The Clinical Utility of Zinc Transporter 8 Autoantibody Measurement in Diabetes

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Abstract

Maturity onset diabetes of the young (MODY) is caused by single gene mutations that are of autosomal dominant inheritance. Mutations are highly penetrant, and patients often develop a phenotype similar to type 1 or type 2 diabetes. Glucokinase, Hepatic nuclear factor 1a and 4a mutations consists of 80% of MODY cases. Approximately 1% of patients with diabetes have MODY, and it is often misdiagnosed. Diagnosis is important as patients with MODY often have a good prognosis and glycaemic control if they are treated appropriately. The aim of this thesis was to explore the use of islet autoantibodies, in particular a new autoantibody against Zinc Transporter 8, as biomarkers to identify MODY.

A literature review of MODY and its important subtypes are discussed. It highlights the major mutation that cause MODY and the management of patients with MODY is also explored. Islet autoantibodies will also be reviewed in the same chapter, with a discussion on established autoantibodies and ZnT8 autoantibodies in relation to type 1 diabetes.

Chapter 1 aims to investigate whether ZnT8 autoantibodies are similar to established autoantibodies against GAD and IA-2 as a biomarker in differentiating T1D patients from MODY patients. The prevalence of ZnT8 autoantibodies in MODY patients and the effect of disease duration on antibody prevalence and discriminative power would also be investigated.

In Chapter 2, a study was performed to investigate whether islet autoantibodies are useful in the MODY referral setting in ruling out patients for genetic testing. This is a way to rationalise genetic testing at the Exeter molecular genetics referral service. Additionally, other biomarkers will also be investigated, namely C-peptide levels and Type 1 Diabetes Genetic Risk score. Results from the study will have implications to how MODY is diagnosed at the referral service.

A discussion of the findings of each chapter, implications and plans for future research will be explored in chapter 3.

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Finally, I would like to thank my parents for their continued support and encouragement despite us living 6000 miles apart. I would like to dedicate this thesis to them.

Abbreviations

ATP	Adenosine Triphosphate
AUC	Area Under the Curve
BMI	Body Mass Index
DASP	Diabetes Antibody Standardisation Programme
ELISA	Enzyme-Linked Immunosorbent Assay
GAD	Glutamic Acid Decarboxylase
GCK	Glucokinase
GLUT2	Glucose Facilitative Transporter 2
HDL	High Density Lipoprotein
HNF1A	Hepatocyte Nuclear Factor-1 alpha
HNF4A	Hepatocyte Nuclear Factor-4 alpha
HNF1B	Hepatocyte Nuclear Factor-1 beta
hsCRP	High Sensitivity C-reactive Protein
IA-2	Protein Tyrosine Phosphatase-related protein Islet Antigen 2
ICA	Islet Cell Antibodies
IPF1	Insulin Promoter Factor 1
IQR	Inter Quartile Range
IRR	International Reference Reagent
ISPAD	International Society of Paediatric and Adult Diabetes
LR	Likelihood Ratio
MODY	Maturity Onset Diabetes of the Young
NPV	Negative Predictive Value
OHA	Oral Hypoglycaemic Agent
PPV	Positive Predictive Value
ROC	Receiver Operating Characteristic Curve
ZnT8	Zinc Transporter 8

Structure of thesis

The aim of this thesis is to investigate whether autoantibodies against ZnT8 (ZnT8A) is a useful biomarker in the diagnosis of MODY. This will be investigated alongside established antibodies against glutamic acid decarboxylase (GADA) and protein tyrosine phosphatase islet antigen-2 (IA-2A).

The introduction is a review of MODY and islet autoantibodies. The first part discusses the clinical features, diagnosis and treatment of MODY. The second part provides an overview on GADA and IA-2A, their relationship with disease activity in type 1 diabetes, and how they are measured. ZnT8A will also be reviewed, discussing the importance of ZnT8 in insulin secretion and the current understanding of ZnT8A on T1D disease activity.

Chapter 1

The next chapter aim to explore the clinical utility of ZnT8A in MODY diagnosis. It had been shown that patients with MODY have low prevalence of established islet autoantibodies, namely GADA and IA-2A. The results from the previous study show that islet autoantibodies can be used as rulling out test, i.e, the chance of a patient having MODY with a positive antibody test is low.ZnT8 autoantibody is a relatively new test compared to established antibodies, and its clinical utility in differentiating T1D from MODY patients is unknown.

The aim of this chapter is to study the prevalence of ZnT8A in patients with MODY and whether they are able to discriminate T1D from MODY patients compared to GADA and IA-2A in a case control study.

Chapter 2

Although islet autoantibodies had been shown to have clinical utility in discriminating T1D from MODY patients, previous studies were performed in a retrospective case control setting. In this chapter we aim to investigate whether islet autoantibodies are useful in ruling out patients from molecular genetic testing in the referral setting. A consecutive patient cohort taken from the Exeter molecular genetics referral service is studied, with islet autoantibody levels compared between MODY patients and patients without a genetic diagnosis. Although not the main focus of the chapter, the clinical utility of other

biomarkers, such as C-peptide and the type 1 diabetes genetic risk score, was also investigated.

Chapter 3

The major findings from the previous two chapters are discussed, along with the strength and limitation of the study, clinical implication and future research within the area.

Introduction

Maturity Onset Diabetes of the Young and Islet Autoantibodies

1 – Maturity Onset Diabetes of the Young (MODY)

1.1 – Biology of MODY

Maturity Onset Diabetes of the Young is characterised by a monogenic mutation causing a familial, non-insulin dependent diabetes mellitus. The clinical description of the first distinct cases by Tattersall in 1920s found that these patients often have disease of autosomal dominant inheritance. Patients described in the original cohorts were a mixture of young-onset and maturity-onset diabetes, and therefore the term maturity onset diabetes of the young was coined at the time (1).

In recent years, new molecular genetic techniques allowed the identification of genes that are involved in the pathogenesis of MODY. It is now known that MODY is caused by single gene mutations that are highly penetrant which leads to a diabetic phenotype. Over 10 different genetic mutations have been identified since Tattersall's discovery of the disease, and the classification of gene mutations enabled further understanding of the phenotypes and clinical characteristics caused by MODY-related mutations. This includes the age of onset, level of hyperglycaemia, complications, and treatment prognosis. The most common MODY genotypes includes heterozygous mutations of glucokinase (*GCK*) gene, hepatocyte nuclear factor 1a (*HNF1a*) and hepatocyte nuclear factor 4a (*HNF4a*), which accounts for 80% of MODY cases (2). Patients with these mutations have differing clinical characteristics, with different responses to treatment.

1.2 – Glucokinase (*GCK*)

Mutation to the glucokinase (*GCK*) gene disrupts the beta cells' ability to sense glucose at normal homeostatic levels. *GCK* is an enzyme that allows the phosphorylation of glucose to glucose-6-phosphate after its entry into the beta cell through the GLUT-2 channel. Glucose-6-phosphate undergoes glycolysis and metabolised into pyruvate, which enters the citric acid cycle within the mitochondria for the production of ATP. Increasing levels of ATP closes ATP-dependent potassium channels and opens voltage-gated calcium channels, causing an influx of calcium (3,4). This triggers insulin exocytosis into the luminal space. In many ways, GCK acts as a "glucose sensor" for insulin

exocytosis as it controls the amount of glucose-6-phosphate that enters the insulin exocytosis pathway (5).

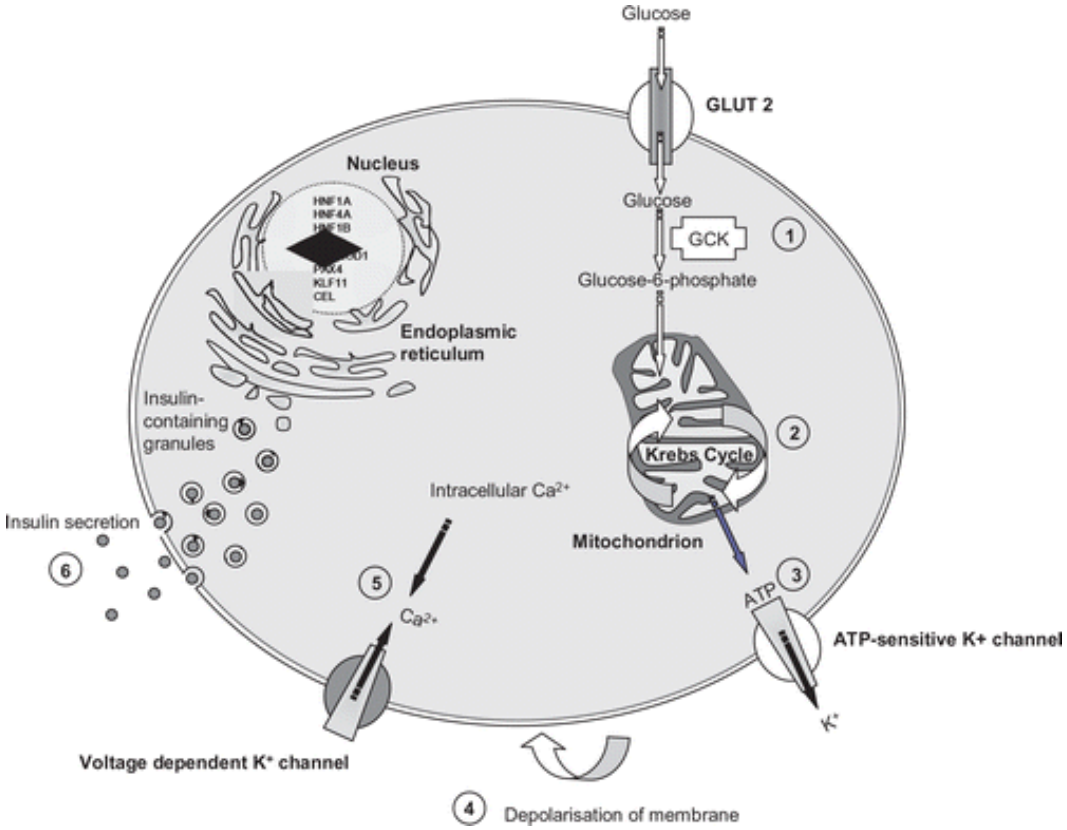

Figure 1: A diagram representing insulin secretion within the beta cell. Glucose enter the beta cell through the GLUT2 transport. Glucose then undergoes phosphorylation by GCK into Glucose-6-phosphate and enters the glycolytic pathway which forms pyruvate. Pyruvate enters the citric acid (Kreb's) cycle in the mitochondria, leading to production of adenosine triphosphate (ATP). Increasing ATP closes ATIP-sensitive potassium channels, leading to the depolarisation of the beta cell membrane and an influx of calcium ions within the cell, which triggers insulin exocytosis. The most common genetic mutations occur in the GCK gene and HNF1a and HNF4a genes within the beta cell nucleus. (Reproduced from McDonald TJ, Ellard S. Maturity onset diabetes of the young: identification and diagnosis. Ann Clin Biochem. 2013 Sep 1;50(5):403–

A heterozygous loss of function mutation of *GCK* results in decreased levels of glucose phosphorylation within the beta cell, which have effects on insulin exocytosis. Compared to the beta cell in normal individuals, patients with GCK mutations require a higher glucose threshold before insulin exocytosis is triggered. Previous studies have demonstrated a decrease in insulin secretion rates over a normal glucose range of 5-9 nmol and reduced sensitivity to glucose in the beta cells of patients with heterozygous GCK mutations (6).

Patients with heterozygous mutations of GCK presents with a mild hyperglycaemia of 5.5 – 7.5 mmol with minimal symptoms. Glycated haemoglobin (Hba1c) levels are usually fairly constant and vascular complications are rare, even in patients with longstanding disease. Due to these characteristics, patients with GCK mutations are often diagnosed incidentally with a higher than normal Hba1c test (3,7). Although clinical symptoms of these disease are mild without further sequelae (8), it is important to diagnose GCK-MODY correctly. Clinicians may treat patients with GCK-MODY unnecessarily with insulin or OHA if they misdiagnosed them as having type 1 or type 2 DM, which carries unnecessary side effects. This is especially true in younger populations, where type 1 DM is prevalent.

1.3 – Hepatic nuclear factor 1a (*HNF1a*)

In contrast to GCK-MODY, mutations to *HNF1a* causes longstanding diabetes mellitus associated with vascular complications. HNF1a is a transcription factor that is important in the expression of genes during embryonic development, and it is expressed in the pancreas, kidneys and intestines (9). In the mature beta cell, HNF1a is important in beta cell development and the secretion of insulin. Animal models with HNF1a mutations showed reduced gene expression affecting glucose transport within the beta cell, with reduced expression of insulin (9,10). The gene is localised in chromosome 12, with the mutation being highly penetrant (11).

Patients with HNF1a-MODY have slightly different clinical characteristics compared with GCK-MODY. These patients tend to be normoglycaemic at birth, with lower BMI and lower prevalence of hypertension compared to patients with type 2 diabetes. They are also diagnosed younger, with median age of diagnosis under the age of 25 years old (3,7). Unlike MODY caused by GCK mutations, patients with *HNF1a* mutations tend to develop microvascular and macrovascular complication from poor glycaemic control due to continuing decline of beta cell function and decreasing insulin levels (12). Patients with HNF1a mutations often have symptoms of diabetes and are usually non-insulin dependent, with persistent C-peptide production of more than 200 umol even after the honeymoon phase of 3 years (7,13).

Although HNF1a-MODY carries serious complication and disease burden, patients with the disease shows marked response to sulphonylureas, an oral

hypoglycaemic agent. It had been shown in case reports that patients with *HNF1a* were sensitive to the effects of sulphonylureas (14). Further randomised crossover trials had shown that patients with HNF1a-MODY had a 5.2 fold greater response than to the biguanide metformin compared to patients with type 2 DM (15). The proposed mechanism of such a response had been attributed to the nature of the defects seen in HNF1a mutations. Mouse models with *HNF1a* mutations had been shown to have decreased insulin release and impaired glucose metabolism, due to decrease ATP production. Sulphonyureas bind to the ATP sensitive potassium channels, leading to the closure of the channel and calcium influx into the beta cell, ultimately causing insulin release. As the problems caused by *HNF1a* mutation and ATP production are higher upstream in the insulin release pathway, sulphonylureas bypasses these problems to facilitate insulin release (15). Interestingly, patients who had previously been on longstanding insulin can be switched to sulphonylureas without further complications, although some patients with long standing diabetes may require insulin therapy (16).

As sulphonylureas are shown to be effective, it is vital not to misdiagnosis HNF1a-MODY. It is recommended that patients with HNF1a-MODY be put on a low dose of sulphonylurea, for example, gliclazide, at a dose of 20 – 40 mgs per day (3,7).

1.4 – Hepatic nuclear factor 4a (*HNF4a*)

HNF4a forms part of a network of transcription factors within the beta cell, along with HNF1a-MODY, therefore patients with HNF4a-MODY present similarly as patients with HNF1a-MODY. Like HNF1a-MODY, patients with HNF4a-MODY often present before the age 25 years old, although a portion of patients do present at a later age (3). There is a good treatment response to sulphonylureas in patients with HNF4a-MODY, therefore a low dose sulphonylurea is recommended in the treatment of HNF4a-MODY.

Additionally, it is important to diagnose HNF4a-MODY correctly as it may complicate the management of pregnancy. *HNF4a* mutations was found to be associated with macrosomia, with a median birthweight increase of 790g in patients with HNF4a-MODY (17). Transient hypoglycaemia has also been observed in patients with *HNF4a* mutations (17). These are important features

of HNF4a-MODY as they may complicate the perinatal period of patients with the disease.

Genetic Mutation	Function	Key characteristics	Treatment
Glucokinase (*GCK*)	Phosphorylates glucose to form glucose-6-phosphate	• Mild hyperglycaemia of 5.5 – 7.5 mmol with minimal symptoms • Stable HbA1c with levels < 7.5% • Minimal complications of diabetes	Rarely requires treatment
Hepatic Nuclear Factor 1a (*HNF1a*)	Transcription factor of pancreatic beta cells	• Poor glycaemic control due to decline of beta cell function • Persistent fasting C-peptide production • Microvascular and macrovascular complications	Sensitive to sulphonylureas
Hepatic Nuclear Factor 4a (*HNF4a*)	Transcription factor of pancreatic beta cells	• Similar to MODY caused by HNF1a mutations • Associated with macrosomia and transient neonatal hypoglycaemia	Sensitive to sulphonylureas

Table 1: Summary table of key characteristics of most common subtypes of MODY

1.5 – Other forms of MODY

HNF1b-MODY is caused by a rarer mutation of the *HNF1b* gene leading to a distinct form of MODY. Similar to *HNF1a* and *HNF4a*, *HNF1b* form part of the transcription factors within the beta cell. *HNF1b* is expressed in a wide range of organ, including the pancreas and kidneys. There is variable penetrance with *HNF1b*, and family history may not always be present as de novo mutations had been found to be present in up to 50% of patients (18–20). Unfortunately, the benefits of sulphonylureas are not seen in patients with HNF1b-MODY, with patients experiencing hyperinsulinaemia and insulin resistance. Although patients usually require insulin therapy, insulin requirement is usually low (21).

Although *HNF1b* is closely related to *HNF1a* and *HNF4a*, there are some distinct clinical characteristics seen in HNF1b-MODY. As *HNF1b* is important in the development of the pancreas and beta cell function, patients with *HNF1b* mutation had been shown to have pancreatic atrophy, mild exocrine insufficiency and low birth weight (20). *HNF1b* is a rare cause of neonatal diabetes, and due to the fact that *HNF1b* affects early foetal development and is expressed in multiple organs, extra-pancreatic features are often seen. The most common extra-pancreatic manifestation of HNF1b-MODY is a syndrome of Renal Cysts and Diabetes. This affects the patients' renal function significantly, and only 6% of HNF1b-MODY patients will have normal renal function (19).

Besides from HNF1b-MODY, other rarer forms of MODY had been identified. They impact the function of the beta cell in different ways, from beta cell development regulated by the transcription factor *IPF1* (22) to the mis-folding of insulin seen in *INS*-mutation MODY (23).

1.6 – Importance of diagnosis

Although the prevalence of MODY is low, accounting for 0.6% to 2% of all diabetes cases, it is important to attain the correct diagnosis as the right treatment can improve outcomes. Patients with GCK-MODY rarely requires treatment, and *HNF1a* and HNF4a-MODY can be well managed on low dose sulphonylurea. This means that patients can avoid unnecessary insulin treatment. It has been shown that patients with MODY can switch from insulin to sulphonylureas with no deterioration glycaemic control (24). Qualitative studies suggests that MODY patients felt fearful and anxious when they switched from insulin therapy to low dose sulphonylureas, which was possibly due to anxiety surrounding the treatment change and the patient's reliance on insulin therapy (25). This further supports the need to improve diagnosis of patients with suspected MODY to avoid unnecessary insulin therapy. Furthermore, family members of MODY patients are at greater risk of developing MODY due to the nature of the disease. Early identification and management of MODY can reduce the development of microvascular and macrovascular complications that could arise from *HNF1a* and HNF4a-MODY.

2 – Diagnosis of MODY and the use of islet autoantibodies

Although the recent advances in molecular genetics have made testing more accessible, it is still expensive. Molecular genetic testing remains the gold standard for the diagnosis of MODY in the form of Sanger sequencing and more recently Next Generation Sequencing. However, these techniques are still labour intensive as the results requires expertise to interpret (26). Therefore, potential biomarkers should be used to aid the diagnosis of MODY, such as islet autoantibodies. As MODY is a disease of genetic aetiology, patients with MODY are not expected to have positive islet autoantibodies. As such, islet autoantibodies can be used as a way to exclude patients from a diagnosis of MODY. It had been shown previously that the prevalence of established autoantibodies against glutamic acid decarboxylase (GADA) and protein tyrosine phosphatase islet antigen-2 (IA-2A) in MODY patients were low, with positivity rates being similar to control populations (<1%). GADA and IA-2A were also able to differentiate T1D patients from MODY patients, with a combined autoantibody sensitivity and specificity of 99% and 82% respectively (27). Patients with positive islet autoantibodies can be ruled out from genetic testing as the chance of them having MODY is increasingly low.

2.1 – Background of Islet autoantibodies

Islet autoantibodies are a hallmark of Type 1 diabetes, and since their discovery, had formed an essential part of diabetes mellitus diagnosis and classification. Autoantibodies are antibodies created by the immune system that targets self-antigens. In health, the immune system is trained to recognise self-antigens and direct antibodies against foreign, unrecognised antigens. In type I diabetes mellitus (T1D), autoantibodies are directed against beta cells within the islets of Langerhans of the pancreas. Infiltration of cytotoxic T-cells and macrophages contributes to the destruction of the beta-cells, leading to cell death and insulin deficiency (28,29). As a result, patients experience symptoms of hyperglycaemia, polyuria and polydipsia, along with micro and macrovascular complications. The discovery of islet autoantibodies allowed us to further our understanding of the disease and allow the classification of diabetes

2.1.1 – Islet cell cytoplasmic antibodies (ICAs)

First discovered by Botazzo et al in 1974, Islet cell cytoplasmic autoantibodies (ICAs) was the first islet antibody that demonstrated autoimmunity basis in type

I diabetes. Botazzo performed a study looking at patients with diabetes mellitus along with multiple endocrine deficiencies associated with organ-specific autoimmunity, including patients with Addison's disease, Hashimoto's thyroiditis and pernicious anaemia. Antibodies bound to human and rat pancreatic tissue was found, and the presence of antibodies correlated with disease activity in patients requiring insulin (30). These antibodies were later known as ICAs. Although ICAs were the first antibodies to be identified, they were difficult to quantify and measure as they bound non-specifically to human islet tissue. Other groups went on to study the components of ICAs (cite). Although studies have shown that ICAs can predict the development T1D (31), other antibodies have superseded the use of ICAs in the investigation of DM.

2.1.2 – Glutamic acid decarboxylase antibodies (GADA)

Autoantibodies against a 65kDa isoform of glutamic acid decarboxylase (GAD65) was discovered in 1990 as a major autoantigen. It was observed that some patients with a rare disease called stiff-man syndrome also seemed to have T1D. Patients with stiff-man syndrome have measurable GAD antibodies (GADA) (32). Baekkeskov et al used [^{35}S]-methionine labelled rat islets and sera from patients with stiff-man syndrome to study antibodies involved in the disease process. They found that antibodies within the sera cross-reacted with the pancreatic islet tissue (33). This effectively demonstrated that autoantibodies seen in patients with T1D was essentially GADA within the pancreas.

GAD65 seemed to be expressed within neurone and the pancreas. It catalyses a reaction which leads to the formation of gamma-aminobutyric acid (GABA), which had been shown to be important in beta cell signalling and autocrine functions. Experiments showed that blocking GABA receptors in human islets inhibited insulin secretion, suggesting a link between GABA and B-cell signalling (34). Although GABA is known for its inhibitory functions, especially within neurone, it had been shown that activating GABA channels within the pancreas causes an influx of chloride within the beta cell, leading to cell depolarisation and insulin secretion (34).

The presence of GADA are closely related to disease activity of T1D. Studies found that GADA was measurable even in young patients with newly diagnosed T1D (35). This was support by later studies, which found that GADA were found

in two thirds of children with newly diagnosed T1D within the Finnish Paediatric Diabetes register (36). Studies also show that titres of GADA in patients with positive antibodies predicted subsequent antibody positivity (37).

GADA is widely measured as part of the diagnostic workup for T1D. Assays have been developed and investigated in international laboratory workshops such as the Islet Autoantibodies Standardisation Programme (IASP) (formerly known as the Diabetes Autoantibodies Standardisation Programme). Traditionally, GADA measured in radioimmunoassay (RIA) format achieved the highest sensitivity (77%), specificity (95%) and diagnostic utility (ROC AUC = 0.93) (38). In later IASPs, enzyme linked immunosorbent assay (ELISA) format of the test improved and achieved similar diagnostic value and utility compared to RIA formats (39).

2.1.3 – Protein tyrosine phosphatase islet antigen-2 antibody (IA-2A)

An antibody that was targeted a protein tyrosine phosphatase-like protein was discovered in subsequent years after the discovery of GADA. The antibody was isolated in cloning complementary DNA (cDNA) fragments in the sera of patients with insulin-dependent diabetes (40). Sequencing of the protein found that it was closely related to the enzyme protein tyrosine phosphatase (PTP), but did not show any PTP enzymatic activity. The molecule was subsequently named PTP islet antigen-2 (IA-2). Like GAD65, IA-2 mRNA seemed to be highly expressed in both brain and pancreatic tissue. Experiments suggest that IA-2 is important in insulin secretion (41), however further studies need to be performed to investigate its functions.

IA-2 antibodies (IA-2A) are highly related to disease activity in T1D. A study investigating islet autoantibodies prevalence in childhood onset T1D found that IA-2A was present in 75% of cases studied. They also found that the risk of developing T1D was highly related to the number of positive autoantibodies (35). Interestingly, IA-2A levels were significantly higher in younger patients compared to older patients with T1D. This is in contrast to GADA, where the reverse is true, and the prevalence of GADA increased with disease duration (35). IA-2A positivity had also been shown to predict future T1D. In non-affected siblings of patients with T1D, there was more progression to diabetes in the presence (58%) than in the absence of IA-2A (10%) (42). This reiterates the fact that IA-2A is closely related to T1D disease activity.

Due to the relationship between IA-2A and disease activity, it had been used as a marker of disease activity. Like GADA, assays had been developed to measure IA-2A, and this was investigated in IASP. IA-2A assays achieved a median sensitivity of 57% and specificity of 99% in the first IASP if measured in a RIA format, with relatively good diagnostic utility (ROC AUC = 0.77) (38). ELISA format of the test, like GADA, did not perform as well originally, however subsequent IASP workshops found that ELISA methods improved over time, with ROC-AUC increased from 0.81 to 0.85, similar to RIA assays, albeit with a slightly lower sensitivity (65% vs 70%) (39). Due to its high specificity, IA-2A is a useful test in evaluating patients with possible T1D.

2.2 – Zinc transporter 8 antibody (ZnT8)

2.2.1 – Background history and discovery

More recently, antibodies against the cation efflux channel Zinc transporter 8 (ZnT8) was identified as a potential target involved in the autoimmunity of T1D. ZnT8 antibodies (ZnT8A) were discovered after screen looking for possible autoantigens in the sera of newly diagnosed T1D patients and pre-diabetic populations. Due to the fact that ZnT8A were present in sera of patients with T1D who otherwise had negative GADA and IA2A, it was suggested that ZnT8A were an independent marker of T1D (43).

2.2.2 – ZnT8 receptor function

ZnT8 regulates the levels of zinc within the beta cell, which is essential to eventual insulin secretion. ZnT8 belongs to a family of cation efflux channels that is expressed throughout human tissue. Currently two families of zinc transporters had been identified, ZnT and Zrt-, Irt-like proteins (ZIP), with at least 10 members in the ZnT family (44,45). ZnT8 is solely expressed in the pancreas, and allows the efflux of zinc into secretory granules within the beta cell containing insulin (46).

Zinc performs special functions within the beta cell, and its interaction with insulin is vital for insulin storage and secretion. After insulin is cleaved from proinsulin, it is stored in granules as monomers and dimers within the beta cell. Zinc transporter 8 causes the efflux of zinc into the luminal space, which is essential to the eventual secretion of insulin. Zinc interacts with insulin to form Zn-insulin hexamers, leading to crystallisation of insulin (46). These hexamers

are less soluble and less prone to enzymatic breakdown, which allows the insulin to stay in a relatively stable state during storage. Storing insulin in such a way increases the storage efficiency of insulin within secretory granules. As insulin is secreted, zinc is co-secreted, which decreases hepatic insulin clearance, prolonging the activity of insulin.

2.2.3 – Measurement of ZnT8A

Similar to GADA and IA-2A, ZnT8A assays were developed and its efficacy was investigated. The first IASP workshop that investigated ZnT8A assay efficacy was held in 2007, with participating laboratories using a RIA method to measure ZnT8 antibodies. This achieved a median sensitivity of 55%, a specificity of 99% and a relatively good diagnostic utility. Assays were improved during the second IASP in 2009, achieving higher sensitivity (63%) with similar specificity to the previous tests. Recently, commercially available ELISA systems had been manufactured to measure ZnT8A, and this achieved similar sensitivity and specificity compared to RIA methods (47).

2.2.4 – Relation to disease activity and progression

As ZnT8 is solely expressed in the beta cell, ZnT8A is closely related to disease activity due to its tissue specificity. ZnT8A correlated weakly with levels of IA-2A but not insulin antibodies and GADA. The same study group looked at ZnT8A levels in samples of first-degree relatives of T1D patients and individuals from the general population with a high-risk HLA genotypes for T1D and found that ZnT8A levels preceded the development of T1D by many years (43).

Study of patients with new onset T1D with 2.5 year follow up 6 weeks after diagnosis compared to a cross-sectional study group of patients with longstanding diabetes revealed that ZnT8A were present at the time of diagnosis. Interestingly, similar to IA-2A, ZnT8A titres decreased progressively alongside C-peptide levels, suggesting a weaning of autoimmunity, possibility due to the decreasing number of beta cells within the pancreas (48,49). ZnT8A also demonstrated low persistence over time. Patients with longstanding T1D also had lower levels of ZnT8A compared to patients with recent onset disease. However, although the same study illustrated an statistically significant inverse relationship between antibody levels and age of onset, the correlation between two variables are weak ($r^2 = 0.02$) (48).

As islet autoantibodies are associated with disease activity in T1D, they are useful in aiding the diagnosis of T1D in cases were the diagnosis is uncertain. Patients with T1D often have multiple antibody positivity at diagnosis. It had previously been shown that around 70% of patients with T1D would have three to four positive islet autoantibodies at the time of diagnosis, with less than 10% of patients having only one positive islet antibody positive (35,50). The association of islet autoantibodies with T1D can help to classify patients into subtypes of diabetes especially close to diagnosis. Since a positive islet autoantibody test point towards a diagnosis of T1D, it can theoretically be used as a test to rule out other forms of diabetes, such as T2D or MODY.

3 – Biomarkers to aid the diagnosis MODY

As mentioned, GADA and IA2A can be used as a way to exclude patients from a diagnosis of MODY. However, ZnT8A had recently been discovered, and its prevalence in MODY patients and its clinical utility in discriminating T1D and MODY is unknown. It would be of interest to study ZnT8A in relation to GADA and IA-2A as they may be helpful in the diagnosis of MODY.

Besides from islet autoantibodies, other biomarkers are known to be useful in the diagnosis of MODY. These include the Type 1 diabetes genetic risk score (T1D-GRS), Connecting peptide (C-peptide), high density lipoprotein (HDL) and high sensitive C-reactive protein (HsCRP), which have been shown to aid the diagnosis of MODY (51–53). Most studies investigating these biomarkers were performed in retrospective case control studies, and have shown good diagnostic utility in discriminating T1D / T2D from MODY. However, no data is available on the diagnostic utility of these biomarkers in a prospective setting.

4 – Aims and Objectives

The aim of this thesis is to investigate whether ZnT8A is a useful biomarker in the diagnosis of MODY. This will be investigated alongside established antibodies GADA and IA-2A.

The main objectives are:

1. To assess the prevalence of ZnT8A in patients with genetically confirmed MODY compared to patients with T1D. The prevalence of ZnT8A will also be compared to the prevalence of GADA and IA-2A in both groups. This will be carried out in a retrospective case control study.

2. To assess the diagnostic utility of GADA, IA-2A and ZnT8A in discriminating non-MODY patients from MODY patients in a referral setting. The diagnostic utility of other biomarkers, namely C-peptide and T1D-GRS, would be investigated within this study. This will be investigated in a prospective cohort study.

References

1. Tattersall RB, Fajans SS, Arbor A. A Difference Between the Inheritance of Classical Juvenile-onset and Maturity-onset Type Diabetes of Young People. Diabetes. 1975 Jan 1;24(1):44–53.

2. Shields BM, Hicks S, Shepherd MH, Colclough K, Hattersley AT, Ellard S. Maturity-onset diabetes of the young (MODY): how many cases are we missing? Diabetologia. 2010 Dec 1;53(12):2504–8.

3. McDonald TJ, Ellard S. Maturity onset diabetes of the young: identification and diagnosis. Ann Clin Biochem. 2013 Sep 1;50(5):403–15.

4. Hattersley AT, Turner RC, Patel P, O'Rahilly S, Hattersley AT, Patel P, et al. Linkage of type 2 diabetes to the glucokinase gene. The Lancet. 1992 May 30;339(8805):1307–10.

5. Matschinsky F, Liang Y, Kesavan P, Wang L, Froguel P, Velho G, et al. Glucokinase as pancreatic beta cell glucose sensor and diabetes gene. J Clin Invest. 1993 Nov;92(5):2092–8.

6. Byrne MM, Sturis J, Clément K, Vionnet N, Pueyo ME, Stoffel M, et al. Insulin secretory abnormalities in subjects with hyperglycemia due to glucokinase mutations. J Clin Invest. 1994 Mar;93(3):1120–30.

7. Hattersley A, Bruining J, Shield J, Njolstad P, Donaghue KC. The Diagnosis and Management of Monogenic Diabetes in Children and Adolescents. Pediatr Diabetes. 2009 Sep;10:33–42.

8. Steele AM, Shields BM, Wensley KJ, Colclough K, Ellard S, Hattersley AT. Prevalence of Vascular Complications Among Patients With Glucokinase Mutations and Prolonged, Mild Hyperglycemia. JAMA. 2014 Jan 15;311(3):279–86.

9. Shih DQ, Screenan S, Munoz KN, Philipson L, Pontoglio M, Yaniv M, et al. Loss of HNF-1alpha function in mice leads to abnormal expression of

genes involved in pancreatic islet development and metabolism. Diabetes. 2001 Nov;50(11):2472–80.

10. Wang H, Maechler P, Hagenfeldt KA, Wollheim CB. Dominant-negative suppression of HNF-1alpha function results in defective insulin gene transcription and impaired metabolism-secretion coupling in a pancreatic beta-cell line. EMBO J. 1998 Nov 16;17(22):6701–13.

11. Yamagata K, Oda N, Kaisaki PJ, Menzel S, Furuta H, Vaxillaire M, et al. Mutations in the hepatocyte nuclear factor-1α gene in maturity-onset diabetes of the young (MODY3). Nature. 1996 Dec;384(6608):455.

12. Isomaa B, Henricsson M, Lehto M, Forsblom C, Karanko S, Sarelin L, et al. Chronic diabetic complications in patients with MODY3 diabetes. Diabetologia. 1998 Apr;41(4):467–73.

13. Juszczak A, Pryse R, Schuman A, Owen KR. When to consider a diagnosis of MODY at the presentation of diabetes: aetiology matters for correct management. Br J Gen Pract. 2016 Jun;66(647):e457–9.

14. Pearson ER, Liddell WG, Shepherd M, Corrall RJ, Hattersley AT. Sensitivity to sulphonylureas in patients with hepatocyte nuclear factor-1alpha gene mutations: evidence for pharmacogenetics in diabetes. Diabet Med J Br Diabet Assoc. 2000 Jul;17(7):543–5.

15. Pearson ER, Starkey BJ, Powell RJ, Gribble FM, Clark PM, Hattersley AT. Genetic cause of hyperglycaemia and response to treatment in diabetes. Lancet Lond Engl. 2003 Oct 18;362(9392):1275–81.

16. Shepherd M, Shields B, Ellard S, Rubio-Cabezas O, Hattersley AT. A genetic diagnosis of HNF1A diabetes alters treatment and improves glycaemic control in the majority of insulin-treated patients. Diabet Med. 26(4):437–41.

17. Pearson ER, Boj SF, Steele AM, Barrett T, Stals K, Shield JP, et al. Macrosomia and Hyperinsulinaemic Hypoglycaemia in Patients with Heterozygous Mutations in the HNF4A Gene. PLoS Med [Internet]. 2007

Apr [cited 2018 Jul 25];4(4). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1845156/

18. Yorifuji T, Kurokawa K, Mamada M, Imai T, Kawai M, Nishi Y, et al. Neonatal diabetes mellitus and neonatal polycystic, dysplastic kidneys: Phenotypically discordant recurrence of a mutation in the hepatocyte nuclear factor-1beta gene due to germline mosaicism. J Clin Endocrinol Metab. 2004 Jun;89(6):2905–8.

19. Bingham C, Hattersley AT. Renal cysts and diabetes syndrome resulting from mutations in hepatocyte nuclear factor-1beta. Nephrol Dial Transplant Off Publ Eur Dial Transpl Assoc - Eur Ren Assoc. 2004 Nov;19(11):2703–8.

20. Edghill EL, Bingham C, Slingerland AS, Minton J a. L, Noordam C, Ellard S, et al. Hepatocyte nuclear factor-1 beta mutations cause neonatal diabetes and intrauterine growth retardation: support for a critical role of HNF-1β in human pancreatic development. Diabet Med. 2006 Dec;23(12):1301–6.

21. Pearson ER, Badman MK, Lockwood CR, Clark PM, Ellard S, Bingham C, et al. Contrasting Diabetes Phenotypes Associated With Hepatocyte Nuclear Factor-1α and -1β Mutations. Diabetes Care. 2004 May 1;27(5):1102–7.

22. Stoffers DA, Zinkin NT, Stanojevic V, Clarke WL, Habener JF. Pancreatic agenesis attributable to a single nucleotide deletion in the human IPF1 gene coding sequence. Nat Genet. 1997 Jan;15(1):106–10.

23. Edghill EL, Flanagan SE, Patch A-M, Boustred C, Parrish A, Shields B, et al. Insulin Mutation Screening in 1,044 Patients With Diabetes: Mutations in the INS Gene Are a Common Cause of Neonatal Diabetes but a Rare Cause of Diabetes Diagnosed in Childhood or Adulthood. Diabetes. 2008 Apr 1;57(4):1034–42.

24. Shepherd M, Pearson ER, Houghton J, Salt G, Ellard S, Hattersley AT. No Deterioration in Glycemic Control in HNF-1α Maturity-Onset Diabetes of

the Young Following Transfer From Long-Term Insulin to Sulphonylureas. Diabetes Care. 2003 Nov 1;26(11):3191–2.

25. Shepherd M, Hattersley AT. ‘I don’t feel like a diabetic any more’: the impact of stopping insulin in patients with maturity onset diabetes of the young following genetic testing. Clin Med. 2004 Mar 1;4(2):144–7.

26. Ellard S, Lango Allen H, De Franco E, Flanagan SE, Hysenaj G, Colclough K, et al. Improved genetic testing for monogenic diabetes using targeted next-generation sequencing. Diabetologia. 2013 Sep;56(9):1958–63.

27. McDonald TJ, Colclough K, Brown R, Shields B, Shepherd M, Bingley P, et al. Islet autoantibodies can discriminate maturity-onset diabetes of the young (MODY) from Type 1 diabetes. Diabet Med. 2011 Sep 1;28(9):1028–33.

28. Willcox A, Richardson SJ, Bone AJ, Foulis AK, Morgan NG. Analysis of islet inflammation in human type 1 diabetes. Clin Exp Immunol. 2009;155(2):173–81.

29. Morgan NG, Leete P, Foulis AK, Richardson SJ. Islet inflammation in human type 1 diabetes mellitus. IUBMB Life. 2014;66(11):723–34.

30. Bottazzo G, Florin-Christensen A, Doniach D. Islet-cell antibodies in diabetes mellitus with autoimmune polyendocrine deficiencies. The Lancet. 1974 Nov 30;304(7892):1279–83.

31. Velluzzi F, Secci G, Sepe V, Klersy C, Shattock M, Foxon R, et al. Prediction of type 1 diabetes in Sardinian schoolchildren using islet cell autoantibodies: 10-year follow-up of the Sardinian schoolchildren type 1 diabetes prediction study. Acta Diabetol. 2016 Feb 1;53(1):73–9.

32. Solimena M, Folli F, Aparisi R, Pozza G, De Camilli P. Autoantibodies to GABA-ergic Neurons and Pancreatic Beta Cells in Stiff-Man Syndrome. N Engl J Med. 1990 May 31;322(22):1555–60.

33. Baekkeskov S, Aanstoot H-J, Christgai S, Reetz A, Solimena M, Cascalho M, et al. Identification of the 64K autoantigen in insulin-dependent diabetes

as the GABA-synthesizing enzyme glutamic acid decarboxylase. Nature. 1990 Sep;347(6289):151.

34. Braun M, Ramracheya R, Bengtsson M, Clark A, Walker JN, Johnson PR, et al. γ-Aminobutyric Acid (GABA) Is an Autocrine Excitatory Transmitter in Human Pancreatic β-Cells. Diabetes. 2010 Jul;59(7):1694–701.

35. Bingley PJ, Bonifacio E, Williams AJK, Genovese S, Bottazzo GF, Gale EAM. Prediction of IDDM in the General Population: Strategies Based on Combinations of Autoantibody Markers. Diabetes. 1997 Nov 1;46(11):1701–10.

36. Knip M, Siljander H, Ilonen J, Simell O, Veijola R. Role of humoral beta-cell autoimmunity in type 1 diabetes. Pediatr Diabetes. 2016 Jul 13;17(S22):17–24.

37. Bingley PJ, Boulware DC, Krischer JP. The implications of autoantibodies to a single islet antigen in relatives with normal glucose tolerance: development of other autoantibodies and progression to type 1 diabetes. Diabetologia. 2016;59:542–9.

38. Bingley PJ, Bonifacio E, Mueller PW. Diabetes Antibody Standardization Program: First Assay Proficiency Evaluation. Diabetes. 2003 May 1;52(5):1128–36.

39. Törn C, Mueller PW, Schlosser M, Bonifacio E, Bingley PJ, Participating Laboratories. Diabetes Antibody Standardization Program: evaluation of assays for autoantibodies to glutamic acid decarboxylase and islet antigen-2. Diabetologia. 2008 May;51(5):846–52.

40. Rabin DU, Pleasic SM, Shapiro JA, Yoo-Warren H, Oles J, Hicks JM, et al. Islet cell antigen 512 is a diabetes-specific islet autoantigen related to protein tyrosine phosphatases. J Immunol. 1994 Mar 15;152(6):3183–8.

41. Cai T, Hirai H, Zhang G, Zhang M, Takahashi N, Kasai H, et al. Deletion of IA-2 and/or IA-2β Decreases Insulin Secretion by Reducing the Number of Dense Core Vesicles. Diabetologia. 2011 Sep;54(9):2347–57.

42. Decochez K, Leeuw ID, Keymeulen B, Mathieu C, Rottiers R, Weets I, et al. IA-2 autoantibodies predict impending Type I diabetes in siblings of patients. Diabetologia. 2002 Dec 1;45(12):1658–66.

43. Wenzlau JM, Juhl K, Yu L, Moua O, Sarkar SA, Gottlieb P, et al. The cation efflux transporter ZnT8 (Slc30A8) is a major autoantigen in human type 1 diabetes. Proc Natl Acad Sci. 2007 Oct 23;104(43):17040–5.

44. Eide DJ. Zinc transporters and the cellular trafficking of zinc. Biochim Biophys Acta BBA - Mol Cell Res. 2006 Jul 1;1763(7):711–22.

45. Cousins RJ, Liuzzi JP, Lichten LA. Mammalian Zinc Transport, Trafficking, and Signals. J Biol Chem. 2006 Aug 25;281(34):24085–9.

46. Li YV. Zinc and insulin in pancreatic beta-cells. Endocrine. 2014 Mar 1;45(2):178–89.

47. Lampasona V, Schlosser M, Mueller PW, Williams AJK, Wenzlau JM, Hutton JC, et al. Diabetes Antibody Standardization Program: First Proficiency Evaluation of Assays for Autoantibodies to Zinc Transporter 8. Clin Chem. 2011 Dec 1;57(12):1693–702.

48. Wenzlau JM, Walter M, Gardner TJ, Frisch LM, Yu L, Eisenbarth GS, et al. Kinetics of the Post-Onset Decline in Zinc Transporter 8 Autoantibodies in Type 1 Diabetic Human Subjects. J Clin Endocrinol Metab. 2010 Oct 1;95(10):4712–9.

49. Vaziri-Sani F, Oak S, Radtke J, Lernmark Å, Lynch K, Agardh C-D, et al. ZnT8 autoantibody titers in type 1 diabetes patients decline rapidly after clinical onset. Autoimmunity. 2010 Dec 1;43(8):598–606.

50. Sabbah E, Savola K, Ebeling T, Kulmala P, Vähäsalo P, Ilonen J, et al. Genetic, autoimmune, and clinical characteristics of childhood- and adult-onset type 1 diabetes. Diabetes Care. 2000 Sep;23(9):1326–32.

51. Patel KA, Oram RA, Flanagan SE, De Franco E, Colclough K, shepherd M, et al. Type 1 Diabetes Genetic Risk Score: a novel tool to discriminate monogenic and type 1 diabetes. Diabetes. 2016 Jul;65(7):2094–9.

52. McDonald TJ, Shields BM, Lawry J, Owen KR, Gloyn AL, Ellard S, et al. High-Sensitivity CRP Discriminates HNF1A-MODY From Other Subtypes of Diabetes. Diabetes Care. 2011 Aug 1;34(8):1860–2.

53. McDonald TJ, McEneny J, Pearson ER, Thanabalasingham G, Szopa M, Shields BM, et al. Lipoprotein composition in HNF1A-MODY: Differentiating between HNF1A-MODY and Type 2 diabetes. Clin Chim Acta. 2012 May 18;413(9):927–32.

Methods

1 – Islet Autoantibodies

1.1 – GADA and IA-2A measurements:

Samples were measured at the Academic Department of Blood Sciences at the Royal Devon and Exeter Hospital. GADA and IA-2A were measured using ELISA assays on a Dynex DS2 automated ELISA system. The positivity of titres cut offs were determined after testing control patients (n = 1559) Control subjects were between the age of 18 and 75, did not have a diagnosis of diabetes, and had an Hba1c of 6.0%. Islet autoantibodies were considered positive if titre levels were above the 99th centile of the control samples. 99th centile cutoff for GADA is 64 WHO units/mL and IA-2A is 7.5 WHO units/mL.

1.2 – ZnT8A measurements:

Samples were measured similar to GADA and IA-2A using the ELISA method above. Titre cutoffs were defined differently as there were differences in ZnT8 antibody titres between control subjects at different sets of age groups. An age specific cut off was applied to ZnT8A titre measurements. ZnT8A were considered positive if levels were above the 99th centile at age specific cutoffs. 99th centile cutoff for ZnT8A is 126 WHO units/mL (age < 30 years) / 20 WHO units/mL (age ≥ 30 years).

2 – Type 1 diabetes genetic risk score

The Type 1 diabetes genetic risk score (T1D-GRS) was measured in the study within Chapter 4. The score was computed using the number of risk alleles across 30 common T1D single nucleotide polymorphisms (SNPs), with variants from both HLA and non-HLA loci. They were selected based on variants that were strongly associated with T1D as described in existing studies. Each variant was weighted based on their effect on T1D genetic risk from previous literature. A GRS was generated as the sum across SNPs of the number of risk increasing alleles (0, 1 or 2) at that SNP multiplied by the ln(odds ratio) for each allele divided by the number of alleles (cite). The HLA-DR3 and HLA-DR4 haplotypes were weighted using imputed haplotypes. The sum of the score signifies a person's risk for T1D. Genotyping of SNPs was performed using the KASP assay by LGC Genomics (Hoddesdon, UK).

3 – C-peptide measurement

Serum C-peptide was measured in the study within Chapter 4. C-peptide analysis was performed using the Roche Modular Analytics Cobas 601 immunoassay analyser (Roche Diagnostics, Mannheim, Germany). An antigen-antibody-antigen sandwich complex was formed by reacting one biotinylated anti-C-peptide specific monoclonal mouse antibody and a second monoclonal antibody to Cpeptide labelled with a ruthenium complex with 20uL serum sample of C-peptide. Separation is achieved via interaction of biotin and streptavidin attachment to paramagnetic microparticles (solid phase). The detection system employs electrochemiluminescence with ruthenium trisbipyridyl as the label. Electrochemiluminescence occurs at 620 nm and readings are taken by the photomultiplier tube (PMT). The intensity of light signal is proportional to the concentration of C-peptide in the serum. The assay was calibrated using Roche C-peptide CalSet calibration material (Roche Diagnostics, Mannheim, Germany), traceable to WHO International Reference Reagent (IRR) for C-peptide of human insulin for immunoassay (IRR code 84/510). Quality Control was performed on each day of analysis using low level (0.67 nmol/L) and high level (3.33 nmol/L) PreciControl MultiAnalyte.

4 – MODY genetic sequencing

Genomic DNA was extracted from whole blood using standard procedures and the promoter, all coding regions and intron/exon boundaries of the *GCK, HNF1A* and *HNF4A* genes were amplified by PCR. Amplicons were sequenced using the Big Dye Terminator Cycler Sequencing Kit v3.1 (Applied Biosystems, Warrington, UK) according to manufacturer's instructions and reactions were analysed on an ABI 3730 Capillary sequencer (Applied Biosystems, Warrington, UK). Sequences were compared with the reference sequences (NM_000162.4 for *GCK*, NM_000545.6 for *HNF1A* and NM_175914.4 for *HNF4A*) using Mutation Surveyor v5.0.1 software (SoftGenetics, State College, PA).

Chapter 1

The addition of zinc transporter 8 autoantibodies to established islet autoantibodies improved discrimination of MODY from type 1 diabetes close to diagnosis

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Acknowledgement of co-authors and contributions to paper

Tim McDonald and the Academic department of Blood Sciences at the Royal Devon and Exeter Hospital were involved with data collection and laboratory analysis of biochemistry tests for the study. Kevin Colclough was involved in genetic analysis of patient samples. Beverley Shields advised on data analysis with Andrew Hattersley.

I undertook data analysis with the assistance of Beverley Shields. I wrote the manuscript with advice from Kashyap Patel. Revisions suggested by co-authors were incorporated into the manuscript.

Abstract

Introduction: Antibodies against 65 kilodalton isoform of glutamic acid decarboxylase (GAD) and protein tyrosine phosphatase-related islet antigen-2 (IA-2) have previously been shown to be useful tests in differentiating Type 1 Diabetes (T1D) from MODY. Zinc transporter 8 antibody (ZnT8A) is a relatively new islet autoantibody, and its clinical utility in differentiating T1D from MODY has not been described. The aim of this study was to determine the prevalence of ZnT8A in a cohort of patients with genetically confirmed MODY and to investigate whether ZnT8A can be used to differentiate MODY from T1D and the additive value above established islet antibodies GADA and IA-2A.

Method: GADA, IA2A and ZNT8A were measured in 997 individuals, with 294 (29.6%) patients having genetically confirmed MODY and 703 (70.4%) patients having T1D. Antibody titre cutoffs were established at the 99th centile of 1559 control subjects. We compared the positivity rates of antibodies between MODY and T1D patients.

Results: ZnT8A was positive in 148/703 patients (21.1%) with T1D and 2/294 (0.7%) patient with MODY ($p<0.001$). ZNT8A increased the number of individuals with two or more positive antibodies by 26.3% (calculated as Net Reclassification Improvement Index, n=36 to n=66, p=<0.0001). GADA and IA2A were detected in 227/703 (32.3%) and 271/703 (38.5%) T1D patients respectively. In patients with MODY with positive antibodies, 1/137 (0.7%) patients had measureable ZnT8A and 1/137 (0.7%) had measurable GADA. Another patient had a combination of ZnT8A and GADA. No MODY patients had detectable IA-2A antibodies or more than two positive antibodies. The prevalence of islet autoantibodies decreases as increasing disease duration.

Conclusion: In conclusion, we have shown that ZnT8A prevalence is low in MODY patients, similar to GADA and IA-2A. ZnT8A should be used in conjunction with GADA and IA-2A as a routine test before molecular genetic testing. This should be performed closer to diagnosis preferably as this enhances its ability to discriminate T1D from MODY.

1 – Introduction

Maturity-onset diabetes of the young (MODY) is a rare form of diabetes caused by highly penetrant autosomal dominant mutations in a single gene. MODY accounts for approximately 3% of diabetes diagnosed less than 30 years of age (1). The most common form of MODY results from mutations within the glucokinase (*GCK*) gene and the genes encoding transcription factors hepatocyte nuclear factor 1a (*HNF1a*) and 4a (*HNF4a*), together accounting for over 80% of MODY cases (2). Identifying patients with MODY is important as it defines the appropriate treatment; patients with GCK-MODY do not require any treatment, whereas patients with HNF1A/HNF4A MODY are sensitive to sulphonylurea tablets (3, 4).

Despite the clinical implication of making a correct diagnosis of MODY, there is a significant delay for a correct genetic diagnosis and 40% are often misdiagnosed and mistreated as Type 1 Diabetes (T1D) (5-7). This misdiagnosis is due to overlapping clinical features of MODY and T1D and highlights the need for tests that can aid in identifying patients with a higher probability of having MODY.

Islet autoantibodies (GADA and IA-2A) have been shown to have utility to aid clinicians differentiate MODY from T1D (1, 8). The prevalence of detectable islet antibodies against the 65 kDa isoform glutamate acid decarboxylase (GAD65) and tyrosine phosphatase islet antigen 2 (IA-2A) in MODY patients is the same as non-diabetic populations (~1%), compared to 80-90% in T1D at diagnosis (8). Therefore, the presence of autoantibodies can robustly rule out MODY and reduce the need for genetic testing. GADA and IA-2A assays are now widely available to clinicians and these autoantibodies can be measured easily, reproducibly and with a higher sensitivity and specificity than traditional pancreatic islet cell antibody (ICA) (9, 10).

Autoantibodies against Zinc transporter 8 (ZnT8A) is the most recent T1D related autoantibody to be described. It can be used to identify patients with T1D with sensitivity and specificity to GADA and IA-2A (11, 12). However, there are no studies reporting ZnT8A in MODY and the utility of ZnT8A in addition to GADA and IA-2A to differentiate MODY from T1D remains unknown.

The aim of this study was to determine the prevalence of ZnT8A in a cohort of patients with genetically confirmed MODY and to investigate whether ZnT8A can be used to differentiate MODY from T1D against established islet antibodies GADA and IA-2A. In addition, we aim to explore the impact of disease duration on the diagnostic utility of islet autoantibodies at differentiating MODY from T1D

2 – Methods

2.1 – Study design and participants:

We conducted retrospective cross-sectional case control study of 997 individuals, 294 patients with genetically confirmed MODY: (102 *GCK*, 121 *HNF1A*, 57 *HNF4A*, 14 *HNF1b*) and 703 patients diagnosed with T1D.

The Type 1 diabetes cohort (clinical diagnosis of type 1 diabetes and on insulin from diagnosis) were all research participants from three research studies and the clinical characteristics were collected during recruitment. 229 were from the TIGI study (IRAS 141756), 309 from DARE (REC ref 2002/7/118), 117 samples were from UNITED (IRAS 51251). In addition, 48 samples for patients with T1D were taken from the Islet Autoantibody Standardization Programme (IASP).

We had plasma from 294 patients with genetically confirmed MODY, where whole-blood EDTA samples were sent for the routine genetic testing to Exeter Molecular Genetics Department, The Royal Devon and Exeter Hospital, UK from 2012 to 2015. Clinical characteristics for the MODY patients were collected from clinical referral form.

2.2 – Islet autoantibodies measurement

Analysis of islet autoantibodies (GADA, IA2A, ZNT8A) was performed by the Academic department of Blood Sciences at the Royal Devon and Exeter Hospital. We measured GADA, IA2A and ZnT8A in serum for T1D and MODY cohort. Serum was prepared by adding calcium chloride solution and thrombin to EDTA plasma to initiate clotting as described previously (8).

GADA, IA-2A and ZnT8A were measured using ELISA assays (RSR Limited, Cardiff, U.K.) on a Dynex DS2 automated ELISA system (Launch Diagnostics, Longfield, U.K.). Antibody titre cut-offs were established after testing 1559 control subjects without a diagnosis of diabetes between the age of 18 and 75, along with an Hba1c of less than 6.0% (42 mmol/mol). Antibody results were considered positive if titre exceeds the 99th percentile limit of controls. The 99th centile for GADA is ≥ 64 World Health Organization units/mL, IA-2A ≥ 7.5 World Health Organization units/mL, ZnT8A ≥126 World Health Organization units/mL (age < 30 years) / ≥20 World Health Organization units/mL (age ≥ 30 years)). The laboratory participates in the Islet Autoantibody Standardisation Programme.

2.3 – MODY genetic sequencing

Genomic DNA was extracted from whole blood using standard procedures and the promoter, all coding regions and intron/exon boundaries of the *GCK, HNF1A* and *HNF4A* genes were amplified by polymerised chain reaction (PCR). Big Dye Terminator Cycler Sequencing Kit v3.1 (Applied Biosystems, Warrington, UK) was used to sequence amplicons. Reactions were analysed on an ABI 3730 Capillary sequencer (Applied Biosystems, Warrington, UK). Sequences were compared with the reference sequences (NM_000162.4 for *GCK*, NM_000545.6 for *HNF1A* and NM_175914.4 for *HNF4A*) using Mutation Surveyor v5.0.1 software (SoftGenetics, State College, PA).

2.4 – Statistical analysis

Antibody positivity rates were compared between MODY patients and T1D patients. Mann-Whitney U-test was used to determine statistical significance of clinical characteristics between T1D and MODY patients. Fisher's exact test was used to determine the effect of single antibodies in differentiating T1D from MODY. Positive likelihood [sensitivity/(1 – specificity)] and negative likelihood values [(1 – sensitivity) / specificity] were calculated for single and multiple islet autoantibody positivity. Net reclassification improvement (NRI) index was used to compare the efficacy between established antibodies (GAD and IA2) and triple antibodies (GAD, IA2 and ZnT8) (13).

The effect of duration on antibody positivity was explored based on 25th, 50th and 75th centile of diabetes duration of the whole cohort. Data was cleaned using *Stata Statistical Software: Release 14*. (College Station, TX: StataCorp) and *Rstudio* (RStudio, Boston, MA). Graphs were created using ggplot2 package within RStudio (14).

3 - Results

3.1 – Patient Characteristics

Overall, patients with T1D had a higher Hba1c (median 68, interquartile range (IQR) 58 – 81 mmol/mol vs 50, 45 – 60 mmol/mol, p = < 0.0001) and longer disease duration (median 13 , IQR 5 – 25 years vs 3, 0 – 16 years, p = < 0.0001) compared to MODY patients within this cohort. Age of diagnosis was similar between both groups (median 21, IQR 13 – 34 years vs 21, 16 – 31 years, p = 0.83).

3.2 – ZnT8 antibody prevalence in T1D and MODY close of diagnosis (<2 years duration)

The prevalence of ZNT8A was 50% (57/114) in T1D and 0.7% (1/137) in MODY patients. The prevalence of ZNT8A in patients with T1D was higher than GADA (50% vs 48.2%) (p = 0.7) and modestly higher than IA-2A (50% vs 66.7%) (p = < 0.001).

3.3 – Additional benefit of ZnT8 islet autoantibody in T1D and MODY patients close of diagnosis (< 2 year duration)

Overall 99/114 (86.8%) patients with T1D and 2/137 (1.5%) patients with MODY had positive islet autoantibodies. Testing of ZnT8A identified an additional 23/721 (3.2%) of T1D patients who were previously negative for GADA and IA-2A. In MODY patients, only 1/137 (0.7%) patient had positive ZNT8A and 1/137 (0.7%) GADA. No MODY patients had two positive antibodies (See figure 1). The patient characteristics of antibody positive MODY cases for the whole cohort are summarised in table 1.

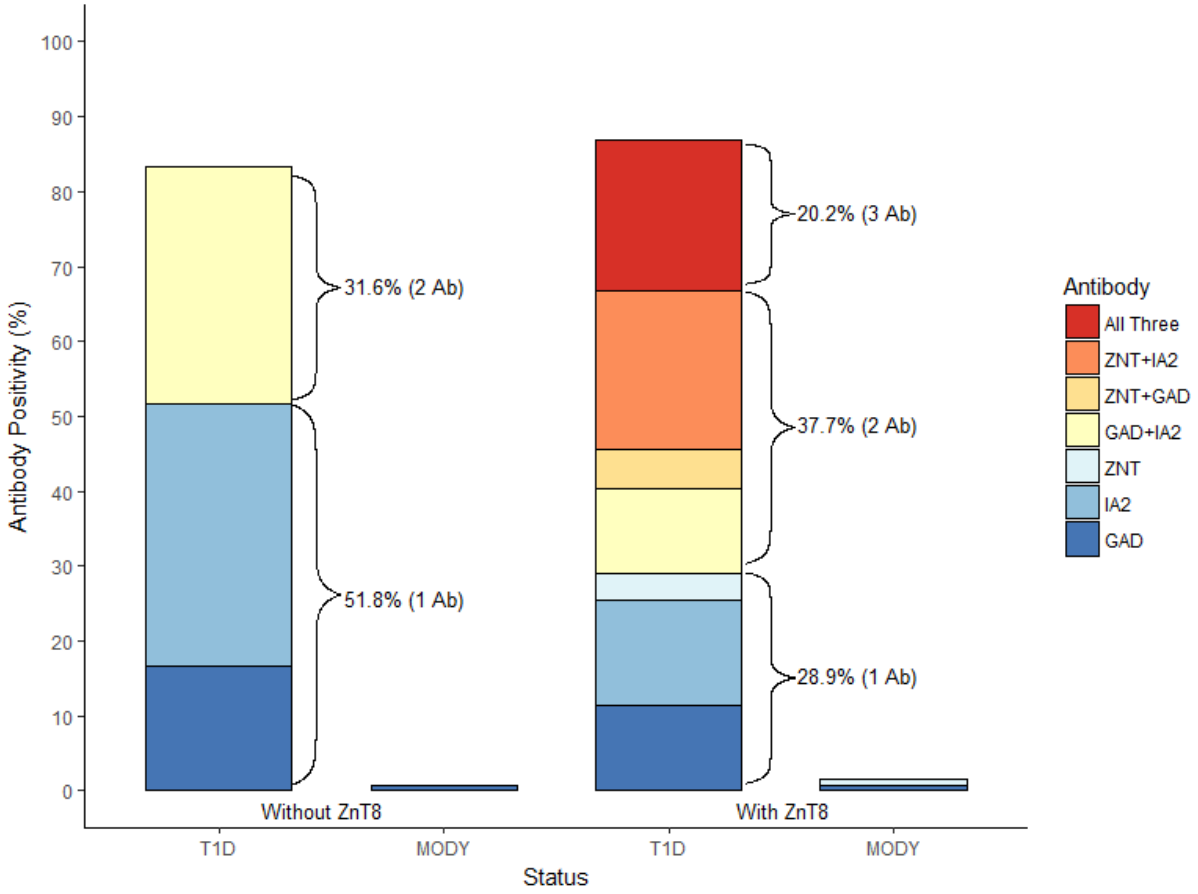


Figure 1. Bar chart showing the percentage of antibody positivity between T1D (n=114) and MODY (n=137) patients with disease duration less than two years

Genetic Mutation	**Positive Antibody**	**Phenotype**	**Initial Treatment**	**Current Treatment**	**Age of Diagnosis**	**Time to insulin**	**Hba1c (mmol/mol)**	**C-peptide level (pmol/L)**	**Clinical Features**
HNF4a p.R114W	GADA	Diabetic	Diet	Insulin (basal bolus)	64	N/A	69	119	Ischaemic heart disease, peripheral vascular disease, retinopathy, and neuropathy
HNF1b p.R276*	GADA+ZnT8A	Diabetic	Insulin (basal bolus)	Insulin (basal bolus)	25	at diagnosis	63	45	Treated as type 1, on insulin throughout diabetes, stage 4 kidney disease
HNF1b p.R295H	ZnT8A	Diabetes & Renal Cysts	Insulin (basal bolus)	Insulin (basal bolus)	4	at diagnosis	66	Not available	Treated as type 1, on insulin throughout diabetes, renal cysts found antenatally

Table 1. Clinical characteristics of MODY antibody-positive patients within the whole cohort

3.4 – Differentiating MODY from T1D in short disease duration

Negative ZnT8A had sensitivity of 99.3 % and specificity of 50.0% at discriminating MODY from T1D. This was similar to a negative GADA autoantibody test (sensitivity of 99.3% and specificity of 48.2%). The sensitivity and specificity of a negative IA-2A test was higher compared to ZnT8A (sensitivity of 100% and specificity of 66.7%). The combined sensitivity and specificity of GAD, IA-2A and ZnT8A is 98.5% and 86.8% respectively. The addition of ZnT8A antibodies increased the number of patients with two or more antibodies by 26.3% (calculated as NRI index, $p < 0.0001$)

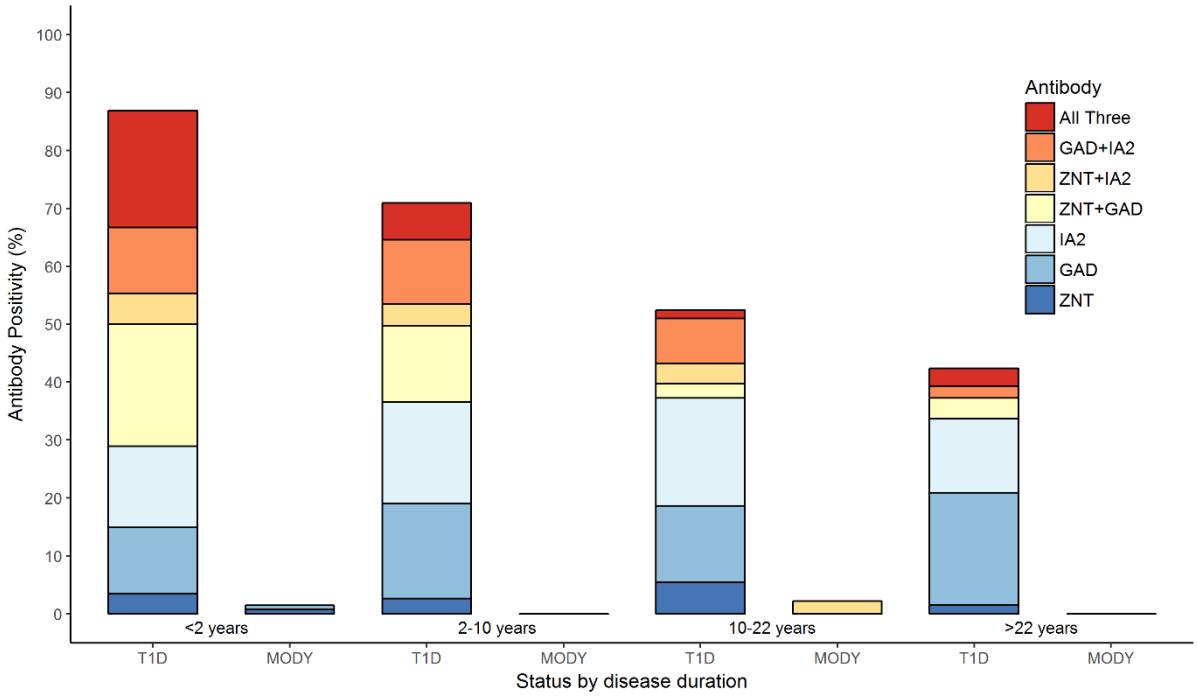
This equates to a positive likelihood ratio for identifying MODY from T1D in short duration disease of 7.5 for three negative antibodies and a negative likelihood ratio of 0.02 and < 0.0001 for one and two or more detectable antibodies respectively. Based on a MODY prevalence of 3% (1:33) in patients with diabetes diagnosed under the age of 30, this would decrease the probability to 1:1923 for one positive antibody, and 1:25645 for two or more antibodies.

3.5 – The prevalence of islet autoantibody is dependent on duration of diabetes

In order to assess the effect of diabetes duration on antibody positivity, we divided the whole cohort into quartiles of disease duration (<2 years, 2 – 10 years, 10 – 22 years, and >22 years). We found 86.8% (99/114) of T1D patients with disease duration less than two years were positive for antibodies. This falls to 70.9 % (134/189), 52.5 % (107/204) and 42.3% (83/196) at disease duration between 2 to 10 years, 10 to 22 years, and >22 years respectively (see figure 2). Antibody prevalence remains low in MODY patients, with positivity rates of 0.7 to 1.5% across all durations.

The prevalence of GADA, IA-2A and ZnT8A fell as disease duration lengthened in patients with T1D. The prevalence of GADA was higher in patients with shorter disease duration (<2 years) compared to patients with longer disease duration (>22 years) (48.2% to 24.5%, difference 23.7%, p= <0.0001, 95% CI 12.6 – 34.3%). A fall in prevalence is also seen in IA-2A (66.7% to 21.4%,

difference 45.3%, p= <0.0001, 95% CI 34.3 – 56.3%) and ZnT8A (50% to 8.2%, difference 41.8%, p= <0.0001, 95% CI 31.2 – 52.5%). The prevalence of ZnT8A after 22 years was lower compared to GADA (8.2% vs 24.5%, p= <0.0001) and IA-2A (8.2% to 21.4%, p= <0.0001) (see figure 3). Subsequently, the specificity of islet autoantibody tests in differentiating MODY and T1D decreases in patients with longer disease duration, with a specificity of 86.8% and 42.3% in patients with < 2years and >22 years disease duration respectively. Sensitivity remains similar in all groups, ranging from 97.8% to 100% and the NPV also remains high across all durations.



	0-2 yrs	2-10 yrs	10-22 yrs	>22 yrs
T1D (n)	114	189	204	196
MODY (n)	137	61	46	50
Sensitivity(%)	98.5	100	97.8	100
Specificity(%)	86.8	70.9	52.5	42.3
PPV(%)	7	3.3	2	1.7
NPV(%)	99.9	99.9	99.9	99.9

Figure 2. Bar chart and table showing the percentage of antibody positivity between T1D and MODY patients and diagnostic utility by disease duration

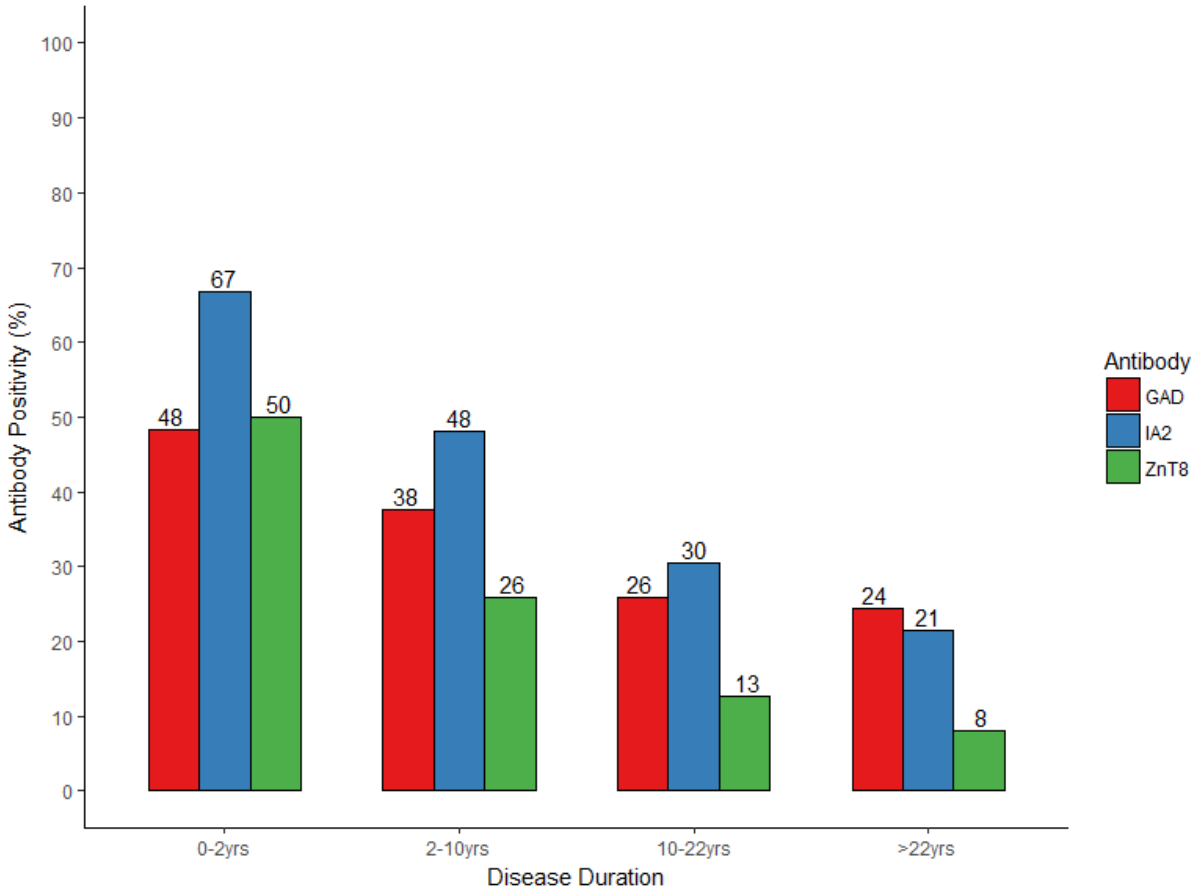


Figure 3. Bar chart showing individual antibody positivity rates in patients with T1D across disease duration (in quartiles)

4 – Discussion

ZnT8A were able to differentiate T1D patients from MODY independently, with sensitivity and specificity comparable to GADA and IA-2A. We have shown that prevalence of ZnT8A is around 1% in patients with MODY, equivalent to the prevalence within control populations. Although ZnT8A modestly increased the number of patients who were previously antibody negative, they were able to increase the number of patients who were positive for a single antibody to multiple antibody positivity by 26.3%.

Maximum diagnostic utility of islet autoantibodies in differentiating MODY from T1D was seen close to diagnosis, with the prevalence of antibodies decreasing with time. This was especially true for ZnT8A and IA-2A. ZnT8A was evidently lowest in T1D patients with the longest disease duration within this cohort, compared to GADA and IA-2A, possibly due to the decreasing number of antigens present within the beta cell over time. It is important to note that the prevalence of islet autoantibodies in MODY patients remained low across different durations within this cohort.

The three MODY patients with positive GADA and / or ZnT8A may have developed concomitant T1D, which was supported by their clinical characteristics. Firstly, the patients were insulin-dependent, and two of the patients required insulin at diagnosis. Secondly, the serum C-peptide levels for two of the patients were below 200 pmol/L, suggesting a decreased insulin production as seen in patients with T1D. The data here suggests the high likelihood that these patients may have developed T1D. Alternatively, the MODY patients may represent the 1% of the population with positive islet autoantibodies without associated pathology.

This is to our knowledge the first formal study which investigated the prevalence and diagnostic utility of ZnT8A in MODY patients in relation to GADA and IA-2A. This study shows that the prevalence of islet autoantibodies, including ZnT8A, is low in MODY patients. Previously we have measure the serum of 500 patients with genetically confirmed MODY (8). Along with this cohort of patients presented in this study, we have now measured the serum of over 800

individuals with MODY, and had consistently found low prevalence of islet autoantibodies. This reiterates the fact that islet autoantibodies do not form part of the disease process in classical forms of MODY. Other studies in the past, mainly in the form of case reports, have suggested that autoimmunity is rare in MODY (15-18). A study based on a German-Austrian registry cohort with MODY patients reported an islet autoantibody prevalence rate of 17% in MODY patients. However, not all patients within the registry received MODY genetic testing, as 20% of the patients within the registry were not tested. This meant that some patients could have been misclassified as having MODY. It is possible that autoantibody-positive patients within this registry have T1D.

The strength of this study include the large cohort of MODY patients, allowing the examination of duration effects on the positivity rates of islet autoantibodies at different time points. We have also measured ZnT8A along with GAD and IA2 and compared their prevalence between T1D and MODY patients, and the serum was analysed in a single laboratory. Our antibody reference ranges were derived from a standard control population, making our reference cut-offs robust. Finally, we have explored the effect of disease duration on the prevalence of islet autoantibodies between T1D and MODY patients within this study which had been impossible previously because patients were studied close at diagnosis.

There are several limitations to our study. As this study was set up as a case control, it was a retrospective analysis of the data available. The study on the effects of disease duration was based on a cross-sectional analysis across the study cohort, meaning we were not able to study the effect of disease duration in the same patients over time as longitudinal data was unavailable. We also could not exclude the possibility that patients within this cohort were pre-screened for antibodies before they were referred to our genetic laboratory, which could explain the low prevalence in our study group. However, if the analysis within this study was restricted to only probands (n = 183) who previously were not screened for autoantibodies, the antibody prevalence rate was 0.01%, lower than the expected prevalence in control populations

The results of this study have several clinical implications. Since islet autoantibodies is a sign of autoimmunity in T1D, we would not be expected to

observe positive autoantibodies in patients with MODY. Therefore, islet autoantibodies could be considered as a “ruling out” test for patients with suspected MODY. The results here also show that the increasing number of positive islet autoantibodies decreases the probability of a MODY diagnosis, signifying that more islet autoantibodies should be measured when investigating patients with suspected MODY. This means that ZnT8A should be measured alongside GADA and IA-2A to increase the diagnostic utility of islet autoantibodies as a whole. In addition, since the maximum diagnostic utility of islet autoantibodies is achieved when they are measured close to diagnosis, we suggest that clinicians should measure islet autoantibodies as close to diagnosis as possible when the diabetes subtype is uncertain or if MODY is suspected, as this would enhance their ability in differentiating T1D from MODY.

As this is a case control study, the next step would be to investigate whether islet antibodies are useful in the referral setting. Other biomarkers, such as Connecting peptide, MODY prediction model, and Type 1 diabetes genetic risk scores could also be investigated, which may have additive value to islet autoantibody before genetic testing is performed. It would also be clinically useful to perform a longitudinal study of T1D and MODY patients looking at the effects of disease duration on the prevalence of islet autoantibodies. This would provide a better picture on islet autoantibodies prevalence over time.

In conclusion, we have shown that ZnT8A prevalence is low in MODY patients, similar to GADA and IA-2A. ZnT8A should be used in conjunction with GADA and IA-2A as a routine test before molecular genetic testing. This should be performed closer to diagnosis preferably as this enhances its ability to discriminate T1D from MODY.

References

1. Shields BM, Shepherd M, Hudson M, McDonald TJ, Colclough K, Peters J, et al. Population-Based Assessment of a Biomarker-Based Screening Pathway to Aid Diagnosis of Monogenic Diabetes in Young-Onset Patients. Diabetes Care. 2017;40(8):1017-25.

2. Ellard S, Bellanné-Chantelot C, Hattersley AT, Group EMGQNM. Best practice guidelines for the molecular genetic diagnosis of maturity-onset diabetes of the young. Diabetologia. 2008;51(4):546-53.

3. Hattersley A, Bruining J, Shield J, Njolstad P, Donaghue KC. The Diagnosis and Management of Monogenic Diabetes in Children and Adolescents. Pediatric Diabetes. 2009;10:33-42.

4. Pearson ER, Starkey BJ, Powell RJ, Gribble FM, Clark PM, Hattersley AT. Genetic cause of hyperglycaemia and response to treatment in diabetes. Lancet. 2003;362(9392):1275-81.

5. Pihoker C, Gilliam LK, Ellard S, Dabelea D, Davis C, Dolan LM, et al. Prevalence, characteristics and clinical diagnosis of maturity onset diabetes of the young due to mutations in HNF1A, HNF4A, and glucokinase: results from the SEARCH for Diabetes in Youth. J Clin Endocrinol Metab. 2013;98(10):4055-62.

6. Gandica RG, Chung WK, Deng L, Goland R, Gallagher MP. Identifying monogenic diabetes in a pediatric cohort with presumed type 1 diabetes. Pediatr Diabetes. 2015;16(3):227-33.

7. Johansson BB, Irgens HU, Molnes J, Sztromwasser P, Aukrust I, Juliusson PB, et al. Targeted next-generation sequencing reveals MODY in up to 6.5% of antibody-negative diabetes cases listed in the Norwegian Childhood Diabetes Registry. Diabetologia. 2017;60(4):625-35.

8. McDonald TJ, Colclough K, Brown R, Shields B, Shepherd M, Bingley P, et al. Islet autoantibodies can discriminate maturity-onset diabetes of the young (MODY) from Type 1 diabetes. Diabetic Medicine. 2011;28(9):1028-33.

9. Bingley PJ, Bonifacio E, Mueller PW. Diabetes Antibody Standardization Program: first assay proficiency evaluation. Diabetes. 2003;52(5):1128-36.

10. Törn C, Mueller PW, Schlosser M, Bonifacio E, Bingley PJ, Laboratories P. Diabetes Antibody Standardization Program: evaluation of assays for autoantibodies to glutamic acid decarboxylase and islet antigen-2. Diabetologia. 2008;51(5):846-52.

11. Wenzlau JM, Juhl K, Yu L, Moua O, Sarkar SA, Gottlieb P, et al. The cation efflux transporter ZnT8 (Slc30A8) is a major autoantigen in human type 1 diabetes. Proc Natl Acad Sci U S A. 2007;104(43):17040-5.

12. Wenzlau JM, Walter M, Gardner TJ, Frisch LM, Yu L, Eisenbarth GS, et al. Kinetics of the post-onset decline in zinc transporter 8 autoantibodies in type 1 diabetic human subjects. J Clin Endocrinol Metab. 2010;95(10):4712-9.

13. Pencina MJ, D'Agostino RB, Sr., D'Agostino RB, Jr., Vasan RS. Evaluating the added predictive ability of a new marker: from area under the ROC curve to reclassification and beyond. Stat Med. 2008;27(2):157-72; discussion 207-12.

14. Wickham H. Ggplot2 : elegant graphics for data analysis. New York: Springer; 2009. viii, 212 p. p.

15. Aguilera E, Casamitjana R, Ercilla G, Oriola J, Nicoletti F, Gomis R, et al. Clinical characteristics, β-cell function, HLA class II and mutations in MODY genes in non-paediatric subjects with Type 1 diabetes without pancreatic autoantibodies. Diabetic Medicine. 2005;22(2):137-43.

16. Bowden SA, Hoffman RP. Triple diabetes: coexistence of type 1 diabetes mellitus and a novel mutation in the gene responsible for MODY3 in an overweight adolescent. Pediatric Diabetes. 2008;9(2):162-4.

17. Miura J, Sanaka M, Ikeda Y, Watanabe C, Nakagami T, Iwasaki N, et al. A case of Type-1 diabetes mellitus formerly diagnosed as maturity-onset diabetes of the young (MODY) carrying suggestive MODY3 gene. Diabetes Research and Clinical Practice. 1997;38(2):139-41.

18. Gach A, Wyka K, Pietrzak I, Wegner O, Malecki MT, Mlynarski W. Neonatal diabetes in a child positive for islet cell antibodies at onset and Kir6.2 activating mutation. Diabetes Research and Clinical Practice. 2009;86(2):e25-e7.

Chapter 2

Islet autoantibodies can be used to rationalise genetic testing for MODY in a NHS genomics laboratory referral pipeline

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Acknowledgement of co-authors and contributions to paper

Tim McDonald and the Academic department of Blood Sciences at the Royal Devon and Exeter Hospital were involved with data collection and laboratory analysis of biochemistry tests for the study. Kevin Colclough was involved in genetic analysis of patient samples. Beverley Shields advised on data analysis with Andrew Hattersley.

I undertook data analysis with the assistance of Beverley Shields. I wrote the manuscript with advice from Kashyap Patel. Revisions suggested by co-authors were incorporated into the manuscript.

Abstract

Introduction: Maturity Onset Diabetes of the Young (MODY) is caused by a single gene mutation inherited in an autosomal dominant fashion. The disease comprises of 3-4% of all diabetes in patients under 30 years old. However, the correct genetic diagnosis is important as it defines treatment. We aimed to investigate if islet autoantibodies could be used as a test to rationalise MODY genetic testing in an NHS referral setting. In addition, we compared the diagnostic utility of islet autoantibodies with C-peptide and the Type 1 diabetes genetic risk score (T1D-GRS) both of which have been indicated as useful tests to rule out patients from MODY genetic testing.

Method: Triple islet autoantibodies (GADA, IA-2A and ZnT8A) and C-peptide were measured on 834 consecutive patients referred for MODY testing with whole-blood sampled between 2012 and 2015. Positivity of islet antibodies were based on the 99th centile of 1500 non-diabetic controls. Every patient had GCK, HNF1a and HNF4a genes sequenced. We compared the positivity rates of antibodies in patients with and without an established genetic diagnosis.

Result: Islet autoantibodies were able to exclude 20.9% patients from unnecessary MODY genetic testing. The exclusion rate increased to 32.2% in a sub-analysis of patients who were on insulin treatment, i.e, 1 in 3 non-MODY patients could have been excluded from MODY genetic testing. There was no additional diagnostic utility in C-peptide and T1D-GRS in excluding patients from MODY genetic testing.

Conclusion: The combination of GADA, IA-2A and ZnT8A were able to exclude 20-30% of patients from MODY genetic testing in the referral setting. C-peptide and T1D-GRS were not additive on top of islet autoantibodies in excluding patients from genetic testing. As the measurement of islet autoantibodies was a cost effective way of rationalising genetic testing, it would be embedded in the diagnostic pathway at the genetics referral service in Exeter.

1- Introduction

Maturity Onset Diabetes of the Young (MODY) is caused by a single gene mutation inherited in an autosomal dominant fashion. The mutation is highly penetrant, and phenotypically similar to more common forms of diabetes, namely type 1 and type 2 diabetes (T1D and T2D). Mutations of the glucokinase gene (*GCK*), hepatic nuclear factor 1a (*HNF1a*) and 4a (*HNF4a*) comprises of 80% of MODY diagnoses (1).

MODY is relatively rare, comprising of 3.6% of all diabetes in patients under 30 years old (2). However, the correct genetic diagnosis is important as it defines treatment, patients with *GCK* mutations rarely require treatment, and patients with *HNF1a* and *HNF4a* mutations can be well managed on low dose sulphonylurea, with good glycaemic control (3-5). Despite this, the disease is often misdiagnosed, with more than 80% of patients initially being treated as either type 1 (T1D) or type 2 diabetes (T2D) (6). A diagnosis of MODY is suspected if a patient lack clinical characteristics of T1D and T2D with a family history of diabetes in one parent and first-degree relatives of that affected parent or a mild stable fasting hyperglycemia which does not progress (5). Genetic testing should be considered in these patients to guide diagnosis.

Molecular genetics remains the gold standard for the diagnosis of MODY and despite decreasing costs with improved technology, it is still not possible to implement wide spread genetic testing for MODY. The molecular genetics laboratory at the Royal Devon and Exeter NHS Foundation Trust is internationally recognised for its MODY testing service. The laboratory has made a genetic diagnosis in over 4000 patients over the past 20 years and processes over 1000 referrals for MODY testing each year. This can be an expensive process, and as a result, biomarkers, such as islet autoantibodies, C-peptide, and Type 1 genetic risk score (7-9) have been investigated to help identify MODY in young onset diabetes and have been found to be useful in rationalising genetic testing.

It is worth investigating the use of biomarkers in rationalising genetic tests in an atypical group of patients referred for molecular genetic testing as there are

clinical and financial implications. Clinically, biomarkers can be used to aid the diagnosis of patients with suspected MODY so that they could be correctly treated. Financially, it is important not to test patients who are unlikely to have MODY as molecular genetic testing still remains costly.

We have previously shown that there is a low prevalence of autoantibodies against GAD65 isoform of glutamate decarboxylase (GADA) and tyrosine phosphatase-related protein islet antigen 2 (IA-2A) in MODY patients and its discriminative power in differentiating T1D patients from MODY (7). Recently, we showed that autoantibodies against the zinc transporter 8 (ZnT8A) can also discriminate T1D patients from MODY patients. However, these studies were retrospective case control in design, and not performed in the prospective referral setting. In addition, the use of other biomarkers, namely C-peptide and Type 1 diabetes genetic risk score (T1D-GRS), has potential use in ruling out patients for MODY genetic testing but their efficacy have not been investigated in the prospective referral setting.

The aim of this study was to investigate if islet autoantibodies were able to exclude patients in the referral setting as a way to rationalise MODY genetic testing. In addition, we aimed to explore whether C-peptide and T1D-GRS were useful biomarkers along antibodies in ruling out patients from MODY genetic testing.

2- Method

2.1 Study participants

834 consecutive patients were referred to the Exeter molecular genetics service, with whole blood available. Islet autoantibodies (GADA, IA-2A, ZnT8A) and C-peptide were measured analysed by the Academic department of Blood Sciences Department at the Royal Devon and Exeter Hospital. All patients had *GCK*, *HNF1a* and *HNF4a* genes sequenced. Clinical characteristics were taken from genetic test referral forms. Referral forms indicate whether patients had autoantibody tests before being referred.

2.2 Islet autoantibodies measurement

GADA, IA-2A and ZnT8A were measured using ELISA assays (RSR Limited, Cardiff, U.K.) on a Dynex DS2 automated ELISA system (Launch Diagnostics, Longfield, U.K). Antibody titre cutoffs were established after testing 1559 control

subjects without a diagnosis of diabetes between the age of 18 and 75, along with an Hba1c of less than 6.0%. Islet antibodies were considered positive if levels were above the 99th centile of the non-diabetic control subjects (GADA ≥ 64 World Health Organization units/mL, IA-2A ≥ 7.5 World Health Organization units/mL, ZnT8A ≥126 World Health Organization units/mL (age < 30 years) / ≥20 World Health Organization units/mL (age ≥ 30 years)).

2.3 Type 1 diabetes genetic risk score measurement

T1D-GRS was generated using 30 single gene polymorphisms (SNPs) as previously described (10). Briefly, the score was computed using the number of risk alleles across 30 common T1D single nucleotide polymorphisms (SNPs), with variants from both HLA and non-HLA loci. They were selected based on variants that were strongly associated with T1D as described in existing studies. Each variant was weighted based on their effect on T1D genetic risk from previous literature. A GRS was generated as the sum across SNPs of the number of risk increasing alleles (0, 1 or 2) at that SNP multiplied by the ln(odds ratio) for each allele divided by the number of alleles (10). The HLA-DR3 and HLA-DR4 haplotypes were weighted using imputed haplotypes. The sum of the score signifies a person's risk for T1D. Genotyping of SNPs was performed using the KASP assay by LGC Genomics (Hoddesdon, UK).

2.4 C-peptide measurement

C-peptide analysis was performed on the Roche Modular Analytics E170 immunoassay analyser (Roche Diagnostics, Mannheim, Germany). An antigen-antibody-antigen sandwich complex was formed by reacting one biotinylated anti-C-peptide specific monoclonal mouse antibody and a second monoclonal antibody to Cpeptide labelled with a ruthenium complex with 20uL serum sample of C-peptide. Separation is achieved via interaction of biotin and streptavidin attachment to paramagnetic microparticles (solid phase). The detection system employs electrochemiluminescence with ruthenium trisbipyridyl as the label. Electrochemiluminescence occurs at 620 nm and readings are taken by the photomultiplier tube (PMT). The intensity of light signal is proportional to the concentration of C-peptide in the serum. The assay was calibrated using Roche C-peptide CalSet calibration material (Roche Diagnostics, Mannheim, Germany), traceable to WHO International Reference Reagent (IRR) for C-peptide of human insulin for immunoassay (IRR code

84/510) (11). Quality Control was performed on each day of analysis using low level (67 pmol/L) and high level (3.33 pmol/L) PreciControl MultiAnalyte.

2.5 MODY genetic sequencing

Genomic DNA was extracted from whole blood using standard procedures and the promoter, all coding regions and intron/exon boundaries of the *GCK, HNF1A* and *HNF4A* genes were amplified by PCR. Amplicons were sequenced using the Big Dye Terminator Cycler Sequencing Kit v3.1 (Applied Biosystems, Warrington, UK) according to manufacturer's instructions and reactions were analysed on an ABI 3730 Capillary sequencer (Applied Biosystems, Warrington, UK). Sequences were compared with the reference sequences (NM_000162.4 for *GCK*, NM_000545.6 for *HNF1A* and NM_175914.4 for *HNF4A*) using Mutation Surveyor v5.0.1 software (SoftGenetics, State College, PA).

2.6 Statistical analysis

Islet autoantibody positivity rates were compared between patient groups. Fisher's exact test was used to determine the effect of single antibodies in differentiating non-MODY from MODY. C-peptide and T1D-GRS levels were also compared between patient groups, with data presented as median and interquartile range (IQR). Mann-Whitney U-test was used to determine statistical significance between C-peptide and T1D-GRS levels as they are not normally distributed, determined by the Shapiro-Wilk's test. Data was cleaned using *Stata Statistical Software: Release 14*. (College Station, TX: StataCorp LLC). Statistical analysis was performed using *RStudio* (RStudio, Inc., Boston, MA) and the creation of graphs was performed using ggplot2 package within RStudio (12).

3 - Results

3.1 Patients characteristics

834 consecutive patients were referred to the Exeter molecular genetics service between 2012 to 2015. 188 (23%) of participant had a genetic diagnosis of MODY (78 *GCK*, 65 *HNF1a,* 29 *HNF4a,* 11 *HNF1b*), and 646 (77%) of patients with no genetic cause found (non-MODY). Baseline characteristics were different between non-MODY and MODY patients, with MODY patients being younger (median 25 vs 18 years, $p < 0.0001$), a lower BMI (median 24.8 vs 23.05 $kg/m^{2,}$ $p < 0.0001$), and a lower Hba1c (median 62 vs 50 mmol/mol, $p < 0.0001$) at diagnosis. Although non-MODY patients generally had a longer disease duration compared to MODY patients before they were diagnosed, this was not statistically significant (median 3 vs 2 years, $p = 0.48$). Further clinical characteristics are shown in table 1.

	non-MODY	**MODY**
n	646	188
Female (n)	336 (55%)	111 (61%)
BMI (kg/m2)	25 (22 – 29)	23 (20 – 27)
Age at diagnosis (years)	25 (16 – 34)	18 (14 – 25)
Disease duration (years)	3 (1 - 9)	2 (0 – 12)
Hba1c (mmol/L)	62 (48 – 84)	50 (44 – 60)
Exeter MODY Probability Score	33 (6 – 58)	75 (46 – 76)
Patients on insulin (n)	239 (37%)	28 (15%)
Patients on Oral Hypoglycaemic Agent (OHA)	74 (11%)	62 (33%)
Patients on Insulin and OHA treated (n)	49 (8%)	4 (2%)
Patients on Diet Treatment (n)	40 (6%)	20 (11%)

Table 1: Clinical characteristics of patient cohort in this study. Results are in median with the interquartile range (IQE) in parentheses unless stated otherwise.

3.2 Differentiating MODY from non-MODY

3.2.1 Islet autoantibodies

Islet autoantibodies were able to exclude 21% patients from unnecessary MODY genetic testing. A total of 135/646 (20.9%) non-MODY patients had positive islet autoantibodies (Figure 1). Overall, the combination of GADA and IA-2A excluded 18.9% of patients from genetic testing. ZnT8A along with GADA and IA-2A excluded an additional 1.4% of patients from genetic testing. ZnT8A increased to number of patients from single to multiple antibody positivity by 3.3% ($p < 0.0001$). The autoantibody positivity rates in non-MODY patients increased to 32.2% in a sub-analysis of patients who were on insulin treatment, i.e, 1 in 3 non-MODY patients could have been excluded from MODY genetic testing. Islet autoantibodies were also able to exclude 14.3% of non-insulin

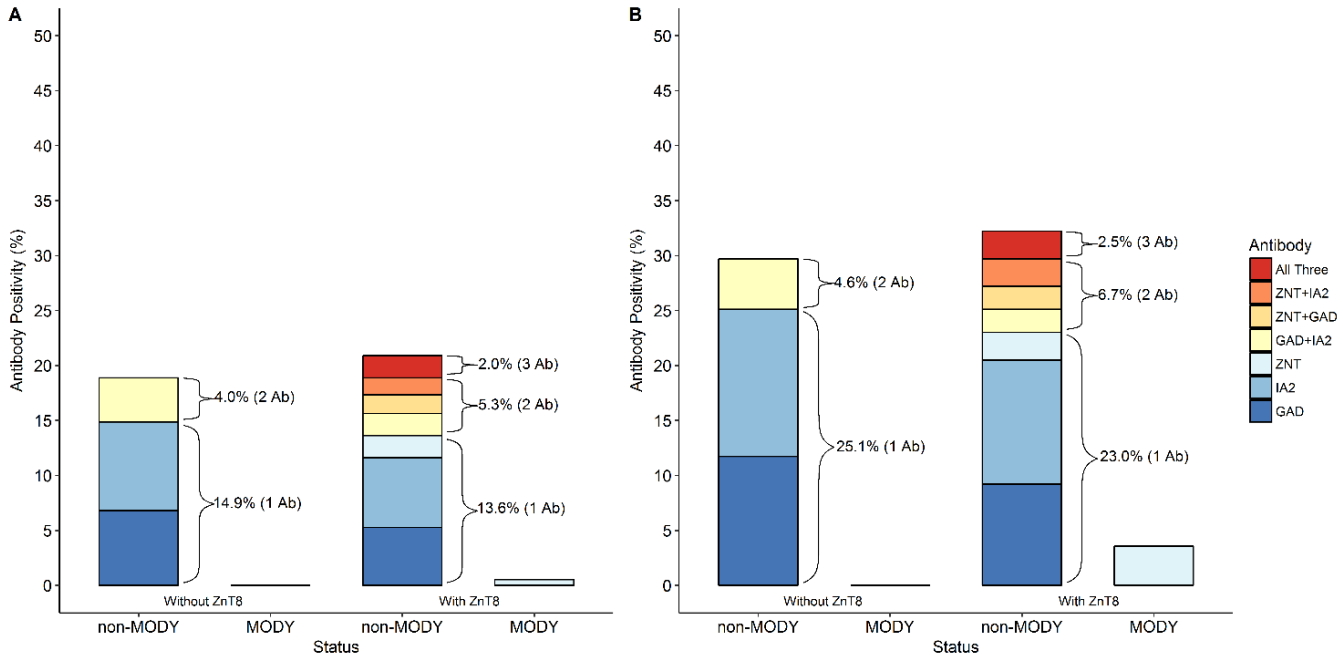

Figure 1: (A) Stacked bar chart showing percentage of antibody positivity between non-MODY and MODY patients. (B) Stacked bar chart showing percentage of antibody positivity between insulin treated non-MODY and MODY patients.

treated patients from genetic testing. Interestingly, non-MODY patients within this group had a median disease duration of 1.5 years (IQE 0 – 5 years). Table of antibody positivity between both groups are shown in table 2. Only 1/188 (0.5%) MODY patient was found to be positive to ZnT8. The area under the receiver operator curve (ROC-AUC) for combined islet antibodies was 0.6013.

	non-MODY		MODY	
	Insulin treated	Non-insulin treated	Insulin treated	Non-insulin treated

Table 2: Number of patients with positive islet autoantibodies, split by disease and treatment status.

GADA only	22	12	0	0
IA-2A only	27	14	0	0
ZnT8A only	6	7	1	0
GADA+IA-2A	5	8	0	0
IA-2A+ZnT8A	5	6	0	0
GADA+ZnTA	6	4	0	0
All Three antibodies	6	7	0	0
No antibody	162	349	27	160

Clinicians notified the genetic service on the referral form if a patient had routine islet autoantibodies measured prior to their referral. A total of 314/646 (48.6%) non-MODY patients and 74/188 (39.4%) MODY patients had islet antibodies measured externally. Out of these patients, 101/646 (15.6%) non-MODY patients had positive GAD and IA2 autoantibodies. No patients had ZnT8A measured prior to their referral. In addition, no MODY patients had positive islet autoantibodies prior to referral.

3.2.2 C-peptide diagnostic utility

There is a slight decrease in C-peptide levels in patients as disease duration lengthens (Figure 2). However, there was no difference in C-peptide levels between non-MODY patients compared to MODY patients at referral (median 564 pmol/L, Interquartile range (IQR) 280 – 958 pmol/L vs 483, 285 – 752, p = 0.08). There was low diagnostic utility in using C-peptide to differentiate non-MODY from MODY patients, with the area under the receiver operator curve (ROC-AUC) of 0.53 (Figure 3). In a sub-analysis of patients who were on insulin treatment, C-peptide levels remained similar between non-MODY and MODY patients, with higher median C-peptide levels in MODY patients compared to non-MODY patients without statistically significance and low diagnostic utility (median 262 pmol/L, IQR 79 – 553 pmol/L, vs 251, 181 – 451, p = 0.55, ROC-

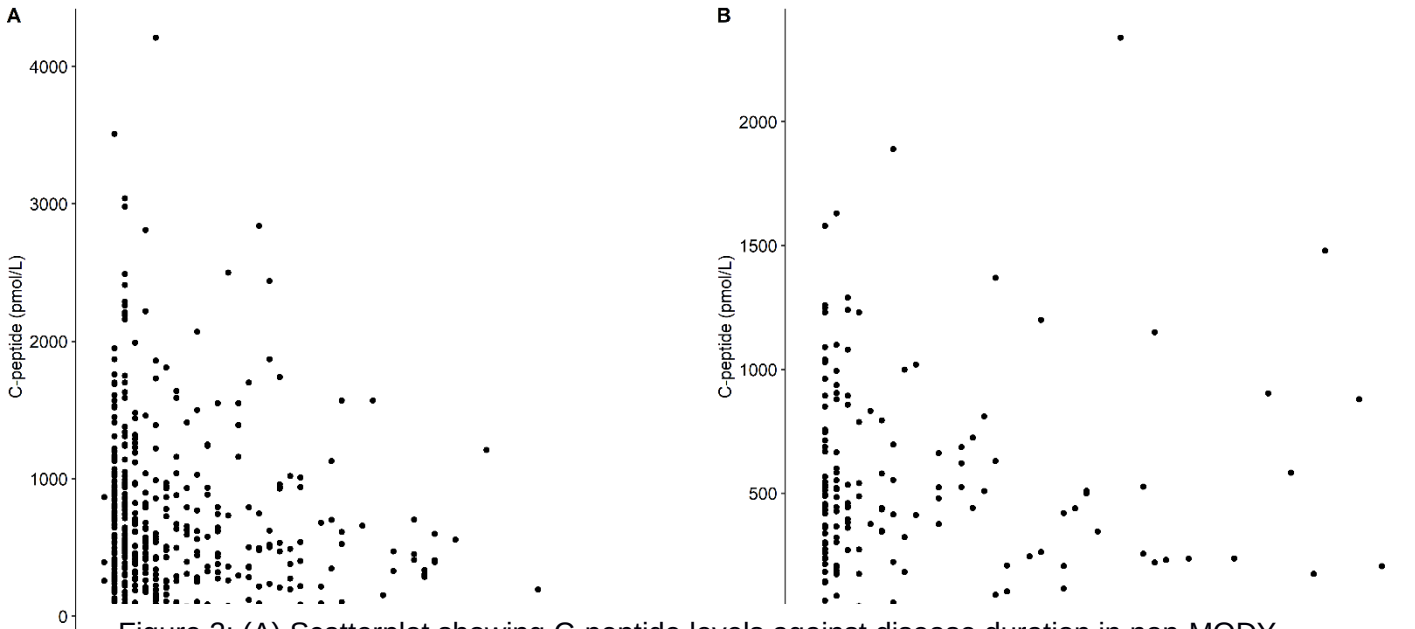

Figure 2: (A) Scatterplot showing C-peptide levels against disease duration in non-MODY patients. (B) Scatterplot showing C-peptide levels against disease duration in MODY patients.

AUC =0.54).

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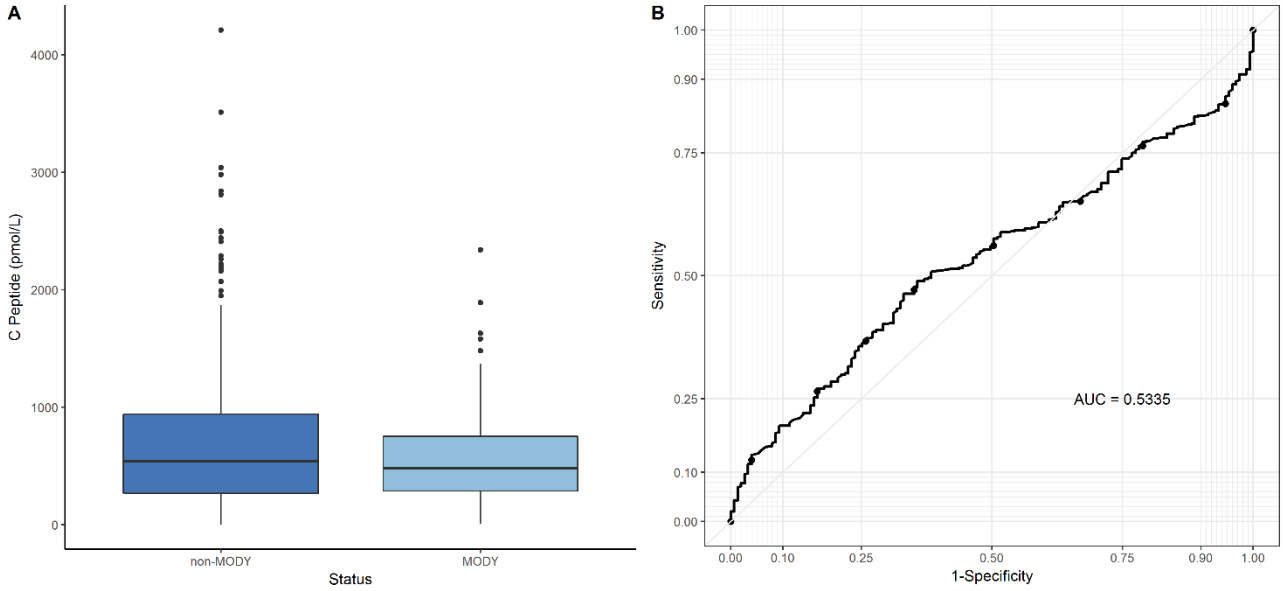

Figure 3: (A) Boxplot showing C-peptide levels between non-MODY and MODY patients. (B) ROC graph of C-peptide with AUC.

3.2.3 Type 1 genetic risk score diagnostic utility

T1D-GRS was not useful in differentiating between non-MODY and MODY patients at referral. The median T1D-GRS in non-MODY patients was higher compared to MODY patients with statistical significance (median 0.24, IQR 0.22 – 0.27, vs 0.23, 0.22 – 0.25, p = <0.0001), however the diagnostic utility of T1D-GRS in differentiating non-MODY from MODY patients was modest (ROC-AUC = 0.62) (Figure 3). Results remained similar in a sub-analysis of patients who were on insulin treatment, with statistically significant T1D-GRS but low diagnostic utility in differentiating non-MODY patients from MODY patients (median 0.26, IQR 0.23 – 0.28 vs 0.24, 0.22 – 0.26, p = 0.01, ROC-AUC = 0.65.)

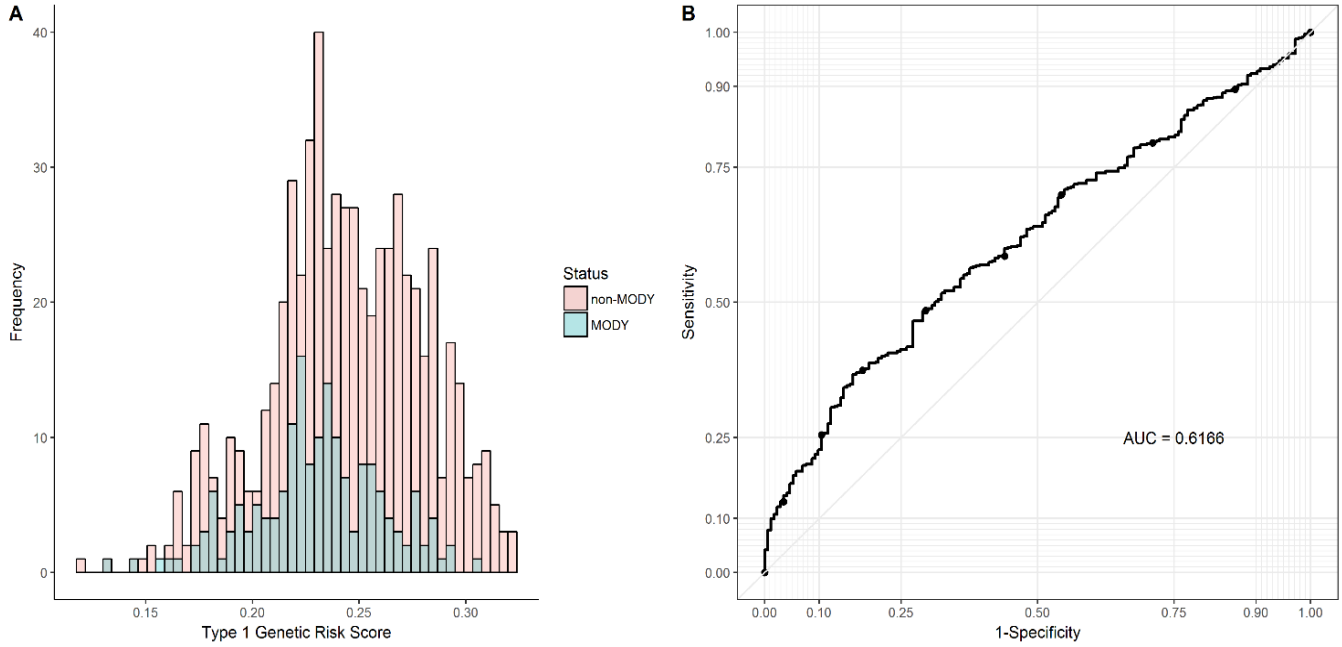

Figure 4: (A) Histogram showing T1D-GRS levels between non-MODY and MODY patients (B) ROC graph of T1D-GRS with AUC.

3.3 - Combined biomarker utility

Due to the strength of islet autoantibodies in differentiating non-MODY patients from MODY patients, we investigated whether C-peptide and / or T1D-GRS provided further diagnostic discrimination in patients with negative islet autoantibody tests. Overall, C-peptide and T1D-GRS did not provide additional diagnostic discrimination in antibody negative patients. C-peptide levels were higher in non-MODY patients compared to MODY with statistical significance (median 618, IQR 340 – 1020 vs 480 (285 – 752) pmol/L, p = 0.0008), however there was very little diagnostic utility (ROC-AUC = 0.6016) above islet antibodies. Similarly, there was no additional diagnostic utility in insulin treated antibody negative patients (median 399 pmol/L, IQR 135 – 670 pmol/L vs 251 pmol/L , 181 – 451 pmol/L, p = 0.48, ROC-AUC = 0.5727).

T1D-GRS levels were comparable between non-MODY patients and MODY patients with modest statistical significance and did not provide further diagnostic utility (0.23 (0.22 – 0.26) vs 0.23 (0.22 – 0.25), p = 0.02, ROC AUC = 0.57). Likewise, in insulin treated antibody negative patients, GRS levels and diagnostic utility remained similar with no additive diagnostic benefit (0.24 (0.22 – 0.27) vs 0.24 (0.22 – 0.26), p = 0.25, ROC AUC = 0.58).

4 - Discussion

The results of this study shows that islet autoantibodies can be used to rationalise genetic testing for MODY. We found that the combination of GADA, IA-2A, and ZnT8A were able to exclude up to 21% of patients from MODY genetic testing, which increases to 32% if islet autoantibodies were measured in patients who were on insulin treatment. Although the additional benefit of ZnT8A in identifying patients who were previously antibody negative was small, it modestly increased the number of patients who were single autoantibody positive to multiple antibody positivity. This decreases the probability of MODY and increases our confidence in excluding patients from MODY genetic testing. In addition, despite the fact that the net discrimination of islet autoantibody was not high, with a ROC-AUC of 0.6013, islet autoantibodies still has good negative predictive value, as islet autoantibody positive rates in MODY patients are low.

The islet autoantibody positive rates in non-MODY patients was lower compared to previous studies. This is possibly due to the fact that the cohort referred to the Exeter genetics referral service represent a very atypical group of patients with young onset diabetes. They have been identified by their clinicians as having feature that may be indicative of MODY, including assessment of islet autoantibodies and C-peptide in some cases. Therefore, there would be a mixture of T1D and T2D patients within the non-MODY cohort. The lower positive rates of islet autoantibodies could also be explained by the fact that islet autoantibodies were not measured close to diagnosis in a proportion of patients within this cohort, since the positive rates of islet autoantibodies decreases as disease duration lengthens. This would also explain the lower positive rates of ZnT8A compared to GADA and IA-2A within this cohort, as the positive rate of ZnT8A seems to decrease more over time compared to the two established autoantibodies. Nonetheless, islet autoantibodies were still able to exclude 20 to 30% of patients from MODY genetic testing. Interestingly, in a sub-analysis of patients who were not on insulin treatment, islet autoantibodies were also able to exclude 14% of patients from MODY genetic testing. This shows that islet autoantibodies can be used as a biomarker to rationalise genetic testing.

We did not find additional diagnostic utility of C-peptide in ruling out patients from MODY genetic testing in the referral setting. Levels of C-peptide were not

statistically different between non-MODY and MODY patients, and the ROC-AUC was too low to use as a rule out test, even in those who were insulin treated. This may highlight the fact that non-MODY patients that were referred to the genetics service were predominately patients with type 2 diabetes. Evidence of this included more non-MODY patients being on oral hypoglycaemic agents or diet treatment with longer disease duration, and relatively low levels of islet autoantibodies.

T1D-GRS did not perform as well as what we described in the referral setting. Previously, we found that T1D-GRS showed discriminative power between MODY and T1D patients, with scores being higher in patients with T1D compared to MODY patients with relatively good diagnostic utility (ROC-AUC = 0.87), which was in contrast to the results in this study. Although T1D-GRS was statistically higher in non-MODY patients compared to MODY patients within this study, there was a lack of diagnostic utility when the cohort was compared as a whole. It was possibly due to the fact that our previous study was done in a case control setting, with T1D-GRS of pre-selected patients having a T1D phenotype (diagnosed under 17 years of age, insulin treated and islet autoantibody positive) compared to patients with MODY, whereas the cohort within this study consists of a mixed population of T1D, T2D and MODY. It is also important to note that the mean T1D-GRS in MODY patients in our previous paper was 0.231, which was identical to the results seen in MODY patients within this cohort. However, the mean T1D-GRS of patients with T1D previously described was 0.279, which was significantly higher compared to the scores of non-MODY patients within this study. This highlights the heterogeneous group of patients that were referred to our genetics service, reiterating the fact that there was a mixture of patients with T1D and T2D within the non-MODY group. In addition, by comparing non-MODY patients who were phenotypically similar to T1D (in accordance with Royal College of General Practitioners Diabetes Classification guidance) with MODY patients within this study, the diagnostic utility of T1D-GRS increases, with a ROC-AUC of 0.75.

This is to our knowledge the first study which investigated whether biomarkers were useful in rationalising genetic testing in the referral setting. Novel biomarkers in differentiating non-MODY and MODY patients were also investigated, namely ZnT8 autoantibodies in combination with established islet

autoantibodies and the T1D-GRS. Whilst most studies that investigated the use of islet autoantibodies in differentiating non-MODY patients from MODY patients were done retrospectively or presented in case studies (7, 13-16), this study was done prospectively in the referral setting. This study also contained the largest cohort of patients referred prospectively for MODY genetic testing. In addition, serum sent to the genetics service was analysed within a single laboratory and all patients had standardised genetic testing. The islet autoantibody reference ranges were derived from a control population, making the results more reliable. Clinicians also notified the genetics service on referral forms if they were previously screened for islet autoantibodies prior to their referral, and patients were not excluded on the basis of positive autoantibodies to minimise bias.

There were several limitations to our study. The study looked at classical MODY, namely *GCK*, *HNF1a*, *HNF4a* and *HNF1b*. However, the study did not take into account whether islet autoantibodies and biomarkers were able to exclude patients from classical forms of other less common forms of monogenic diabetes, such as *INS* and Peroxisome proliferator-activated receptor gamma (*PPARG*) mutations, which were excluded from the study. Secondly due to the nature of the referral service, there were low numbers of MODY patients who were treated with insulin when they were referred, meaning we could be underestimating the discriminative power of C-peptide and T1D-GRS in insulin treated patients within this study. However, there was significant overlap of C-peptide distributions between non-MODY and MODY patients, meaning that C-peptide levels would not be significantly higher even if we increased the number of insulin treated MODY patients within the sub-analyses.

Islet autoantibodies represents a cost effective way in ruling out patients from genetic testing, as a triple antibody test cost £27 compared to £700 for targeted capture. This meant the service could have saved £90855 by rationalising genetic testing using islet autoantibodies. Based on these result, we believe all three islet autoantibodies should be measured in all patients who would be referred to the service for genetic testing. The results here can also be applied to other MODY referral centres. More studies are required to investigate whether islet autoantibodies, C-peptide and T1D-GRS is useful in the setting of a diabetes clinic in secondary care.

In conclusion, we have shown that the combination of GADA, IA-2A and ZnT8A were able to exclude 20-30% of patients from MODY genetic testing in the referral setting. C-peptide and T1D-GRS were not additive on top of islet autoantibodies in excluding patients from genetic testing. As the measurement of islet autoantibodies was a cost effective way of rationalising genetic testing, it would be embedded in the diagnostic pathway at the genetics referral service in Exeter.

References

1. Ellard S, Bellanné-Chantelot C, Hattersley AT, Group EMGQNM. Best practice guidelines for the molecular genetic diagnosis of maturity-onset diabetes of the young. Diabetologia. 2008;51(4):546-53.
2. Shields BM, Shepherd M, Hudson M, McDonald TJ, Colclough K, Peters J, et al. Population-Based Assessment of a Biomarker-Based Screening Pathway to Aid Diagnosis of Monogenic Diabetes in Young-Onset Patients. Diabetes Care. 2017;40(8):1017-25.
3. Pearson ER, Starkey BJ, Powell RJ, Gribble FM, Clark PM, Hattersley AT. Genetic cause of hyperglycaemia and response to treatment in diabetes. Lancet. 2003;362(9392):1275-81.
4. McDonald TJ, Ellard S. Maturity onset diabetes of the young: identification and diagnosis. Ann Clin Biochem. 2013;50(Pt 5):403-15.
5. Hattersley A, Bruining J, Shield J, Njolstad P, Donaghue KC. The Diagnosis and Management of Monogenic Diabetes in Children and Adolescents. Pediatric Diabetes. 2009;10:33-42.
6. Shields BM, Hicks S, Shepherd MH, Colclough K, Hattersley AT, Ellard S. Maturity-onset diabetes of the young (MODY): how many cases are we missing? Diabetologia. 2010;53(12):2504-8.
7. McDonald TJ, Colclough K, Brown R, Shields B, Shepherd M, Bingley P, et al. Islet autoantibodies can discriminate maturity-onset diabetes of the young (MODY) from Type 1 diabetes. Diabetic Medicine. 2011;28(9):1028-33.
8. Besser RE, Shepherd MH, McDonald TJ, Shields BM, Knight BA, Ellard S, et al. Urinary C-peptide creatinine ratio is a practical outpatient tool for identifying hepatocyte nuclear factor 1-{alpha}/hepatocyte nuclear factor 4-

{alpha} maturity-onset diabetes of the young from long-duration type 1 diabetes. Diabetes Care. 2011;34(2):286-91.

9. Patel KA, Oram RA, Flanagan SE, De Franco E, Colclough K, Shepherd M, et al. Type 1 Diabetes Genetic Risk Score: A Novel Tool to Discriminate Monogenic and Type 1 Diabetes. Diabetes. 2016;65(7):2094-9.

10. Oram RA, Patel K, Hill A, Shields B, McDonald TJ, Jones A, et al. A Type 1 Diabetes Genetic Risk Score Can Aid Discrimination Between Type 1 and Type 2 Diabetes in Young Adults. Diabetes Care. 2016;39(3):337-44.

11. Bristow AF, Das RE. WHO international reference reagents for human proinsulin and human insulin C-peptide. J Biol Stand. 1988;16(3):179-86.

12. Wickham H. Ggplot2 : elegant graphics for data analysis. New York: Springer; 2009. viii, 212 p. p.

13. Aguilera E, Casamitjana R, Ercilla G, Oriola J, Nicoletti F, Gomis R, et al. Clinical characteristics, β-cell function, HLA class II and mutations in MODY genes in non-paediatric subjects with Type 1 diabetes without pancreatic autoantibodies. Diabetic Medicine. 2005;22(2):137-43.

14. Bowden SA, Hoffman RP. Triple diabetes: coexistence of type 1 diabetes mellitus and a novel mutation in the gene responsible for MODY3 in an overweight adolescent. Pediatric Diabetes. 2008;9(2):162-4.

15. Miura J, Sanaka M, Ikeda Y, Watanabe C, Nakagami T, Iwasaki N, et al. A case of Type-1 diabetes mellitus formerly diagnosed as maturity-onset diabetes of the young (MODY) carrying suggestive MODY3 gene. Diabetes Research and Clinical Practice. 1997;38(2):139-41.

16. Gach A, Wyka K, Pietrzak I, Wegner O, Malecki MT, Mlynarski W. Neonatal diabetes in a child positive for islet cell antibodies at onset and Kir6.2 activating mutation. Diabetes Research and Clinical Practice. 2009;86(2):e25-e7.

Discussion

Discussion

This thesis assessed the diagnostic utility of zinc transporter 8 (ZnT8) autoantibodies compared to autoantibodies against glutamic acid decarboxylase (GADA) and protein tyrosine phosphatase islet antigen-2 (IA-2A) in discriminating type 1 diabetes (T1D) from Maturity Onset Diabetes of the Young (MODY). This thesis also investigated whether islet autoantibodies, Connecting peptide (C-peptide) and the type 1 diabetes genetic risk score (T1D-GRS) are useful in rationalising genetic testing in the prospective referral setting by ruling patients out for MODY genetic testing.

This chapter provides a discussion of the findings of previous chapters, implications, strength and limitations, along with plans for future research.

Chapter 1: The addition of Zinc Transporter 8 autoantibodies to established islet autoantibodies improves discrimination of MODY from T1D close to diagnosis

It had been shown that the prevalence of GADA and IA-2A were low in patients with MODY, with a prevalence of <1% within MODY patients, similar to non-diabetic controls (1). However, ZnT8A had never been studied in this context as it was a relatively novel biomarker for T1D compared to established autoantibodies GADA and IA-2A. Studies investigating ZnT8A were mainly performed in the context of T1D diagnosis and classification, and not its discrimination of T1D from MODY (2–4). Furthermore, the previous paper also only studied patients close to diagnosis, and did not investigate the effects of disease duration on the prevalence and discriminative power of islet autoantibodies.

The aim of this chapter was to determine the prevalence of ZnT8A in relation to established islet autoantibodies GADA and IA-2A in patients with T1D compared to patients with MODY. The discriminative power of ZnT8A in differentiating T1D from MODY and whether ZnT8A offered additive

discriminatory effects along with GADA and IA-2Awas studied. Lastly, the effect of disease duration on prevalence and discriminative power of all three autoantibodies was also studied.

Main result:

ZnT8A were able to discriminate T1D patients from MODY independently, with sensitivity and specificity comparable to GAD and IA-2A. The results here show that the prevalence of ZnT8A in MODY patients were similar to non-diabetic control, at a level of 1% in concordance with the prevalence of GADA and IA-2A. Although ZnT8A only modestly increased the number of patients who were previously antibody negative to single antibody positivity, they were able to significantly increase the number of patients who were previously positive for single antibody to multiple antibody positivity.

Islet autoantibody prevalence remains low in patients with MODY, supporting the results found in our previous study (1). Around 800 individuals with genetically confirmed MODY in both studies had islet autoantibodies assessed in our unit, with a consistently low prevalence of islet autoantibodies. This reiterates the fact that biomarkers of autoimmunity do not form part of the disease process in MODY. This has also been supported by case reports and family studies (5–7). A study based on cohorts within a German and Austrian registry with MODY showed an autoantibody prevalence of 17% within MODY patients, although the study did not publish details on islet autoantibody assays or the reference range thresholds used. The study also defined MODY using clinical features (non-insulin-dependent diabetes with no or unexpectedly low insulin requirement and the absence of signs of insulin resistance such as acanthosis nigricans or marked obesity) and did not confirm the diagnosis of MODY using a genetic test in up 20% of the patients (8). It is possible that the patients defined as MODY had T1D, which may explain the higher prevalence of islet autoantibodies.

Islet autoantibodies achieved the highest diagnostic utility in discriminating T1D from MODY when they were measured closer to diagnosis. This is due to the decreasing prevalence of all three islet autoantibodies in patients with T1D as disease duration increased. This is most evident in ZnT8A and IA-2A, although a drop in prevalence was also seen in GADA. In patients with the longest

disease duration at 22 years, ZnT8A prevalence was evidently lower compared to GADA and IA-2A. Previously, studies suggested that islet autoantibody levels were highest within patients with young onset T1D, and cross-sectional analysis of showed that autoantibody levels decreased over time, possibly due to a weaning of autoimmunity (9). This may be explained by the decrease of the overall number of antigens present within the beta cell over time, associated with the lowering number of beta cells. However, this does not necessarily explain the difference between the types of autoantibody as their prevalence seem to vary at different time points.

Clinical Implications

The results of this chapter suggests that islet autoantibodies should be used as a test while investigating patients with a suspected diagnosis of MODY, with ZnT8A autoantibodies measured in addition to GADA and IA-2A. Since the increasing number of positive islet autoantibodies decreases the probability of a MODY diagnosis, islet autoantibodies could be considered as a “ruling out” test for patients with suspected MODY. A single positive islet autoantibody test in a patient with suspected MODY warrants further clinical investigations before molecular genetic testing. However, if the patient is positive for multiple autoantibodies, genetic testing should not be performed since the probability of a MODY diagnosis would be very low.

In addition, based on the results from this chapter, islet autoantibodies should be measured as close to diagnosis as possible if MODY is suspected. The falling of islet autoantibody prevalence affects its discriminatory power in differentiating T1D from MODY, as less T1D patients were positive for autoantibodies. Although islet autoantibodies would also be useful in patients with longer disease duration, it is important to acknowledge that the discriminative power of islet autoantibody decreases over time.

Strength and limitations:

This is the first study which investigated the prevalence of ZnT8A in MODY patients, with comparisons made between GADA and IA-2A. The sera used within this study was analysed in a single laboratory, and the antibody reference ranges were derived from a standard control population, making reference thresholds more robust. Finally, as mentioned above, the study investigated a

large cohort of MODY patients and T1D patients, allowing the examination of duration effects on the prevalence of islet autoantibodies.

Several limitations exist within this study, including the study design and the study method. This study was performed as a retrospective case control study, meaning prospective data on patients were unavailable. Patient groups, especially patients with T1D, were pre-selected and pre-defined before they were analysed within the study cohort due to the design of the study. We were also unable to select patients within the IASP cohort based on insulin requirement, as anonymised samples were sent to our laboratory for the programme. The study of disease duration and its effects on islet autoantibodies prevalence and discriminative power was based on a cross-sectional analysis across the study cohort, meaning we were unable to study the effect of disease duration in the same patients as longitudinal data is unavailable.

Lastly, the possibility that patients within this cohort were pre-screened for antibodies before they were referred to our genetic laboratory could not be excluded, which could explain the low prevalence in our study group. However, if the analysis within this study was restricted to only probands (n = 183) who previously were not screened for autoantibodies, the antibody prevalence rate was 0.01%, lower than the expected prevalence in control populations.

Future areas of research

As this study was performed as a retrospective case control study, it would be important to investigate whether islet autoantibodies remains useful in the prospective referral setting. Other biomarkers, such as C-peptide and the T1D-GRS, should also be investigated, as previous case control studies suggested their use as a ruling out test similar to islet autoantibodies (10,11). It would also be of interest to recruits patients with a newly diagnosed diabetes with annual assessment of islet autoantibodies to investigate the rate of change that occurs with disease duration and compared this with MODY patients matched by the same disease duration.

Chapter 2: Islet autoantibodies are useful in rationalising genetic testing for MODY in a NHS genomics laboratory referral pipeline

ZnT8A in addition with GADA and IA-2A had been shown to be useful in discriminating T1D from MODY in the case control setting, as shown in Chapter 3. However, the diagnostic utility of islet autoantibodies in a referral setting had never been studied. It is important to determine this as the results would affect the referral pipeline at the Exeter molecular genetics service, which is a national referral centre for MODY and receives around 1000 referrals for MODY testing each year. Islet autoantibodies can be used as a way to rationalise genetic testing at the referral service by excluding patients from testing due to the costs associated with genetic testing.

In addition, other biomarkers such as Connecting peptide (C-peptide) and the Type 1 diabetes genetic risk score (T1D-GRS) had been previously shown to have some use in differentiating T1D from MODY patients (1). However, this had never been studied in the context of the referral setting, and studies were mainly performed in a retrospective case control analysis.

The aim of this chapter was to determine whether islet autoantibodies were able to exclude patients from MODY genetic testing in a consecutive patient cohort as a way to rationalise genetic testing. This chapter also aimed to investigate whether there was any diagnostic value with C-peptide and T1D-GRS in ruling out patients from MODY genetic testing in the referral setting.

Main Result

In this study, we found that islet autoantibodies were able to exclude 21% of patients from MODY genetic testing, meaning that 1 in 5 patients could have avoided genetic tests. This percentage increased in patients who were on insulin treatment to 33%. Although the additional benefit of ZnT8A in identifying individuals who were previously antibody negative was small, it modestly increased the number of patients who were single autoantibody positive to multiple antibody positivity. This increases our confidence in excluding patients

from genetic testing, as the increasing number of positive antibodies decreases the probability of MODY. Important to note that clinicians refer patients with an atypical presentation of diabetes, such as a patient with a T1D phenotype and not requiring a standard dose of insulin, which means that the study cohort may have a mix of patients with T1D, T2D and MODY. Even so, islet autoantibodies were able to exclude 20-30% of patients from MODY genetic testing. Interestingly, islet autoantibodies could have excluded close to 15 of non-insulin treated non-MODY patients from genetic testing (oral hypoglycaemic agent or diet treated). Inaccuracies in referral forms sent to the genetic service may explain this, with clinicians entering incorrect treatment data into referral forms. This means that some patients could be incorrectly classed as non-insulin treated due to clinician error. Alternatively, this result could also be explained by the mixed cohort of patients referred to the genetics referral service. Patients within the honeymoon period of T1D may require variable amounts of insulin as they continue to have a decreasing number of functioning beta cells. However, they would have measurable islet autoantibodies at this time as this reflects the underlying autoimmune processes leading to beta cell dysfunction. Therefore, this may affect a clinician's decision in referring a patient to the Molecular genetics service. The level of islet autoantibodies is lower in this study compared to the results in the previous chapter since the cohort of non-MODY patients are not pre-selected to fit a T1D criteria, with a possibility of a predominance of patients with T2D within the non-MODY group. This may be supported by the fact that more non-MODY patients were on oral hypoglycaemic agents or diet treatment with longer disease durations.

C-peptide did not provide further diagnostic value in excluding patients from MODY genetic testing. It is thought that persistent C-peptide is an important clinical feature in the diagnosis of MODY (12). C-peptide reflects endogenous insulin secretion, as it is cleaved to from proinsulin to form insulin in a 1:1 ratio (13). Theoretically, C-peptide levels would be lower in patients with T1D compared to MODY, due to endogenous insulin deficiency associated with T1D.

The level of C-peptide was not significantly different between non-MODY and MODY groups within this study. The area under the receiver-operator curve (ROC-AUC) was also very low (ROC-AUC = 0.53) to justify its use in the referral setting. This may be due to the fact that a majority of patients in the non-MODY

group had T2D. C-peptide levels between patients with T2D and MODY would be similar since endogenous insulin production would be present in both conditions, unlike T1D. A sub-analysis of C-peptide levels in patients on insulin treatment was performed, however, levels of C-peptide between both groups remained similar with a lack of diagnostic utility.

Like C-peptide, T1D-GRS was not additive in ruling out patients from MODY genetic testing. It was found previously that T1D-GRS can discriminate between patients with MODY and T1D. Previous studies showed that T1D has a strong genetic component, and a score was generated as a way to quantify a person's genetic risk of T1D, based on single nucleotide polymorphisms (SNPs) genotyping data (10). As expected, the T1D-GRS was higher in patients with T1D compared to MODY with good diagnostic utility (ROC-AUC = 0.87) (11). However, that was shown in a case control setting with pre-defined study groups. In this study, although T1D-GRS was statistically higher in non-MODY patients compared to MODY patients, diagnostic utility was not achieved, with a ROC-AUC of 0.62 Similar to C-peptide, this results remained the same in a sub-analysis of patients on insulin treatment, with a modest gain of diagnostic utility (ROC-AUC = 0.65). Again, this highlights the heterogeneous group of patients referred to the genetic service.

Clinical Implications

Currently, a clinical features based approach is used to rationalise genetic testing i.e a clinical scientist / geneticist decides whether a patient has genetic testing based on clinical experience and judgement. Islet autoantibodies are only measured on patients who are on insulin treatment since insulin requirement is associated with T1D and islet autoantibodies would likely be positive before genetic testing is performed. Given that non-insulin treated patients also had positive islet autoantibodies, the results from this chapter suggest that all patients should receive islet autoantibody testing regardless of their clinical features. This approach would be able to capture all patients referred regardless of treatment status and would also eliminate some human biases when rationalising genetic testing at the referral service. Islet autoantibodies are also a cost effective way to rationalise genetic testing. Since a triple antibody test (GAD, IA2 and ZnT8) cost £27 compared to £700 for targeted capture genetic testing, meaning that the service could save £90855

by rationalising genetic testing using islet autoantibodies based on this patient cohort.

The results from this chapter also suggest that serum C-peptide measurement and the T1D-GRS should not be used in the genetic referral service. The test does not provide additional benefit as a ruling out test compared to islet autoantibodies in the referral service. Whether these tests are useful in the setting of a clinic has yet to be elucidated.

Strength of study

This is the first study that investigated whether islet autoantibodies, C-peptide and T1D-GRS have diagnostic utility in ruling out patients from MODY genetic testing in the referral setting. The use of novel biomarkers ZnT8A and the T1D-GRS were also investigated. This study was also performed in the prospective referral setting, which studied a large cohort of patients referred prospectively for MODY genetic testing. In addition, all patients within this cohort had standardised genetic testing (*GCK, HNF1a* and *HNF4a* genes sequences), and sera were analysed in a single laboratory.

Limitations and Future areas of research

Due to the nature of the referral service, there were low numbers of MODY patients treated with insulin when they were referred, meaning the study could be underestimating the discriminative power of C-peptide and T1D-GRS in sub-analyses of insulin treated patients within this study. This was mainly due to some clinicians not providing treatment data on referral forms when their patients were referred. In order to capture data from every patient referred to the genetic service, an electronic referral form with mandatory fields should be created, where clinicians have to input a minimum amount of data before the referral form can be sent to the genetic service. This would be beneficial for both research purposes and the running of the genetics service. It would also be useful to investigate whether the data from the electronic referral forms could be transferred directly into the electronic patient database held at the genetic service. This would minimise issues with missing data within the database and reduce human error when transferring patient data from the current paper referral forms in to the patient database.

This study did not investigate whether biomarkers, especially islet autoantibodies, were able to exclude patients from genetic testing compared against rarer forms of monogenic diabetes, such as conditions caused by mutations to the *INS* or Peroxisome proliferator-activated receptor gamma (*PPARG*) genes. It would be of interest to look at the prevalence of islet autoantibodies in patients with rare forms of MODY referred to the genetic service compared to patients without a genetic diagnosis to see if islet autoantibodies retain its clinical utility. In addition, since this study was performed in the context of the referral setting, the results may not represent what happens in the setting of a diabetes clinic. Biomarkers investigated within this study may perform differently due to the difference in patient presented in the clinic setting. A study can be performed to look at a cohort of patients with newly diagnosed diabetes (T1D or T2D) with biomarkers measured and compared to newly diagnosed genetically confirmed MODY patients.

Although serum C-peptide was not useful within this study in discriminating non-MODY from MODY patients, it would be of interest to investigate the diagnostic utility of urine C-peptide : Creatinine ratio as a rule out test in the referral setting, as case control studies have suggested that the test has clinical utility in the diagnosis of MODY (14,15).

References

1. McDonald TJ, Colclough K, Brown R, Shields B, Shepherd M, Bingley P, et al. Islet autoantibodies can discriminate maturity-onset diabetes of the young (MODY) from Type 1 diabetes. Diabet Med. 2011 Sep 1;28(9):1028–33.

2. Wenzlau JM, Juhl K, Yu L, Moua O, Sarkar SA, Gottlieb P, et al. The cation efflux transporter ZnT8 (Slc30A8) is a major autoantigen in human type 1 diabetes. Proc Natl Acad Sci. 2007 Oct 23;104(43):17040–5.

3. Wenzlau JM, Walter M, Gardner TJ, Frisch LM, Yu L, Eisenbarth GS, et al. Kinetics of the Post-Onset Decline in Zinc Transporter 8 Autoantibodies in Type 1 Diabetic Human Subjects. J Clin Endocrinol Metab. 2010 Oct 1;95(10):4712–9.

4. Vaziri-Sani F, Oak S, Radtke J, Lernmark Å, Lynch K, Agardh C-D, et al. ZnT8 autoantibody titers in type 1 diabetes patients decline rapidly after clinical onset. Autoimmunity. 2010 Dec 1;43(8):598–606.

5. Miura J, Sanaka M, Ikeda Y, Watanabe C, Nakagami T, Iwasaki N, et al. A case of Type-1 diabetes mellitus formerly diagnosed as maturity-onset diabetes of the young (MODY) carrying suggestive MODY3 gene. Diabetes Res Clin Pract. 1997 Nov 1;38(2):139–41.

6. Aguilera E, Casamitjana R, Ercilla G, Oriola J, Nicoletti F, Gomis R, et al. Clinical characteristics, β-cell function, HLA class II and mutations in MODY genes in non-paediatric subjects with Type 1 diabetes without pancreatic autoantibodies. Diabet Med. 2005 Feb 1;22(2):137–43.

7. Bowden SA, Hoffman RP. Triple diabetes: coexistence of type 1 diabetes mellitus and a novel mutation in the gene responsible for MODY3 in an overweight adolescent. Pediatr Diabetes. 2008 Apr 1;9(2):162–4.

8. Schober E, Rami B, Grabert M, Thon A, Kapellen T, Reinehr T, et al. Phenotypical aspects of maturity-onset diabetes of the young (MODY diabetes) in comparison with Type 2 diabetes mellitus (T2DM) in children and adolescents: experience from a large multicentre database. Diabet Med. 2009 May 11;26(5):466–73.

9. Bingley PJ, Bonifacio E, Williams AJK, Genovese S, Bottazzo GF, Gale EAM. Prediction of IDDM in the General Population: Strategies Based on Combinations of Autoantibody Markers. Diabetes. 1997 Nov 1;46(11):1701–10.

10. Oram RA, Patel K, Hill A, Shields B, McDonald TJ, Jones A, et al. A Type 1 diabetes genetic risk score can aid discrimination between Type 1 and Type 2 diabetes in young adults. Diabetes Care. 2016 Mar;39(3):337–44.

11. Patel KA, Oram RA, Flanagan SE, De Franco E, Colclough K, shepherd M, et al. Type 1 Diabetes Genetic Risk Score: a novel tool to discriminate monogenic and type 1 diabetes. Diabetes. 2016 Jul;65(7):2094–9.

12. Hattersley A, Bruining J, Shield J, Njolstad P, Donaghue KC. The Diagnosis and Management of Monogenic Diabetes in Children and Adolescents. Pediatr Diabetes. 2009 Sep;10:33–42.

13. Clark PM. Assays for Insulin, Proinsulin(S) and C-Peptide, Assays for Insulin, Proinsulin(S) and C-Peptide. Ann Clin Biochem. 1999 Sep 1;36(5):541–64.

14. Besser REJ, Shepherd MH, McDonald TJ, Shields BM, Knight BA, Ellard S, et al. Urinary C-Peptide Creatinine Ratio Is a Practical Outpatient Tool for Identifying Hepatocyte Nuclear Factor 1-α/Hepatocyte Nuclear Factor 4-α Maturity-Onset Diabetes of the Young From Long-Duration Type 1 Diabetes. Diabetes Care. 2011 Feb 1;34(2):286–91.

15. Besser RE, Shields BM, Hammersley SE, Colclough K, McDonald TJ, Gray Z, et al. Home urine C-peptide creatinine ratio (UCPCR) testing can identify type 2 and MODY in pediatric diabetes. Pediatr Diabetes. 2013 Jan 4;14(3):181–8.