	18.5% % (n=4)	
Mixed / Other	4.7% (n=24)	7.4%(n=2)	
Male	48.4%	37.0%	0.248
Children (<18yrs)	8.8%	0	
%Currently insulin-treated	99% (507)	70.4 % (19)	**<0.001**
Median age at diagnosis (years)	15 (9-23)	25 (20-28)	**<0.001**
Median duration (years)	20.8 (8.8-35.4)	15.5 (7.3-19.5)	0.092
Median age at recruitment (years)	37.2 (26.6-50.7)	37.1 (32.8-45.4)	0.547
BMI k/m^2(aged≥16 years)	25.3 (22.6-29.2)	28.7 (24.4-37.1)	**0.003**
C-peptide ≥200 pmol/L	5.9% (n=29)	78.3% (n=18)	**0.000**
Pancreatic antibody positive	70.5%	92.6%	**0.013**
Parental history diabetes	26.2% (n=128)	82.6% (n=19)	**0.000**

Individuals biochemically (C-peptide ≤27pmol/L) and immunologically (antibody positive) defined as having type 1 diabetes in those with complete C-peptide and antibody results.

Individuals who were not referred with a type 1 diabetes diagnosis, but in whom the criteria were met for type 1 diabetes, were significantly more likely to be from SA or AC ethnicity, were less likely to be insulin-treated, were older at diagnosis, had higher BMI's and were more likely to have a history of parental diabetes than people who were referred as type 1 diabetes. As the definitions of type 1 incorporated C-peptide

and antibody results, the percentage with positive antibodies was also higher in the people not referred as type 1 diabetes, but defined as having type 1. Two of the 27 individuals had undetectable C-peptide levels and were antibody negative.

Examining the pancreatic autoantibody status in those reclassified as having type 1 diabetes, the following was observed;

- 25 / 27(93%) of individuals were antibody positive
- Of these 25 individuals
    - 21 were positive to a single antibody
    - 4 were positive to two antibodies
    - None were positive to all three antibodies
- Of the 21 positive to a single antibody
    - 7 (33%) were positive to GAD alone
    - 2 (9.5%) were positive to IA-2 alone
    - 12 (57%) were positive to ZnT8 alone
- Of the 4 individuals with two antibodies
    - 1 was positive to GAD and IA-2
    - 3 were positive to GAD and ZnT8

5.3 Impact of Ethnicity on the Clinical and Biochemical Features of the Type 1 Cohort

In the next analysis, the impact of ethnicity was assessed on the features of the entire cohort classified as type 1 diabetes on the basis of C-peptide (≤27 pmol/L) and antibody results (positive to one or more antibodies). The clinical features are described in table 5.4. There were significant differences in the clinical phenotype; both SA and AC participants tended to be younger at recruitment and therefore had significantly shorter durations than WE individuals. However, no differences in the age at diagnosis was observed across any ethnic group. Also of note, no differences were observed between BMI across ethnicities, although SA women had higher waist:hip ratios.

There were also higher proportions of people not currently insulin treated in the SA group compared to the WE group. Parental history of diabetes was also observed to be much higher in the SA group than WE.

Table 5.4: Comparison of the type 1 diabetes group (defined using C-peptide <27 pmol/L and/or positive pancreatic antibodies) by ethnicity

Parameter	WE	SA	p-value *	AC	p-value **	p-value ***
n	408	78		43		
Born in the UK	311 (85%)	48 (62%)	**<0.001**	26 (61%)	**<0.001**	**<0.001**
Age at diagnosis (years)	15 (10-23)	18 (8-24)	0.59	15 (9-24)	0.85	0.84
Duration (years)	22.7 (11.3-37)	14.5 (7.4-22.8)	**0.0002**	14.6 (4.3-22.7)	**0.001**	**0.0001**
Current age (years)	39.6 (27.7-52.4)	33.3 (24.3-41.3)	**0.0003**	31.1 (22.7-43.9)	**0.002**	**0.0001**
BMI[#] (kg/m^2)	25.3 (22.9-29.3)	25.4 (22.6-29.0)	0.54	25.3 (23.1-28.9)	0.99	0.83
Female waist circumference(cm)	83 (75-95)	89 (79-92)	0.44	83 (74-94)	0.91	0.74
Female waist:hip	0.83 (0.77-0.89)	0.91 (0.85-0.93)	**0.0006**	0.89 (0.79-0.93)	0.09	**0.0012**
Male waist circumference(cm)	91 (82.6-102)	89.5 (82.7-99)	0.31	82 (80-89)	**0.03**	0.07
Male wasit:hip	0.92 (0.86-0.99)	0.93 (0.87-0.99)	0.37	0.90 (0.86-0.95)	0.86	0.65
% Parent affected	23.1%	54.6%	**<0.001**	27.8%	0.53	**<0.001**
% Currently not on insulin	1.2%	7.8%	**<0.001**	4.7%	0.08	**0.002**
% Diagnosed <25 years	80	75	0.34	81	0.89	0.61

*WE, white European; SA south Asian; AC African-Caribbean. [#]BMI calculated in those ≥16 years at * p-value for WE vs SA; ** p-value for WE vs AC; *** p-value WE vs SA vs AC. Significant p values in bold.*

On comparison of biochemical features by ethnic group (see table 5.5), AC individuals were noted to have higher HbA1c than WE along with higher HDL's and lower triglycerides. Also of note, SA and AC individuals were more likely to have C-peptide

concentrations above 200 pmol/L and were more likely to be antibody positive at assessment. The relationship of these results to potential confounders are explored in the next section.

Table 5.5: Biochemical Features Individuals Defined as type 1 by Ethnic Group

Parameter	WE	SA	p-value *	AC	p-value **	p-value WE vs SA vs AC
n	408	78		43		
% fasting	78.1	70.1	0.13	74.4	0.586	0.3
HbA1c (mmol/mol)	62 (55-71)	62 (53-78)	0.85	69 (58-80)	**0.0297**	0.10
Total cholesterol mmol/L	4.4 (3.9-5)	4.4 (3.6 -5)	0.22	4.6 (4.1-5.3)	**0.0458**	**0.0461**
HDL cholesterol mmol/L	1.6 (1.3-2.0)	1.38 (1.2-1.7)	**0.0009**	1.8 (1.5-2.0)	**0.0151**	**0.0001**
Triglycerides mmol/L	0.8 (0.6-1.2)	0.9 (0.7-1.2)	0.32	0.71 (0.53-1.03)	**0.0467**	0.06
LDL cholesterol mmol/L	2.3 (1.8-2.9)	2.2 (1.8-2.9)	0.79	2.57 (1.97-3.1)	0.1118	0.25
C-peptide (pmol/L)	3 (3-24)	3 (3-204)	**0.0196**	3 (3-38)	0.0926	**0.0253**
%C-peptide ≤27	80.9	68.5	**0.017**	68.3	0.056	**0.018**
% C-peptide ≥200 pmol/L	6.5%	26%	**0.000**	14.6%	**0.041**	**0.000**
% Antibody positive	68 (n=271)	79% (n=62)	0.05	88 (n=37)	<0.001	**0.007**
Glucose mmol/L	9.8 (6.9-12.6)	9.2 (5.9-13)	0.24	9.2 (6.2-13.8)	0.7549	0.49

*Type 1 defined by C-peptide<27 pmol/L and/or antibody positive; WE, white European; SA south Asian; AC African-Caribbean. * P-value for WE vs SA;** p-value for WE vs AC; *** p-value WE vs SA vs AC. Significant p values in bold.*

SA and AC individuals had shorter durations and younger ages at recruitment, but similar ages at diagnosis. BMI was similar across all ethnic groups, but SA and AC individuals had higher waist:hip ratios. SA individuals were more likely to have a parent affected and more likely to not currently be on insulin treatment. AC individuals had higher HbA1c, higher HDL and lower triglycerides than WE individuals

5.4 Predictors of Antibody Status

As antibody positivity appeared to differ with ethnicity, univariate logistic regression was undertaken to determine the variables that had an impact on antibody status. From this initial analysis, the following variables were found to significantly associate with antibody status; duration, current age, age at diagnosis, BMI and ethnicity. Migration status (born in the UK or not) did not show an association with antibody status, however as there is evidence of different antibody profiles depending on country of origin(30), it was also included. Using multivariable logistic regression, the significant variables were incorporated into a regression model (see table 5.6).

Table 5.6: Multivariable Linear Regression of Independent Predictors of Pancreatic Autoantibody Status (positive versus negative)

Independent predictor of Antibody Status	Odds Ratio	p-value
Ethnic group compared to white		
South Asian	1.16	0.668
African-Caribbean	1.81	0.259
Duration	0.94	<0.0001
BMI	0.98	0.489
UK born versus born abroad	1.13	0.681
Multivariable model p<0.0001		

Odds ratios are presented, with a level above 1 indicating antibody positivity to be more likely, along with the associated p-value.

By incorporation of other potential confounding variables into the model, the significant effects of AC ethnicity on antibody positivity were found to be accounted for by differences in duration between the ethnic groups

5.5 Comparison of Cohort with Detectable versus Undetectable C-peptide

To further explore the impact of ethnicity on type 1 diabetes phenotype, the group was separated by C-peptide status, into those with detectable circulating C-peptide (>27pmol/L) and those without (≤ 27pmol/L), regardless of antibody status. The features of the two groups are shown in table 5.7.

Table 5.7: Comparison of the Type 1 Diabetes Group by C-peptide Status

	C-peptide ≤27pmol/L	C-peptide >27 pmol/L	p-value
Meets criteria for 'type 1 diabetes'	n=414	n=119	
White	318 (80.9%)	75 (19.1%)	**0.018**
South Asian	50 (68.5%)	23 (31.5%)	
African-Caribbean	28 (68.3%)	13 (31.7%)	
Mixed / Other	18 (72%)	7 (28%)	
% Male	46.6	47.9	0.805
% Children (<18yrs)	7.9	10.9	0.953
%Currently insulin-treated	100%	89.1	**<0.0001**
Median age at diagnosis (years)	13 (8-21)	22 (16-26)	**<0.0001**
Median duration (years)	25.2 (15.5-37.6)	4.1 (1.2-12)	**<0.0001**
Median age at recruitment (years)	41 (28.8-52.4)	28.7 (22.7-36)	**<0.0001**
BMI k/m^2(aged≥16 years)	25.6 (22.8-29.3)	24.6 (22.5-28.7)	0.2837
% Pancreatic antibody positive	62.3	100	**<0.0001**
% Parental history diabetes	27%	31%	0.42
% Born in the UK	80	78.1	0.668

WE, white European; SA south Asian; AC African-Caribbean. #BMI calculated in those ≥ 16 years at recruitment. Significant p values in bold.

Those with undetectable C-peptide were more likely to be of WE ethnicity than those with detectable. They were diagnosed younger, with longer durations and were more likely to be on insulin treatment at recruitment.

5.6 Characteristics of Those with Undetectable C-peptide by Ethnicity

To examine subtle differences in type 1 diabetes phenotype by ethnicity, the type 1 group was separated to examine those with undetectable C-peptide and those with detectable C-peptide as distinct groups. In this section, the clinical features of

individuals who had an undetectable C-peptide (≤27pmol/L) are explored by ethnicity, shown in table 5.8. The biochemical features are depicted in table 5.9.

SA and AC participants were significantly younger at recruitment, but had similar ages at diagnosis. No differences were observed in the BMI, but higher waist:hip ratios were observed in SA and AC women, but not men. As observed in other analyses, SA participants were more likely to have an affected parent with diabetes.

HbA1c was found to be higher in AC participants, who also had higher HDL levels and lower triglycerides compared to WE recruits. The percentage who were antibody positive was noted to be higher in AC individuals compared to WE, however as duration was shorter in AC participants, multiple variable regression as undertaken to determine if ethnicity was impacting on antibody positivity rates or if these changes were accounted for by other factors. Using multivariable regression analysis, the impact of AC ethnicity on antibody positivity was accounted for fully by the difference observed in duration, in turn accounted for by the younger age at recruitment.

Table 5.8: Comparison of Individuals with Undetectable C-peptide, by Ethnicity

Parameter	WE	SA	p-value *	AC	p-value **	p-value ***
n	318	50		28		
Born in the UK	85%	64%	**<0.001**	57%	**<0.000**	**<0.001**
Age at diagnosis (years)	13 (9-21)	11 (5-24)	0.3668	13 (7.5-21)	0.9567	0.6698
Duration (years)	27.6 (16.3-38.9)	17.2 (10-27.3)	**0.0001**	18.1 (10.1-26.4)	**0.0026**	**0.0001**
Current age (years)	44 (30.1-53.7)	33.5 (21.5-40.7)	**<0.0001**	34 (27.3-44.1)	**0.0053**	**0.0001**
BMI# (kg/m^2)	25.9 (23.2-29.6)	26.2 (22.4-29.3)	0.4644	24.6 (22.3-27.0)	0.0868	0.1906
Female waist circumference(cm)	83 (74-94)	89 (76-92)	0.6072	77.5 (73.2-89.6)	0.1978	0.3445
Female waist:hip	0.83 (0.77-0.89)	0.91 (0.86-0.93)	**0.0016**	0.88 (0.77-0.9)	0.2153	**0.0045**
Male waist circumference(cm)	95 (85-104)	95 (86-100)	0.3876	78 (73.6-82)	0.0130	0.0387
Male waist:hip	0.95 (0.87-0.99)	0.93 (0.91-0.99)	0.8305	0.92 (0.83-1.0)	0.7879	0.9367
% Parent affected	23.2%	50%	**<0.001**	26.9%	0.666	**0.001**
% Currently not insulin-treated	0	0		0		
% Diagnosed <25 years	85	82	0.556	86	0.909	0.827

*WE, white European; SA south Asian; AC African-Caribbean. #BMI calculated in those ≥16 years at recruitment. *p-value WE vs SA; ** p-value WE vs AC; *** p-value WE vs SA vs AC. Significant p values in bold.*

Table 5.9: Biochemical Features of the Individuals with Undetectable C-peptide compared by Ethnic Group

Parameter	WE	SA	p-value *	AC	p-value **	p- value ***
n	318	50		28		
HbA1c (mmol/mol)	62 (55-71)	63.5 (54-78)	0.48	71 (61-84)	**0.0089**	0.0311
Total cholesterol mmol/L	4.4 (3.9-5)	4.5 (3.7-4.9)	0.40	4.6 (4.3-5.3)	0.0807	**0.12**
HDL cholesterol mmol/L	1.62 (1.3-2)	1.5 (1.3-1.8)	0.07	1.9 (1.7-2.3)	**0.0007**	**0.0003**
Triglycerides mmol/L	0.82 (0.62-1.2)	0.83 (0.62-1.0)	0.42	0.6 (0.5-0.8)	**0.0002**	**0.0009**
LDL cholesterol mmol/L	2.2 (1.9-2.9)	2.2 (1.9-2.9)	0.92	2.4 (1.9-3.0)	0.5120	0.80
% Antibody positivity	59	68	0.24	81	**0.023**	**0.046**
Glucose mmol/L	10.3 (7.1-13.1)	10.4 (6.2-13.4)	0.33	10.3 (6.1-13.9)	0.78	0.62

*WE, white European; SA south Asian; AC African-Caribbean. * p-value for WE vs SA; ** p-value for WE vs AC; *** p-value WE vs SA vs AC. Significant p values in bold.*

5.7 Characteristics of Those Classified as Type 1 Diabetes with Detectable C-peptide

People with C-peptide >27 pmol/L and therefore, who were all antibody positive, were further analysed for the effect of ethnicity on phenotype. The clinical features are examined in table 5.10. As before, SA and AC participants were more likely to be born abroad. Age at diagnosis, duration and age at recruitment were however all non-significantly different between ethnic groups. The AC individuals appeared to have higher BMI compared to WE. Unlike in the other categories waist:hip ratio was not different. Once again SA individuals were more likely to have an affected parent with diabetes and not be insulin treated.

Table 5.10: Comparison of people with type 1 diabetes and a detectable C-peptide, by ethnicity

Parameter	WE	SA	p-value *	AC	p-value **	p-value ***
n	75	23		13		
Born in the UK	87	65.5	**0.002**	69.2	**0.110**	**0.008**
Age at diagnosis (years)	22 (16-26)	23 (18-27)	0.3039	18 (10.5-24.5)	0.1392	0.1611
Duration (years)	3.88 (1-10)	8.4 (1.9-15.6)	0.2941	2.1 (1.9-12.7)	0.8614	0.5387
Current age (years)	28.2 (23-35.9)	31.7 (23.6-41.0)	0.3543	24.4 (12.8-31.7)	0.1464	0.1848
BMI# (kg/m^2)	24.3 (22.0-28.1)	24.4 (22.3-28.4)	0.9533	30.1 (24.9-32)	**0.0202**	0.0549
Female waist circumference(cm)	83 (76-103)	89 (82-92)	0.6818	111 (94-128)	0.076	0.128
Female waist:hip	0.83 (0.78-0.91)	0.88 (0.82-0.94)	0.1781	0.95 (0.77-1.1)	0.2937	0.2610
Male waist circumference(cm)	82 (76-88)	85 (79-90)	0.5732	86.3 (81-94)	0.3242	0.5706
Male wasit:hip	0.86 (0.79-0.91)	0.89 (0.85-0.93)	0.1873	0.89 (0.86-0.95)	0.1367	0.1877
% Parent affected	20.8%	65%	**<0.0001**	30%	0.512	**<0.0001**
% Currently not on insulin	4%	36%	**0.001**	15	0.102	**0.006**
% Diagnosed <25 years	67	61	0.544	75	0.607	0.688

*WE, white European; SA south Asian; AC African-Caribbean. #BMI calculated in those ≥16 years at recruitment. *p-value WE vs SA; ** p-value WE vs AC; *** p-value WE vs SA vs AC. Significant p values in bold.*

The biochemical features of the people with detectable C-peptides are shown in table 5.11. No differences in the HbA1c was observed. The previous higher HDL and lower triglycerides in AC individuals with undetectable C-peptide was not observed. In contrast, SA people had significantly lower HDL and higher triglycerides than WE people. Median C-peptide levels were also significantly higher in the SA group compared to WE individuals, however duration was no different.

Table 5.11: Biochemical Features of Individuals with Detectable (C-peptide>27 pmol/L and antibody positive), compared by Ethnic Group

Parameter	WE	SA	p-value *	AC	p-value **	p-value ***
n	75	23		13		
% Fasting	81	78	0.766	69	0.331	0.622
HbA1c (mmol/mol)	58 (51-72)	62 (48-90)	0.5885	60 (55-69)	0.7040	0.8299
Total cholesterol mmol/L	4.5 (3.9-4.9)	4.2 (3.4-5.3)	0.3323	4.6 (3.9-5.5)	0.5535	0.493
HDL cholesterol mmol/L	1.5 (1.1-1.9)	1.1 (0.9-1.5)	**0.0045**	1.7 (1.4-1.8)	0.6786	**0.0088**
Triglycerides mmol/L	0.8 (0.6-1.1)	1.3 (0.8-1.6)	**0.012**	0.9 (0.8-1.4)	0.1204	**0.0218**
LDL cholesterol mmol/L	2.4 (2.0-3.2)	2.4 (1.8-2.9)	0.5157	2.5 (2.4-3.3)	0.3277	0.4259
C-peptide (pmol/L)	132 (54-303)	417 (209-878)	**0.0001**	114 (64-426)	0.6979	**0.0005**
Glucose mmol/L	8.6 (6-11.5)	7.3 (5.6-10.7)	0.6595	8.7 (7.3-9.4)	0.9428	0.9032

*WE, white European; SA south Asian; AC African-Caribbean. * p-value for WE vs SA; ** p-value for WE vs AC; *** p-value WE vs SA vs AC. Significant p values in bold.*

To further explore the higher C-peptide observed in SA individuals, the following analyses were undertaken:

1) Comparison of proportion of C-peptide levels between 27-200 pmol/L in WE vs SA.

WE people with detectable C-peptide were split unevenly between those with levels 28-199 pmol/L (n=51/75, 68%) and those ≥200 pmol/L (n=24/75, 32%). In contrast people of SA ethnicity were more likely to have C-peptides ≥200pmol/L (19/23, 73%) than in the 28-199 pmol/L range (n=4/23, 17%), p<0.0001). However, duration was not different.

2) Comparison of proportion on sulphonylurea therapy

As more people in the SA group were not insulin-treated compared to WE, the numbers who were on sulphonylurea therapy, an insulin secretagogue, was examined, to determine if this accounted for the higher observed C-peptide levels. In

the WE group 1/75 (1.3%) individuals classified as type 1 diabetes with a detectable C-peptide, was treated with a sulphonylurea. In contrast 3/23 (13%), p=0.013 were treated with sulphonylurea treatment. However, with these 4 individuals who were sulphonylurea-treated removed from the analysis of C-peptide levels, the significant difference was still observed; WE individuals had median C-peptide of 129 pmol/L (54-277) vs SA 365 (214-780) p=0.0005.

3) Multivariable regression

Multivariable linear regression analyses were undertaken to determine the factors influencing C-peptide levels and whether the effect of SA ethnicity was independent of these. Using univariate linear regression, the following variables were identified as predictors of C-peptide levels; ethnicity, age at diagnosis, BMI, waist:hip ratio, waist circumference, triglyceride level, HDL level, currently insulin-treated and parental history of diabetes. Duration, current age, fasting status, HbA1c, total cholesterol, LDL cholesterol and whether or not born in the UK did not significantly predict C-peptide levels. The significant predictors were incorporated into a multi-variable model as depicted in table 5.12 and figure 5.2.

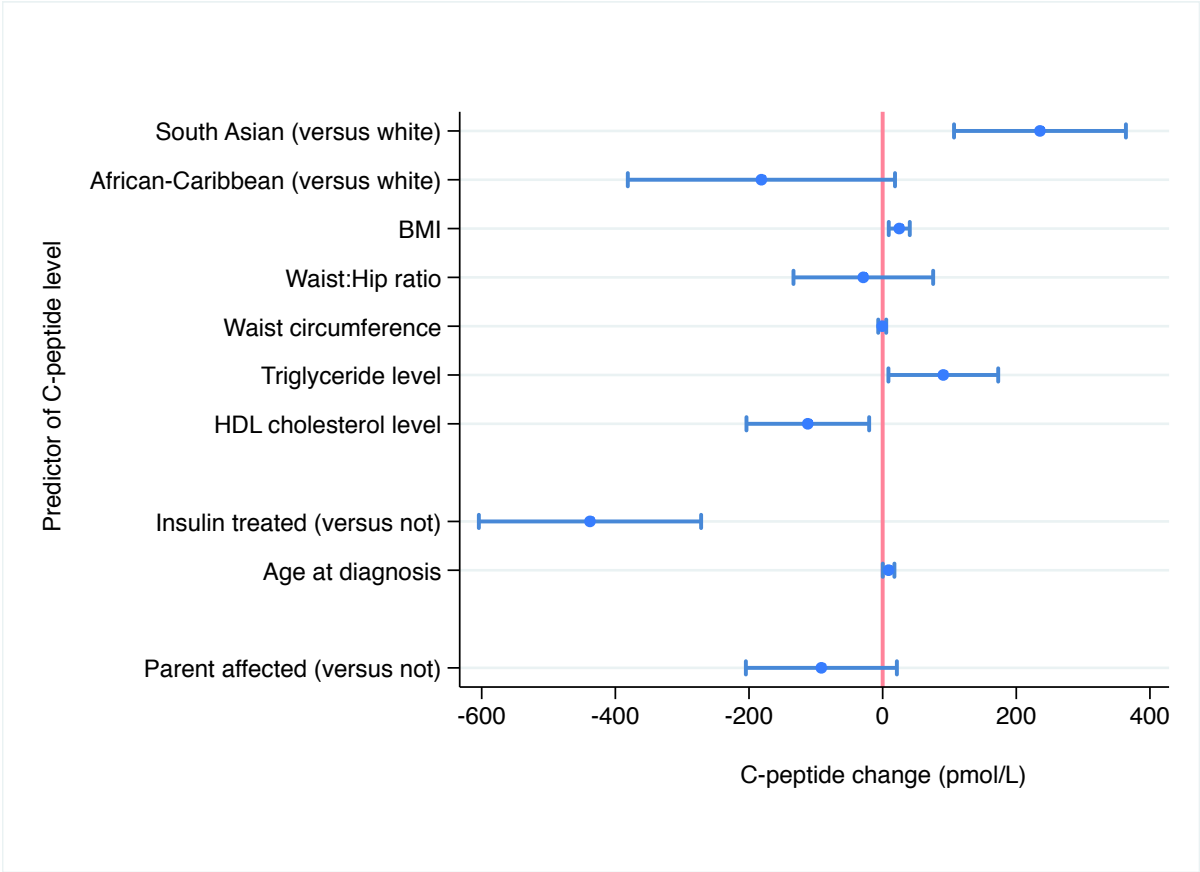
Table 5.12: Multivariable Regression Model Incorporating Significant Predictors of C-peptide in People Defined as having Type 1 Diabetes with Detectable C-peptide (i.e. antibody positive)

Independent predictor of C-peptide (n=84 observations)	Coefficient	T-statistic	p-value
Ethnic group compared to white			
South Asian	+235	3.65	**<0.0001**
African-Caribbean	-181	-1.81	0.075
BMI	+25	3.15	**0.002**
Waist: Hip ratio	-29	-0.55	0.583
Waist Circumference	-0.62	-0.21	0.835
Triglyceride level	+91	2.20	0.063
HDL cholesterol level	-112	-2.43	**0.018**
On insulin treatment	-438	-5.25	**<0.0001**
Age at diagnosis	+9	+2.04	**0.045**
Having a parent affected	-92	-1.62	0.110
Multivariable model p<0.0001, Adjusted R^2 0.639			

A positive coefficient signifies the predictor (higher levels or presence of) is associated with a higher C-peptide level. Significant p values in bold.

As C-peptide was not normally distributed, multiple variable regression was repeated on log transformed data and the same significant results were ascertained. To aid interpretation, non-log transformed data are presented.

Figure 5.2: Mean Coefficients of C-peptide and 95% confidence interval of mean change when incorporated into a multivariable linear regression model



On incorporation of multiple variables, regression analysis revealed that SA ethnicity, BMI, triglyceride levels, HDL cholesterol level, insulin treatment and age at diagnosis were all independent predictors of C-peptide.

To assess the impact of the varying proportions of individuals with C-peptide between 28-199pmol/L and ≥200pmol/L a second multivariable regression analysis was undertaken incorporating only individuals with C-peptide ≥200pmol/L.

Table 5.13: Multivariable Regression Model Incorporating Significant Predictors of C-peptide in Individuals Defined with Type 1 Diabetes with C-peptide >200pmol/L (i.e. antibody positive)

Independent predictor of C-peptide (n= 38)	Coefficient	T-statistic	p-value	Log transformed C-peptide		
				Coefficient	T-statistic	**p-value**
Ethnic group compared to white						
South Asian	+206	2.19	**0.038**	+ 0.18	1.03	0.312
African-Caribbean	-314	-1.47	0.152	-0.33	-0.86	0.399
BMI	+35	2.83	**0.009**	+0.04	1.83	0.079
Waist: Hip ratio	+55	0.48	0.635	+0.03	0.16	0.877
Waist Circumference	-4	-0.93	0.359	-0.004	-0.56	0.579
Triglyceride level	+60	0.93	0.360	+0.09	0.81	0.423
HDL cholesterol level	-202	-1,78	0.087	-0.51	-2.45	**0.021**
On insulin treatment	-342	-3.03	**0.005**	-0.53	-2.59	**0.015**
Age at diagnosis	+7	0.75	0.459	+0.01	0.72	0.479
Having a parent affected	-156	-1.42	0.168	-0.07	-0.34	0.740
Multivariable model p=0.0001, Adjusted R^2 0.559						

A positive coefficient signifies the predictor (higher levels or presence of) is associated with a higher C-peptide level.

However, when this anaylsis was repeated with log-transformed data, the significance of south Asian ethnicity and BMI was lost, whilst HDL became significantly associated with C-peptide levels. However, it should be noted the number of observations for those with C-peptide >200 pmol/L is small at n= 38.

5.8 Characteristics of Those Antibody Positive by Ethnicity

In this section the features of those who were antibody positive are examined. Antibody positivity was observed in 72.2 % of the entire cohort defined as having type 1 diabetes (C-peptide<27 pmol/L or antibody positive). In the first analysis the people who were found to be antibody positive were compared to those who were antibody negative (see table 5.14).

Table 5.14: Comparison of Characteristics of Individuals with Type1 Diabetes by Antibody Status

	Antibody Negative	Antibody Positive	p-value
Meets criteria for 'type 1 diabetes'	n= 151	n=392	
White	n=125 (83%)	68% (271)	
South Asian	n=16 (11%)	16% (62)	**0.007**
African-Caribbean	n=5 (3%)	9% (37)	
Mixed / Other	n=5 (3%)	6% (22)	
% Male	53	45	0.089
% Children (<18yrs)	6	6.8	0.767
%Currently insulin-treated	100	97	**0.028**
Median age at diagnosis (years)	12 (6-20)	17 (11-24)	**<0.0001**
Median duration (years)	32.7 (22-42.4)	16 (6-28.3)	**<0.0001**
Median age at recruitment (years)	48.8 (36.1-56.6)	33.4 (25.9-45.8)	**<0.0001**
BMI k/m^2(aged≥16 years)	26.5 (23.8-29.9)	25.1 (22.6-28.9)	**0.011**
% Parental history diabetes	22.6%	30.4	0.086
% Born in the UK	81.3	78.5	0.495

People who were antibody negative were significantly younger at diagnosis and older at recruitment, making the duration significantly longer. BMI was also noted to be slightly higher in the antibody negative group. The ethnic distribution also varied with more people of non-white ethnicity in the antibody negative group.

5.8.1 Antibody Positivity by Type

The 394 individuals who were antibody positive on testing, were examined further to investigate type of antibody and combinations of antibody positivity, by ethnicity. The breakdown of antibody results in those positive is depicted in table 5.15.

Table 5.15: Percentage breakdown of antibody type in those defined as type 1 diabetes, with positive antibodies

	Number out of 394 tested positive	%
GAD positive*	295	75
IA-2 positive*	161	41
ZnT8 positive*	138	35
GAD alone	148	34
IA-2 alone	37	9.4
Znt8 alone	44	11
GAD + IA-2	71	18
GAD + ZnT8	41	10.4
IA2 + ZnT8	18	4.6
GAD+ IA2 + ZnT8	35	8.8

**Denotes positive antibody result alone or in combination*.

5.8.2 Impact of Ethnicity on Antibody Positivity.

Table 5.16 displays the clinical phenotype of individuals who were antibody positive, by ethnic group. Few differences were observed in the clinical phenotype, with similar ages at diagnosis and durations.

Table 5.16: Comparison of people with type 1 diabetes who were antibody positive, by ethnicity

Parameter	WE	SA	p-value *	AC	p-value **	p-value ***
n	271	62		37		
% Born in the UK	86 (202)	63 (39)	**<0.0001**	59 (22)	**<0.0001**	**<0.0001**
Age at diagnosis (years)	17 (12-24)	20 (9-25)	0.766	17.5 (9.5-24)	0.461	0.726
Duration (years)	16.7 (6.1-29.9)	12.7 (6.9-18.9)	0.036	14.6 (2.8-23.0)	0.173	0.061
Current age (years)	33.9 (26.8-47.9)	32.8 (24.3-40.5)	0.028	31.1 (24.4-44.4)	0.132	0.044
BMI# (kg/m^2)	25.1 (22.6-28.9)	25.2 (22.3-28.4)	0.660	26.4 (23.8-21.2)	**0.463**	0.659
Female waist circumference(cm)	84 (75-95)	89 (77-91)	0.696	86 (73.4-100)	0.990	0.941
Female waist:hip	0.84 (0.78-0.89)	0.91 (0.85-0.93)	0.005	0.87 (0.80-0.93)	0.278	0.016
Male waist circumference(cm)	87.3 (82-100)	88.5 (81.1-96.5)	0.539	82 (80-89)	0.119	0.268
Male waist:hip	0.9 (0.84-0.97)	0.92 (0.86-0.97)	0.424	0.90 (0.86-0.95)	0.693	0.694
% Parent affected	24 (61)	58 (36)	**<0.0001**	32 (10)	0.287	**<0.0001**
% Currently not on insulin	1.5 (4)	10 (6)	**0.001**	5.4 (2)	0.105	**0.003**
% Diagnosed <25 years	77 (208)	74 (45)	0.587	81 (36)	0.635	0.738

*WE, white European; SA south Asian; AC African-Caribbean. #BMI calculated in those ≥16 years at recruitment. *p-value WE vs SA; ** p-value WE vs AC; *** p-value WE vs SA vs AC. Significant p values in bold.*

The biochemical phenotype of people who were antibody positive by ethnicity is shown in table 5.17. The major finding was that SA and AC were less likely to be IA-2 positive. Lipid profile changes were as previously described.

Table 5.17: Biochemical Features of Individuals with Type 1 Diabetes, who were Antibody Positive, Compared by Ethnic Group

Parameter	WE	SA	p-value *	AC	p-value **	p-value ***
n	271	62		37		
% Fasting	76	72	0.278	76	0.689	0.542
HbA1c (mmol/mol)	62 (54-70)	61 (53-76)	0.954	69 (60-80)	0.0077	0.033
Total cholesterol mmol/L	4.5 (4.0-5.0)	4.5 (3.6-5.0)	0.261	4.6 (4.0-5.3)	0.277	0.239
HDL cholesterol mmol/L	1.6 (1.3-2.0)	1.3 (1.1-1.7(	**0.0001**	1.8 (1.5-2.0)	0.034	**0.0001**
Triglycerides mmol/L	0.8 (0.6-1.2)	0,9 (0.7-1.3)	0.203	0.7 (0.5-1.0)	0.057	**0.048**
LDL cholesterol mmol/L	2.4 (1.9-2.9)	2,4 (1.9-2.9)	0.892	2.5 (2.0-3.0)	0.376	0.689
C-peptide (pmol/L)	3 (2-42)	3 (3-243)	0.123	3 (3-73)	0.621	0.304
Glucose mmol/L	9.7 (6.7-12.6)	8.9 (5.7-12.5)	0.201	9 (6.2-13.8)	0.906	0.447
% GAD positive	76	69	0.305	81	0.466	0.397
% IA2-positive	45	29	**0.02**	10	**0.036**	**0.013**
% ZnT8-positive	59	51	0.353	40	0.054	0.134

*WE, white European; SA south Asian; AC African-Caribbean. * p-value for WE vs SA; ** p-value for WE vs AC; *** p-value WE vs SA vs AC. Significant p values in bold.*

5.9 Discussion

5.9.1 Key Findings

- Significantly more WE participants met the criteria for type 1 diabetes than SA or AC individuals.
- 4% of individuals meeting the criteria for type 1 diabetes, did not have a clinician-assigned diagnosis of type 1 diabetes.
    - These individuals were more likely to be of SA or AC ethnicity, were older at diagnosis and had higher BMI's.
- On comparing the impact of ethnicity on the phenotype of type 1 diabetes, the following was found;

    - No difference in age at diagnosis or BMI
- Ethnicity showed no association with antibody status and the differences in antibody positivity by ethnicity were fully accounted for by the variability in duration.
- When examining C-peptide levels in those with detectable C-peptide
    - SA ethnicity, BMI, age at diagnosis and triglyceride levels, all associated with higher C-peptide concentrations in people classified as type 1.
    - Whilst, having a parent affected, being on insulin treatment and having a high HDL all associated with lower C-peptide concentrations.

5.9.2 Proportions with Type 1 Diabetes, by Ethnicity

More WE people in the young-onset cohort met the criteria for type 1 than SA and AC individuals. This may reflect a genuinely higher percentage of younger-onset type 2 diabetes in the SA and AC ethnic diagnosed before the age of 30 years or it may be a recruitment bias favouring type 2 diabetes cases, given the primary aim was to detect cases of MODY. It remains to be seen if this persists when the study is fully recruited, however the disparity is likely to be genuine based on findings from other surveys. Systematic surveys of diabetes types in people with young-onset diabetes tend to be focused on paediatric populations, where type 2 diabetes is usually less common, however differences in the relative proportions of type 1 and type 2 diabetes have been observed. For example, in the SEARCH study from the US examining people aged under 20 with diabetes, 85% of WE children aged between 10 and 19 years had type 1 diabetes and 15% type 2 diabetes. In contrast the AC group was split 42% and 58% and Asian-Pacific islanders 30% type 1 and 70% type 2 diabetes(230). The relative incidences of type 2 diabetes also increased exponentially with every 5-year increase in age in the non-white ethnic groups in that study. Therefore, the proportions of diabetes types observed in the current cohort may well be expected, given the higher threshold for age-at-diagnosis applied in the current study and the increasing likelihood of type 2 diabetes with age.

5.9.3 Features of Those with Undetected Type 1 Diabetes

Of the people defined as having type 1 diabetes using the MY DIABETES study criteria, approximately 4% (n=27) were not referred with a type 1 diabetes diagnosis.

This group was therefore potentially misclassified and entry into the study enabled reclassification of their diabetes type. That the group who were reclassified, were older at diagnosis, with higher BMI's and more features in commons with type 2 diabetes, illustrates the overlapping phenotypic features between type 1 diabetes and type 2 and replicates findings in other cohorts that gave attempted to identify misclassified individuals(12,28,29,231). This in turn signifies the difficulties clinicians face making this diagnosis in people who do not have the typical features of type 1 and also reflects underlying clinician-bias. However, as C-peptide levels were higher in this group, the classification of type 1 diabetes in this group, was based predominantly on the pancreatic autoantibody result and this may not be wholly appropriate.

Single antibody positivity in people diagnosed at older age may have less significance as a proportion of individuals in any population will be positive to antibodies and never develop diabetes, based on the derivation of antibody reference ranges in non-diabetic control. However, it is noteworthy that 4 of the individuals were also positive to two antibodies. It is known from studies in relatives of individuals with type 1 diabetes that single antibody positivity carries less risk of progression to type 1 diabetes than those with multiple antibodies present(166,232,233). However the significance of a single positive antibody in someone with established diabetes is not clearly investigated, although data from the UKPDS study does demonstrate that those individuals positive to GAD progressed to insulin requirement faster, however other specific antibodies were not tested (234). The GAD titres observed were high, however the ZnT8 titres were in the low positive range. The clinical significance of antibody titre is unclear at present.

5.9.4 Clinical Features of Type 1 Diabetes

Age at diagnosis and BMI are two factors that still tend to be used to help classify individuals with type 1 diabetes, even though the latter in particular has been shown to be insensitive (28). This analysis has shown that age and BMI are not different across ethnicities. Therefore, it should be expected that SA and AC people presenting with type 1 diabetes, do so in a similar way ?to WE. Equally, ethnicity itself should not should not favour one type over another.

There were also higher proportions of people not currently insulin treated in the SA group compared to the WE group, probably signifying diagnostic difficulty or bias in this group. Parental history of diabetes was also observed to be much higher in the SA group than WE making it clear that the parental history of diabetes is insensitive at delineating type 1 form other subtypes. A history of parental diabetes was consistently higher in SA people referred for genetic testing. The negative predictive value of a parental history of diabetes is likely to be high in south Asian individuals, but the positive predictive value low in signifying MODY.

5.9.5 Biochemical Features of Type 1 Diabetes

Lower triglycerides and higher HDL in the AC group, were similar to the cardio-protective profile previously described in AC people without diabetes(235,236) and likely relate to ethnic differences in hepatic lipase activity(237). Lipid profiles in type 1 diabetes have been explored in a limited number of studies and findings in people with young-onset type 1 diabetes have demonstrated a relatively normal lipid profile or even an anti-atherogenic profile (174,238,239). Abnormal lipid profiles associated with atherogenic risk were identified in people with other factors, for example suboptimal glucose control, smokers and central obesity (174,239). With the exception of the AC group as outlined above, there were no differences in the WE and SA lipid profiles, except when looking exclusively at the sub-group with detectable C-peptide. In that analysis, SA individuals had higher triglycerides and lower HDL levels than WE. This was the same analysis that revealed higher C-peptide levels in the SA group and fits with an overall insulin resistance phenotype. Of course, it is difficult to draw real conclusions from this analyses as statin use, and smoking history were not recorded in the current study.

Examining specifically those with a detectable C-peptide in the type 1 group, some additional difference was observed. Predictably all individuals had much shorter durations and unlike in the Type 1 group as a whole, the duration was not significantly different between WE, SA and AC participants. This suggests that the differences outlined above simply signify a disparity in more SA and AC of recent diagnoses being recruited, but there is not a clear explanation for why and this may disappear when fully recruited.

SA people in the detectable C-peptide group, were significantly less likely to be on insulin treatment even though age and diagnosis and BMI were not significantly different between ethnic groups. The SA individuals also had an insulin-resistance like profile with lower HDL, higher triglycerides but no differences in waist circumference, waist: hip ratio or BMI.

5.9.6 Predictors of C-peptide

Most interestingly, significantly higher C-peptide circulating concentrations were associated with SA ethnicity but not WE ethnicity. This association was however, not observed in the AC group. As outlined in Chapter 4, AC ethnicity in people who were eligible for genetic testing was associated with lower C-peptide. In contrast, in the current analysis of people who were antibody positive and defined as type 1 diabetes, SA ethnicity was associated with higher C-peptide levels. There were, however, significant differences in the proportions of people recruited with C-peptide concentrations between 28-199 and those ≥200pmol/L between the SA and WE groups. A secondary multivariable regression analysis looking only at people with C-peptide ≥200 pmol/L showed that SA ethnicity did not associate with higher C-peptide levels compared to WE, when log-transformed. However, the numbers of observations are small in this analysis and it will be repeated one the cohort is fully recruited. The difference was also not accounted by variable use in sulphonylurea therapy.

This finding is of interest as it suggests that SA people produce more C-peptide in the early stages of type 1 diabetes than other ethnic groups. This also appears to be associated with a more insulin resistant phenotype with the higher triglycerides and lower HDL observed in SA people compared to WE. Insulin resistance in type 1 diabetes has been shown to result in higher C-peptides (24), but this has not been reported specifically as a SA phenotypic observation and represents a novel finding in this study. It is possible that the coexistence of insulin resistance unmasks type 1 diabetes at a higher level of beta cell function than in those, with normal insulin sensitivity.

Of course, it could be argued that these individuals with higher C-peptides are antibody positive, but don't have type 1 diabetes and it will remain to be seen if this finding

persists as larger numbers are recruited, particularly as the numbers in the current analysis are small.

5.9.7 Antibody Status by Ethnicity

Antibody positivity appeared to be higher in the AC group when examining the whole type 1 diabetes group, however on logistic regression this was accounted for by the shorter duration and younger age at recruitment of the AC group. It is unclear why both the AC and SA group were younger at recruitment, this disparity may, with further recruitment be addressed. At present, although the odds ratio is higher, being born in the UK had no significant effect on antibody positivity.

As there is some suggestion from published studies that second generation migrants (born in the UK) adopt the local type 1 diabetes risk of the country they move to (18–20) it may be expected that antibody positivity would be higher in people born in the UK, but this was not the case.

Examining the antibody positive group alone revealed very few differences across ethnic groups. Proportions of IA-2 antibody positivity were lower in SA and AC groups even though durations were similar. Antibody positivity in type 1 diabetes has been shown to be lower in SA and AC people in a variety of studies performed in native countries (33,196). However, studies in migrant groups have shown similar levels of GAD positivity and lower levels of IA-2 positivity, as demonstrated in the current analysis (30,240). Studies into antibody positivity and type 1 diabetes in non-white populations have been limited by several factors including; less than complete testing of all known pancreatic auto-antibodies, persistent use of islet cell immunofluorescence assays and assessment in cross-sectional studies, rather than in incident cases.

5.9.8 Implications for Clinical Practice

Taken together the data in the chapter demonstrate a broadly similar type 1 diabetes phenotype when assessed cross-sectionally in systematically classified WE, SA and AC individuals in the UK. This means that ethnicity per se should not influence clinicians when classifying type 1 that is defined strictly on the basis of biochemically and immunological results.

These data have also helped to put a figure on people with type 1 diabetes that were misclassified. The 4% figures is higher than reported in a study of incident type 1 diabetes cases (2%), but that was likely to be lower than the real figure as it only assessed reclassification within the first year post-diagnosis(231). People who were misclassified were older, more likely to be from an ethnic minority group and had higher BMI's. Current NICE guidelines do not recommend routine testing of C-peptide or autoantibodies to define type 1 diabetes, except in grey cases for example people with BMI >25 or over the age of 50 years(25). These data suggest that further phenotyping is warranted in people who are older, from minority ethnic groups and overweight. Interestingly, the average BMI in this cohort was above 25 kg/m^2 in all analyses of the type 1 cohort, suggesting that NICE cut-offs may need to be reconsidered.

That SA ethnicity was associated with higher C-peptide levels is an interesting signal that warrants further investigation. For now, it suggests that a higher C-peptide level in someone of SA ethnicity alone, should not go against a diagnosis of type 1 diabetes and other aspects of the phenotype should be explored. Fundamentally, although most people with SA and AC ethnicity and systematically defined type 1 diabetes were treated with insulin, the proportion who were not on insulin was higher in these groups compared to WE ethnicity. The key clinical message is that insulin should not be withheld in those with an unclear phenotype, whilst this is being investigated.

5.9.9 Limitations and Future Work

The absolute numbers in each ethnic group still remain small and so it is unclear if these signals will persist with full recruitment. The cross-sectional nature of the study also makes it a challenge to draw definitive conclusions about phenotype, which would be best confirmed at diagnosis. However, this is also a strength as it enables classification to be undertaken on the basis of C-peptide, which may be uninterpretable around diagnosis.

Future work on the cohort planed includes genotyping individuals classified as type 1 diabetes on the basis of HLA haplotypes. This is likely to be done using the published

genetic risk score and will help correlate phenotype to HLA haplotype and antibody status by ethnicity, which has not been done systematically previously. There is also an opportunity to follow-up people with detectable C-peptide in 3-5 years to assess decline in C-peptide and features associated with it.

Second void fasting urine samples were collected during study visits, to enable measurement of C-peptide to creatinine ration (UCPCR). These will be analysed and compared to fasting serum C-peptide levels, to establish relationships in each ethnic group and explore the utility of UCPCR by ethnicity.

5.9.10 Conclusion

Studying type 1 diabetes by ethnicity using a systematically defined criteria for type 1 diabetes has revealed few differences in the phenotype with similar ages at diagnosis, proportions who are antibody positive and similar BMI. Misclassification of type 1 diabetes was more likely to occur in older people at diagnosis, those who were overweight and who were more likely to be from SA or AC ethnicity. Type 1 diabetes if therefore phenotypically similar in UK ethnic groups and the diagnosis should be considered equally in people with young-onset diabetes from WE, SA and AC ethnic groups. SA people seem to favour a more insulin-resistance phenotype concomitantly present with type 1 diabetes and this warrants further investigation, particularly to determine if insulin resistance unmasks type 1 diabetes earlier in the south Asian individuals.

Chapter 6 : Investigations into the Pathogenicity of the Novel *HNF1A* Variant, p.A251T

The contents of this chapter have been presented at the American Diabetes Association and are currently being written up for peer-reviewed publication.

Misra S, Hassanali N, Smith C, Colclough K, Ellard S, Hattersley A, et al. Homozygous loss of function HNF1A variant resulting in sulfonylurea-responsive early-onset diabetes. Diabetes. 2016;65 (Supp 1:A360-A431)

6.1 Introduction

In chapter 4 the results of genetic testing on the cohort were presented. One of the participants (MD-IC-004) was found to be homozygous for the novel *HNF1A* gene

variant p.Ala251Thr (NM_000545 c.751G>A; p.A251T, hereafter referred to as A251T). It was unclear from preliminary investigations whether this variant was pathogenic - disease-causing - or a benign variant of no significance to *HNF1A* function. It is important to be able to classify variants in this way, as treatment and on-going management may vary if a mutation can be confirmed, clarity around uncertain diagnoses can be provided to affected individuals and family member testing can be initiated.

Homozygous mutations in *HNF1A* are thought to be embryonic lethal (241). The finding of this homozygous variant with the associated phenotype, was therefore of interest. If this variant was indeed responsible for the diabetes phenotype observed in MD-IC-004, then this would be highly unusual, the first reported case of a homozygous *HNF1A* mutation and probably signify that the mutation was hypomorphic – a mutation that modestly reduces, but does not completely eliminate gene functionality. Typical heterozygous *HNF1A* mutations exert their phenotypic effects due to haploinsufficiency (242–244). In the case of a hypomorphic mutation, it would follow that in a heterozygous state the mutation may result in no phenotype at all or have variable penetrance, however in the homozygous state, presentation would be similar to a bona fide heterozygous MODY mutation, because both copies of the allele would be modestly reduced in function, analogous to the haploinsufficiency of a heterozygous mutation. Hypomorphic mutations have been reported in other monogenic diabetes inheritance patterns, but not in *HNF1A* (245).

Determining pathogenicity of a variant is now the biggest challenge in the interpretation of exome-based sequencing data and of huge importance in the clinical context, where incorrect interpretation can have erroneous consequences (182). Assigning pathogenicity is a particular issue in genes associated with monogenic diseases such as MODY, where distinguishing between variants that are clearly disease-causing versus those that impair protein function or are neutral, is problematic (183). This issue is further impacted by the fact that many of the variants found in the common MODY genes are novel or arise *de novo*, making it impossible to draw conclusions or gain insights from other affected individuals (183,246).

A variety of guidelines, both national and international, have attempted to standardise the approach to investigating variant pathogenicity using *in silico* modelling, database searches and other parameters to assist with classification and to also standardise notation (182,184,185). However, despite these techniques, it is still often difficult to confidently assign pathogenicity. In these cases, further work may be necessary to shed light on variant functionality. These may be clinical studies in the individual or family members or by use of reliable *in vitro* functional or biochemical assays that assess the effect of the variant in a cellular system model. Even then, confident assignment of pathogenicity may not always be possible due to the limitations of these techniques.

To investigate this highly unusual case further, a series of *in silico*, clinical and *in vitro* investigations were undertaken and these are outlined in detail in this chapter, as follows:

- Baseline clinical studies
- Initial investigations into pathogenicity
- Further structural analysis *in silico*
- *In vitro* functional assays
- *In vivo* functional investigations

6.2 Hypothesis

The p.A251T variant is a hypomorphic mutation, which in the heterozygous state has variable penetrance, but in the homozygous state, has an effect comparable to a typical heterozygous *HNF1A* MODY mutation.

6.3 Baseline Clinical Studies

MD-IC-004 is a 28-year-old male of white Turkish ethnicity. He was diagnosed at the age of 16 years, with presumed type 1 diabetes and started on twice daily intermediate-acting insulin injections (Humalog Mix 25). He was diagnosed after a short duration of symptoms, as his identical twin also had a diagnosis of type 1 diabetes, made at the age of 12 years (see family pedigree, figure 6.1). It emerged his

twin had previously been genotyped as a child and the homozygous p.A251T variant, identified. However, the family had been lost to follow-up and no further investigations had been undertaken.

The variant was confirmed in our proband and targeted next generation sequencing, as per the MY DIABETES study protocol, revealed no mutation in any other diabetes-related gene (full list of genes sequenced in chapter 3).
At the study visit assessment, the proband was still on Humalog Mix 25 BD, his glycaemic control was not ideal as he was experiencing frequent hypoglycaemia with impaired awareness. His BMI was 27.8 kg/m^2. Biochemical results and continuous glucose monitoring from a baseline clinic appointment are displayed in table 6.1 and figure 6.2.

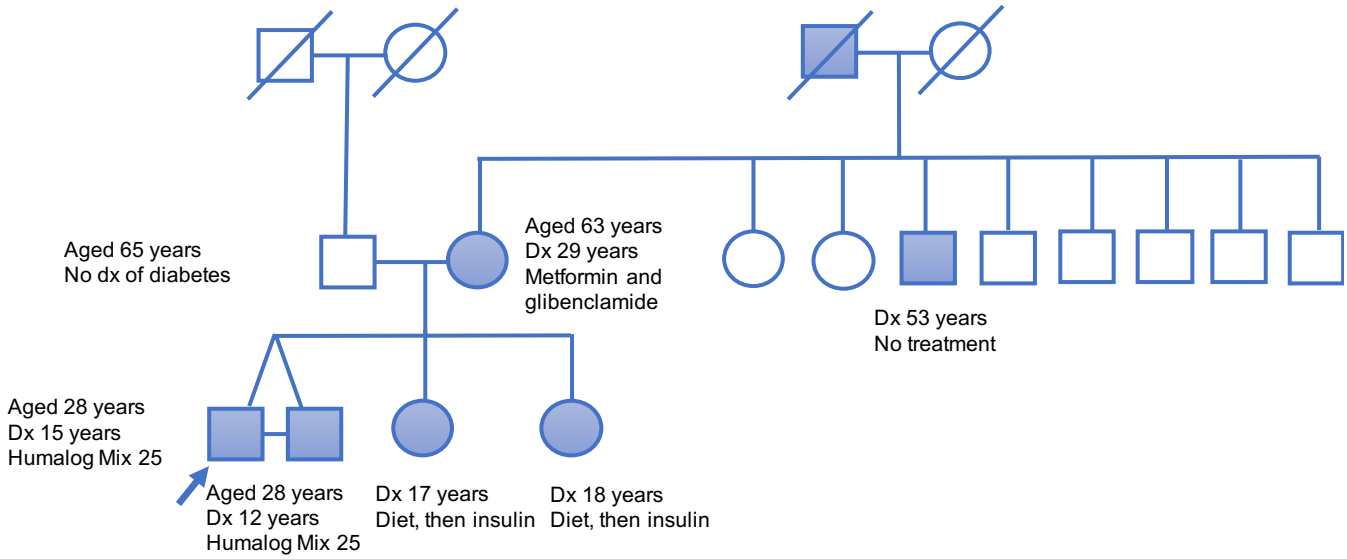
After ascertainment of his *HNF1A* genotype, his family members were asked to attend clinic for cascade testing. Both parents were found to be heterozygous for the A251T variant. There was no reported consanguinity, however both parents were from the same village in Turkey. His sisters are currently being contacted for genotyping.

Table 6.1: HbA1c and C-peptide Results in Proband and Family Members

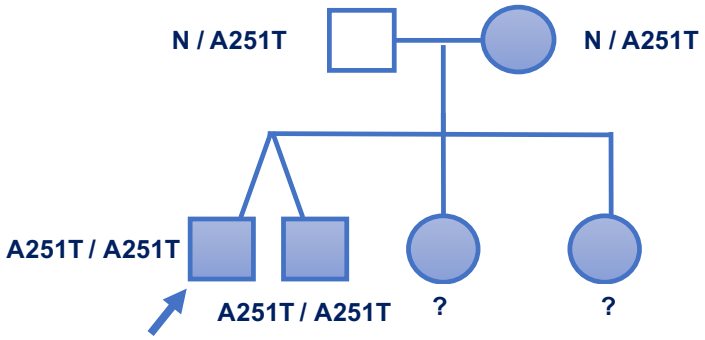
	HbA1c (mmol/mol)	C-peptide (pmol/L)	Treatment
Proband	58	622 (fasting)	Humalog Mix 25
Identical twin	67	263	Humalog Mix 25
Mother	72	502	Metformin and glibenclamide
Father	46	651	Nil

Figure 6.1: Family Pedigree and Genotype

a)



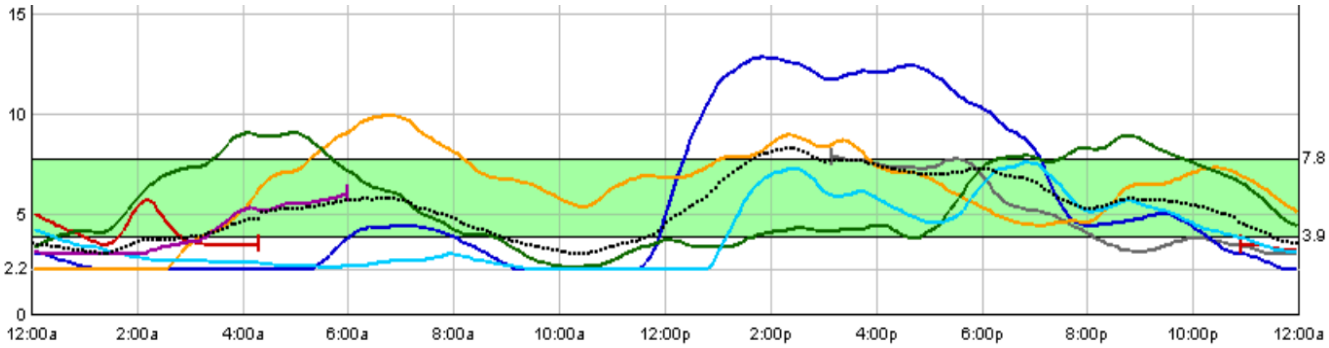
b)



a) Family pedigree of MD-IC-004, with associated treatment and approximate age at diagnosis of affected individuals. Family members with diabetes are filled in blue. b) Where established, the genotype of affected individuals, is additionally displayed. N, normal allele; A251T, variant allele; Dx, diagnosed.

Continuous glucose monitoring using an iPro2 sensor (Medtronic, Northridge, CA), confirmed significant hypoglycaemia (see figure 6.2) on current treatment and doses of insulin were reduced. His mean blood glucose was 5.2 mmol/L with a standard deviation of 2.6 mmol/L.

Figure 6.2: Retrospective Continuous Glucose Monitoring



Undertaken over 7 days in the proband, due to impaired hypoglycaemia awareness, using the iPro sensor (Medtronic, Northridge, CA). Each coloured line indicates a different day of glucose monitoring.

6.4 Initial Investigations into Pathogenicity

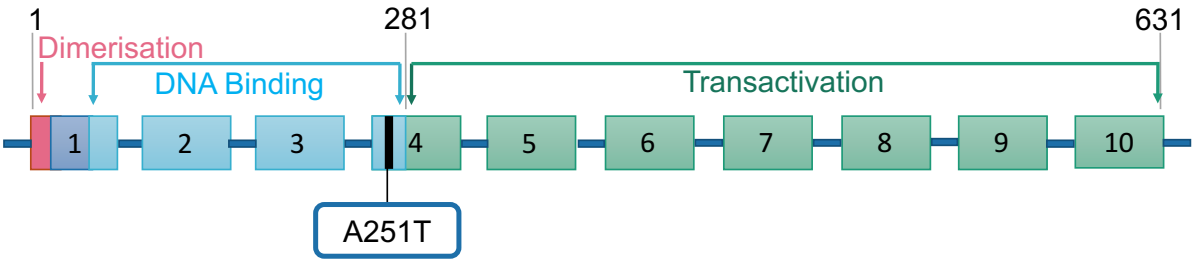
Initial investigations into pathogenicity were undertaken in line with national guidelines as described in this section.

The *HNF1A* variant found, substitutes a non-polar alanine for an uncharged polar threonine at amino acid position 251 of the HNF1A protein.

As shown in figure 6.3, this variant occurred in the DNA-binding domain of the *HNF1A* gene. The DNA-binding domain comprises two regions, a POU (Pit 1 -Oct 1 -Unc 86) specific domain (POU_S, residues 91-181) and a homeodomain (POU_H, residues 203-279) (100). These domains have specific sequences that bind to and recognise the complimentary DNA motif. A251T is in the POU_H domain.

Figure 6.3: Schematic of HNF1A Gene

a)



b)



Part a) displaying exons and functional domains. Variant A251T was situated in exon 4 of the DNA binding domain. DNA binding domain shown in more detail in part b).

6.4.1 Database Searches

The Human Gene Mutation Database (HGMD) (247) and other databases including the database of single nucleotide polymorphisms, dbSNP (248), Exome Aggregation Consortium (ExAc) (220,249), Exome Variant Server (250) and 1000 genomes Nation Genetics Research Laboratory were searched (251). The variant was not listed in any of these databases except for HGMD. The variant had been previously published in 2013 as part of a study investigating the role of plasma glycans in the identification of *HNF1A* MODY (178). In the reported case, the individual was heterozygous for the A251T variant. Limited clinical details were available, except for the fact the proband was Croatian, diagnosed aged 45 years, treated with metformin and a sulphonylurea for 25 years. The individual had 11 non-diabetic off-spring and their mother and sister developed diabetes, but over the age of 65 years.

6.4.2 Species Conservation

Species conservation measures the degree to which amino acid properties at any given position are evolutionarily conserved (185). Essential sites for protein function

are likely to be invariant across species (highly conserved), where as a variant sites across species can, by definition, accommodate a degree of substitution. Therefore, missense variants encountered at highly conserved sites are likely to be pathogenic (252). Conservation can be scored using tools such as ConSurf (253–255).
Multiple sequence alignment was undertaken to compare orthologs across species.

The A251 residue was compared across the following species; Human, Chimp, Mouse, Rat, Horse, Cat, Dog, Cow, Armadillo, Opossum, Platypus, Chicken, Zebra Finch, Frog, Zebrafish, Pufferfish, Tetraodon. It was conserved in all species except the Tetraodon, where the alanine was found to be a leucine residue. The tetraodon belongs to the PufferFish genus and the finding of a single species lacking conservation if of little significance. Therefore, it was concluded that the A251 residue is highly conserved across vertebrates.

6.4.3 *In Silico* Predictions

Using web-based applications, missense variants can be classified based on the predicted effects of the variant. Each methods prediction should not be taken in isolation and it is expected that several methods are used to predict, as they often lack agreement. Broadly speaking four main approaches exist; those based on sequence conservation methods, those on protein sequences, structural methods and splicing-predictions (185,256).
In the former, programs such as SIFT(J Craig Venter Institute, La Jolla CA) (257,258) and Align-GVGD (International Agency for Research on Cancer hosted by the World Health Organization (259,260) are used to undertake multiple sequence alignments across species, as above, and this information is combined with the changes in the physiochemical properties from the amino acid substitution and it's predicted effect on function to assign pathogenicity. For the variant p.A251T, both SIFT and Align-GVGD, (accessed through Alamut Visual 2.7 (Biosoftware, Rouen, France), predicted the variant to be 'likely pathogenic'.
Protein sequence methods were also deployed, including PolyPhen-2 (261,262). This program predicts the effect of the amino acid substitution on the subsequent protein and assesses the degree of similarity. Grantham scores (259), which assess the 'biochemical distance' between amino acids, can also be incorporated into decision-

making. Scores range between 5 and 215, with higher scores predicting a more radical change in amino acid properties. Scores can then be classified according to predicted pathogenicity. Using both PolyPhen-2 and Grantham scores, the p.A251T variant was predicted to be likely pathogenic and uncertain, respectively.

6.4.4 Splicing Predictions

Using modelling software, it can be determined whether the amino acid substitution creates a cryptic splice site. Or if the variant is close to a splice site, it can determine any potential consequences of the variant to nearby splicing. Using a variety of programs (accessed through Alamut Visual 2.7 Biosoftware, Rouen, France), the p.A251T variant was not predicted to create a cryptic splice site or affect splicing. Basic investigations into pathogenicity are summarised in table 6.2.

Table 6.2: Summary of Findings from Basic Investigations into the Pathogenicity of A251T

	Overall: Variant of unknown significance
Mutation Database searches (HGMD professional)	Previously reported: Heterozygous p.A251T variant identified in a patient diagnosed aged 43 years, treated with Metformin and sulphonylurea for 25 years from diagnosis (178)
Sequence Variant Database	Not listed in dbSNP, Exome Variant Server or 1000 genomes
Amino Acid Change	Alanine (non-polar) to Threonine (uncharged polar)
Species conservation	Conserved in 16 out of 17 orthologs, including 10 mammalian orthologs
In silico prediction	Likely pathogenic: SIFT, PolyPhen2 & Align-GVGD Uncertain: Grantham Distance
Cryptic Splice Site	Not predicted to create splice site

HGMD, Human gene mutation database; dbSNP, database of single nucleotide polymorphisms

6.5 Further Structural Analysis *In Silico*

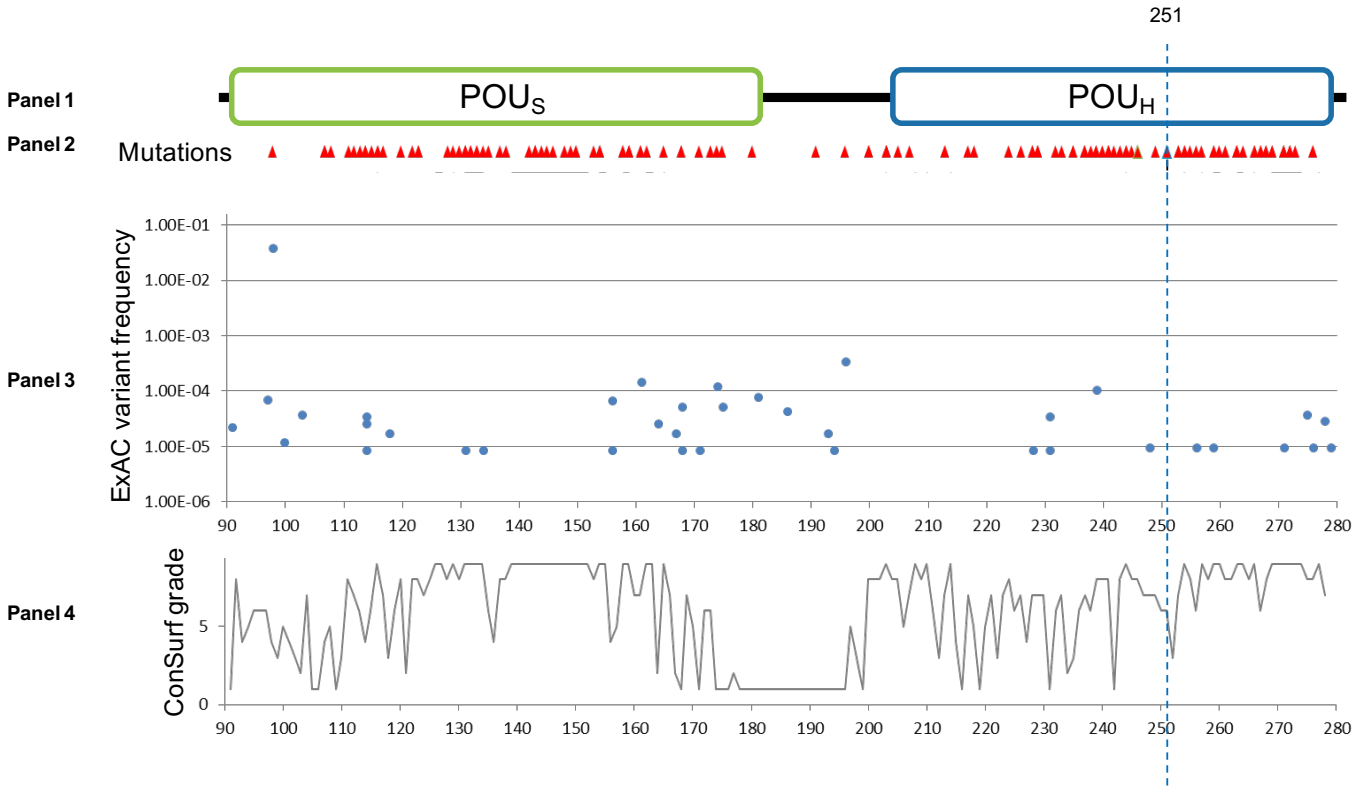
Recognising the interest in the variant, a more detailed *in silico* analysis of the structural effects of A251T was undertaken by a collaborator Mr Richard Caswell, Exeter Molecular Genetics laboratory. Results of these are presented below.

6.5.1 A251 Region Variation

The A251 region of the *HNF1A* gene was explored in sequence and mutation databases. The functional importance of this region was supported by the finding of many reported mutations in this area on HGMD (see figure 6.4) and a relatively low frequency of polymorphisms in ExAc, as might be predicted for a gene associated with a monogenic condition that is dominantly inherited. ConSurf (255), a database of conservation scores for any region, was also used and shown in figure 6.4. Taken

together it was concluded that this region is functionally important and highly conserved, suggesting that variants encountered are more likely to be deleterious to *HNF1A* function.

Figure 6.4: Schematic of the HNF1A DNA Binding Domains and the Location of the A251 Residue



Mutations confirmed in the Human Gene Mutation Database (HGMD) are depicted by red triangles in panel 2. Panel 3, shows the frequency of variants found in the DNA-binding domain on ExAc and panel 4, depicts ConSurf scores for this region (range 1-9, lowest to highest conservation).

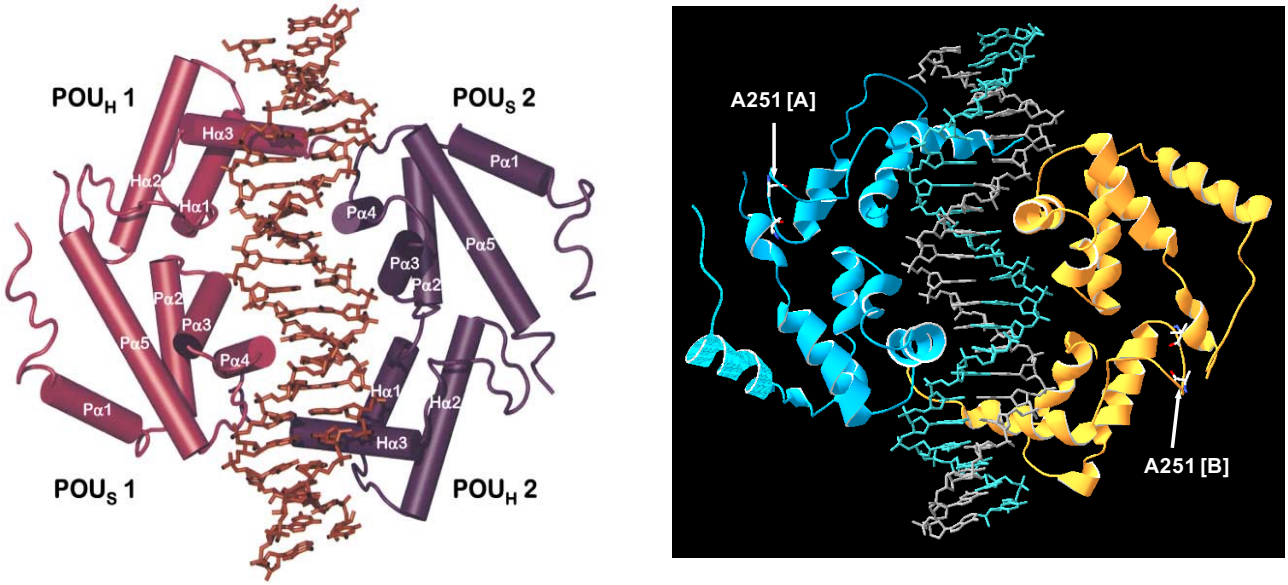
6.5.2 Structural Modelling

The crystal structure for the *HNF1A* homeodomain in complex with DNA was previously reported (263). From structural modelling undertaken by Richard Caswell, the A251 residue was found to be in a region that forms the interface between the POU_S and POU_H domains of the DNA-binding domain (see figure 6.5). Along with other residues (V173, F177, V246 and P248) A251 is described as forming a hydrophobic patch between the two domains. These residues were therefore

examined in the ExAc and HGMD databases, to determine if poylmorphisms or mutations have been described in any of the residues. Only the P248 residue was found in the ExAc database and this was reported in a single heterozygous individual. In contrast, as would be expected based on results so far for this region, MODY-causing mutations were described in V173, V246 and indeed A251 (previously published see table 6.2).

Figure 6.5: Domain Structure of HNF1A

a) b)



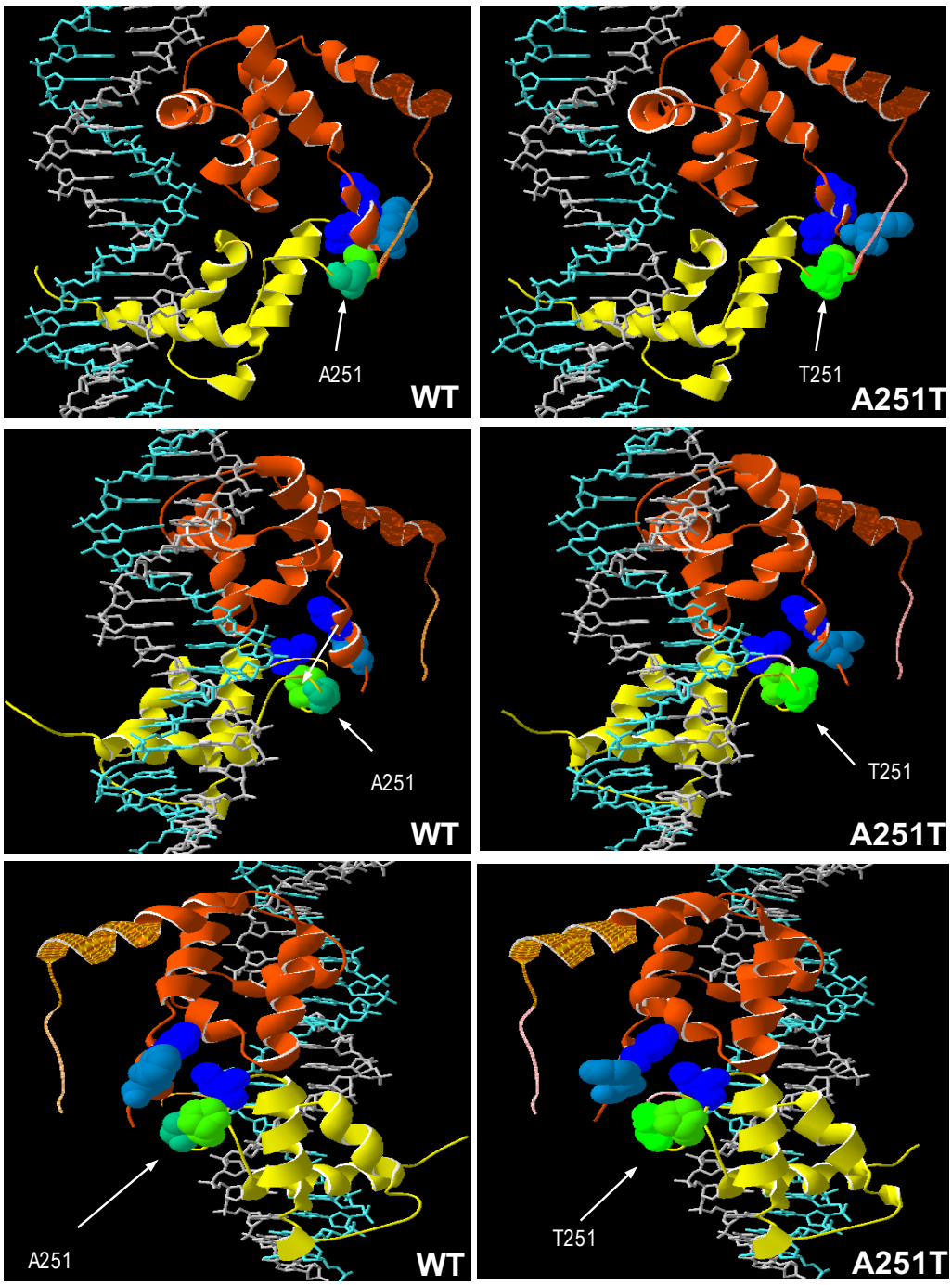
Part a) Indicating the location of the POU-specific (POU_S) and homeodomain (POU_H) regions and b) the crystal structure of two monomers of HNF1A (blue and yellow) bound to DNA (turquoise and grey), with residue A251 in stick format, demonstrating that the position interfaces POU_H and POU_S domains. (reproduced from (263) *with Elsevier copyright permission).*

The Swiss-Model server (Biozentrum, Switzerland) (264) was further used to predict the changes of the A251T variant on the overall HNF1A structure. Using a variety of models, the A251T variant was not found to significantly change protein structure. However, one of the limitations of this modelling process, is that the effect on protein folding is not taken into consideration.

Detailed inspection of the A251 residues did, however, show that the substitution changed the physiochemical properties of the interface, due to packing of the side

chains or hydrophobicity. The A251T substitution resulted in a small increase in polarity and side chain volume, demonstrated by the Grantham distance of the A to T substitution of 58 (scores >50 are generally considered to be potentially damaging). The substitution also displaced the side chain of the F177 residue. These changes are depicted in figure 6.6.

Figure 6.6: Modelling HNF1A Structural Effect of A251T Variant



Effects of the T substitution compared to A at position 251. Each panel shows a different rotation. In each case wild-type (WT), with the alanine in position 251 on the left and variant (A251T) on the right. The POU_S and POU_H domains are brown and gold respectively; residues forming the hydrophobic patch (173, 177, 246, 248 and 251) are shown as spherical and are coloured by hydrophobicity (dark blue = most hydrophobic, brown = most polar)

In summary this more detailed structural analysis showed that;

- the A251 residue was found at the interface of the POU_S/POU_H domains, a region that is intolerant to substitutions, as evidenced by the high density of HGMD mutations and low frequency of ExAC variants in this area.
- considering the relative position of A251 within the interface, structural modelling suggested that the molecular effects were likely to be, if anything, mildly detrimental.
- these findings were inconclusive but did support the hypothesis that the A251T variant studied, could be a hypomorphic mutation, however further studies were needed to confirm this.

6.6 In vitro Functional Assays

In vitro functional studies were undertaken to further elucidate the effect of the A251T variant on *HNF1A* function.

6.6.1 Background

A series of assays have been described to investigate transcription factor variants, such as those in *HNF1A,* based on published studies (265–267). These include an assessment of transactivation potential (the transcriptional activity of HNF1A), protein production and DNA-binding. The background to these methodologies are described here.

6.6.1.1 Luciferase Assay

Genetic reporter assays utilise the ability of a reporter gene (for example *Firefly*) to produce a product (for example luciferase), which can then be measured by assessment of its bioluminescent activity. A vector containing a promoter (for example the albumin promoter with a specific *HNF1A* recognition sequence) and the *Firefly* reporter gene can be transfected into a cell line, such that the amount of luciferase produced is dependent on the activity of the promoter. An additional vector containing

a gene of choice, e.g. *HNF1A* can be co-transfected. Now the ability of the albumin promoter (if the cell does not express HNF1A itself) to produce luciferase is based on the amount of HNF1A protein generated and its ability to transactivate the albumin promoter by binding to its recognition sequence.

In this way, by co-transfection of the luciferase expressing vector with HNF1A vectors containing either variant or wild-type (WT) HNF1A, the different effects of the HNF1A variants on transactivation can be ascertained. See figure 6.7.

However, effects observed may be due to other variables, for example cell number and transfection efficiency. To correct for these variables, a third vector expressing a control luciferase reporter gene can also be transfected. Classically, a *Renilla* luciferase vector with an sv40 (simian-virus 40) promoter is co-transfected with other experimental vectors. The cell will produce *Renilla* luciferase and this will be a marker of cell number and transfection efficiency. At the end of the assay the proportion of Firefly luciferase can be corrected for Renilla luciferase activity as an internal control.

6.6.1.2 Protein expression studies

Ascertaining the effect of the variant on *HNF1A* protein expression adds a further piece of information. Specific mutations may interfere with expression of HNF1A protein, but not with its transactivating potential. Therefore, combining these functional aspects, provides further insights into the effect of the variant. Using an immunoblot technique, HNF1A protein can be identified and measured, as previously published (223).

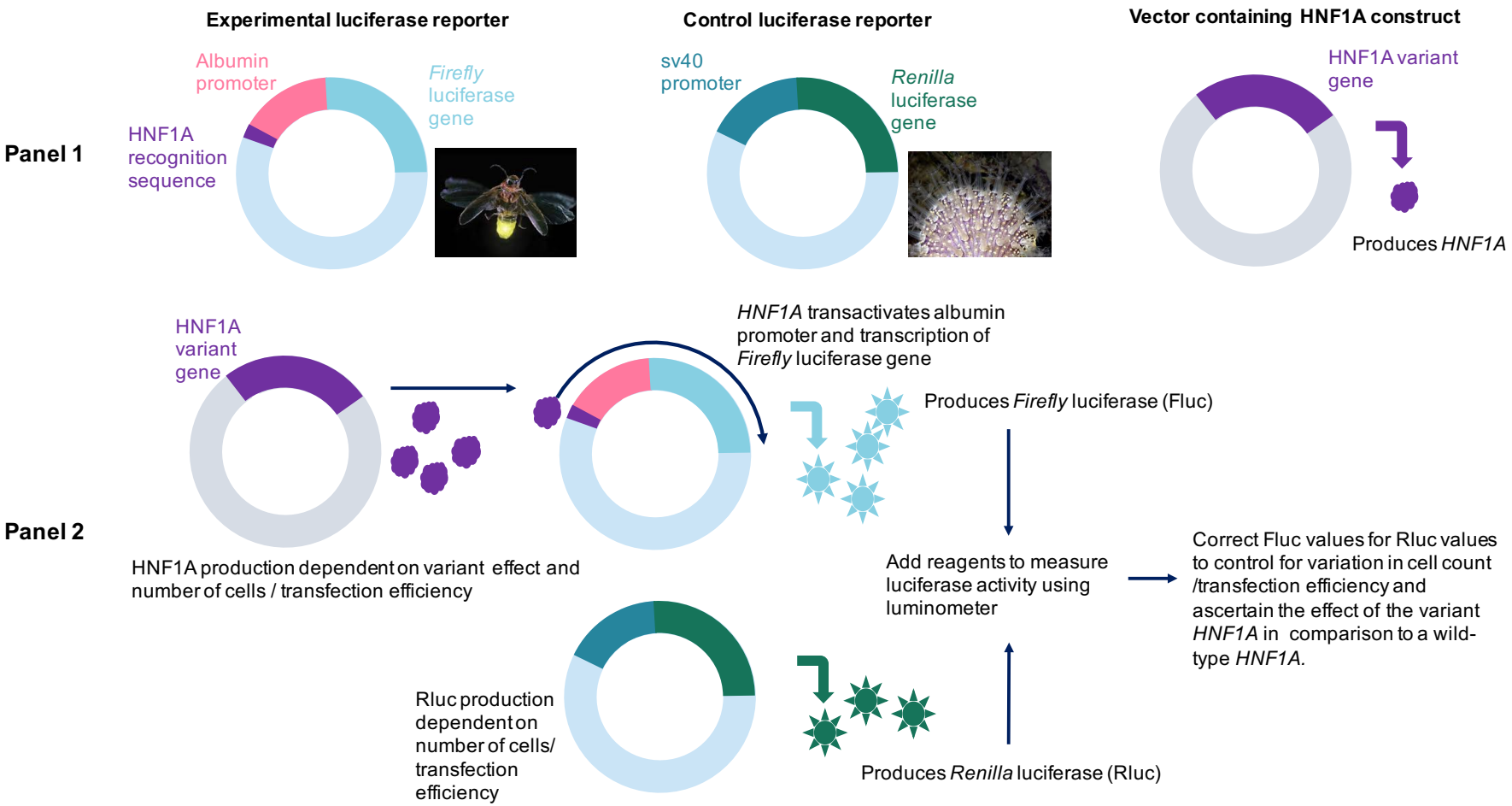
6.6.1.3 DNA-binding studies

DNA binding studies assess how well a transcription factor binds to its recognition sequence in double stranded DNA oligonucleotide probes. This component is assessed in luciferase assays too, as described above, to initiate transactivation of the luciferase gene, HNF1A must first bind to its recognition sequences. However, transactivation may be reduced for a variety of reasons other than DNA binding and therefore it is critical to assess DNA-binding in isolation too.

An electromobility shift assay (EMSA) allows DNA binding to be assessed by the electrophoresis of DNA-protein complexes. In the presented studies, HNF1A variant

or wild-type protein was incubated with DNA probes designed to replicate the palindromic sequences that HNF1A recognises. These mixes were then electrophoresed on a gel. To prove that it was HNF1A binding to the DNA probe an additional DNA mix containing wild-type HNF1A was incubated with an HNF1A primary antibody. The resulting complex formed is much larger and shifts in the gel. Comparison of band intensity between wild-type and variant complexes with DNA, allow for quantification of the extent of DNA binding to the variant HNF1A.

Figure 6.7: Steps in a Dual Luciferase assay



Panel 1 depicts each of the plasmid constructs transfected and the genes incorporated. Panel 2 illustrates the steps in the generation of luciferase and its relation to HNF1A or cell effects

During a research attachment in collaboration with Professor Anna Gloyn, in Oxford University, detailed functional investigations were undertaken by myself.

6.6.2 Hypothesis

Using a series of functional assays, A251T will be only mildly impaired in function when compared to wild type *HNF1A* and established MODY mutations.

6.6.3 Methods

The methods described here have been developed in the Gloyn laboratory over years of work in this area and where acknowledged, members of the Gloyn team were responsible for the specific work outlined.

To investigate the effect of the A251T variant, experiments were conducted comparing A251T to wild type (WT) HNF1A, to a synonymous variant, H179H (that should be comparable to WT) and also to variants with a confirmed MODY-causing phenotype or with published functional data; P112L (266), R203H (214), T260M (268) and E508K (269). In each experiment a negative control, where no HNF1A was expressed (Empty vector), was also used. Data on each of the variants used as internal controls are outlined in table 6.3.

The Gloyn lab had previously undertaken initial functional characterisation of the A251T variant using Luciferase assays, based on the heterozygous case previously outlined (178). In those initial experiments, transactivation was assessed using the human albumin promoter sequence and variants in the PrK5 plasmid and this had demonstrated a 20% reduction of the A251T variant of transactivation potential compared to WT (80% of WT).

Since those assays the team changed methodology to align with a concurrent project running the lab, to use the rat albumin promoter and variants in the pcDNA3.1 plasmid. Experiments described here, were therefore conducted in line with this new protocol.

However, as will be seen later, experiments were also repeated using the original parameters to check comparability.

Table 6.3: Description of Variants Transfected in Functional Assays

Variant name / location	Rationale	Effect size (if known)		
		T	DB	P
P112L Pro112Leu DNA-binding domain	Known MODY mutation with confirmed severe deleterious effect on transactivation, DNA-binding and protein expression (266).	15-30%	30-40%	
H179H His179His DNA-binding domain	Synonymous variant that should have similar results to WT, used as an internal control to demonstrate that mutant generation itself has no effect on functional assays.	=WT	=WT	=WT
R203H Arg203His DNA-binding domain	Known MODY mutation with confirmed deleterious effect on transactivation, DNA-binding and protein expression (unpublished).	60%		
A251T Ala251Thr DNA-binding domain	Test variant. Transactivation result based on previous internal lab work (270).	80%		
T260M Thr260Met DNA-binding domain	Known MODY mutation with confirmed deleterious effect on transactivation, DNA-binding and protein expression (unpublished).	20%		
E508K Glu508Lys Transactivation domain	Low frequency variant in Latino population associated with increased type 2 diabetes risk, with confirmed reduction in transactivation potential (269).	35-45%	=WT	53%

WT, wild-type; T, transactivation potential; DB, DNA-binding; P, protein expression. Percentages of WT are given, where WT is 100%.

6.6.3.1 Generation of Mutant and Wild-type constructs

The WT and variant/mutant *HNF1A* constructs were created using site directed mutagenesis (work undertaken by Dr's Amanda J Bennett and Neelam Hassanali over preceding years in the Gloyn lab). Briefly, reactions were performed using the QuikChange II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA,

USA) to introduce single nucleotide changes into *HNF1A* cDNA (NCBI Entrez Gene BC104910.1). Each construct was then cloned into the pcDNA 3.1/HisC vector (ThermoFisher, Waltham, MA, USA) using appropriate restriction sites. These constructs were then introduced into competent E.coli cells through transformation, cultured on agar-plates overnight and DNA extracted and sequenced for confirmation. In the experiments outlined in this chapter, mutant and WT plasmids were cultured from DMSO stocks of the originally transformed bacteria by myself. DNA extraction was then undertaken using the PureYield MidiPrep plasmid system (Promega, Madison, WA, USA) into 50 μL of distilled water and DNA was quantified using the Nanodrop 2000 spectrophotometer (ThermoScientific, Waltham, MA, USA).

6.6.3.2 Cell Lines and Cell Preparation

HeLa cells were selected for experimentation as they lack endogenous *HNF1A*, enabling the homozygous state of the A251T mutant to be replicated following transfection. HeLa cells were maintained in accordance with established published (266) and laboratory protocols, these protocols are fully described in appendix 8 and a brief overview is provided here.

For Luciferase experiments, HeLa cells were seeded in 24-well plates at a cell density of $4x10^5$/ well in 500 μL volume of Dulbecco's modified Eagle's Media (DMEM, ThermoScientific, Waltham, MA, USA). For Western blot and electromobility shift assays (EMSA), 10 cm dishes were used and HeLa cells were seeded at $2x10^6$ cells/dish in 10 mls of DMEM. In both experiments, cells were incubated for 24 hours prior to transfection and transfected at between 70-90% confluency. See appendix 9 for full details.

6.6.3.3 DNA Mixes and Transfection protocol

- Dual Luciferase Assay Kit (Promega, Madison, WA, USA)

DNA mixes for transfection were prepared in a standardised format to ensure in each case a total of 1 μg DNA was transfected per well. Each DNA mix was prepared to enable transfection in triplicate (3 technical replicates). Each mix contained:

- 500 ng promoter (rat or human albumin in pGL3-basic vector containing the albumin promoter, the *HNF1A* recognition sequence and the *Firefly* luciferase sequence)
- 10 ng pRL-SV40, simian-virus 40 (control reporter vector encoding *Renilla* luciferase gene),
- 250 ng WT or variant DNA, in pcDNA3.1 (or PrK) vector.
- 240 ng empty plasmid (or 490 ng for negative control) in pcDNA3.1 (or PrK)
- water to make the total volume 70 μL

DNA mixes were then incubated with Opti-MEM reduced serum media (Gibco/Life Technologies, Waltham, MA, USA) and Lipofectamine 2000 (Invitrogen, Waltham, MA, USA) before transfection (fully described in appendix 9).

- Combined Western Blot / EMSA assay

A combined protocol enabling cell lysates from one transfection experiment to be used for both Western Blot and EMSA analysis was used and is shown in full in appendix 10. Briefly, DNA mixes were prepared such that 5 μg of each variant was aliquoted into a tube and water added to a total volume of 50 μL. DNA mixes were then incubated with Opti-MEM reduced serum media and Lipofectamine 2000 before transfection.

6.6.3.4 Transactivation Studies

Transcriptional activity of wild-type (WT) and variants were assessed using the Dual Luciferase Assay System (Promega, Madison, WI, USA), performed in three technical replicates and at least three biological replicates on cells of varying passage. Following transfection, cells were harvested at 24 hours and media removed. Using passive lysis buffer, plates were placed on a plate-shaker for 30 minutes to lyse cells. Plates were then stored at -80°C for at least 4 hours. After plates were defrosted the luciferase assays was performed on 96-well plates using 10 μL of cell lysate from each well, combined with 75 μL of Luciferase assay reagent and 75 μL of Stop & Glo reagent (from the Luciferase kit). Finally, bioluminescence was measured in each well using the Enspire plate reader. Luminescence values of *Firefly* luciferase were normalised to *Renilla* activity and expressed as a percentage of WT.

6.6.3.5 Western Blot and EMSA Protocol

The full protocol can be found in appendix 10. Briefly, 24 hours post-transfection, 10 cm dishes were scraped with phosphate buffered saline and collected into Eppendorf tubes. Cells were lysed using 1 x protein lysis buffer containing a protease inhibitor and lysis buffer, incubated on ice and centrifuged at 13,000 rpm. Supernatant was collected and aliquots of the supernatant were quantified using Bradford reagent.
Protein lysate aliquots were then used for Western Blot of EMSA analysis.

- Western Blot

Using Bradford quantified protein lysates, the volume required of each variant and WT lysate to equate to 10 μg of total protein, was calculated. Appropriate volumes of lysates were then denatured on an 80-degree heat block, cooled and combined with a loading buffer before being loaded onto a pre-cast gel (BioRad Criterion TGX Stain-Free Protein Gel). The gel was electrophoresed in running buffer for 20 minutes at 280 volts.
After the run, the gel was activated on the Chem Doc Imager (Bio-Rad laboratories). Proteins were then transferred to a membrane using the Bio-Rad Trans-Blot turbo pack. The blot was visualised to check success of transfer, blocked for 1 hour with 5% milk solution and finally incubated overnight in 5% milk solution with primary antibodies to HNF1A and tubulin (Santa-Cruz biotechnology). The following morning, the blot was washed and incubated with secondary antibodies for 1 hour. Blots were then visualised on the Bio-Rad ChemiDoc. Protein bands were quantified using Image Lab densitometry software (Bio-Rad). HNF1A protein bands were expressed as a ratio to tubulin bands and variants were then expressed as a ratio to WT.

- EMSA

A fresh aliquot of the same Bradford quantified lysate was defrosted for the EMSA assay using the LI-COR Odyssey Infrared EMSA kit (Bio-Rad). A full protocol is available in appendix 10. Briefly, forward and reverse oligonucleotides (DNA fragments) to a specific HNF1A sequence were ordered with end labelled infrared

dyes, as below. Oligonucleotides were annealed on a heat block to form double-stranded DNA oligonucleotides prior to the EMSA assay.

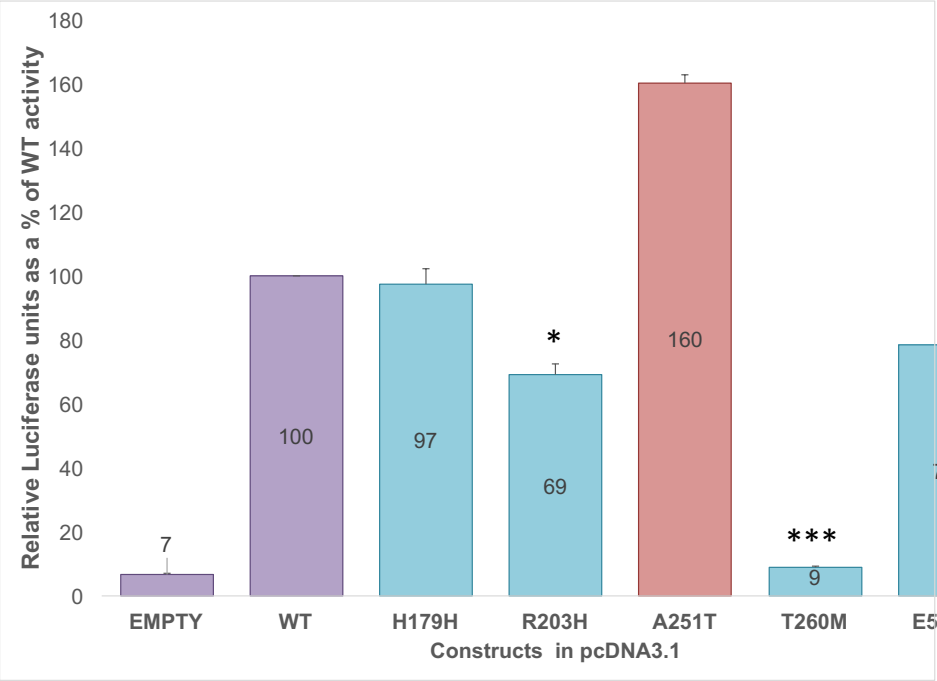
5'CY5-TGT**GGTTAATG**ATCTACAGTTATTGGT-3'
5'CY5-ACCAATAACTGTAGAT**CATTAACC**ACA-3'

On the day of the experiment, lysates were thawed and binding reactions prepared, which included appropriate buffers (as per kit instructions), lysate from WT or variant transfections and the DNA probe (pre-prepared as above). In addition, a second WT binding reaction was set up, which included HNF1A primary antibody for the supershift assay that was incubated at room temperature for 30 minutes prior to addition of the DNA probe. Once DNA probe was added binding reactions were incubated at room temperature and protected from light for 20 minutes. A pre-cast non-denaturing gel (Criterion 5% in Tis Borate EDTA buffer, Bio-Rad) was pre-run at 100 volts during the binding reaction. After the binding reaction was complete, samples were loaded onto the gel and run at 100 volts for at least 1 hour, protected from light. After the run was complete, gels were visualised on the Chemi-Doc imager and densitometry was undertaken using Image-Lab software. Band intensity of variants was expressed as a proportion of the WT band.

6.6.4 Functional Assay Results

In the first set of luciferase experiments, the rat albumin promoter and WT/ variants in pcDNA 3.1 were used to assess transactivation of A251T. These results (see figure 6.8) showed an increase in transactivation of A251T compared to WT, though this did not reach significance. This was in marked contrast to the original data (see table 6.3), which demonstrated a 20% reduction in comparison to WT (80% of WT). The result was confirmed on 3 biological replicates.

Figure 6.8: Transcriptional Activity of Wild-type and Variant HNF1A in pcDNA3.1 Plasmids, using the Rat Albumin Promoter Reporter Construct

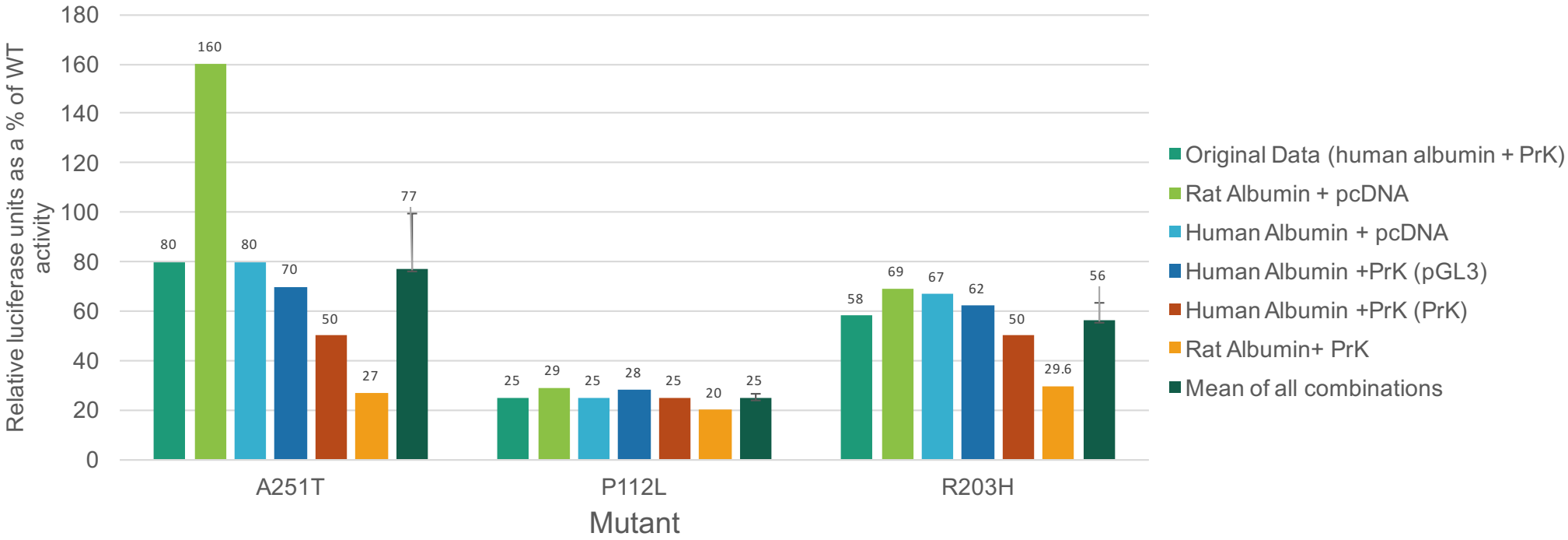


*Blue bars represent positive controls, which have previous documented effects on transcriptional activity. Data shown is the mean (SEM) of n=3 biological replicates, each with n=3 technical replicates). A two-tailed students t-test was used to compare the mean variant activity to WT, with significance *p<0.05, **p<0.01, ***p<0.001.*

As these assays were undertaken using a different plasmid and promoter to the original experiments, a crossover experiment that assessed the effect of the rat albumin and human albumin promoters along with constructs in the pcDNA 3.1 and pRK-5 plasmid was undertaken (figure 6.9). This experiment consisted of 3 technical replicates from a single biological replicate.

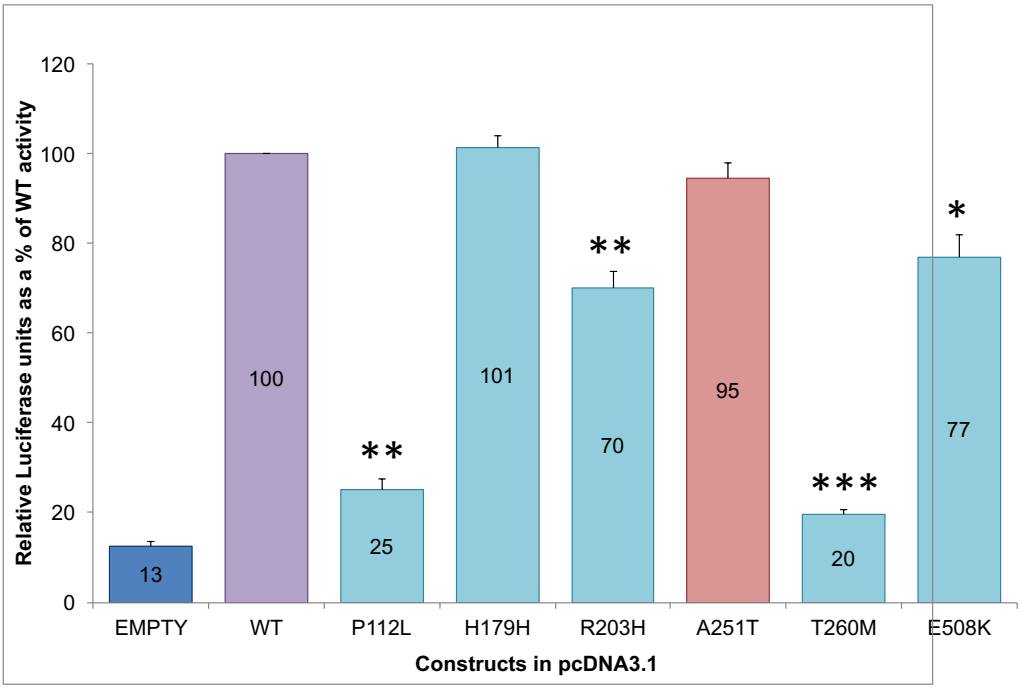
Figure 6.9: Transcriptional activity of A251T, P112L and R203H using either the PrK or pcDNA plasmids and rat albumin or human albumin promoters, in HeLa cells

The human albumin with PrK plasmid experiment (blue and red bars), was conducted twice, once with a pGL3 empty vector and once with PrK empty vector. Each experiment was repeated in three technical replicates on a single biological replicate.



Considerable variability in the transactivation potential of A251T was observed depending on the constructs and promoter used. However, P112L and R203H were less variable across experiments. This suggested a specific A251T effect that remained unexplained. It was noted that using the human albumin promoter and PrK plasmid with pGL3 empty vector, the activity was 70% of WT and this was comparable to original data (see previous table 6.3). The use of the human albumin promoter with pcDNA was also comparable and as protein expression and EMSA analyses were underway with use of the pcDNA 3.1 vector, for continuity, the following constructs were selected for luciferase experiments: pcDNA3.1 plasmid for WT and variant constructs and human albumin promoter in pGL3-basic vector with the *Firefly* reporter. Results of 4 biological replicates using these constructs are shown in figure 6.10.

Figure 6.10: Transcriptional Activity of Wiild-type and Variant HNF1A in pcDNA3.1 Plasmids, using the Human Albumin Promoter Reporter Construct



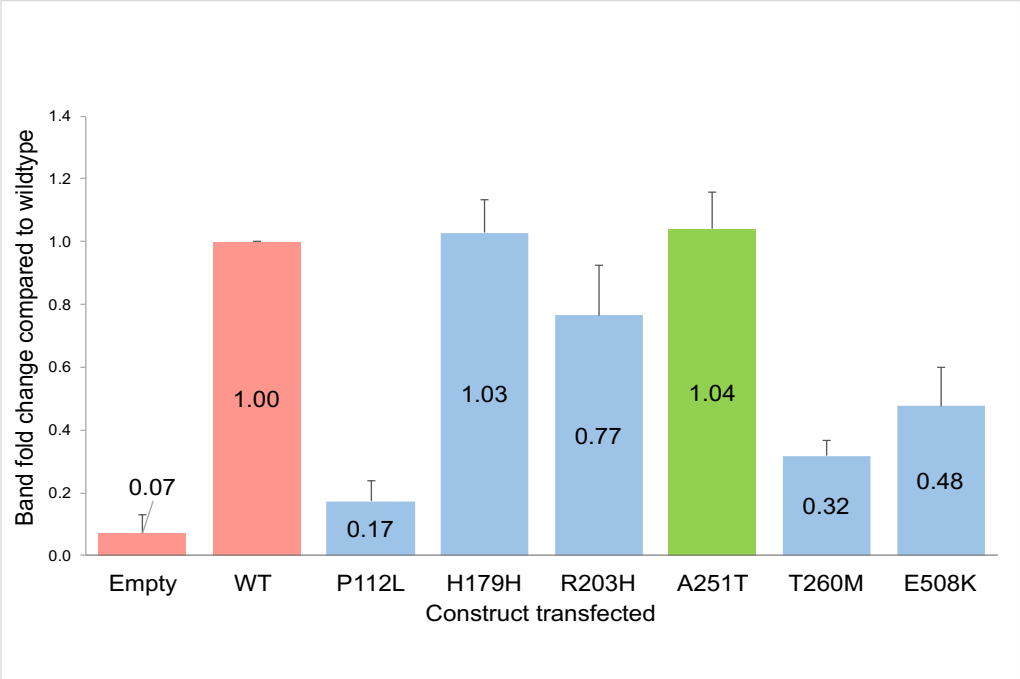
*Blue bars represent positive controls, which have previous documented effects on transcriptional activity. Data shown is the mean (SEM) of n=3 biological replicates, each with n=3 technical replicates). A two-tailed students T-test was used to compare the mean variant activity to WT, with significance *p<0.05, **p<0.01, ***p<0.001.*

These data demonstrated that there was no significant difference in the transactivation potential of A251T compared to WT HNF1A protein. Control variants performed as expected based on previous data available.

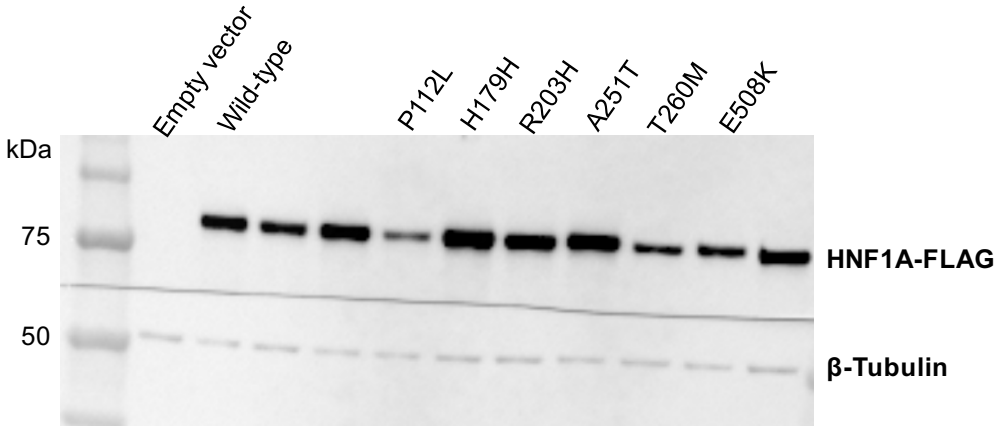
Results of protein expression experiments are shown in figure 6.11.

Figure 6.11: Protein Expression Levels of Wild-type and Variant HNF1A in pcDNA3.1 Transfected in HeLa cells

a)



b)

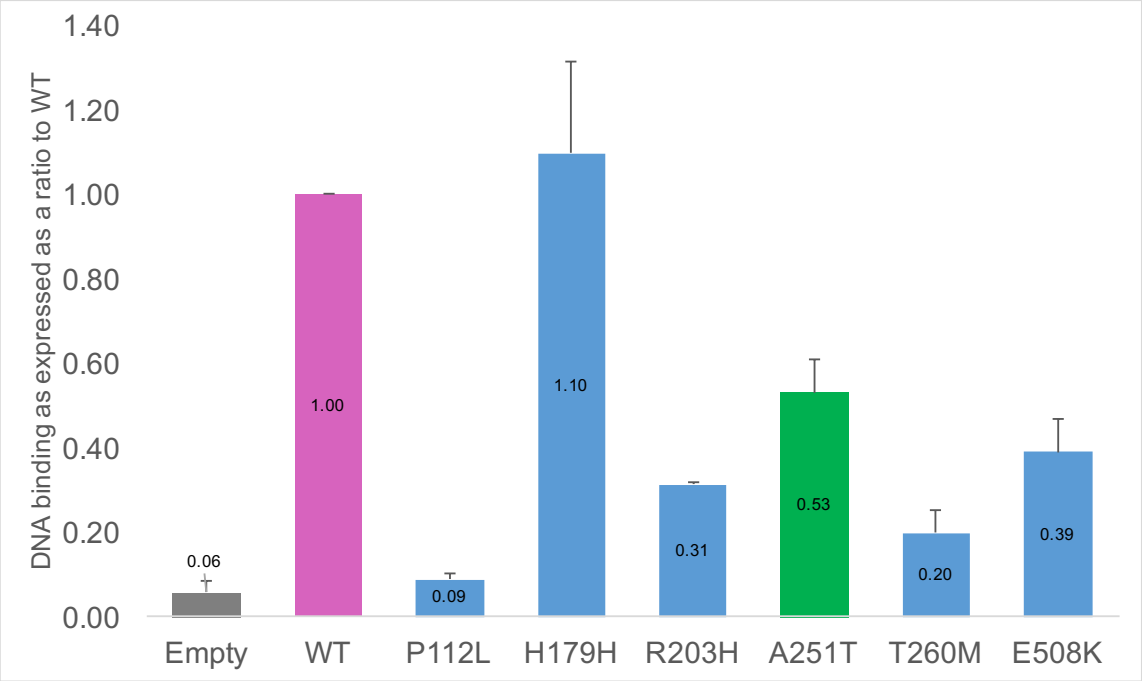


*Part a) Band fold change compared to WT: Blue bars represent positive controls with previous documented effects on transcriptional activity. HNF1A expression was corrected to tubulin and variants were expressed as a ratio to WT. Data shown is the mean (SEM) of n=3 biological replicates. A two-tailed students t-test was used to compare the mean variant activity to WT, with significance *p<0.05, **p<0.01, ***p<0.001. b) the Western blot is depicted from one of the biological replicates.*

In comparison to wild-type HNF1A, the A251T variant demonstrated no reduction in protein expression. H179H was not significantly different to WT, as predicted. MODY mutations such as P112L and T260M performed similar to internal experiments and E508K data was comparable to published reports of 0.53 compared to WT (269). The results of DNA-binding experiments using EMSA's are shown in figure 6.12.

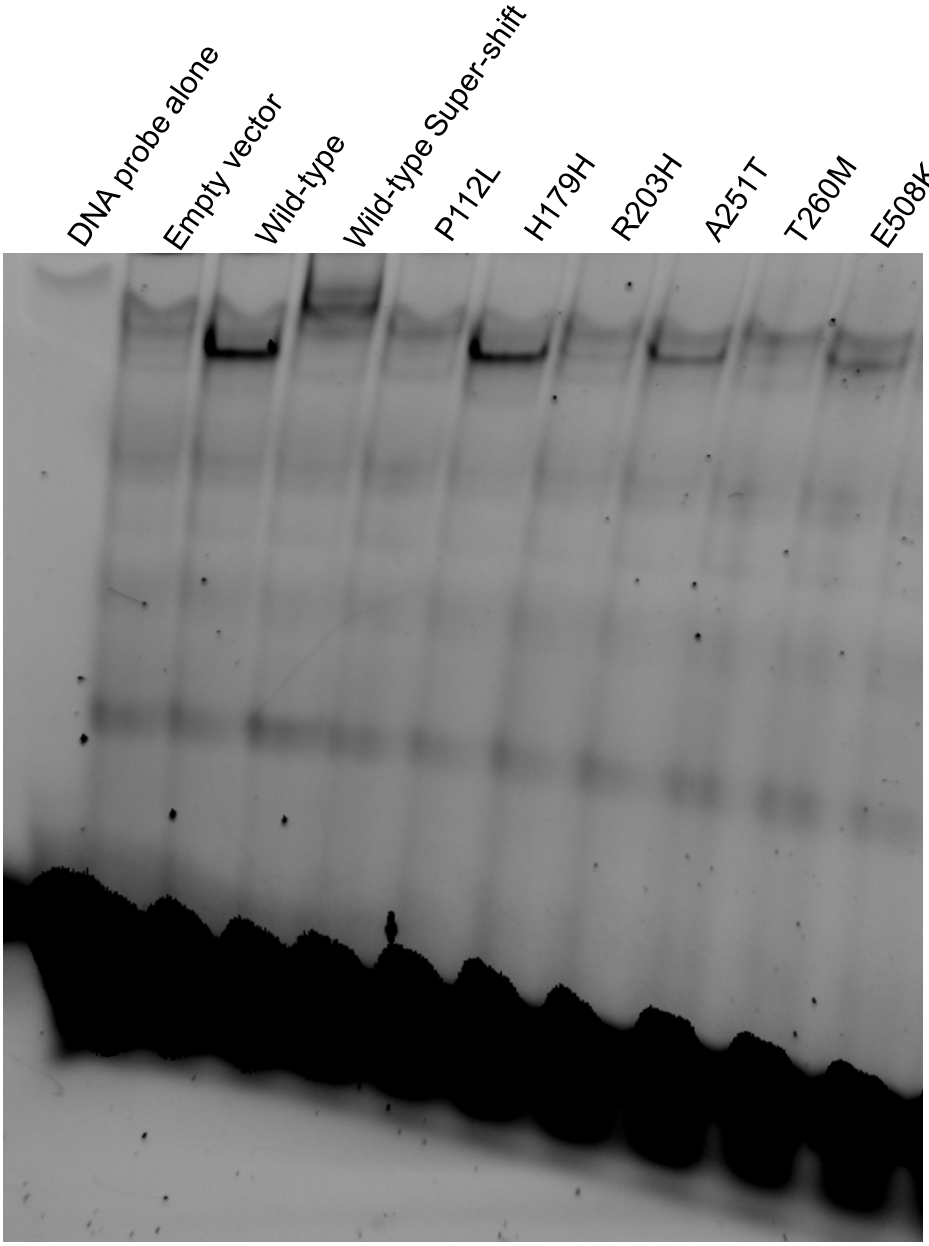
Figure 6.12: a) Electromobility Shift Assay Comparing DNA Binding of Wild-type and Variant Constructs from HeLa cell Protein Lysates and b) example gel

a)



*Part a) DNA-binding activity normalised to WT of n=3 biological replicates. Blue bars represent positive controls. A two-tailed students t-test was used to compare the mean variant activity to WT, with significance *p<0.05, **p<0.01, ***p<0.001.*

b)

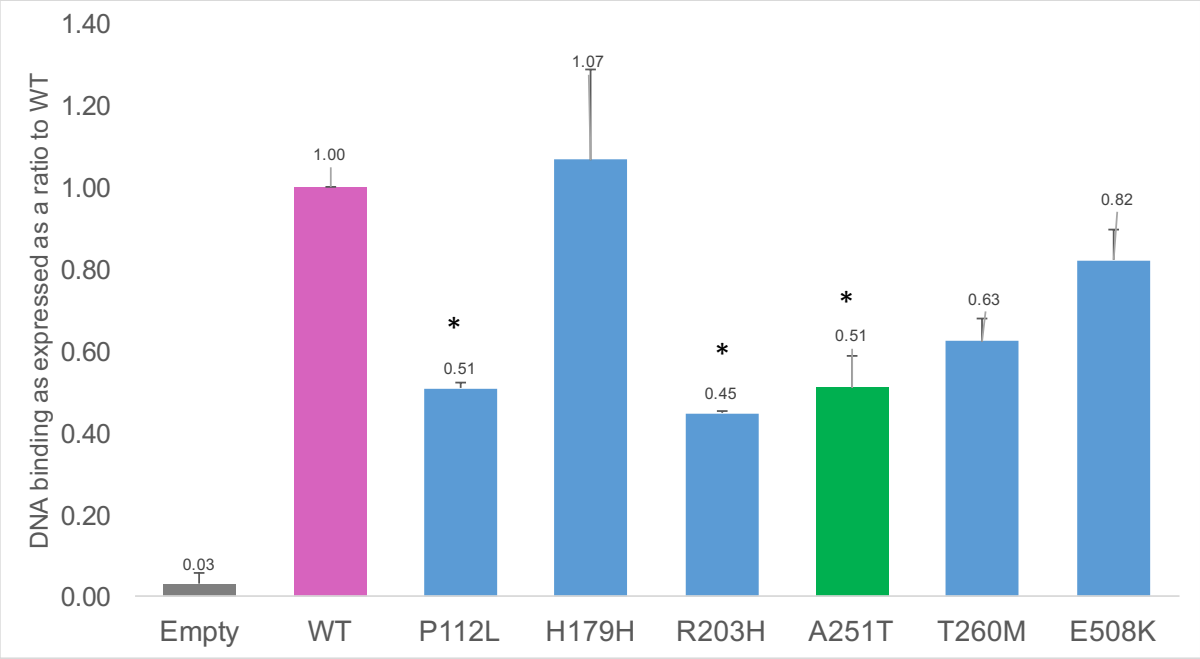


b) the super-shift gel is depicted from one of the biological replicates as an example

A251T demonstrated a significant reduction in DNA-binding compared to WT. However, this reduction was milder (47% reduction) than the severe MODY mutations reported in the DNA-binding domain; P112L (91% reduction) and T260M (80% reduction).

To determine if these effects were related to the proportion of HNF1A in each experiment used to assess DNA binding, activity was divided by the HNF1A expression based on western blot analysis. This protein corrected EMSA activity is depicted in figure 6.13.

Figure 6.13: HNF1A Protein Expression-corrected Electromobility Shift Assay Comparing DNA Binding of Wild-type and Variant Constructs from HeLa Cell Protein Lysates



*All activity normalised to WT. Blue bars represent positive controls. N=3 biological replicates. A two-tailed students t-test was used to compare the mean variant activity to WT, wit significance *p<0.05, **p<0.01, ***p<0.001.*

When corrected for HNF1A protein expression levels, the reduction in DNA-binding observed in the A251T variant was still statistically significant. The P112L variant has a published DNA-binding activity between 30-40% of WT, results were therefore comparable. However, the approach of correcting in this way, may be flawed as HNF1A protein expression, when reduced, may signify reduced DNA binding, thus results may be falsely increased using this technique.

In summary results from *in vitro* functional data, have shown that compared to wild-type HNF1A, A251T has comparable transactivation potential and protein expression,

but modestly reduced DNA-binding. Variability was observed in the transactivation potential of A251T depending on the experimental system and this requires further investigation (see discussion). Taken together these results support only a mildly deleterious effect of A251T on the HNF1A functional domains assessed and support the hypothesis that the A251T variant is a hypomorphic mutation.

6.7 In vivo Functional Assays

With the results of the *in vitro* data and *in silico* modelling, there was clearly a potential that the hypothesis regarding A251T was correct. However, further proof of this would lie in results of *in vivo* studies within the proband. Specifically, high-sensitivity C-reactive protein (hsCRP) and response to sulphonylurea studies were undertaken.

6.7.1 High Sensitivity C-Reactive Protein

As discussed in chapter 1, the HNF1A transcription factor also transcriptionally activates C-reactive protein (CRP), an acute phase response protein produced in the liver. Studies in people with confirmed *HNF1A* MODY mutations have shown that CRP levels are low in comparison to people with type 2 diabetes, a pro-inflammatory state, and other types of diabetes including type 1 and non-HNF1A MODY mutations (176,177,271). However, a high-sensitivity assay must be used to ensure the assay can measure to a very low level, beyond those assays used for standard CRP measurement.

Serum samples from the proband, his parents and identical twin were used to measure hsCRP on a Roche/Hitachi Cobas C sytem, using an immunoturbidometric assay. The lower limit of detection was 0.15mg/L. The results of this along with genotype of each individual is depicted in table 6.4.

Table 6.4 High sensitivity C- reactive protein levels in A251T kindred

	Genotype	CRP mg/L
Proband	A251T / A251T	<0.15
Identical twin	A251T / A251T	<0.15
Mother	Normal / A251T	0.2
Father	Normal / A251T	1.4

As can be seen, both homozygous individuals had undetectable hsCRP levels in keeping with an *HNF1A* MODY phenotype, whilst the parents who are heterozygotes, demonstrated variable, but not undetectable levels of hsCRP. The result in the father in particular, is much higher than would be expected in someone with an *HNF1A* mutation. These results, again, support the hypothesis of a hypomorphic mutation; homozygotes of A251T behave similarly to bona fide MODY mutations, but heterozygotes have no HNF1A phenotype.

6.7.2 Sulphonylurea Trial

Demonstrating sulphonylurea sensitivity such that a transition off insulin can be achieved and maintained is perhaps one of the most compelling pieces of evidence, when investigating pathogenicity of an *HNF1A* variant. It is not without its limitations as a proportion of mutations are not reportedly sensitive and with long-duration of HNF1A MODY, beta-cell function may decline such that insulin is required anyway (104). The proband attended the day unit for a trial dose and observation to monitor for the development of hypoglycaemia, since it was unclear whether a homozygous HNF1A variant would be more or less sensitive to treatment.

Study protocol

The proband was advised to take Humalog Mix 25 insulin the night before the study as usual and attended the unit the following morning fasting, without taking the morning Humalog Mix 25 dose.

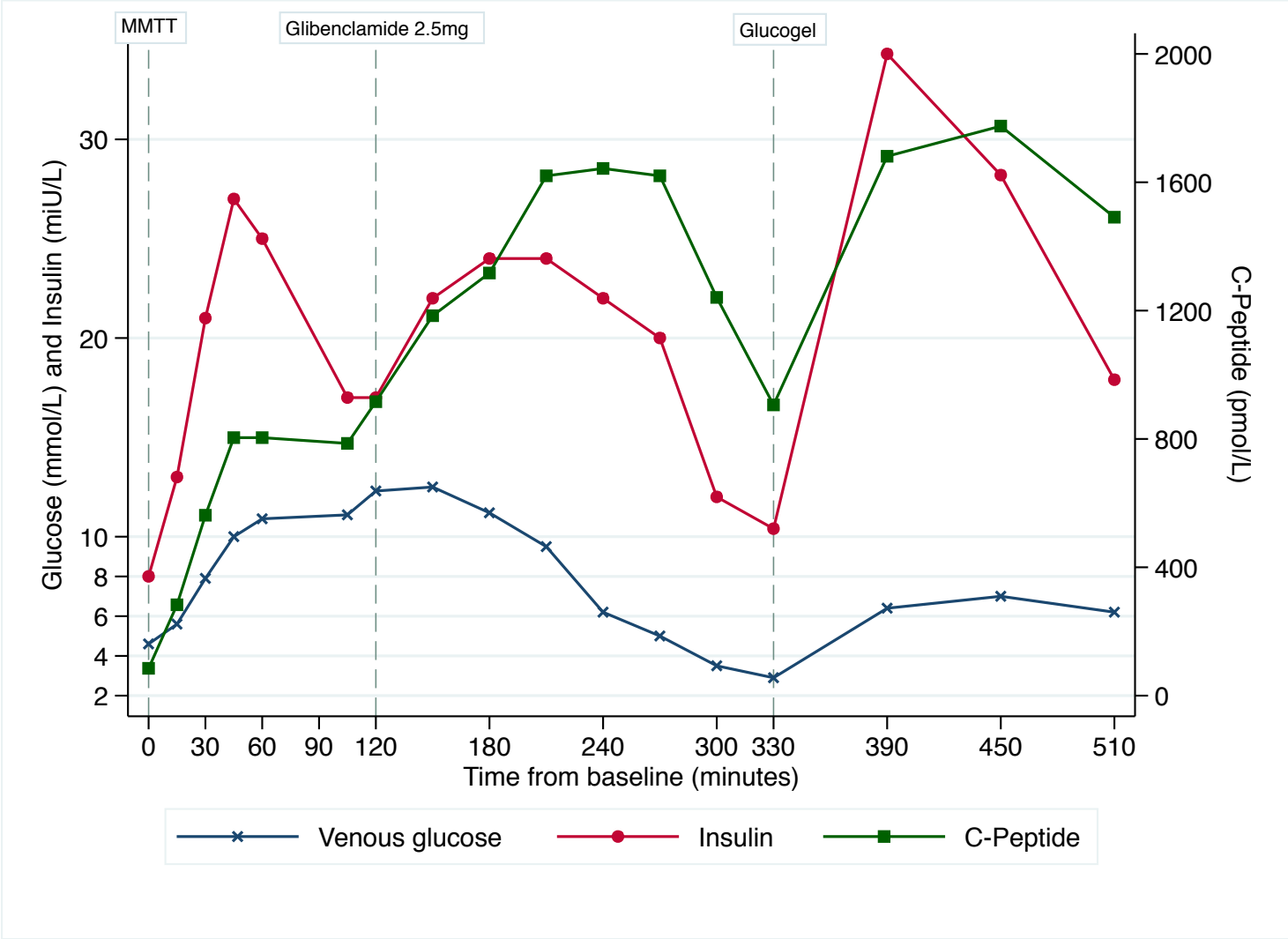
A mixed meal tolerance test (MMTT) was undertaken at time 0 minutes and samples for insulin, C-peptide and glucose were collected. The MMTT was conducted according to local protocols. Briefly, 6ml/kg of Ensure Plus (13.8 g protein, 10.8 g fat, 44.4 g carbohydrates, 220 ml, Abbott laboratories, Abbott Park, IL) was calculated to a maximum volume of 360ml. The proband was instructed to drink the contents over a maximum of 5 minutes. Blood samples were then collected at regular intervals until 2-hours post-ingestion.

At 2 hours after the MMTT, a 2.5mg glibenclamide dose was administered and glucose, C-peptide and insulin levels were measured for the rest of the day.

The proband also commenced real-time continuous glucose monitoring using a DexCom G4 device (San Diego, CA), which was provided as an extra precaution to mitigate against hypoglycaemia.

Results of glucose, C-peptide and insulin values are depicted in figure 6.14.

Figure 6.14: Composite Graph of Glucose, C-peptide and Insulin During a Mixed-Meal Tolerance Test and Glibenclamide Test Dose



As shown in the graph, despite not taking his morning insulin dose, the proband arrived with a normal blood glucose value. After ingesting the mixed meal, there was a rapid rise in blood glucose reaching a peak of 12.5 mmol/L. This was accompanied by a rise in insulin and C-peptide levels, signifying endogenous beta-cell function. However,

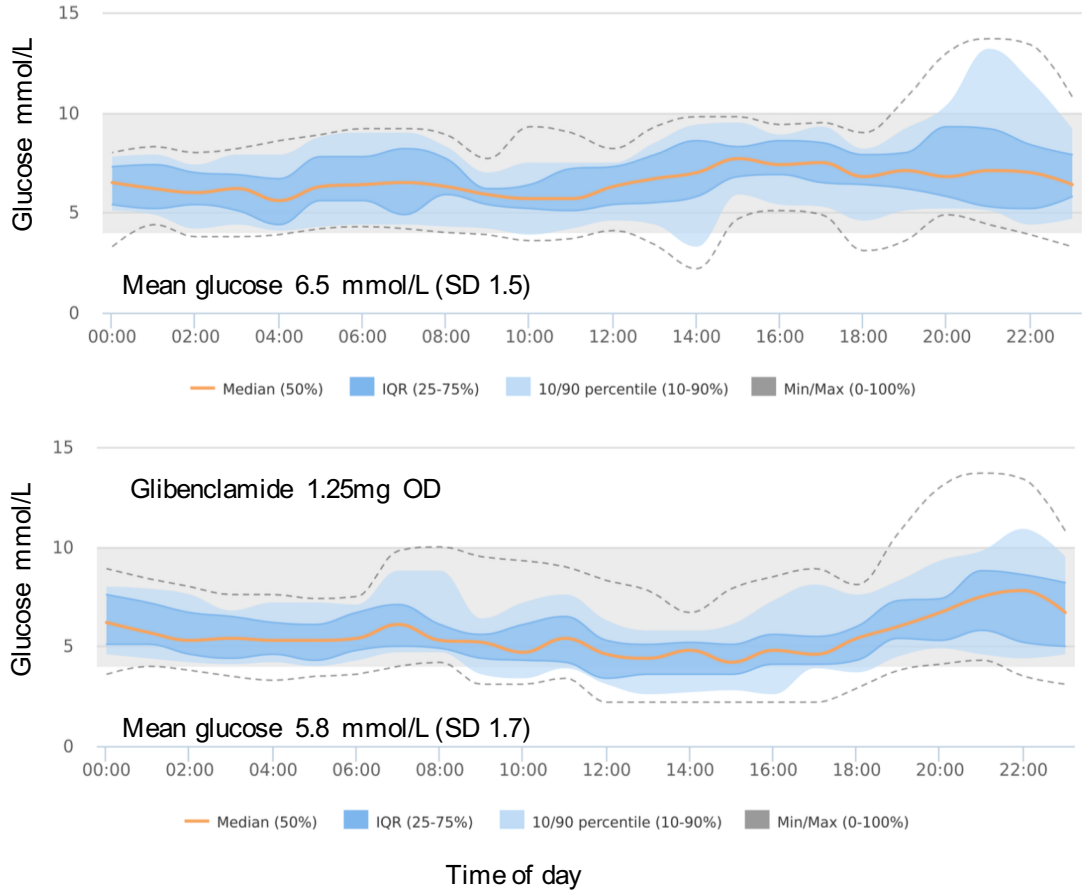
insulin levels began to drop even though glucose had not returned to its baseline level. The glucose levels plateaued, but did not return to baseline.

After ingestion of the glibenclamide test dose, there was a further increase in insulin and C-peptide, beyond the peak values seen during the mixed meal, even though no further food was ingested. The insulin and C-peptide rise appeared to be more sustained and levels began to drop after glucose values reduced. However, glucose levels reduced beyond the fasting level and the participant experienced symptoms of hypoglycaemia with a blood glucose value of 2.9mmol/L. Oral glucose was therefore administered (Glucogel 25g, BBI Healthcare, Brigend UK). This resulted in an even higher rise in C-peptide and insulin than seen before and glucose values returned to normoglycaemia, but not in the hyperglycaemic range.

These results, clearly demonstrate that the proband was sensitive to sulphonylurea therapy and experienced hypoglycaemia on a very low dose 2.5mg. He was therefore discharged on a dose of 1.25mg OD and reported no hypoglycaemia over the following 7 days.

This was confirmed on his real-time continuous glucose monitoring, shown in figure 6.15 before and after commencement of glibenclamide.

Figure 6.15: Ambulatory Blood Glucose Profiles Recorded from DexCom G4 Real-time Continuous Glucose Monitor



Upper panel, whilst insulin treated with BD Humalog Mix 25 and lower panel whilst on glibenclamide 1.25 mg OD. SD, standard deviation.

Despite transitioning off insulin, no change in the mean blood glucose values or standard deviations were observed.

After this transition off insulin, the probands' identical twin was also changed from insulin to sulphonylurea therapy with similar success. The two older sisters, now insulin-treated, were invited for cascade testing.

6.8 Discussion

6.8.1 Chapter Summary

The investigations into the pathogenicity of the A251T variant are summarised as follows;

- The homozygous A251T genotype, but not heterozygous genotype, correlated with an early-onset diabetes phenotype
- *In silico* structural predictions supported the hypothesis that the A251T variant would result in mild changes at the interface where the residue is situated in the DNA-binding domain of the HNF1A protein, but not a severe phenotype.
- The A251 residue is sited in a highly conserved area of the *HNF1A* gene with few reported polymorphisms and several confirmed mutations.
- *In vitro* studies supported that compared to wild-type HNF1A protein, the A251 variant has normal transactivation potential, normal protein expression, but significantly lower DNA-binding affinity.
- *In vivo* studies confirmed that the homozygous genotype, but not heterozygous genotype is associated with low hs-CRP levels and the homozygous genotype results in sulphonylurea sensitive phenotype.

Taken together these findings support the hypothesis that the A251T variant is indeed a hypomorphic mutation, however additional studies could be undertaken to investigate this further (see below).

This is the first report of a homozygous hypomorphic *HNF1A* mutation resulting in a MODY phenotype.

Though the first description of a homozygous *HNF1A* mutation is an interesting finding, more than that, this variant sheds light on the grey distinction between *HNF1A* variants that are classed as risk variants for type 2 diabetes and at the other end of the spectrum, those that are confirmed mutations impairing function. In the heterozygous state, this mutation appeared to have no effect or variable penetrance, as the probands father was non-diabetic (although, by HbA1c criteria, is in the pre-diabetes range) and the mother, developed diabetes after a history of gestational diabetes and was overweight. She clearly also did not demonstrate sensitivity to sulphonylureas, as she was on 10 mg dose of glibenclamide with suboptimal glycaemic control. Further confirmation of genotype-phenotype correlation will come from studies in the two older siblings, who have been invited to attend the unit for investigations. The prediction would be that, they too would be homozygotes for the A251T variant, given the young-age of onset, however it is possible that they are heterozygotes with perhaps some

other risk factor for developing diabetes. Given both parents are carriers, the chances of each offspring being homozygote is 25%. It is therefore not impossible that three successive pregnancies resulted in homozygous offspring, however confirmation is awaited.

Given that the parents are from the same region of Turkey, one possibility is that this is a private mutation or a variant found in that region only. This would certainly explain why the variant was not reported in any of the variant databases searched. This scenario may be analogous to the Canadian Oji-Cree population, who were found to harbour a private mutation, G319S in the *HNF1A* transactivation domain (272) that significantly increased the odds ratio for developing type 2 diabetes with a homozygous genotype predicting younger-age at onset. This was later shown to be due to aberrant splicing, however effects on transactivation were not thought to be at a level seen in classical HNF1A-MODY mutations (273).

Deciding what constitutes an abnormal functional response in *in vitro* studies, is a hugely challenging area and is likely to be gene-specific. The results presented in this chapter used a study design that compared unknown variants to internal positive controls for known MODY mutations and those with published data. It was important to select mutations that were clearly disease-causing.

Even so, there is a large degree of variability in functional assays, which are often difficult to unpick, methodologically. In this analysis, there has been considerable variation in the transactivation potential of A251T depending on the construct and promoter selected. This issue requires further investigation. The rat and human albumin promoters are highly conserved but do have a number of base pair changes (274). The HNF1 binding site in the promoter is also highly conserved but some base pairs are different, as outlined below.

Human albumin promoter HNF1 binding	A G T T A A T A A T C T A C A
Rat albumin promoter HNF1 binding	G G T T A A T G A T C T A C A

It is possible that these variations account for differences in transactivation encountered when using human or rat albumin promoters, but further work is needed

to clarify this. As the variability was only observed with A251T, in the limited number of constructs assessed in this way, it is possible that the 251 nucleotide position itself interacts with the different promoter sequences.
Because of the variability of assays, it is therefore preferable to look at the relationship on unknown variants to those that are known, as conducted in this study. Looking at the A251T variant it can be concluded that DNA-biding is reduced compared to wild-type HNF1A, but not to the extent observed in a severe mutation, such as P112L or T260M. However, this approach still has its limitations, as it is conceivable that a severe mutation may have relatively normal DNA binding, because another functional domain is affected. It is also likely that confidence in results can increase with further biological replicates

6.8.2 Functional Effects of A251T

It is unclear why, despite demonstrating A251T transactivation to be comparable to wild-type, there was still significantly reduced DNA-binding activity on the EMSA. Typically, a mutation in the DNA-binding domain would be expected to have reduced DNA binding and because of that, reduced transactivation potential, given the first step in the transactivation assay, is DNA-binding. There are a few plausible explanations;

- first, titration studies to determine if there was a dose-dependent effect in A251T transactivation were not undertaken. Concentrations of wild-type and variant DNA added were based on previously internally optimised protocols. However, it is possible that by transfecting too much A251T construct any subtle reductions in transactivation were masked by the over-production of variant protein. Titration studies therefore need to be undertaken to fully investigate this.
- second, it has been shown that transactivation potential is variable depending on the system used. This suggests something specific about A251T as it has not been observed with other variants anecdotally. However, groups publishing studies in this area tend to replicate their data in the same experimental models, so it could be that this is more of a generic issue, highlighting the vast

differences between such models. Comparisons of more variants in cross-over experiments using different plasmids and promoters are therefore required.

- finally, if this is a genuine effect, and it must be noted that in the experimental system used, the finding was highly reproducible with tight error bars, it may be that there are co-factors that in a cellular system can assist or synergistically work with the A251T HNF1A protein to overcome potential defects in transactivation potential, which given our hypothesis, should be mild. However, in the relatively artificial EMSA-setting, which only tests binding to the DNA oligonucleotide, these factors are not present and therefore DNA-binding is affected.

Clearly functional studies in isolation, are unlikely to provide a clear answer and the result must be interpreted with other functional modalities taken into consideration. It is likely that in the future, similar to *in silico* predictions, *in vitro* assays may result in a composite score of likelihood of pathogenicity.

That the proband was able to transfer off insulin completely and maintain near-normal glucose on glibenclamide, is a very compelling piece of evidence in favour of A251T being a hypomorphic mutation.

HNF1A MODY mutations are characteristically sensitive to sulphonylurea therapy and hypoglycaemia on relatively low dose, is commonly observed making it an excellent treatment, at low-dose, for affected individuals (82). The mechanism by which *HNF1A* haploinsufficiency leads to reduced insulin secretion is not completely understood, but is thought to result from the reduction in ATP generation at the end of glycolysis, mediated through intermediary proteins presumably transactivated by HNF1A protein (103).

Sulphonylurea compounds bind to the potassium-sensitive ATP channel of the beta-cell and close it independently of ATP, thus resulting in membrane depolarisation, calcium influx and insulin secretion.

It was unclear whether the homozygous A251T phenotype would be more, less or equally sensitive to sulphonylureas than a standard heterozygous MODY mutation. People with *HNF1A* mutations have higher insulin increments after a sulphonylurea challenge compared to people with type 2 diabetes, have higher fasting insulin levels

and are more insulin sensitive despite being BMI and age-matched (83,275). The hypoglycaemia encountered, logically results from this large increment on a background of higher insulin sensitivity, unlike people with type 2 diabetes who have a smaller increment on the background of higher fasting insulin and lower insulin sensitivity. In fact, in one study, when given a tolbutamide challenge, homeostatic model assessment (HOMA B) increased by 310% in people with HNF1A MODY compared to 55% in people with type 2 diabetes (p=0.001) (83). Interestingly, in this seminal study, the reduction in plasma glucose observed in the people with *HNF1A* mutations varied between 3.3mmol/L and 6.2 mmol/L, however this was not explained by covariates such as duration of diabetes, beta-cell function , insulin sensitivity or mutation type or site. It is therefore plausible that this variability in glucose response could be a mutation-specific.

The proband clearly had a successful response to a sulphonylurea challenge and became significantly hypoglycaemic on the lowest prescribed dose of 2.5mg. It is difficult to conclude that he was more sensitive as a homozygous case, but it certainly seems likely that his response was similar to someone with a typical heterozygous pathogenic mutation. Studies in his heterozygous parents, may help, but with their older age and higher BMI's there will be potential confounders.

6.8.3 Further work

The work on this interesting variant is on-going and future clinical studies will involve further familial segregation studies on the siblings, as yet to be tested and if homozygous, transition to sulphonylurea therapy.
Transactivation studies will also be repeated using DNA-titration experiments as outlined above.

One functional domain not assessed in the *in vitro* studies to date is an assessment of the nuclear localisation of the variant HNF1A protein. These studies investigate whether the variant HNF1A is transported successfully into the nucleus, or if, because of aberrant function, it predominates in the cytosol. Immunofluorescence techniques have been used successfully previously, however are qualitative (266). The lysates

collected from Western and EMSA experiments outlined in this chapter, have an aliquot saved to enable fractionation of nuclear and cytosolic contents. These fractions will then be compared between wild-type and variant HNF1A. These assays are planned in coming months.

Finally, to further study this variant it would be logical to use more advanced techniques such as genome editing using CRISPR-Cas9 technologies (276), which would enable the variant to be introduced at a genomic level in a cell line and would circumvent the impact of the variability observed depending on transfection models. Another alternative being explored is the possibility of obtaining skin fibroblasts from the proband, one of his parents and if possible a family member with a normal genotype. These fibroblasts could be reprogrammed to form induced pluripotent stem cells, which could then be directed towards a beta-cell line. By comparison of beta-cell lines from the homozygous, heterozygous and normal allele family members, further insights could be gained into the functional aspects of this novel and interesting variant. These cutting-edge techniques may shed further light on the A251T variant *in vivo* (277).

Further work outlined is being incorporated into future grant applications and will be undertaken in collaboration with relevant experts.

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With thanks to;

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Chapter 7 :Overall Summary and Conclusion

This thesis has presented results from a series of studies designed to investigate the phenotype of young-onset diabetes across UK white, south Asian and African-Caribbean ethnic groups. The particular subtype of diabetes an individual has influences treatment and correct classification is essential to ensure appropriate management. There is increasing recognition and understanding that even within the two major types of diabetes, type 1 and type 2 diabetes, there is clinical heterogeneity, which may be underpinned by a combination of genetic and environmental factors and may influence treatment choice and response. Additionally, there are other distinct types of diabetes that, despite being individually less common, cumulatively account for a significant proportion of cases. These other forms may have different management approaches meaning there is real clinical benefit to the individual in making a diagnosis. This can be either through commencing appropriate treatment or through prediction of progress and complications.

The impact of ethnicity on diabetes phenotype, progression and presentation has been under-studied in the UK. The challenges with assessing the interplay between ethnicity and diabetes subtype often relate to the applicability of studies in native migrant countries compared to the UK, where differential environmental exposures might be present (19,199), and also to the genetic heterogeneity of individuals in one so-called ‘ethnic group’, which may be more genetically diverse than the genetic ancestry of white European individuals (202).

In an attempt to initiate systematic investigations assessing the impact of ethnicity of diabetes phenotype and recognition, to address the disparity in the extent of classification difficulties across ethnic groups and to evaluate the utility of some of the approaches developed to improve classification in white populations, in south Asian and African Caribbean groups, a series of systematic studies were undertaken.

Study 1

In the first study, the MODY database in Exeter was interrogated to determine the proportion of referrals by ethnic group, the likelihood of finding a mutation and to explore the phenotype of people with and without mutations in white and south Asian ethnic groups. This database holds clinical information and genetic testing results on

all people referred for testing to this centralised molecular genetics laboratory since its inception. MODY had been described in a handful of people from south Asian backgrounds in the UK and accounted for only 0.5% of all referrals in that study from 2006 (142). The major finding, was that in contrast to the reported number of referrals in south Asian individuals, they now accounted for 7.3% of all referrals, significantly more, but possibly still fewer than would be predicted based on the frequency of type 2 diabetes. The detection rate of finding a positive mutation was significantly lower in the south Asian group at 12.6% versus 29% in the white European group. Whilst it was unclear what led to the clinical referral for testing, this result suggested that the criteria being used were either less sensitive at detecting MODY in south Asian individuals or there were fewer cases in general in south Asian individuals, perhaps due to the relative increased frequency of young-onset type 2 diabetes. Interestingly, the phenotype of people with *HNF1A* and *HNF4A* mutations were broadly similar across ethnic groups, however in those without mutations, the SA individuals were more likely to meet traditional criteria for testing; diagnosed younger, more likely to not be treated with insulin and have a parent affected than white European referrals. It was unclear if this was all accounted for by referral bias.

Appreciating the limitations of this retrospective dataset that inherently relies on clinician referrals, it was clear that a more systematic assessment needed to be undertaken. There was also a need and opportunity to define other subtypes of diabetes in ethnic groups presenting at young-age. To effect any changes in the approach to MODY diagnosis in other ethnic groups, a clear understanding of the phenotype of other types of diabetes was required and these data were also lacking for ethnic groups.

Study 2

Focussing on the south Asian and African Caribbean ethnic groups, the two largest in the UK, the MY DIABETES study was initiated and continues. The aim of the study was to establish the frequency of MODY in people diagnosed before 30 years of age in each ethnic group and to compare to a white European ethnic group. People were phenotyped used fasting C-peptide and pancreatic auto-antibodies to determine those that had type 1 diabetes and those eligible for genetic testing. Genetic testing on all

known diabetes genes was then undertaken. In the interim analysis of the 728 individuals recruited, there were significant differences in the proportion of people eligible for testing, by ethnicity. However, when genetic testing was undertaken, there was no observed difference in the detection rate by ethnic group with the overall detection rate of finding a mutation of 9.5%.

This detection rate was comparable to other studies that have used similar criteria (140), but significantly lower than the detection rate observed in the previous study (190). Critically, mutations in known MODY genes were found in south Asian and African-Caribbean ethnic groups. Although variants of unknowns significance were detected, the mutations reported in the ethnic groups were all previously encountered. The suggestion that the lower frequency of MODY in ethnic groups is due to mutations that are novel, is therefore not borne out from the current data. This finding suggests that the differential detection rates from the first retrospective study are accounted for by referral bias, as the systematic criteria applied in the MY DIABETES study has eradicated this bias and detected MODY equally across ethnic groups.

Of course, these finding are from an interim analysis and the absolute numbers in each ethnic group that had a MODY mutation are small. It is therefore prudent to be cautious in the interpretation of these data. Nevertheless, if these findings persist with full recruitment, they suggest that the systematic approach to testing in ethnic groups, devoid of clinical information, may be a better approach, accepting that more people may need to be tested.

The MY DIABETES study also offered the opportunity to systematically explore other types of diabetes by ethnicity. The major finding was that type 1 diabetes phenotype varied little by ethnic group with similar ages at diagnosis, body mass index and rates of antibody positivity by ethnic group. Classification issues occurred more frequently in people from south Asian or African-Caribbean groups, demonstrating the diagnostic challenge clinicians face.

Of interest south Asian ethnicity in type 1 diabetes was associated with a higher C-peptide level than white ethnicity, which was not accounted for fully by differences in lipid profile, BMI or duration. One explanation is that C-peptide levels are higher because of inherent insulin resistance. If replicated once fully recruited, this is an important point as clinically, higher C-peptide might be encountered in south Asian

individuals with type 1 diabetes and so cut-offs for C-peptide that define type 1 diabetes, may need to be ethnicity specific.
The need for ethnic specific reference ranges is also supported by the finding that in those who met the criteria for genetic testing, African-Caribbean ethnicity was independently associated with a lower fasting C-peptide level. However, African-Caribbean ethnicity was not associated with lower c-peptide levels in those who were pancreatic auto-antibody positive.

There are some interesting early signals in this interim analysis of the MY DIABETES cohort that require confirmation once the study is fully recruited.

There are also some additional analyses and studies that would further assist with phenotypic characterisation that it has not been possible to undertake to date. These include:

- Evaluation of the MODY probability calculator by ethnic group, once all groups fully recruited
- Evaluation of hs-CRP as a biomarker to differentiate HNF1A MODY from other forms of diabetes across ethnicities
- Assessment of various C-peptide cut-offs in differentiating the different types of diabetes, once fully recruited
- Evaluation of the relationship between urine C-peptide to creatinine ratio and fasting serum C-peptide, by ethnic group.
- Use of the type 1 diabetes genetic risk score in the entire cohort to evaluate its performance against diabetes defined using C-peptide and antibodies, by ethnic group.

The cross-sectional nature of the MY DIABETES study has been a strength, but is also a limitation as we cannot be certain about phenotype around diagnosis. There is scope in future work to undertake prospective studies; this is already being undertaken for type 1 diabetes (ADDRESS-2 study), but people who have residual C-peptide in the MY DIABETES study could also be followed-up to look at predictors of C-peptide loss, both in type 1 and type 2 diabetes, by ethnicity. A body of work, looking in more

detail at the phenotype of slim south Asian and African-Caribbean individuals with diabetes that is not type 1, is planned for future projects.

Study 3

One of the findings from an early MY DIABETES recruit was a homozygous HNF1A variant (A251T) in a Turkish kindred. A homozygous change in *HNF1A* was thought to be embryonic lethal and had not be encountered previously. That an individual existed with a homozygous change, suggested that the A251T variant was a hypomorphic mutation, which mildly affected protein function in the heterozygous state, with incomplete penetrance. However, in the homozygous state may present similar to a typical pathogenic HNF1A mutation. This case highlighted the difficulty in classifying variants encountered in genes associated with monogenic diseases. This now represents one of the biggest challenges in the interpretation of genomic information from sequencing.

Standard approaches to assigning variant pathogenicity, were inconclusive and so a series of *in vivo* and *in vitro* assays were undertaken. In the experiments undertaken the A251T variant had comparable transactivation potential to the wild-type HNF1A protein and similar amounts of protein expression, however there was a significant reduction in DNA binding, which was not as severe as those reported in standard HNF1A mutation, but lower than wild-type nonetheless. These findings supported that the A251T was functionally compromised, but only mildly so and was in keeping with the hypothesis of a hypomorphic mutation.

In the next set of studies, the proband's insulin treatment was withheld and they were given a sulphonylurea challenge. *HNF1A* mutations are typically sensitive to sulphonylurea therapy and variant pathogenicity can be supported by demonstration of this. The proband was able to completely transition off insulin and was also noted to have undetectable high sensitivity C-Reactive Protein levels – another functional assessment – but not in his heterozygous parents. Taken together there was evidence to support that the A251T is a hypomorphic mutation, however further work is needed to move forward with this, as outlined in chapter 6. The finding of this unusual variant in someone of Turkish ethnicity highlights the limitations of variant interpretation, which

can sometimes remain inconclusive. It also demonstrates that in depth phenotyping, as undertaken in this case, can lead to beneficial treatment changes and ensure people are treated precisely for their type of diabetes.

Conclusion

In conclusion, the findings from the body of work outlined in this thesis, demonstrate that ethnic-specific approaches are needed to be able to sensitively and specifically delineate subtypes of diabetes, by ethnic group. MODY has been detected in all ethnic groups and with a similar detection rate. The type 1 diabetes phenotype is broadly similar, in contrast to studies elsewhere.

Further assessment of detection rates will be needed when the study is complete and at that point, specific guidance around changes to clinical practice may be possible. Until then, the current findings highlight similarities and differences in diabetes phenotype in different subtypes, defined using a systematic approaches. These data are novel, impactful and should be taken into consideration when classifying individuals.

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Appendices

Appendix 1: Manuscripts

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RESEARCH LETTER

South Asian individuals with diabetes who are referred for MODY testing in the UK have a lower mutation pick-up rate than white European people

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Abbreviations
SA South Asian
WE White European

To the Editor: Maturity onset diabetes of the young (MODY) is an autosomal dominantly inherited form of diabetes that has been extensively described in white populations, but diagnosed with lower frequency in other ethnic groups. Mutations in the *GCK*, *HNF1A* and *HNF4A* MODY genes have been reported in a variety of ethnic groups [1–3], but there are few data on prevalence in non-white ethnic groups. MODY was first reported in a UK South Asian (SA) cohort in a systematic survey of childhood diabetes [4]. A 2006 study revealed a lower than expected frequency of referrals for MODY testing in the UK SA population [5]. The frequency and characteristics of MODY in non-white European populations is important for the precise diagnosis of young-onset diabetes.

We undertook a retrospective analysis of UK referrals for MODY testing to determine if referrals for individuals of SA origin had increased since that reported in the 2006 study and to investigate the pick-up rate and clinical features of MODY mutations in this population.

The UK MODY database, held centrally at the Royal Devon and Exeter National Health Service (NHS) Foundation Trust, is populated prospectively from a standardised request proforma that includes ethnicity. Proband referrals (>1 year old at diagnosis) from 1996 to November 2015, in whom sequencing for mutations in the most common MODY genes (*HNF1A*, *HNF4A*, *HNF1B* or *GCK*) was requested, were selected. Only variants classified as pathogenic or likely pathogenic were included. Assignment of pathogenicity was based on UK best practice guidelines [6]; including findings from established variant/mutation database and literature searches, details of familial cosegregation and identification of clinical features characteristic of the MODY gene mutation. Additionally, data from in silico prediction models (SIFT, PolyPhen2, AlignGVGD, Grantham distance accessed using AlaMut Visual 2.7, Interactive Biosoftware, Rouen, France) were used, along with details of amino acid species conservation and predictions of effects on splicing. Finally, variants in the Exome Aggregation Consortium [7] and 1000 genomes [8] databases with a minor allele frequency >0.01, were excluded.

Ethnicity was categorised as white European (WE) or SA based on UK census classification [9]. The SA group included people from India, Pakistan and Bangladesh or in whom ethnic origin was stated to be ‘Asian’ and excluded mixed ethnicity.

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A total of 4688 proband referrals were identified and ethnicity was recorded in 4010 cases. White European referrals accounted for 3472 of 4010 (86.6%). 293 cases referred were in people of SA ethnicity (7.3%), and 81 referrals (2.0%) were from African or Caribbean backgrounds (see Table 1).

1180 probands had MODY mutations, with a mutation pick-up rate of 25.2% (1180/4688). The mutation pick-up rate was 29.1% (1011/3472) in the WE group and 12.6% (37/293) in the SA group ($p < 0.001$) (see Table 1). The pick-up rate in children (≤12 years) referred for testing did not differ between ethnic groups; 32.6% in WE vs 26.7% in SA children, $p = 0.356$ (data not shown).

Phenotypes of those with and without confirmed MODY mutations are displayed in Table 2. Generally, SA MODY patients with *GCK* mutations were diagnosed earlier than WE patients (6.5 vs 20 years, $p < 0.0001$); however, when only considering diagnosis in children under 12 years old, there was no significant difference in age at diagnosis. Referrals from both ethnic groups had similar proportions of a history of parental diabetes and were equally likely to be non-insulin dependent.

In both SA and WE individuals, the *HNF1A / HNF4A* MODY phenotypes were similar across all variables reported except for HbA_{1c}, which was higher in the SA group (8.6% [70 mmol/mol] vs 7.2% [55 mmol/mol], $p = 0.004$; see Table 2).

SA people in whom no MODY mutations were found were younger at diagnosis (19 years vs 25 years, $p < 0.0001$) and had a shorter duration of diabetes, compared with WE individuals. BMI was similar across both ethnic groups (WE, 25.3 vs SA 25.0 kg/m^2, $p = 0.217$). However, SA people were more likely to have a parent affected with diabetes (83.6% vs 68.2%, $p < 0.0001$) and were more likely not to be treated with insulin at referral (60.2% vs 50.6%, $p = 0.006$), when compared with WE individuals (see Table 2).

This study is the first to explore the characteristics of individuals of SA ethnicity who have been referred for MODY testing using data from the largest MODY diagnostic service in the UK. It demonstrates a lower pick-up rate of MODY mutations in people of SA ethnicity. In our analysis, SA people accounted for markedly more referrals than previously reported in 2006 (7.3% vs 0.5%) [5]. Whilst this broadly reflects the UK population demographics, because of the higher prevalence of type 2 diabetes in SA people it is suggested that this is still lower than expected [9]. The detection rate for MODY mutations was lower in the SA group than in WE referrals. However, when a mutation was not detected, SA referrals were more likely to meet the clinical criteria for MODY than WE individuals. This may demonstrate a referral bias favouring SA cases that more stringently meet the clinical criteria. However, the results also show that these criteria are not sensitive for the separation of MODY from young-onset type 2 diabetes in this population. For example, SA people without mutations had higher BMIs than those with confirmed MODY mutations, suggesting that the low MODY detection rate in this group may have resulted from a higher prevalence of young-onset, familial diabetes.

When analysing the SA group as a whole, the best predictors differentiating those with MODY (mutations in all genes) from those without a mutation, were a lower BMI and age at onset; BMI was lower in the SA group with MODY mutations than those without mutations (MODY, 21.4 kg/m^2 and no MODY, 25.0 kg/m^2, $p = 0.001$, data not shown), but BMI was generally higher and non-significantly different in the no mutation group when compared with WE people (25.0 vs 25.3 kg/m^2, respectively). The lower BMI and age at diagnosis in SA referrals was primarily observed in the *GCK* mutation group and not seen in the *HNF1A / HNF4A* mutation or 'no mutation' groups. It is likely that these findings reflect a lower referral rate in adults and children over 12 years old since the mutation pick-up rate in both ethnic groups was similar in children under 12 years.

There is limited evidence to support that SA individuals have a lower prevalence of MODY, with only one previous study suggesting fewer *GCK* mutations

Table 1 Breakdown of proband referrals for MODY genetic testing and MODY pick-up rate by ethnic group

Ethnic group	*n*	%	MODY mutation (*n*)	Pick-up rate (%)
All groups	4688		1180	25.2
Individuals with known ethnicity	4010		1083	27.0
White	3472	86.6	1011	29.1
South Asian	293	7.3	37	12.6***
African-Caribbean	81	2	8	9.9
Middle Eastern	47	1.2	5	10.6
South East Asian	21	0.5	6	28.6
Mixed/other	96	2.4	16	16.7
All non-white ethnic groups	538	13.4	72	13.4

Statistical analysis was carried out using a χ^2 test on the two largest ethnic groups, white and South Asian

***$p < 0.001$ vs white individuals

Table 2 Clinical characteristics of referrals with *GCK* or *HNF1A* / *HNF4A* MODY mutations and those without MODY mutations, analysed by ethnic group

Mutation status	No mutation			*GCK*			*HNF1A* / *HNF*4A		
Variable	WE	SA	*p* value	WE	SA	*p* value	WE	SA	*p* value
Total (*n*)	2387	256		358	17		545	15	
Age at diagnosis (years)	25 (16–35)	19 (14–30)	<0.0001***	20 (14–28)	6.5 (4.5–15)	<0.0001***	18.5 (15–25)	16 (14–20)	0.054
Age at diagnosis in children ≤12 years (years)	9 (6–11)	11 (7–11.5)	0.012*	8 (5–10)	5 (3–7)	0.079	11 (9–12)	11 (8–12)	1.000
Current age (years)	35 (25–47)	28 (19–36)	<0.0001***	29 (19–39)	13 (6–17)	0.0044**	37 (26–48)	25 (20–48)	0.108
Duration of diabetes (years)	7 (2–14)	4 (2–9)	<0.0001***	6 (2–11)	2 (0–5)	0.0045**	14 (6–27)	11 (6–28)	0.658
BMI (kg/m^2) in patients aged ≥16 years	25.3 (22.4–29.6)	25.0 (23.0–28.2)	0.217	22.1 (20.1–25.0)	19.7 (18.0–20.3)	0.02*	24.1 (22.0–27.0)	24.0 (21.1–24.6)	0.246
Fasting glucose (mmol/l)	7.4 (6.1–10.1)	7.9 (6.5–10.7)	0.223	6.7 (6.3–7.3)	6.9 (6.5–7.1)	0.918	7.8 (6.3–9.9)	7.3 (6.7–8.2)	0.638
HbA_{1c} (%)	7.4 (6.3–9.1)	7.8 (6.7–9.7)	0.0065**	6.4 (6.1–6.7)	6.6 (6.5–6.9)	0.04*	7.2 (6.3–8.4)	8.6 (8.1–8.8)	0.004**
HbA_{1c} (mmol/mol)	57 (45–76)	62 (50–83)	0.0065**	46 (43–50)	49 (48–52)	0.04*	55 (45–68)	70 (65–73)	0.004**
Meets all three clinical criteria (%)[a]	14.8	31.3	<0.0001***	43.5	85.7	0.002**	44.7	50	0.736
Diagnosed <25 years (%)	43.4	58.6	<0.0001***	61.5	94.1	0.006**	72.1	93.3	0.069
Currently non-insulin treated (%)	50.6	60.2	0.006**	89.7	92.3	0.762	65.7	69.2	0.794
History of parental diabetes (%)	68.2	83.6	<0.0001***	83.5	93.8	0.274	89.9	92.9	0.720

Data are presented as medians and interquartile ranges or %

[a] Calculated using cases where the data set for all parameters is complete

Statistical analysis was carried out using Mann-Whitney U / χ^2 tests

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

occurred in Indian children [10]; however, this study may have been underpowered since the estimated minimum prevalence of MODY (in a predominantly white population) has recently been demonstrated to be 1 in 1000 cases [11]. Thus, it is more likely that the lower mutation pick-up rate in SA individuals compared to WE individuals observed in our study reflects higher prevalence of younger-onset type 2 diabetes, which is supported by our analysis demonstrating similar mutation pick-up rates between ethnic groups in pre-pubertal children.

Our data highlight the need to consider the optimum method for identifying cases of MODY in people of non-white European ethnicity. Approaches such as the MODY probability calculator [12] need to be validated in non-white populations as our data suggest that MODY cases may be more sensitively identified using lower cut-offs for age at diagnosis and BMI.

Referrals for MODY testing in the SA population have increased considerably but may still be under-representative of the general UK population. Meanwhile, despite meeting referral criteria more stringently, the lower MODY mutation pick-up rate for SA individuals suggests that these criteria are not sensitive for separating MODY from younger-onset type 2 diabetes in the UK SA population.

Further work is required in unselected cohorts, to develop ethnic-specific criteria that facilitate the identification of MODY from type 2 diabetes in SA people with young-onset diabetes.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement SM and ATH designed the study. SM collated the data, carried out the analysis and drafted the manuscript. BS assisted with study design and provided support with analysis. KC assisted with data acquisition. DJ, NO, SE and ATH provided supervision, assisted with interpretation of analysis and revised the manuscript. All authors critically reviewed and revised the manuscript and approved the final version. ATH is the guarantor of this work.

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Appendix 2: Example Participant Information Sheet

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Adult Participant Information Sheet

A cross-sectional study to characterise young-onset diabetes in two UK ethnics groups: MY DIABETES STUDY

Chief Investigator: Dr Nick Oliver
Clinical Research Fellow: Dr Shivani Misra

Introduction

You are invited to take part in a research study called **'A cross-sectional study to characterise young-onset diabetes in two UK ethnic groups'.** You have been invited to take part because you were diagnosed with any type of diabetes before the age of 30 years and are of White British / European, South Asian or African & Caribbean ancestry.

Before you decide if you would like to participate, it is important to understand why the research is being done and what it will involve for you.
Please take time to read the following information carefully. Please ask the study team if there is anything that is not clear or if you would like more information, and please take time to decide if you wish to take part.

What is the purpose of the study?

The purpose of this study is to explore and describe the different types of diabetes that exist in people diagnosed at a young age (under 30 years of age) in the UK. We are interested in finding out if any of these individuals have a genetic cause to their diabetes known as maturity onset diabetes of the young (MODY). MODY is rare but may be treated differently to type 1 or type 2 diabetes, the most common types and is currently commonly misdiagnosed as either type 1 or type 2 diabetes. MODY is handed down from generation to generation, so affected individuals usually have a family history of diabetes, but not always.

We're particularly interested in studying people within the UK who are diagnosed at young-age, because very little is known about the characteristics of the different types

of diabetes that exist in this group of people. We know from our clinical experience that it is increasingly difficult to diagnose the type of diabetes someone has. In addition nobody has yet studied if South Asian or African & Caribbean people with diabetes in the UK could have MODY. We know people of European ancestry can get MODY, but we're missing many cases.

By undertaking this study we hope to identify factors that might help us to tell the different type of diabetes in people from different ethnicities, and if any of them have MODY.

The study involves a single visit to our research clinic, lasting approximately one hour, which will involve history taking, a routine examination and blood tests to look at your diabetes control, how much insulin your body still makes and also other things which might help us differentiate the types of diabetes. Blood will also be taken for DNA to look for diabetes genes, but no other form of genetic testing will take place.

About 1500 people will be taking part from in and around London and other areas in the country.

The study has been designed so that we can:

1) Determine if we are missing cases of MODY in people in the UK
2) Describe what characteristics people with young-onset diabetes have
3) Give advice on changing management in anyone in the study diagnosed with MODY
4) Use this information to enable better diagnosis in the future

Do you have to take part?

No. Your participation is voluntary. If you decide not to take part, this will not affect the standard of care that you normally receive. If you do decide to take part, you are free to withdraw at any time and you do not have to give a reason.

What will happen to you if you take part?

If you decide to take part we will arrange a convenient day for you to come to our research clinic on the St. Mary's, Charing Cross or Hammersmith Hospital sites of Imperial College Healthcare NHS Trust for the study visit.

- Study visit

At the study visit we will ask you to attend fasting in the morning. We will then perform some blood tests which will look at routine things like your kidney and liver function, and also more specialist tests to look at your diabetes control, whether you have antibodies (proteins in the blood which damage other cells) to insulin-making cells in your pancreas and also to assess how much insulin your own body makes. A sample

will also be taken for DNA analysis to look for diabetes genes. We'll also ask you to provide a urine sample to see if there is any protein in your urine. We will then undertake a detailed medical questionnaire and perform a routine examination including your height, weight and waist circumference.

We'll also provide you with an additional container to collect urine later on in the day after your main meal which can be posted back to us, and which will help us to determine how much insulin you still make.

What will you have to do?

If you take part, we would like you to attend fasting; this means having nothing to eat or drink for the 10 hours before the appointment, although water is allowed. If you take treatment for diabetes, you would need to delay taking your morning doses on the day of the study and come straight to our clinic in the morning. You will be advised by the study physician about which treatments to delay taking. All treatment can be resumed as normal after the bloods tests are taken on the morning or your study visit.

Are there any risks or discomforts?

We do not anticipate there being any significant risks to your health by taking part in the study. It can be a bit uncomfortable having blood tests but this should be minimal and an experienced practitioner will take your blood.
Because you won't have had your normal diabetes treatment on the morning of the study, your blood sugar may be higher than usual. However, as soon as the study is finished you will take your medications as normal with a provided breakfast and you will be advised about dosing / meals by the study team.

What are the possible benefits of taking part?

The study will give you information about the type of diabetes you have and this could alter your management. You'll also have the chance to talk and discuss your diabetes with a diabetes specialist. Since almost everyone will also undergo genetic testing we may find that you actually have MODY, a genetic cause of diabetes. If we diagnose MODY, or find from investigations that you have a different type of diabetes to your current diagnosis, we will let your own GP and diabetes doctor know, so that changes to your treatment can take place. Importantly if you're diagnosed with MODY at the end of our study, this could have an impact on your family members who may also have the same condition. We may therefore advise that they be tested too.
In most people the type of diabetes diagnosed will probably be correct, and in that case we will have still collected extremely valuable information on young-onset diabetes in European, South Asian and African and Caribbean people in the UK, and using this data we'll undoubtedly be able to improve diagnosis in the future.

Will everyone have genetic testing for MODY?

No. We are trying to identify the different types of diabetes in the study population. It might be very obvious from your blood tests that you have type 1 diabetes, and in this case we would not proceed to test your DNA for MODY. We will however store your DNA anonymised for future diabetes research projects.

What will happen to my medical information?

The study team will handle your personal health information in a confidential manner. Any data collected relating to you will be coded with a specific study code throughout the research and all identifiable data will be kept within Imperial College NHS Trust.

With your permission, we will inform your GP that you are taking part in the study.

Any information you provide will be held in the strictest confidence; however, we have a duty of care to notify you if any of the findings are clinically important. If this is the case, we may inform you and your doctor of the results. If you are enrolled in the study you will be given a unique study ID number. All information that is collected about you will be held on a password-protected computer. All paper copies of study data will be stored under ID number and kept in locked offices within the research facilities. Access to data and samples will be available to the research team only. Information that is clinically relevant will be supplied to your hospital specialist and/or GP.

What happens to my DNA and other samples?

Your DNA will be sent to a genetics laboratory in Exeter, in the same way NHS samples for genetic analysis are handled. Your DNA may then be tested for mutations in known MODY genes and other diabetes-related genes but no other form of genetic testing will take place.
After we've performed our analysis, with your consent, some of your urine and blood samples will be transferred to a college research bank for storage to ensure their safe use in the future. All samples will be given a unique code and all personal information will be kept separate from your data. Scientists analysing samples will only know the unique code and will not know who the sample is from. The research bank will approve the use of the samples and data for future medical research.

What happens if a MODY mutation is found?

If we find a mutation causing MODY, we will contact you and arrange to see you in clinic, so we can discuss the result and any implications on your treatment. It might be that your treatment could be changed, and we'll discuss how best to do this. It may be that some of your family members with diabetes could also have MODY, and we would then ask you to contact them. They would not need to join our study, and genetic testing could be arranged though their NHS diabetes doctor or GP.

What happens if no MODY mutation is found?

Unfortunately, because we still don't know all the mutations that can cause MODY, if your results are negative, it doesn't fully exclude that you have the condition. We will however have very useful, accurate biochemical and clinical information about the type of diabetes you have from the other tests we perform and these can be discussed with you, and we will also contact your general practitioner with your results.

What is the timescale for getting results?

Results of your clinical and biochemical tests will be available fairly quickly and the study doctor can telephone you to explain what these show or speak to you directly within a few weeks of your visit. It may take several months to get results back on your DNA testing though. This is because your samples will be combined with others to be analysed together at a later date. It can also take a lot of time to anaylse the genetic information we obtain. As soon as we have results, we will contact you.

What happens if new information becomes available?

Sometimes during a study new information becomes available that may be important. If any new information becomes available during the study, we will notify you and the team can discuss with you if you wish to continue in the study.

What if I do not want to participate?

You do not have to take part in this study. It is up to you. If you decide to take part you can change your mind at any time and withdraw from the study. You do not have to do anything further and this will not affect your care provided by your general practitioner in any way. If you decide to withdraw you can also ask for your samples to be discarded and for no genetic analysis to be undertaken.

What will happen to the results of the research study?

The results of the research will be used to describe diabetes types and improve detection of genetic forms of diabetes in people diagnosed at young age and, at the end of the study, will be published in scientific journals. You will not be identified in any reports or publication. We will also send you a summary of the study.

Who is organising and funding the research?

The research project is funded by The Diabetes Research & Wellness Foundation and is being led by Dr Shivani Misra at Imperial College, with support from Dr Nick Oliver (Consultant Diabetologist) and Professor Des Johnston (Professor of diabetes) in collaboration with the MODY genetics team at Peninsula Medical School, Exeter led by Professor Andrew Hattersley. The team includes diabetes specialist doctors, researchers and nurses.

Who has reviewed the study?

This study was given a favourable ethical opinion for conduct in the NHS by the National Research Ethics Service committee in Chelsea, London.

What if something goes wrong?

We will take every care during the course of this study. If a problem occurs during the study visits to the clinic, the right facilities will be available to manage it.

Imperial College London holds insurance policies which apply to this study. If you experience serious and enduring harm or injury as a result of taking part in this study, you may be eligible to claim compensation without having to prove that Imperial College is at fault. This does not affect your legal rights to seek compensation.

If you are harmed due to someone's negligence, then you may have grounds for a legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been treated during the course of this study then you should immediately inform the Investigator (Dr Nick Oliver). The normal National Health Service complaint complaints mechanisms are also available to you. If you are still not satisfied with the response, you may contact the Imperial AHSC Joint Research Office.

How you can get more information

If you have any queries about the study or would like to participate, please contact Dr Shivani Misra on 020 7594 2460 or by e-mail at diabetes@imperial.ac.uk

Appendix 3: Study Visit SOP

Standard Operating Procedures for the MY DIABETES Study

Full title: A cross sectional study of young-onset diabetes in two UK ethnic groups
Short Title: MODY in young-onset diabetes from different ethnicities
Study Acronym: MY DIABETES study

Chief Investigator: Dr Nick Oliver
Author: Dr Shivani Misra

Documents referred to:

Protocol MY DIABETES version 2.0 16/5/2014
SOP/MD/Consenting process/ version 1.0
SOP/MD/Sample processing/version 1.0
Pre-Visit UCPCR MY DIABETES
Post-Visit UCPCR MY DIABETES
Case report form version 1.0

Contents

1.0 Identification of participants and initial approach

2.0 Instructions before visit

3.0 Study visit

4.0 Sample Processing

5.0 Contact with co-ordinating centre

6.0 Results and GP Letters

1.0 Identification of participants and initial approach

Participants may be approached from a variety of sources depending on strategies employed at your site. Two common approaches are outlined:

- Contact in diabetes clinics

Members of the diabetes clinical teams sites may approach potential participants directly and give them participant information sheets. If patients are happy to be contacted, members of the research team can contact them after a suitable interval to discuss the study further, assess suitability and book study visits.

- Telephone contact following mail out

Suitable patients may be identified from hospital registries of diabetes patient or other clinical / research databases. These patients can be contacted with site-specific participant information sheets and either be contacted after a suitable interval, or approached if they respond themselves.

In most cases initial contact from the research team will be over the phone. The research team member should clarify that the participant was diagnosed with diabetes *before* the age of 30 years. They should also check the participants self-reported ethnicity to ensure they are from one of the eligible groups (white European, south Asian or African-Caribbean).

The aim of the study should be briefly explained as well as the instructions for the study visit.
If they are happy to participate;

- an appointment for the visit should be made
- instructions provided as outlined in section 2.0

2.0 Instructions prior to study visit

Instructions can be conveyed via e-mail or over the phone.

Day before:

- Participants should fast from the night before (10 hours).

Day of study visit:

-
- Water is allowed on the morning of the visit and they can take any non-diabetes medications as normal. Please see below for information regarding omission of diabetes medications.

- They should also pass urine once at home, as we wish to collect a second fasting urine at the study visit (i.e. not the first sample of urine they pass after waking).

Omission of medications until after the study visit.

- If they take oral hypoglycaemic agents or non-insulin injectable agents, these should be deferred until after the study visit.
- Any long acting insulin injections (e.g. Levemir or Lantus) should be taken at the usual time. Short acting insulin injections taken with breakfast should be omitted as they will be fasting.
- Patients on insulin pumps should continue basal insulin, but should not bolus as breakfast is omitted.

- For patients on other insulin or complex regimes, omission of specific treatment should be discussed with the PI.

- **What happens if they have a hypoglycaemic episode?** It is important to explain to the participant that were they to have a low blood glucose before the study visit, they should stop fasting and treat as they normally would. They should not defer treatment for the study. If they did have a hypoglycaemic episode on the morning of the study, they should record the blood glucose value and how they treated it, and providing they feel well enough can still participate. The information about the hypoglycaemic episode can be recorded by the research nurse, on the CRF.

- **Is it absolutely necessary to fast?** We would like all participants to fast so that comparisons can be made between serum analyses that have been taken in a standardised way. However, some patients will not have fasted due to hypoglycaemia, as above. These patients can still participate and a note should be made in the CRF of the time of the hypoclycaemia and how it was treated. Some participants will find it hard to fast due to problems or concerns with fasting hypoglycaemia. If patients feel they are unable to fast they can still participate and this should be recorded in the CRF. Parents of children recruited to the study may also prefer a non-fasting visit. These exceptions are allowable and should be clearly documented in the CRF. Where possible, however participants should attend fasted.

3.0 Study Visit

Equipment

- Butterfly needle and vacutainer
- Glucometer or ask patient to bring their own
- Blood collection tubes (see SOP/MD/Sample processing/version 1.0)
- Other phlebotomy equipment: steret, plaster, gauze etc.
- Universal container for urine collection
- Boric acid container for urine transfer
- Cryotubes x9
- PathoShield flat pack box for transfer of genetic sample
- Tape measure
- Optional: Belvita Biscuits or equivalent

Study Visit

On arrival check if participant has fasted and if not the timing of last meal and also dosing of any anti-diabetic medication. Record in proforma.

- Check capillary blood glucose on arrival and document in medical enquiry proforma. If blood glucose <4 mmol/L treat with a carbohydrate snack before proceeding, in accordance with Trust guidance.

- Check participant has read the appropriate PIS. Discuss the research project with the participant and give opportunity for questions / further discussion (see SOP/MD/Consenting process/ version 1.0).

- Assign a study number to the participant e.g. MD_XX_001, where XX represents the agreed code for your site.

- If happy to participate ask volunteer to initial and sign the consent form and make 2 copies. Give one copy to the participant and put the second copy in hospital notes (if they have some). The original is kept in the study folder with the completed CRF.

- Once consent has been given, prepare for taking blood tests and the urine sample to allow volunteers to break their fast as soon as possible.

- Take blood at specified volumes in each tube using a vacutainer set.

- Ensure samples for C-peptide / insulin are kept on ice. All other tubes can be left to stand in a rack, however once clotted should be moved to ice. EDTA samples will not clot and do not need to be moved to ice. The fluoride oxalate tube should be inverted 2-3 times and then placed on ice (see SOP/MD/Sample processing/version 1.0 for full details).

- Provide the participant with the universal container and ask them to provide a urine sample. You may wish to do this before the blood test. However both urine and blood tests must be completed before they can break their fast.

- If available provide a snack, and advise that they can take anti-diabetic medication as normal.

- Start to complete enquiry based on questions in the CRF

Measurements

- For anthropometric measures ensure shoes and bulky outerwear removed before weighing (in kg) and measuring height (in metres).

Waist Measurement (WHO method)

- Measurements should be recorded over underwear or light clothing.
- The subject should stand with their arms by their sides.
- The position of the waist is midway between the lower rib margin and the iliac crest.
- Identify the bony landmarks in the mid-axillary line.
  The lower rib margin (bottom of the rib cage).
  The iliac crest (highest bony part of the pelvis).
- Measure the vertical distance between them. Note the midpoint.
- Ask the subject to stand with their feet 25-30cm apart.
- Ensure that the tape measure is at the same level around the body at the midpoint. Gently tighten the tape measure so that it is taut but not too tight.

- Ask the subject to breathe normally and record the measurement to the nearest 0.1cm at the end of normal expiration.
- Record waist circumference.
- Waist should be measured three times and an average taken of the last two measures.

Hip Measurement (WHO method)

- Measurements should be recorded over underwear or light clothing.
- The subject should stand with their arms by their sides.
- Ask the subject to stand with their feet together.
- The measurer should sit to the side of the subject.
- The hips should be measured at the maximum extension of the buttocks, ensure the tape is the same level around the body. Tape measure should be taught but not too tight. Ask the participant to breath out gently
- Record hip circumference to the nearest 0.1cm.
- Hips should be measured three times and an average taken of the last two measures.

Acanthosis Nigricans

- Please note the presence or absence of acanthosis nigricans in axillae and at the nape of the neck. Acanthosis nigricans is a velvety dark brown discolouration as shown in the picture.



After the CRF is completed the participant is free to go.

If the participant did not bring the post-meal UCPCR urine sample collected the day before with them, please provide them with a urine pack that can be posted back along with the appropriate instructions document (Post-visit UCPCR MY DIABETES).

4.0 Sample Processing

Please refer to the following document for comprehensive instructions on how to process samples from the MY DIABETES study: SOP/MD/Samples/v1

5.0 Contact with the coordinating centre

Once local blood and urine test results are completed on the CRF, the CRF along with the consent form should be scanned and e-mailed to the Imperial principal investigator – s.misra@nhs.net.

The coordinating centre will upload your sites accruals to the CRN portal, so please ensure regular contact so that figures are accurate.

6.0 Results and GP letters

Following participation results will become available at different time points.

- Tests performed in the local laboratory should be available within a few days (renal function, lipid profile, liver tests, CRP, urine microalbulinuria and HbA1c). If concerned about any abnormalities, these should be discussed with the local PI.

- The letter informing GP's of patient participation can be sent out shortly after the visit (MY DIABETES GP participation version 2.0) and routine blood results from the local lab can be appended to this letter.

- GAD-65 and IA-2 antibody testing is undertaken at Exeter. The results of these will come back the coordinating centre. These results will be communicated via e-mail to your site (s.misra@nhs.net) using the study code. These results should be viewed by the PI. If antibody results are positive the following standardized letter can be sent to both the GP and participant (Letter_antibody positive).

- C-peptide results will be run at the coordinating centre after they have been collected in batches from your site. There will therefore be a delay of a few months before results of C-peptide analyses are available. Once C-peptide results are available and in conjunction with the antibody test results we will inform your site as to whether the document Letter-peptide negative can be sent to the GP and participant.

- Genetic testing results: The decision to undertake genetic testing will be made by the Imperial College research team on a case by case basis, but will most likely include people who are antibody negative with detectable C-peptide levels. Genetic testing will be undertaken in batches many months after the study visit. The Imperial College team will inform you if testing is to be undertaken and if the results are negative, you may send the standardized letter (Letter_negative mutation) to the participant.

- If genetic testing results reveal a MODY mutation the results will be sent to the PI of the study at local sites, and the PI should arrange to see the participant to inform them of the result and advise on subsequent management.

Appendix 4: Example Consent Form

Consent Form: ADULT PARTICIPANTS

Participant Study Number: …… **Study Number:**REC 13/LO/0944

Study Title: A cross-sectional study to characterise young-onset diabetes in two UK ethnic groups

Participant Initials

1.	I confirm that I have read, or had read to me, and understand the Participant Information Sheet (version 3.0, dated 16th May 2014) for the above study, and have been given a copy to keep. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	
2.	I understand that my participation is voluntary and that I am free to withdraw at any time, without giving a reason, and without my care or legal rights being affected.	
3.	I agree to my GP being informed of my participation in the study and that they may release relevant information from my medical records, including computer-based data.	
4.	I agree to my hospital being informed of my participation in the study and that they may release relevant information from my medical records, including computer-based data. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the research team, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.	
5.	I agree that the samples I have given and the information gathered about me can be stored for use in future projects related to diabetes.	
6.	I agree that my details can be stored on a secure computer database.	
7.	I agree to taking part in the study and I know how to contact the research team.	
8.	I consent to gift my anonymised samples to the Research Bank for future medical research.	
9.	I agree to being contacted with details of future research into diabetes.	

Participant:

-

_____ _____ _____

Print name Signature Date

Investigator:

_____ _____ _____

Print name Signature Date

Appendix 5: Case Report Form

Participant Details					
Date recruited			Date studied		
Study Number	NHS Number		Hospital ID	GP Name	
First Name	Address			GP Address	
Surname					
DOB	Male / Female	Self-reported ethnic origin		Country of birth	
Mobile No: Home No: Work No: E-mail:		Family Ancestral Origin			
		Mother			
		Father			
		Maternal	grandfather		
			grandmother		
		Paternal	grandfather		
			grandmother		
Source of referral		Notes on ethnicity			
Is this participant known to be related to another participant? Yes ☐ If yes, please provide the study number for the relative and how they are related: ……..					

Checklist			
Consent (copy to patient)	Yes ☐	Capillary blood glucose (mmol/L)	
Blood test	Yes ☐	Attended Fasting	Yes ☐ No ☐
UCPCR pack given	Yes ☐	Time last meal (if not fasting)	……
UCPCR sample received	Yes ☐	Date EDTA sent to Exeter	……

Notes

Study Number				**Date**			
Diabetes Diagnosis							
Type according to patient	Type 1 Type 2 Other Don't know	☐ ☐ ☐ ☐	……	Type according to clinician	Type 1 Type 2 Other Don't know	☐ ☐ ☐ ☐	……
Date of diagnosis		Age at diagnosis		Diagnosed in pregnancy?	☐	Known MODY?	☐
Details of diagnosis							
DKA at diagnosis?	Yes ☐ No ☐			DKA subsequently?	Yes ☐ No ☐		
Symptomatic at presentation?	Yes ☐ No ☐	Weight loss Yes ☐		Duration of Symptoms ……..			
Birth weight (give units)		Sensitivity to sulphonylureas?		Yes ☐			
Notes							

Treatment history									
Initial Treatment (please circle)	Diet Only	Yes ☐	No ☐	Tablets	Yes ☐	No ☐	Insulin	Yes ☐	No ☐
Duration of initial treatment ……months…….years									
Current treatment									
Diet Only	Yes	No							
Tablets	Yes	No		**Insulin**	Yes	No			
	Metformin	☐		Rapid acting	☐				
	Sulphonylurea	☐		Long acting	☐				
	Thiazolidinediones	☐		Mix	☐				
	DPPIV inhibitor	☐		N° insulin injections /day					

	Other		1	2	3	4	5	6	Pump
Non-insulin injection			Total dose 24 hrs			…….			
	GLP-1 agonist	☐	Time to insulin from diagnosis						
Notes			Immediately	☐					
			Not Immediately	<12 months ☐					
				>12 months ☐					

Study Number		**Date**	
Relevant Medical History			
Early Deafness (<40 years)	Yes ☐ No ☐ Don't know ☐	Hypertension	Yes ☐ No ☐ Don't know ☐
Learning difficulties	Yes ☐ No ☐ Don't know ☐	Diabetic Nephropathy	Yes ☐ No ☐ Don't know ☐
Renal Cysts?	Yes ☐ No ☐ Don't know ☐	Macrovascular disease?	Yes ☐ No ☐ Don't know ☐
Diabetic retinopathy?	Yes ☐ No ☐ Don't know ☐	Diabetic neuropathy?	Yes ☐ No ☐ Don't know ☐

Family history of diabetes			
Mother	Yes ☐ No ☐ Don't know ☐	Father	Yes ☐ No ☐ Don't know ☐
Maternal grandmother	Yes ☐ No ☐ Don't know ☐	Paternal grandmother	Yes ☐ No ☐ Don't know ☐
Maternal grandfather	Yes ☐ No ☐ Don't know ☐	Paternal grandfather	Yes ☐ No ☐ Don't know ☐
Number children	……..	No of siblings	……..
No children with diabetes	……..	No of siblings with diabetes	……..

Family Tree / Notes on family history (optional)

Points to note: Large babies in family members, hypoglycaemia or diabetes in babies, deafness or short stature. If family member affected make a note of approximate age at diagnosis and whether on insulin or tablet-treated.

Anthropometric measurements			
Height (m)		Current weight (kg)	
Waist circumference (cm)		Hip circumference (cm)	
Acanthosis nigricans? Yes ☐ No ☐			

Study Number					
Results			**Date**		
U&E's		**Lipids**		**Diabetes**	
Na+ mmol/L		t.Chol mmol/L		HbA1c mmol/mol	
K+ mmol/L		Trigs mmol/L		fasting glucose mmol/L	
Creat umol/L		HDL mmol/L		fasting insulin miu/L	
eGFR		LDL mmol/L		fasting C-peptide pmol/L	
CRP mg/L		Ratio		GAD antibodies	
LFT's				IA-2 antibodies	
ALP				**Urine**	
ALT				Fasting UCPCR	
Bilirubin				Post-meal UCPCR	
Albumin				Microalbuminuria mg/mmoL	

Additional Notes

Completed By	
Name	
Signed	
Date	

Appendix 6: Sample Processing SOP

A guide to the MY DIABETES study sample processing
SOP Title: SOP/MD/Samples/v1

This document is intended for use by research teams at sites undertaking the MY DIABETES research study and outlines the key steps in sample processing.

Number	Process				Tick
BLOOD SAMPLES At the end of the visit the following tubes should be filled. L=perform in local lab, C=latest clinic visit S=save, SA= send away					
1)	Tube number	Preservative	Volume (min)	Tests	
	1 x yellow top	SST/gel separator	2ml	Renal, lipids, LFT's, CRP (L /C)	
	1 x small purple	EDTA	2ml	HbA1c (L/C)	
	1 x grey top	Fluoride oxalate	1ml	Glucose (L)	
	1 x large purple	EDTA	7-10mls	DNA extraction and antibodies (SA)	
	4 x large red	Plain tube	6 mls	Serum save (S)	
2)	Samples for chemistry, HbA1c and glucose should be labelled and processed in local lab along with the sample for microalbuminuria (see below). If there is no funding for local analysis of these bloods please discuss with coordinating centre..				
3)	**Processing the DNA sample** • The DNA sample will need to be labelled (hand-written) with the participants full name and DOB, study ID, date of sample and 'MY DIABETES study'. • The EXETER blood form should be completed with the information requested. • The UN3373 compliant PathoShield flat box should be constructed as per instructions. • The EDTA sample should be encircled by the absorbent swab, placed in the plastic bag and sealed (ensuring all air expelled). The plastic bag along with the EXETER blood form should be placed in the box and the box closed using its flaps. Finally the blue security seal sticker should be placed across the front edge of the box to seal completely. • For extra security use tape to secure other sides of the box. • Please ensure 4 labels are in place on the outside of the box – the address label for Exeter genetics lab, the senders address (on the back), the MY DIABETES study label and the 'FIRST CLASS' study label (we will provide pre-labelled boxes, but please double check). • UN3373 directives state that category B samples (blood and urine) must be transported first class (stamps provided).				

	• If the sample cannot be sent that day, please keep in a fridge until next day (not freezer). Ideally DNA samples should reach Exeter within 72hrs of being taken, so where possible samples should be sent same day. • Only 1 DNA sample can be placed in 1 box (although there is room for more, the postage is paid according to weight). Each sample must be in its own specimen bag and have it's own form.	
4)	**Processing the serum samples** • The 4 plain tubes should be allowed to clot (10-15 minutes) and then centrifuged for 10 minutes. If samples cannot be spun in this time frame, they should be placed on ice until they can be. • Once centrifuged, aliquot serum into 5 cryotubes with 1-1.5mls serum per tube. The cryotubes should be labeled anonymised with the study ID (MD-XX-001), the date of the sample and the word 'serum'. These should then be placed in the MY DIABETES cryobox and frozen at -80 degrees. These samples will be collected periodically by the coordinating centre.	
URINE SAMPLES		
	Processing the fasting urine sample • The participant will provide a urine sample (second void, fasting) in a universal container. • This sample should be split into a boric acid container, leaving ~2 mls behind in the universal container for microalbuminuria analysis locally and the rest in boric acid. • The universal container should be sent to the local lab along with the blood samples as outlined above, for microalbumin measurement. • The boric acid sample should be mixed thoroughly by inversion, and then decanted into 2 cryotubes (filled to capacity). These should be labelled with the study ID code (MD-XX-001), the date of the sample and the letters 'BAUF' which stands for boric acid urine fasting. • These samples should then be placed adjacent to the serum samples in the cryobox and frozen.	
Post-visit		
	Cryobox contents per patient At the end of the study visit, each participant should have a minimum of the following: • 5 x serum containing cryotubes with 1-1.5ml serum • 2 x BAUF (boric acid urine, fasting) •	
	Storage If necessary, cryoboxes will be provided. Therefore 9-10 patients samples can be stored per box. These should be stored at -80 degrees as soon as possible. I will arrange collection of boxes every 3 months.	

Please direct any further queries regarding consenting to Dr Shivani Misra (MY DIABETES study investigator) at s.misra@imperial.ac.uk

Appendix 7: Standardised Results Letters

Patient name

Patient Address

Dr Shivani Misra
Clinical Research Fellow
Diabetes, Endocrinology & Metabolism
Ground Floor Medical School Building
St Mary's Campus
Imperial College London
Norfolk Place
London, W2 1PG
Tel: 020 7594 2691
E-mail: xxx@imperial.ac.uk

Dear patient,

Re: A cross-sectional study to characterise young-onset diabetes in two UK ethnic groups

You recently took part in the above named research project. As part of this you provided us with a blood sample. This sample was tested for a substance called C-peptide, which tells us how much insulin your body still produces.

Your result was: …….. This means you are not producing insulin of your own.

This result is usually seen in people with long standing Type 1 diabetes and means you are extremely unlikely to have the specific genetic type of diabetes that we hope to identify in this project. This means you have now completed your part in this study. However, as previously agreed, we will store your remaining blood samples, including the DNA sample, and may use these in the future for other studies relating to diabetes.

The results of the other tests you had are appended to this letter along with a brief comment, and a copy of this letter along with your results has been sent to your GP.

We would like to take this opportunity to thank you for your involvement in the study. Your contribution will hopefully help us to identify the right series of investigations for ensuring the correct diagnosis and treatment for different types of diabetes in the future.

If you would like to discuss your result, or any other aspect of the project, with a member of the project team, our contact details are given above.
Yours sincerely

Shivani Misra, Clinical Research Fellow
Dr Nick Oliver, Chief Investigator

Patient name

Patient Address

Dr Shivani Misra
Clinical Research Fellow
Diabetes, Endocrinology & Metabolism
Ground Floor Medical School Building
St Mary's Campus
Imperial College London
Norfolk Place
London, W2 1PG
Tel: 020 7594 2691
E-mail: xxx@imperial.ac.uk

Dear patient

Re: A cross-sectional study to characterise young-onset diabetes in two UK ethnic groups

You recently took part in the above names research project. As part of this you provided us with some blood samples. One of these samples was tested for GAD65 and IA2 pancreatic antibodies. These pancreatic antibodies are usually 'positive' in those with Types 1 Diabetes.

Your result was: **GAD65:…… IA2:……**

This result means you are 'positive' to one or more of these pancreatic antibodies which indicates you have Type 1 Diabetes.

This means you have now completed your part in this study. However, as previously agreed, we will store your remaining blood samples, including the DNA sample, and may use these in the future for other studies relating to diabetes.

The results of the other tests you had are appended to this letter along with a brief comment, and a copy of this letter along with your results has been sent to your GP.

We would like to take this opportunity to thank you for your involvement in the study. Your contribution will hopefully help us to identify the right series of investigations for ensuring the correct diagnosis and treatment for different types of diabetes in the future.
If you would like to discuss your result or any other aspect of the project with a member of the research team, our contact details are given above.

Yours sincerely

Nick Oliver, Chief Investigator
Shivani Misra, Clinical Research Fellow

Patient name

Patient Address

Dr Shivani Misra
Clinical Research Fellow
Diabetes, Endocrinology & Metabolism
Ground Floor Medical School Building
St Mary's Campus
Imperial College London
Norfolk Place
London, W2 1PG
Tel: 020 7594 2691
E-mail: xxx@imperial.ac.uk

Dear patient,

Re: A cross-sectional study to characterise young-onset diabetes in two UK ethnic groups

You recently took part in the above named research project. As part of this you provided us with some blood samples, one of which tested negative for pancreatic antibodies. We therefore sent a further blood sample for DNA analysis to look for changes in the genes that most commonly cause Maturity Onset Diabetes of the Young (MODY).

As discussed, we tested your sample for changes in the XXXX genes
Your result is: No changes in these genes were identified

This means we have not found a genetic cause for your diabetes. There are a number of possible explanations for this:

- You have Type 2 diabetes, although this is less common in people below the age of 30 years.
- You have Type 1 diabetes but for some reason your pancreas has not fully stopped working.
- You may have diabetes due to a change in a gene but we have not yet identified this and we may find it in the future.

It is important to continue taking your current diabetes treatment and a copy of this letter will be sent to your GP.

As previously agreed, we will store your remaining blood samples, including the DNA sample, and may use these in the future for other studies relating to diabetes.

The results of the other tests you had are appended to this letter along with a brief comment, and a copy of this letter along with your results has been sent to your GP.

We would like to take this opportunity to thank you for your involvement in the study. Your contribution will hopefully help us to identify the right series of investigations for ensuring the correct diagnosis and treatment for different types of diabetes in the future.
If you would like to discuss your result, or any other aspect of the project, with a member of the project team, our contact details are given above.

Yours sincerely

Shivani Misra, Clinical Research Fellow,
Dr Nick Oliver, Chief Investigator

Appendix 8: HeLa Cell Maintenance

HeLA cell maintenance

Equipment Needed:
75 ml Sarstedt cell culture flask
24-well plate, Sarstedt inc
10 cm dishes, Sarstedt inc
1× phosphate buffered saline (PBS) (Sigma Aldrich)
Dulbecco's modified Eagle's Media (DMEM, ThermoScientific, Waltham, MA, USA
TrypLE Express (Life Technologies)
Tissue culture microscope (Nikon Eclipse T5100)
Foetal calf serum (Gibco, Life Technologies)
Penicillin/streptomycin (Life Technologies).

HeLa cells were spit were split twice weekly (1:10) and maintained in 75 ml tissue culture flasks. Cells were grown in DMEM media with 10% foetal calf serum and 1% penicillin/streptomycin. All cells were grown at 37°C and 5% CO_2.

To split cells for maintenance:

1. Media was removed from the flask using a stripette and cells were washed with 5mls of 1× PBS, which was then removed.
2. 1ml of TrypLE Express was added to the flask after which cells were incubated at 37°C for 5 minutes.
3. Cells were then visually inspected under the tissue culture microscope to check full detachment.
4. To neutralise the TrypLE-Express, 5ml of DMEM was mixed with the cells in the flask.
5. 1 ml of cells was added to 10 mls of fresh DMEM media for continued growth and passage noted.

To split cells for seeding plates:

If cells were being seeded into plates, after point 4, concentration of cells per ml was calculated following cell counting and an appropriate dilution undertaken using DMEM (without antibiotics).

1. Cells were then seeded at the desired concentration and volume of DMEM (with FCS, but no antibiotics) and incubated for 24 hours before transfection.
2. Seeding concentrations were 4 x 10^5 in 500 µl for Luciferase experiments and 2 x 10^6 in 10 mls DMEM for combined Western and EMSA experiments.

Appendix 9: Luciferase Assay Full Protocol

Diabetes Research Laboratories

DRL-SOP
Author: Dr Amanda Bennett
Luciferase Assay protocol

Equipment Needed:
75 ml Sarstedt cell culture flask
24-well plate, Sarstedt inc
Dual Luciferase® Reporter assay system (Promega, Madison, WA, USA)
Enspire plate reader
1× phosphate buffered saline (PBS) (Sigma Aldrich)
Dulbecco's modified Eagle's Media (DMEM, ThermoScientific, Waltham, MA, USA
Opti-MEM reduced serum media (Gibco/Life Technologies, Waltham, MA, USA)
Lipofectamine 2000 (Invitrogen, Waltham, MA, USA)
Enspire plate reader, Perkin Elmer, Life Sciences
TrypLE Express (Life Technologies)
Tissue culture microscope (Nikon Eclipse T5100)

Protocol Day 1

1. Check that the cells are at a suitable confluency (95-100 % visual confluency) and look to be healthy.
    a. If cells aren't growing rapidly enough at the time of plating, they won't be growing optimally during the protein production phase of the experiment
2. Refer to SOP_126 (HeLa cell line maintenance and transfection) for specific instructions for plating cells.
3. Defrost all DNAs required,
    a. including pRL-SV40, pcDNA-EMPTY, pcDNA-HNF1a-WT, pGL3-rat albumin promoter and all variants in pcDNA-HNF1a plasmids, to make up DNA mixes, nano and add concentrations into DNA volume calculations to generate 1µg of DNA. See table 1 at end of protocol for example volumes to add for each DNA mix
4. Aliquot the DNA mixes and add water to make upto 40µl in a 1.5ml Eppendorf, freeze samples at -20C until using on Day 2
5. Using SOP-126 trypsinise flask of cells, split new flask of cells and then count cells. Calculate the volume of cells and media for plating using the example:
    a. Eg count1 (set on Hela cells) = $2.8x10^5$, count2 = $4.0x10^5$. Average = $3.4x10^5$
    b. Calculate volume of cells required per plate (calculate 25 well per 24 well plate, to create an excess)
    c. (Plating density) $4x10^5$ x 25 wells = $1.25x10^6$
    d. Volume of cells required = $1.25x10^6$ / $3.4x10^5$ (average cell count per ml) = 3.7mls

e. Volume of media to add to make up to 12.5mls (25wells x 500µl per well) = 12.5mls – 3.7mls = 8.8mls

6. Into a clean 15 or 50ml falcon add the required volume of cells and media.
7. Label the required number of 24well plates and then shake the cell/media mix to ensure even distribution of cells throughout (cells will settle at the bottom of the tube) and then gently pipette 500ul of cells into each well using a P1000 pipette
    a. Whilst plating cells mix the stock tube of cells after every few well to ensure that all wells get approximately the same number of cells
8. Once all plates have been pipetted place the 24well plates on a cleaned metal tray, in an incubator not the primary cell or viral incubators

Day 2

9. Two time points for the experimental work, so book a cabinet for the start of the transfection and a second slot 4-5hrs after finishing the transfection part, to replace the media
10. Place the DNA mixes into a lidded box and take to the TC area, spray with ethanol before placing in the cabinet. Also bring the lipofectamine from the bug cold room. Set the cabinet up to add Optimem to the DNA mixes and a separate tube for the lipofectamine.
11. Calculate the amount of media/lipofectamine to make up, including an excess of two wells and add to a 15ml or 50ml tube, replace lid and shake well to mix. **NOTE**: Add media to tube first. Lipofectamine must be added to the media. If added first it will adhere to the sides of the tube.
    a. Optimem media 47ul per well x 4 wells x 9 (8 DNA mixes per 24well plate +1 excess) = 1692µl
    b. Lipofectamine – 3ul per 1ug DNA x 4wells x 9 (8 DNA mixes per 24well plate +1 excess) = 108ul
12. Add 160µl of Optimem media to each DNA mix (total = 200µl)
13. Mix the tube of media and lipofectamine and add 200µl of media/lipofectamine to each DNA tube, changing the tip after each addition and discarding in an autoclave bag.
14. Close the lid of the box and shake to mix and quickly spin
15. Leave the DNA mixes/lipofectamine/media tubes in the cabinet for 20mins to form the DNA/lipid complexes
16. Gently drip 100µl of the DNA/lipid complex to the corresponding wells on the 24 well plate, replacing the tip between each new DNA /lipid complex tube. Discard all tips into the autoclave bag
17. Replace the lid of the 24 well plate and replace in the incubator. Discard all residual DNA/lipid complex tubes into the autoclave bag
18. 4-5hrs post completion of the addition of the DNA/lipid complexes: remove the plates from the incubator and in a TC cabinet tilt the plate and remove the media + DNA/lipid complex, using a P1000 pipette and tips, to virkon. Discard the P1000 tip for each set of DNA wells into an autoclave bag

19. Add 500µl of fresh transfection media and replace the 24 well plates in the incubator

Day 3 Refer to DRL-SOP-169 (Lucfirease assay using Enspire instrument) for run protocol

IF LYSING CELLS ONLY:

20. Prepare enough passive lysis buffer for the number of plates/well used and chill on ice (100µl per well + excess) in a 15ml falcon tube. Eg 1 full 24 well plate = 27 wells x 100µl = 2.7mls, round up to 3mls
    a. Passive buffer 5x to add: 3mls/5 = 600µl
    b. Water to add 3mls - 600µl = 2.4mls
21. Remove the test plates from the incubator 24hrs after transfection
22. Place on ice and tilt so that the media collects at the bottom of the well and remove with a pipette to virkon
23. Add 500mls of pre-chilled PBS, swill around the well then tilt the plate, before removing the PBS and any excess media to virkon
24. Add 100ul of the 1x passive lysis buffer to each well, then place on the rocker in the western room to lyse the cells.
25. This can take a variable amount of time, HeLa cells have taken over 60mins to lyse.
    a. The plates can be sealed with parafilm and stored at -20C or -80C at this point, prior to running

IF CARRYING OUT LUCIFERASE RUN:

26. Whilst the cells are lysing calculate amount of LARII and SNG required:
    a. Eg if 10ul of cell lysate = 50µl of each reagent per well + excess 1ml
27. Remove sufficient 1ml aliquots of LARII from the -80C to defrost in an ice bucket, covered to protect the tubes from light
28. Defrost SNG buffer from -20C on ice then make up SNG buffer and SNG concentrate (50x)
    a. 24wells x 50ul + 1ml excess = 2.2mls
        i. 2200ul / 50 = 44ul of the 50x concentrate
        ii. 2200ul – 44ul = 2156ul

29. When the cells have lysed (ie all cells have lost attachment to the base of the well and only clumps of cell debris can be seen under a microscope) prepare the run plate
30. Put the transfection plate on ice and mix the contents of each well and added required amount of lysate to each corresponding well of the run plate (for 10ul lysate +50ul of each luciferase reagent use ½ area 96well plate, Corning)
31. **Proceed using SOP-169 (luciferase run using Enspire)**
32. Once initiated run, parafilm the transfection plate at -20C or -80C

Appendix 10: Combined Western and EMSA Full Protocol



DRL-SOP-193
Author: Dr Amanda Bennett
Western, EMSA and cytosol/nuclear fractionation western combined protocol– HNF1a project

Reagents
DMEM transfection media for HeLa, see SOP126
DNA plasmid including empty plasmid, wildtype HNF1a plasmid and variants HNF1a plasmids
Opti-MEM reduced Serum medium (Gibco/life technologies, 31985-062, 100ml, +HEPES, +2.4Sodium Bicarbinate, + L-Glutamine, stored at 4C in TC fridge, stock in bug lab cold room)
Lipofectamine 2000 (Invitrogen, 11668-500, 15ml, stored at 4C in the drinks fridge)
ddH2O
PBS (Sigma, tablets stored on reagent shelves), 5 tablets in 1L ddH2O, autoclaved
Clarity Western ECL substrate (Bio-Rad, 1705061, 500ml, stored at room temperature in cupboard in western room)
Protein assay dye reagent (Bio-Rad, 500-0006, 450mls, stored at 4C in drinks fridge)
0.1M NaOH (Make from stock NaOH in lab, stored at room temperature on bench)
Western and cytosol-nuclear fractionation western reagents
Lysis buffer 5x (Western, see end of protocol for details, stored in fridge under sink)
Hepes pH 7.8 0.2M (cyto-nucl Western, see end of protocol for details, stored at 4c in fridge under sink)
MgCl2 60mM (cyto-nucl Western, see end of protocol for details, stored at 4C in drinks fridge)
KCl 1M (cyto-nucl Western, see end of protocol for details, stored at 4C in drinks fridge)
IGEPAL10% (v/v) (cyto-nucl Western, see end of protocol for details, stored at room temperature, on bench)
NaCl 4M (cyto-nucl Western, see end of protocol for details, stored at room temperature, on bench)
EDTA 0.5M (cyto-nucl Western, see end of protocol for details, stored at room temperature, on bench)
Dry ice
4–20% Criterion™ TGX Stain-Free™ Protein Gel (26 well, 15 µl Bio-Rad #5678095 or 18 well, 30ul #5678094, stored at 4C in cold room)
TGS 10x (Bio-Rad, 161-0772, 5L, stored at room temperature by western room sink) made to 1x with ddH2O
Trans-Blot® Turbo™ Midi PVDF Transfer Packs (Bio-Rad #1704157, stored at 4C in cold room)
4x Laemmli Sample Buffer (Bio-Rad #1610747, stored at room temperature in cupboard in western room)
Precision Plus Protein™ All Blue Standards (Bio-Rad #161-0373) or Precision Plus Protein™ Dual Color Standards (Bio-Rad #1610374EDU), both stored at -20C in ice-cream freezer, orange box
Dried skimmed milk (use 5% solution made up with TBST, ie 5g in 100mls 1xTBST, stored on reagent shelves)
TBST (see end of protocol for reagents and volumes) all reagents on bench or reagent shelves (Tween20)
Ponceau S solution (Sigma, P7170-1L, stored at room temperature in western room cupboard)
Scalpel and filter paper

HNf1a primary antibody (cyto-nucl western and Western, see end of protocol for details, stored at 4C in drinks fridge primary box)
Tubulin primary antibody (Western, see end of protocol for details, stored at 4C in drinks fridge primary box)
Topoisomerase primary antibody (cyto-nucl western, see end of protocol for details, stored at 4C in drinks fridge primary box)
GAPDH primary antibody (cyto-nucl western, see end of protocol for details, stored at 4C in drinks fridge primary box)
Rabbit anti sheep secondary (see end of protocol for details, stored in aliquots at -20C in ice-cream freezer)
Goat anti Rabbit secondary (see end of protocol for details, stored in aliquots at -20C in ice-cream freezer)

EMSA reagents

Criterion precast gel 5% TBE 1.0mm 18 comb, 30ul, (Bio-rad #345-0048), or 26well comb, 15ul (Bio-rad 345-0049) stored at 4C in cold room
LICOR ODYSSEY INFARED EMSA KIT (Biorad #829-07910, stored at -20C in freezer under bench)
TBE 0.5% (made from concentrate, unopened bottle from DNA lab, used only in Western room)

Equipment

Bio-rad midi gel tank
Power pack
P10, p200, p1000 pipettes and tips
0.5ml and 1.5ml eppendorf tubes (transfection, cell lysate collection, Bradford samples and gel loading)
Cell scrappers
Glass tubes and rack
Chemi-Doc
Trans blot unit
Clingfilm

Protocol

Summary

Day 1 plate HeLa cells at $2x10^6$ per $10cm^2$ dish, see SOP_126 (specifically section for cytosol/nuclear fractionation Western or EMSA). FOR EMSA: if making new aliquots of the probes, make 'Hot' probes, dilute and aliquot. Details in EMSA section Day 7
Day2 transfect cells with 5ug of each variant with lipofectamine 3ul lipo:1ug DNA (ie 15ul per dish)
Day 3 at 24hr, decant media and transfection mix, wash with ice cold PBS, scrap cells from dish using fresh PBS, split cells into 2 x eppendorf's, spin, remove PBS from both tubes, freeze one tube of cells for cyto/nuc fractionation, lyse 2^{nd} tube for western and EMSA, spin, aliquot 3 x 50ul and 10ul for Bradford, freeze all tubes, store lysis buffer for Bradford quantification in fridge
Day 4 Bradford 2ul of protein supernatant, use to calculate volume for each experimental stream, see SOP_192
Day5 Run western, load 5ug of protein, activate, transfer, cut if necessary, block, at 1° antibody
Day 6 remove 1° antibody, wash, add 2° antibody, remove and wash, ECL and image
Day 7 Run EMSA
Day 8 fractionate second aliquot of cells and Bradford
Day9 start fractionation western, load 20ug of cytosol and 20ug of nuclear fraction, activate, transfer, block add 1° antibody
Day 10 remove 1°, wash membrane, add 2° antibody, remove wash, ECL and image

Day 1 plating of cells and DNA preparation for transfection

1. See MMc_SOP126 HeLa cell line maintenance and transfection. Plate Hela cells at density of $2x10^6$ on $10cm^2$ dish in transfection media (DMEM +FCS)
2. Defrost DNA's for transfection, including pcDNA_empty, pcDNA_HNF1a_WT and pcDNA_HNF1a mutagenized plasmids
3. Nano to check concentration, label a 1.5ml Eppendorf for each DNA to be transfected. Aliquot volume equivalent to 5ug of variant or WT DNA, 5ug pcDNA-empty, dilute with ddH2O to make 50ul and store diluted DNA in fridge until following day

Day 2 Transfection of cells

1. Check the plated cells to access growth and to ensure none are showing any signs of infection (such as media colour change or cells death). Check confluency is between 70-90%
2. Pre warm the Optimem media in the waterbath at 37C
3. If not aliquoted on day 1, make up DNA dilution as step 2 and 3 above
4. Add warmed Optimem media to make the DNA aliquots to 500ul
5. Make up OPTImem/lipofectamine mix in a 15ml falcon tube, adding 485ul pre-warmed Optimem media first and then add 15ul lipofectamine for each dish to be transfected plus one (excess), mix tube well
   5 dishes to transfect, make up enough mix for 6 dishes
   = 485ul * 6 (Optimem) + 15ul * 6 (lipofectamine)
   = 3mls
6. Add 500ul of lipo/media to each DNA tube, shake, quick, low speed spin, incubate for 20mins in the cabinet (room temperature)
7. Mix the tube by gently pipetting then add DNA/media/lipofectamine mix (1ml) to each corresponding dish by gently dripping the mix onto the media of each dish, discard the tube and tip into a pot initially then into a double bagged autoclave bag
8. Return to the incubator for 24hrs (from time of adding last DNA mix to the last dish)

Day 3 lysing transfected cells and collecting total protein

1. Prepare 10mls of 1x lysis buffer (for western and EMSA) by aliquoting 8mls of autoclaved ddH2O, add 1 protein inhibitor tablet and 0.924g Sucrose and dissolve. Add 2mls 5x lysis buffer concentrate and 10ul mecaptoethanol and gently mix
2. Prechill the lysis buffer and PBS on ice
3. Label 5 x 1.5ml ('cyto-nuclear', 'lysis', 'Western', 'EMSA1', 'EMSA2', + variant and date) and 1 x 0.5ml ('EMSA-West', variant, date) Eppendorf tubes for each DNA transfected and place on ice
4. At 24hrs after adding the transfection mixes on Day 2, place the dishes on ice, do this in batches if necessary
5. Remove the media from each dish in turn, to virkon. This can either be pipetted or gently invert the dish over a bucket of virkon
6. Add 500ul ice cold PBS, swill gently and remove to virkon, tilt the plate on the ice and pipette off excess PBS to virkon
7. Add 500ul ice cold PBS to each dish, scrap the dish to lift the cells and remove the cells + PBS to a 1.5ml Eppendorf (labelled with plasmid, date, 'cyto-nuclear')
8. Mix cells and PBS and remove 250ul to a second 1.5ml Eppendorf (labelled with plasmid, date, 'Lysis') store the tubes on ice until all dishes have been washed and cells removed
9. Spin the tubes at 1500RPM, 5mins, 4C
10. Replace on ice and remove the PBS to virkon, place all the C/N Western tubes into storage box and freeze at -80C
11. Add 150ul of the lysis buffer to all the tubes labelled 'Lysis'

12. Briefly vortex the tubes, then place on ice for 20mins to lysis
13. Pellet at 13K RPM, 20mins at 4C
14. Remove supernatant to 3rd 1.5ml Eppendorf (labelled with plasmid, date, Western), mix well then remove 2 x 50ul to the 4th and 5th 1.5ml Eppendorf (labelled with plasmid, date, EMSA1 or EMSA2) and 10ul to the 0.5ul Eppendorf (labelled plasmid, date, 'EMSA-West') for Bradford qunatification
15. Use Bradford aliquot for quantification or it can be placed in -20C and stored until Bradford is carried out. The Western, EMSA1 and EMSA2 tubes should be frozen and stored at -20C or -80C for longer term storage
16. Store the remaining lysis buffer (labelled with 'Western lysis buffer, date, initials' at 4-8C until Bradford quantification carried out

Day 4 Bradford quantification

1. See SOP_192 for more extensive quantification protocol
2. Make up enough 1x Bradford reagent in a 15ml falcon tube (1ml per sample to be quantified), with an excess of 2 samples (2mls excess)
3. Label 1 x glass tube for each sample and standard curve samples
4. Create standard curve using by aliquoting 2ul of 1x lysis buffer, 0.1M NaOH, BSA 1mg/ml (see example table below for standards and samples setup)
5. Bradford 2ul of the 10ul sample aliquot, adding 38ul of 0.1M NaOH
6. Add 1ml Bradford reagent to all samples, vortex tubes and leave to stand on the bench for 10mins
7. Start up the VersaMax software (Molecular devices, SoftMax software), open a previous run, set-up and save and rename the document for the run (include experiment type, biological number, date)
8. Aliquot 200ul of each standard and sample in duplicate (only one well for the 0ug standard) onto a clear bottom well plate and carry out run
9. Re-save the run document, select all wells , copy and paste to excel or export the results from plate reader to excel and process using the excel spreadsheet

Day 5 Western

1. Using Bradford data, calculate the volume to load 10ug of total protein, then calculate which gel to use, based on volume of protein + 5ul of loading buffer and DTT, 18well gels can load 30ul, 26 well gels can load 15ul
    a. Each gel should have a protein marker in lane 1, empty pcDNA sample, HNF1a_WT sample
2. Defrost 'Western' protein lysate tubes on ice
3. Pre-heat block in western room to 80C
4. Label 1 x 1.5ml Eppendorf for each sample + 1 to make loading mix
5. Calculate and make up enough loading mix for N + 3 samples, 1ul 1M DTT :4ul Laemmli Sample buffer 4x, mix, quick spin and add 5ul to each 1.5ml eppi.
    If sample volume exceeds 16ul (sample plus loading mix) then more loading mix so that you have Laemmli at ¼ total volume
6. Flick to mix defrosted protein lysate tubes, briefly spin and replace on ice. Add required volume of protein to eppi, seal, quick spin
7. Place all tubes on pre-heated heat block at 80C for 20mins to denature the proteins
8. While the samples are denaturing, set up gel, remove comb and rinse top gently with ddwater, remove tape from the bottom of gel, check that all the wells are properly formed and not damaged and place in tank
9. Add 1x TGX to the loading chamber and fill the tank to the fill line

10. Allow samples to cool (at room temperature or on ice) then spin tubes
11. Use pipette to gently flush out wells, while samples cool
12. Carefully load samples, changing tip between each sample
13. Add 7ul of all blue marker to one lane at the beginning. Add additional marker to differentiate between gels, if running multiple gels
14. Add loading buffer to any empty wells
15. Set power pack to 280V and run for 20mins, or 250V for 30mins
16. While the gel is running, make up 5% milk solution in TBST 1x and set on rocker to fully dissolve
17. When gel has nearly completed it run, check that the Chemi-doc isn't being used and log-on
18. Once gel has finished running, ie the purple fraction of the loading buffer has reached the bottom of the gel turn off the power remove the lid and remove the gel, carefully remove the buffer from the upper chamber
19. Split open the cassette, using either the lid of the tank or the spatula tool. Using the gel tool, carefully cut away the walls of the wells and the thicker footer of the gel
20. Release gel from the plastic into a gel holder containing some of the running buffer, and go to the Chemi-Doc with a blot for each gel run, to activate
21. Clean chemi-doc UV bed with water and then place gel on the bed, start up Image lab software
22. Select New protocol – Single channel – protein gels-stainfree gel-imaging aera-Bio-Rad Criterion, then activate by selecting 'Run Protocol'
23. Whilst gel is being activated, remove tray A from the transblot and clean with water, wiping clean with tissue
24. Once activation run complete, place the righthand tab filters and blot from the blot pack, on the cleaned base of the transfer blot, carefully lay the gel over the blot, trying to keep it as straight as possible, then pack the left hand tab filters on top of the gel. During assemble use the plastic roller to remove air bubbles from the stack
25. Close the lid and place in the transfer blot unit
26. Select 'transblot-turbo-1midi-A' start
    During run save the gel image
27. Once complete remove the left hand tab filters and the gel, place the blot in a gel tray and discard the right hand tab filters
28. Image the blot on the chemi-doc, using blot-chemi Hi sensitivity-imaging area Biorad-Criterion then save the image of the blot
29. Clean the chemi-doc and the transblot tray (base and lid) with water and dry
30. If cutting blot to run with multiple primary antibodies cover the blot with ponceau and place on rocker until bands of protein are visible
31. Use a scalpel to cut the blot, using the ponceau stained bands across the blot as a guide
32. Place the sections of blot in a gel tray and remove the ponceau with water, by swilling, tipping off the water and repeating a few times. Finally wash with 1 x TBST to remove any residual ponceau
33. Cover the blot with 20mls 5% milk solution (dried milk powder dissolved in 1 x TBST) in the plastic tray from the gel, set on rocker in western room for minimum 1hr to block the blot
34. Prepare the primary antibody/ies in 5% milk solution according to the dilution ratio, ie 20ul antibody in 20mls 5% milk solution for 1:1000, see table below for antibody dilution ratios
35. Discard the milk from the gel tray down the sink and replace with the 20mls 5% milk and antibody
36. Cover tray with clingfilm and place on the rocker in the cold room overnight.
    Alternatively place each section of the blot between a custom cut plastic sheet and then carefully heat seal the edges of the plastic, leaving a short side open. Add the milk/antibody to each corresponding bag, carefully remove air bubbles by squeezing them

to the top of the bag and dabbing any milk solution with blue roll. Then seal final side of the bag. Ensure there aren't any leaks and place on the rocker in the cold room overnight

Day 6 Western day 2

1. Remove the blot from the bags, if using and place in a gel tray. The milk and primary antibody can be frozen and reused twice. Cover with 1x TBST and leave on the rocker in the western room for 15mins
2. Discard the TBST down the sink, replace with fresh and leave on rocker for 15mins. Repeat once more (total 3 washes with fresh TBST)
3. Make up the secondary antibody in 5% milk (see below for dilution ratios) and then add to the gel tray (20mls) with the blot or create new bags for the blot, heat seal the sides leaving one short edge open, add the milk/secondary mix (10mls) and seal 4th side
4. Leave on the rocker in the western room for a minimum of 1hr or in the cold room if leaving longer
5. Remove the milk/secondary mix from the blot or from the bags, place in a gel tray and cover the TBST, place on rocker in western room for 3mins (The secondary antibody/milk solution can't be reused)
6. Remove add fresh buffer and rock for 3mins. Repeat 3 times more (total of 5 washes with fresh TBST)
7. Prep for ECL fixing of blot, clean a Perspex or glass sheet, dry thoroughly and lay down on a flat surface
8. Check that Chemi-doc is not in use and open up the Image lab software
9. Prepare 2mls Luminol/enhancer solution + 2mls peroxide solution, using separate stripette for each solution, in a 15ml tube
10. Lay blot on the sheet and gently pipette the ECL mix onto the gel, time 5mins, reapplying the mix to the blot as necessary
11. Using tweezers, pick up the blot and dab to remove any excess ECL mix on a piece of tissue before taking blot to the Chemi-doc to image
12. Go to 'New protocol – blot-chemi Hi Sensitivity-imaging area Bio-Rad Criterion gel-band high intensity – auto exposure or manual exposure' and select time eg 30secs
13. Position the gel and select run protocol
14. Further exposure settings can be used, such as faint band settings, extended exposure times
15. Finally carry out a blot-colorimetry-intense bands run
16. Go to the Tools icon on the left hand side and select merge. Merge the colorimetry and one of the exposure images
17. Save all exposure images, colorimetry image and the merged images. Images can also be exported, select for publication, and change to jpeg (these are smaller files than TIFFs)
18. Remove the blot from the Chemi-doc, clean with water, shut down the software, log off the computer, discard blot
19. Clean the Perspex sheet and any gel trays
20. Refer to SOP_191 for details of using ImageLab for densitometry

Day 7 EMSA

1. The fluorescent EMSA takes approximately 4hrs to carry out. Once a protein lysate aliquot has been used (defrosted) do not re-use for EMSA, structural integrity is imperative for binding the probes.
2. TO BE CARRIED OUT DAY BEFORE RUN OR EARLIER: Resuspend the F and R oligos to 100µM (as stated on data sheet with oligos) using RFW (RNAse-free water)
3. Mix the F and R 1:1 (I do 10uL of F + 10uL of R) into a clean 1.5mL eppy

4. The concentration of this will be 50µM per oligo as you've diluted one with the other
5. Heat this at 100oC for 5min and then switch the heat block off, letting the oligos cool to room temp before you touch them. This can be done at the end of the day and leave the tubes on the block, in the dark to cool overnight
6. Once the oligos are at RT, spin them briefly to get all liquid to the bottom (this is now at 50µM)
7. Make a 1:1000 dilution of the annealed stock (I do 1µL + 999µL RFW) Dilution is now 50nM this is your 'HOT' WORKING STOCK!
8. Aliquot a few 1.5mL Eppys with this, to reduce the number of freeze-thaws (eg 50µL Aliquots)
9. You'll be using 1µL of this per 20µL reaction, which is 20fmol per tube
   NOTE: If you want to make 'cold' probes follow all the steps as above except when you get to Step #5 do a 1:100 dilution of your annealed probes (so the concentration of the cold probes is 500nM). You don't need to aliquot this one.
10. Defrost protein lysate samples and the reagents for the binding reaction on ice (from the LiCor Odyssey kit), see example sample set-up below
11. Label tubes with the sample name and lane number (gel lane number)
12. Always add the water to the tubes first, then the binding reaction mix
13. Add wildtype protein lysate to the 'Supershift' WT tube
14. Then add 1ug of antibody, mix, quick spin of tube and incubate on ice for 30mins
15. During this incubation set up the gel tank, load with 0.5%TBE, remove the gel from the packaging, remove the tape at the bottom, remove the comb, check that all the wells are properly formed and not damaged, rinse the upper buffer chamber with water, place in tank and pre-run the gel at 100V
16. Add the remaining protein lysates to the corresponding tubes
17. Add 'Hot' probe to each tube, mix, quick spin then incubate at room temperature for 20mins (cover tube holder or rack with foil to protect the probe during the binding reaction)
18. Add 1.5ul of loading buffer to each sample tube, flick to mix
19. Stop the pre-run of the gel and load the samples, using different tip for each, add loading buffer to all un-used lanes
20. Run the gel at 100V for 1-1.30hrs until the orange loading buffer has just run off the bottom of the gel
21. Remove the gel cassette from the tank and split it open, user the cutting tool to remove the foot of the gel carefully
22. Clean the base of the Chemi-doc with water and tissue prior to placing the gel on the base
23. Place the gel cassette into the gel tray with some TBE from the tank and image the gel on the Chemi-doc using the correct filter setting (select new-protocol-single channel-blots-CY5-Bio-rad Criterion) set the manual exposure to 120secs, check the position of the gel, Run Protocol
24. Once the image is shown, select a longer exposure period such as 300secs or 600secs
25. Save the image and export the image (export-for publishing-select jpeg or TIFF)
26. Remove the gel from the Chemi-doc and clean the machine
27. Discard the gel in a yellow clinical waste bin bag
28. Refer to SOP_191 for details of using ImageLab for densitometry

MMc_RA_186, MMc_COSHH_186.

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Appendix 12: Ethics Approval

NRES Committee London - Chelsea
HRA
Research Ethics Committee (REC) London Centre
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London Road
London, SE1 6LH

Telephone: 020 7972 2556

20 September 2013

Dr Nick Oliver
Consultant Diabetologist, Honorary Senior Lecturer
Imperial Healthcare NHS Trust, Imperial College London
9th Floor, East Wing
Charing Cross Hospital
Fulham Palace Road
W6 8RF

Dear Dr Oliver

Study title:	**A cross-sectional study to characterise young-onset diabetes in two UK ethnic groups**
REC reference:	**13/LO/0944**
IRAS project ID:	**120070**

Thank you for your letter of 20 August 2013, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the Co-ordinator Mr Thomas McQuillan, nrescommittee.london-chelsea@nhs.net.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Advertisement	1.0	10 April 2013
Covering Letter		03 June 2013
Evidence of insurance or indemnity		29 July 2013
GP/Consultant Information Sheets	1.0	21 March 2013
Investigator CV	Nicholas Oliver	31 May 2013
Letter from Sponsor		05 June 2013
Letter of invitation to participant	1.0	21 March 2013
Other: CV: Desmond G Johnston		31 May 2013
Other: CV: Prof. Andrew T Hattersley		

Other: CV: Shivani Misra		
Other: Participant Brochure MODY	1	10 April 2013
Other: Letter re Funding		07 December 2012
Other: Antibody postive letter	1.0	21 March 2013
Other: C-peptide negative letter	1.0	21 March 2013
Other: Negative MODY results letter	1.0	21 March 2013
Participant Consent Form: Parent/Guardian	2.0	06 August 2013
Participant Consent Form: Adults	2.0	06 August 2013
Participant Consent Form: Children Assent	2.0	06 August 2013
Participant Information Sheet: Children	2.0	06 August 2013
Participant Information Sheet: Young Person	1.0	06 August 2013
Participant Information Sheet: Adults	2.0	06 August 2013
Participant Information Sheet: Parent/Guardian	2.0	06 August 2013
Protocol	1.0	21 March 2013
REC application	120070/460999/1/588	03 June 2013
Response to Request for Further Information		20 August 2013
Summary/Synopsis	1.0	13 October 2014

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

13/LO/0944	**Please quote this number on all correspondence**

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

With the Committee's best wishes for the success of this project.

Yours sincerely



pp
Dr Shelley Dolan
Chair
Email:nrescommittee.london-chelsea@nhs.net

Enclosures: "After ethical review – guidance for researchers" *[SL-AR2]*

Copy to: *Ms Christine Buicke,*