Improving diagnosis of adult-onset diabetes using islet autoantibodies

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PhD in Medical Studies

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Submitted by Sian Louise Grace to the University of Exeter as a thesis for the degree of Doctor of Philosophy in Medical Studies, in November 2022.

I certify that all material in this thesis which is not my own work has been identified and that any material that has previously been submitted and approved for the award of a degree by this or any other University has been acknowledged.

Signature: 

Abstract

The diagnosis of diabetes type in adulthood can be difficult due to overlapping phenotypes and lack of clear classification guidelines. Type 1 and type 2 diabetes have very different treatment and care requirements, and incorrect classification can lead to life-threatening consequences. Islet autoantibodies are biomarkers of the autoimmune pathology of type 1 diabetes and can be used in prediction of risk and classification of diabetes type, however current tests have imperfect specificity and sensitivity, and there are a number of remaining questions for optimal use.

The overall aim of this thesis was to refine the use of islet autoantibody testing in the diagnosis of adult-onset diabetes by:

1. Exploring optimal test thresholds, and whether these are influenced by age.

2. Determining whether islet antibody level has potential utility in patients with clinically diagnosed type 1 diabetes.

3. Examining whether recently developed assays to specific GAD epitopes, isotype and affinity can improve test utility.

In Chapter 1 we provide an introduction to diabetes and the different types, and present some of the difficulties surrounding diabetes classification. Next, we provide a detailed background into islet autoantibodies and review the current evidence for their use in clinical practice and challenges in interpreting their results.

In Chapter 2, we looked at whether the use of age-related positivity thresholds are necessary to improve zinc transporter 8 autoantibody (ZnT8A) assay performance using the commercially available RSR ELISA assay. Our first key finding was that the prevalence of detectable ZnT8A differed between those tested under and over 30 years of age in the general population. ZnT8A age-related positivity thresholds improved the specificity of the assay whilst maintaining sensitivity, and that using one positivity threshold can result in misclassification of diabetes type.

In Chapter 3, we looked at the utility of islet autoantibody level at diagnosis of type 1 diabetes. Our main finding was a bimodal distribution of levels of glutamate

decarboxylase (GADA) and islet antigen-2 autoantibodies (IA-2A) at diagnosis of type 1 diabetes, but not for ZnT8A. Those with high level GADA were older at diagnosis, more likely to be female and to be diagnosed with another autoimmune disease. In contrast, those with high level IA-2A were more likely to be younger at diagnosis and have ZnT8A as additional islet autoimmunity. This was replicated in a second cohort using an alternative method of islet autoantibody assessment. These findings increased our understanding of how islet autoantibody levels at diagnosis are associated with differences in the underlying pathology between age groups at diabetes diagnosis.

In Chapter 4 we looked at GADA epitope specificity, affinity and IgG subclass response in those positive for GADA, clinically diagnosed with type 2 diabetes in adult-hood. In full-length (f-)GADA positive adult-onset diabetes, our novel finding was that testing for truncated (t-)GAD(96-585) autoantibodies stratified risk of progression to early insulin therapy (within 5 years) and identified those with a more type 1 diabetes-like phenotype; lower C-peptide, higher type 1 diabetes genetic susceptibility and positivity for IA-2A. In contrast, testing for f-GADA affinity and IgG subclass response did not stratify risk of progression to early insulin requirement. These findings provide evidence to support the testing for t-GADA in adult-onset patients.

In Chapter 5, we summarize the key findings, the limitations of this work and its implications. Then we present ideas for future research and how to take these findings further.

In summary, here we have provided evidence for the improvement of islet autoantibody testing by taking three different approaches. Firstly, we have shown how using age-related cut-offs improves specificity of a commercially available assay and how these thresholds reduce the risk of misclassification. Secondly, that some differences between child- and adult-onset diabetes are associated with islet autoantibody level and thirdly, that testing for GADA epitope specificity in adult-onset diabetes, can help predict who will require early insulin therapy. Implementation of these findings in clinical testing will improve outcomes for individuals with diabetes.

Contents

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Abbreviations

LADA	Latent Autoimmune Diabetes in Adults
MODY	Maturity-Onset Diabetes of the Young
NICE	National Institute of Health and Clinical Excellence
WHO	World Health Organisation
CRF	Clinical Research Facility
DARE	Diabetes Alliance for Research in England
PRIBA	Predicting Response to Incretin Based Agents in Type 2 Diabetes
GoDarts	Genetics of Diabetes Audit and Research in Tayside Scotland
StartRight	Getting the Right Classification and Treatment From Diagnosis in Adults with Diabetes
ADDRESS-2	After Diabetes Diagnosis Research Support System
UKPDS	United Kingdom Prospective Diabetes Study
GADA	Glutamate decarboxylase 65 autoantibodies
IA-2A	Islet Antigen-2 autoantibodies
ZnT8A	Zinc Transporter 8 autoantibodies
T1D GRS	Type 1 diabetes genetic risk score
T2D GRS	Type 2 diabetes genetic risk score
HLA	Human Leukocyte Antigen
SNP	Single-Nucleotide Polymorphism
HbA1c	Haemoglobin A1c
BMI	Body Mass Index
UNITED	Using pharmacogeNetics to Improve Treatment in Early-onset Diabetes
ICA	Islet cell cytoplasmic autoantibodies
IAA	Insulin autoantibodies
UCPCR	Urinary C-peptide Creatinine Ratio
PPV	Positive Predictive Value
NPV	Negative Predictive Value

Chapter 1:

Introduction

1.1 Introduction Chapter Structure

This chapter is divided into 3 parts.

First the aims and structure of the thesis are stated. We then present a general introduction to diabetes, the different types and present some of the difficulties surrounding diagnosis and classification of diabetes, particularly in adults. Next we focus on islet autoantibodies, providing a detailed background and review the current evidence on their use in clinical practice and the current challenges in interpreting their results. In addition, each chapter has a focused introduction to that topic.

1.2 Aims and structure of thesis

The overall aim of this thesis is to provide evidence on how to improve the use of islet autoantibodies in clinical practice.

This thesis is divided into five chapters.

This chapter (Chapter 1) presents an introduction to diabetes, an overview of the challenges and considerations of using islet autoantibodies in the diagnosis, classification and management of diabetes, and highlights areas where their use could be improved.

Chapter 2 looks at whether different positivity thresholds, based on age, are needed to improve the specificity of ZnT8A testing and apply these to a cohort of mixed diabetes type.

Chapter 3 investigates whether islet autoantibody titre of GADA, IA-2A and ZnT8A at diagnosis of type 1 diabetes can provide additional information to the clinician above their positivity status.

Chapter 4 further characterises the GAD antibodies, commonly detected in adult-onset diabetes, to see if other GADA characteristics can predict early insulin requirement in adult-onset diabetes.

Chapter 5 is a discussion of the main findings and their implications, limitations and future work generated by each chapter.

1.3 General Introduction

1.3.1 Overview of diabetes

Diabetes is a disease in which the body's ability to regulate glucose (sugar in the blood) is impaired leading to hyperglycaemia (increased blood sugar level) which is a cause of serious health conditions such as nephropathy, neuropathy and diabetic retinopathy (1). Type 1 and type 2 diabetes are the two major subtypes of diabetes, with type 2 diabetes being the most prevalent.

1.3.2 Type 1 diabetes

Normal blood glucose levels are maintained by the peptide hormone insulin, produced by beta-cells in the islets of Langerhans of the pancreas. Type 1 diabetes (T1D) is characterised by the progressive autoimmune destruction of beta-cells, resulting in near-absolute insulin deficiency (2). Glucose levels therefore need to be tightly controlled through continuous glucose monitoring and patients are treated with exogenous insulin for life via continuous insulin administration by an insulin pump or multiple daily injections (3; 4). Prompt commencement of insulin therapy at diagnosis of T1D reduces the risk of diabetic ketoacidosis (DKA), where acids build up in the blood (5), which can cause life threatening complications such as cerebral oedema (6). Those that have had DKA have been said to have poorer glycaemic control and less beta cell function in the two years following diagnosis (6). The treatment requirements of T1D are a result of the development of severe endogenous insulin deficiency. Endogenous insulin secretion can be measured by C-peptide as it is produced in equal amounts to insulin. C-peptide can be used to assess endogenous insulin secretion, regardless of whether the patient is receiving exogenous insulin therapy (7). C-peptide measurement is widely available in clinical practice and in patients with longstanding diabetes is the gold-standard test for classifying patients based on their treatment requirements (7). A C-peptide level of <200 pmol/L (non-fasting) is indicative of severe endogenous insulin deficiency, and therefore can indicate T1D. However, high levels of C-peptide are often maintained in T1D at diabetes diagnosis and levels at diagnosis of type 1 and type 2 diabetes can overlap. Therefore, testing at diagnosis of C-peptide is thought to have limited utility, and other biomarkers, such as islet autoantibodies

can assist in classifying diabetes type at diagnosis. Control of T1D needs to be effective and sustained in order to prevent chronic complications (8).

1.3.3 Type 2 diabetes

Type 2 diabetes (T2D) is a progressive metabolic disease where patients usually continue to produce insulin, but at an insufficient level to the body's requirement, due to insults to the beta cells which are likely to be non-autoimmune and multifactorial. Beta cell failure is compounded by insulin resistance, leading to dysregulation of glucose levels (9). This in turn feeds back, leading to the beta cells becoming exhausted and having a reduced capacity to make insulin (10; 11). Patients with T2D can initially be treated successfully with lifestyle changes and/or oral agents for many years (12-14), and remission can be achieved with successful weight loss (15). However, due to the progressive reduction in the beta cells ability to produce insulin, eventually many will require insulin therapy (13).

1.3.4 Slowly evolving immune-mediated diabetes

Formerly known as latent autoimmune diabetes of adults (LADA), slowly evolving immune-mediated diabetes is typically diagnosed in patients that present with the clinical characteristics of T2D, over the age of 35 years and have an islet autoantibody detected (most commonly glutamate decarboxylase autoantibodies (GADA)) (16; 17). This group of patients do not initially require insulin therapy from diagnosis, and can be managed initially with lifestyle changes and/or oral agents like T2D. However, some will progress to severe insulin deficiency and require insulin more rapidly (18). There is controversy on whether this represents true slowly evolving immune-mediated diabetes, or a mixture of those with and without autoimmune aetiology diabetes, due to the imperfect specificity of islet autoantibody testing and the resulting low positive predictive value in the setting of low prior likelihood (discussed in detail in **1.6.3.1**) (19).

1.3.5 Importance of diabetes diagnosis for clinical management

Delays in getting the right treatment for patients due to misdiagnosis of diabetes type can severely affect an individual's diabetes management, resulting in poor glycaemic control and worsening diabetes complications (20; 21). Therefore it is of vital importance to ascertain whether a patient has T1D or T2D, those who

have T1D who are treated as they have T2D (without insulin) can become severely unwell with ketoacidosis, and a person diagnosed as type 1 who does not have this condition can receive a lifetime of unnecessary insulin treatment. Difficulties in obtaining a correct diabetes diagnosis can be attributed to the high prevalence and increasing incidence of T2D in adults, overlapping phenotypes of diabetes types and lack of clear separation by clinical features and biomarkers (22; 23).

1.4 Islet Autoantibodies

1.4.1 Introduction to islet autoantibodies

Autoantibodies to islet cell antigens such as insulin (IAA), glutamate decarboxylase 65 (GADA), islet antigen-2 (IA-2A) and zinc transporter 8 (ZnT8A) are biomarkers of the beta cell autoimmunity that occurs in T1D (24). These islet antigens are primarily found in the insulin secretory granules, however GAD65 is commonly found in a separate microvesicle (Figure 1). Assessment of islet autoantibodies may be used in clinical practice to assist the diagnosis of diabetes subtypes, and may also be potentially used to identify those who are at future risk of developing T1D or progressing rapidly to insulin therapy (25-29). Islet antibody measurement may be increasingly relevant to clinical practice, with recent evidence that clinical features are insufficient to differentiate type 1 from type 2 and monogenic diabetes in many cases, and high reported rates of misclassification (22). Recent guidance now recommends routine testing in all adults diagnosed or suspected to have T1D (30). While islet autoantibodies are not usually measured routinely in those diagnosed with T2D, this has been recommend by some groups (31), and is routinely recommended where there is clinical uncertainty (32). Islet autoantibody measurement is also required before genetic testing for monogenic diabetes (32). In addition, recent advances in islet autoantibody testing and international efforts to improve test standardisation have led to these tests becoming more accessible, reproducible and reliable (33). The Diabetes Antibody Standardisation Program (DASP), set up in 2001 and now continues as Islet Autoantibody Standardisation program (IASP), aimed to standardise islet autoantibody assays and evaluate and improve laboratory performance. Limitations, clinical uses and analytical and clinical considerations of islet autoantibodies and their assessment are discussed in **1.6** and **1.7**.

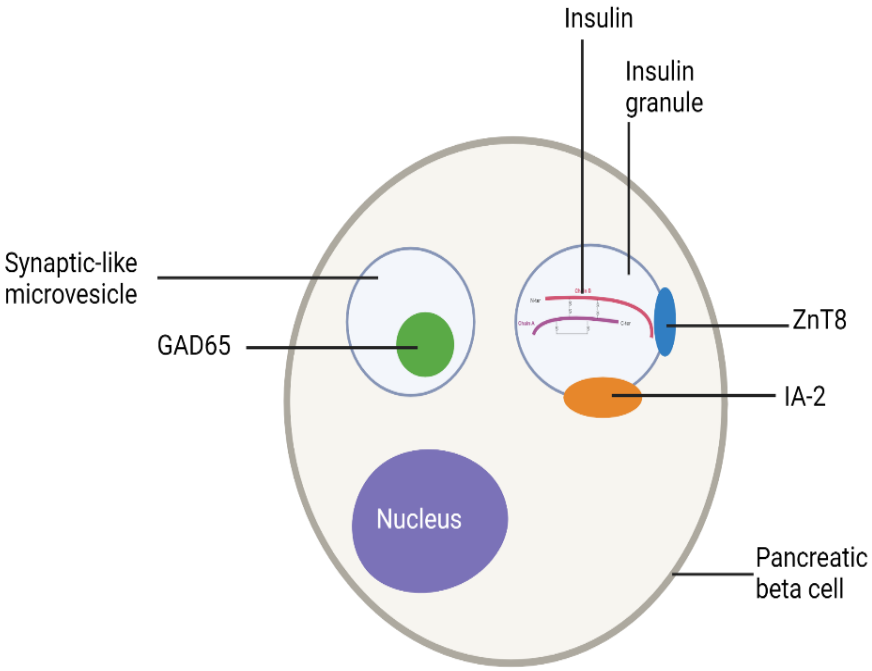

Figure 1: *Schematic diagram of the pancreatic beta cell illustrating the locations of the four major antigens that islet autoantibodies recognise: GAD65 (Glutamate decarboxylase), insulin, IA-2 (Islet antigen-2) and ZnT8 (Zinc transporter 8). (Created with BioRender.com).*

1.4.2 GADA

GADA are the most commonly available islet autoantibody test to clinicians (Figure 2; unpublished data by Cohen and Narendran, Birmingham, U.K.) and have been extensively characterised in terms of their use as sensitive markers of islet autoimmunity and T1D (34). Often reported as one of the first to appear and most prevalent autoantibody at onset of T1D in children (35). They are also being routinely used as a biomarker for autoimmune diabetes in adults across multiple ethnicities (36-38). GADA is also widely used for the recruitment of subjects at high risk of T1D to prevention trials and is essential in screening strategies for T1D. IASP workshops have led to major improvements in GADA assay performances and comparability along with the development of a harmonised radioimmunoassay (RIA) which introduced common working calibrators, units and method and resulted in high concordance between laboratories (39). The most recently published IASP results, suggest that developed non-radioactive tests for GADA assessment have proven to be as good or exceed the

performance of the previous radioactive gold standard assay format for GADA measurement (33).

1.4.3 IA-2A

IA-2A are more commonly detected at diagnosis of T1D in children and adolescents (~60% have detectable IA-2A at diabetes onset) and often appear after seroconversion to GADA or IAA first (38; 40). The prevalence of IA-2A at diagnosis has increased significantly since 1985, and this has been mirrored in raised levels of IA-2A (40), although this was not replicated in a Danish study (41). As with GADA, testing for IA-2A has become increasingly available to clinicians in recent years with the commercially available ELISAs performing comparatively to the harmonised and gold standard RIA. IA-2A was the 3rd commonly available assay at 27 UK hospitals surveyed (Figure 2). Harmonisation of the IA-2A RIA was conducted at the same time as GADA by National Institute of Diabetes and Digestive Kidney Diseases (NIDDK) and The Environmental Determinants of Diabetes in the Young Study (TEDDY), based upon information provided by the IASP working group (42).

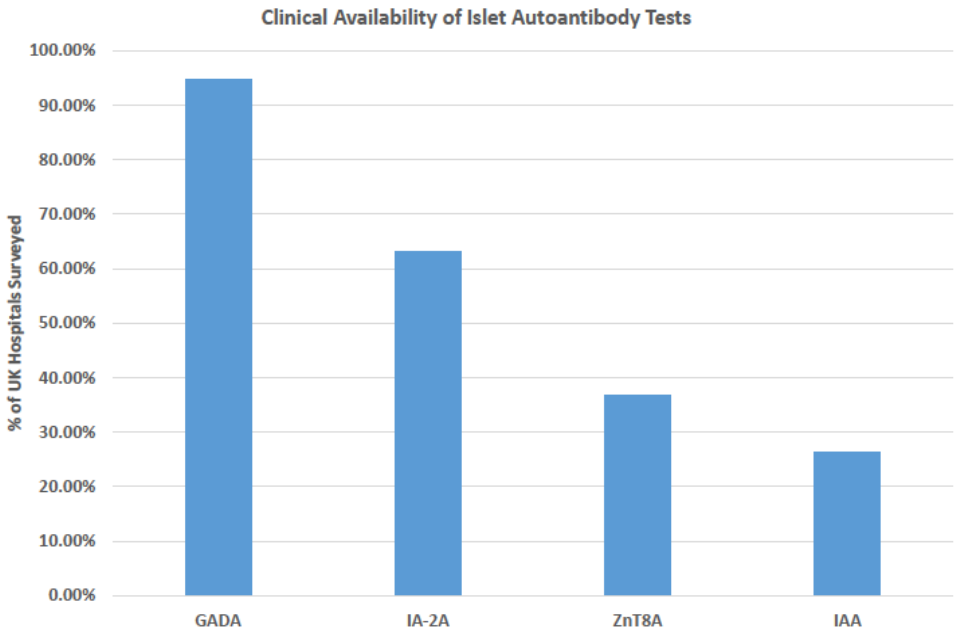

Figure 2: *Bar chart showing the clinical availability of the four major islet autoantibody tests from 27 hospitals (unpublished data by Cohen and Narendran, Birmingham, U.K.)*

1.4.4 ZnT8A

ZnT8A are the latest islet autoantibody test that has become available to clinicians, found in 66-80% of Caucasian patients at diagnosis of T1D (43). ZnT8A have been shown to increase the number of people identified as single or multiple-antibody positive at diabetes onset. Like IA-2A, ZnT8A typically arise later in the pathogenic process than GADA or IAA, are more common in children and adolescents than adults at diagnosis and the prevalence has increased in those diagnosed with T1D under the age of 21 years since 1985 (40). Adding ZnT8A testing to a prior panel of islet autoantibody testing (GADA, IA-2A and IAA), increased the diagnostic sensitivity of islet autoantibody testing at diagnosis of T1D from 90 to 93% (44). ZnT8A assays have been added to the DASP/IASP workshops, and work to standardise ZnT8A measurement is ongoing. However, as they were first characterised in 2007, after many of the major natural history of diabetes studies had begun, ZnT8A is not often fully evaluated because they were not part of the planned strategies (45).

1.4.5 IAA

Whilst the presence of IAAs is frequently detected in children with T1D (>70%), testing in adults is less appropriate due to peak IAA seroconversion occurring in childhood, and therefore testing for IAA in adulthood is not helpful and adds very little to the specificity of islet autoantibody testing panels (46). In childhood-onset diabetes, IAAs can occur as a primary antigen, often occurring in the very young and appearing before GADA (35). Testing for IAA, in addition to GADA, IA-2A and ZnT8A, can provide an autoimmune classification of diabetes type in those previously found to be islet autoantibody negative, and also improve the identification of multiple autoantibody positive children (47). High titre antibodies can develop against exogenous insulin therapy and these can be detected by current IAA assays. Therefore testing for IAAs in someone on insulin therapy for 2 weeks or more is not recommended (48). Even after multiple rounds of DASP/IASP workshops, the concordance of IAA assays between laboratories is low (49). The most sensitive and specific assays are the micro-radioimmunoassays (microRIA) conducted by specialist laboratories, explaining

why fewer clinical laboratories offer this test (50), with only 5 hospitals surveyed offering IAA testing (Figure 2).

1.4.6 Islet autoantibodies, genetics and diabetes

1.4.6.1 Genetics and Diabetes

A complex interplay between genetic and environmental influences drive the development and progression of T1D. Familial and twin studies suggest that ~50% of T1D susceptibility is due to genetic factors (51). This genetic component was initially identified in the 1970s and is commonly measured using single nucleotide polymorphism (SNP) genotyping (52), mainly identified by genome-wide association studies (GWAS) (53). The location of these SNPs are in both the human leukocyte antigen (HLA) and non-HLA regions (54; 55), with the familial heritability of genetic risk in T1D being relatively equal between the two regions (56; 57). The HLA conferring the highest risk of T1D are *(DR3)-DQA1*05-DQB1*02* and *(DR4)-DQA1*03-DQB1*0302* with approximately 80-90% of T1D cases having either haplotype, with 30-40% having both (54). Those at lowest risk of T1D, have an absence of DR3 and/or DR4 and have either one or two of the following protective haplotypes: *(DR15)-DQB1*0602*, *(DR13)-DQB1*0603*, *(DR5)-DQA1*05-DQB1*0301* and *(DR7)-DQA1*0201-DQB1*0303* (58). Assessment of combinations of SNPs, from both HLA and non-HLA regions have led to the development of a T1D genetic risk score (T1D GRS) which can discriminate between patients with T1D and T2D (52), with this discriminative ability being independent and additive to that of islet autoantibodies, BMI and age at diagnosis. The T1D GRS is not yet routinely available to clinicians due to a number of technical and logistical issues that need to be addressed (59). Similarly, genetic studies have identified genetic variants associated with T2D (60), and a GRS for T2D has been produced, however it is less effective at discrimination (52).

1.4.6.2 Genetics and Islet Autoantibodies

In natural history of diabetes studies, IAA have been associated with younger age of onset at seroconversion and the *HLA-DR4-DQ8* genotype, whereas GADA have been associated with an older age of onset at seroconversion, common over a wider age range and the *HLA-DR3-DQ2* genotype (61-63). *HLA-DR4* was

also associated with IA-2A positivity and the *HLA-A*24* genotype was negatively associated with IA-2A and ZnT8A (64-66). However, once an individual was found to be multiple islet autoantibody positive, the *DR-DQ* haplotypes do not appear to influence progression of diabetes (67; 68).

The gene *SLC30A8* encodes for ZnT8, and the common SNP rs13266634 (C/T), causes a non-synonymous modification that changes the C-terminal amino acid 325 (aa325) to either arginine (R) or tryptophan (W) (69). Whilst *SLC30A8* does not confer or influence T1D risk, its main effect in T1D is on ZnT8A specificity, with those positive for ZnT8A having either a tryptophan (ZnT8W) or arginine (ZnT8R) –specific autoantibody response (70). The allele encoding R (homozygous CC genotype) at aa325 (R325) confers a slightly increased risk of T2D (71-73).

1.5 Measuring and characterising islet autoantibodies

1.5.1 Historical ICA Method

Unlike the autoantibodies mentioned above, testing for islet cell cytoplasmic autoantibodies (ICA) uses a technique called indirect immunofluorescence, which occurs on pancreas sections. Positivity is determined through sample titration and comparison to established Juvenile Diabetes Foundation (JDF) standard of known fluorescence intensity, with results expressed as JDF units. The international use of JDF standards improved ICA standardisation and protocol harmonisation between laboratories, but ICA assessment is still subjective (74-76). This technique is technically demanding and difficult to standardise (42). Both GADA and IA-2A have been found to contribute to ICA staining (77; 78), and with autoantibodies to islet autoantigens being reproducibly detected they are currently the best-validated and more widely used tests for islet autoimmunity with ICA assays being largely superseded (25).

1.5.2 Current Methods

With the development of antigen-specific fluid-phase RIAs that were quantifiable and less subjective than ICA assessment, they became the conventional way to measure GADA, IA-2A and ZnT8A. They involve the incubation of serum with the appropriate radionuclide-labelled antigen fragments and subsequent immunoprecipitation of the radiolabelled-antibody complex by Protein G and/or

Protein A Sepharose (PGS and/or PAS, respectively). Radiolabelled-antibody immune complexes are then detected by scintillation detectors where residual radioactivity is expressed as counts per minute (cpm) and are proportional to autoantibody concentration (Figure 3).

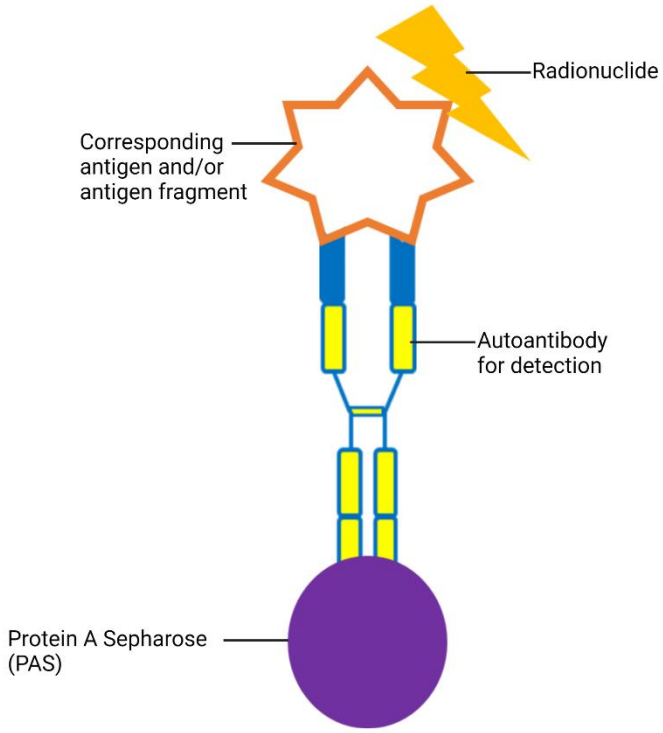

Figure 3: *Schematic diagram of the radioimmunoassay. Autoantibodies in serum bind to 35-S radiolabelled antigen (GADA/IA-2A/ZnT8A) or 125-I radiolabelled antigen (IAA). Immmune complexes are then precipitated using Protein A Sepharose (PAS) to bind the Fc region of the autoantibody. The unbound excess radiolabelled antigen is excluded by serial wash and centrifugation steps. After the addition of MicroScint40 (Perkin Elmer), residual radiation in counts per minute (CPM) is detected on a beta scintillation counter. (Created with BioRender.com).*

Optimisation of RIAs has substantially improved the detection of islet autoantibodies in small quantities of serum. As stated previously, detection of IAA is by microRIAs which now only use ~50µl per test (for a positive with repeat testing), in comparison to up to 600µl previously (79). However, the use of radioisotopes in autoantibody testing limits their long-term sustainability and the adoption of these assays in laboratory and clinical settings. This is due to the radioisotope costs, their short shelf lives (radioactive decay), and the tight regulations that surround radioisotope use regarding storage and disposal procedures for safety and environmental reasons.

Other established methods for detecting antigen-specific autoantibodies that do not rely on radiolabelled-antigen tracers are solid-phase bridge enzyme-linked immunosorbent assays (ELISAs) (80; 81) and electrochemiluminescence (ECL) assays (82-84). Bridge-type ELISAs are commercially available through RSR Limited (Cardiff, U.K.) to measure GADA, IA-2A and ZnT8A. In short, the serum is incubated onto a microplate coated with recombinant antigen (solid-phase) and resultant immune complexes are detected through a biotin-streptavidin-peroxidase system which creates a colourogenic reaction that is detectable by an ELISA plate reader (80; 85; 86). These ELISAs are routinely used, and have a 1-day duration. In contrast, the 2-day ECL assay utilises bivalent autoantibodies in serum to cross-link between an antigen with a Sulfo-TAG (Meso Scale Discovery [MSD], Rockville, MD, USA) and a biotinylated antigen to create immune complexes which are detected by an ECL signal on an MSD Sector Imager 2400 (87).

Whilst ECL and ELISA assay approaches are non-radioactive and have shown high performance (80; 83-87), both have limitations which can impact their use in general population screening and wide-spread clinical use. For ELISAs, the use of a solid-phase can obscure antigenic epitopes for autoantibody binding, and the serum requirement is ~50µl/per test. Low sample volume requirements are necessary where capillary bleeds, from young children, research studies and the general population, are to be utilised. Whilst the ECL assay has a more modest serum requirement (15µl/per test), the serum requires acid treatment, streptavidin-coated plates have to be prepared in advance and per test, two separate antigens need preparation. These extensive processes can be time-consuming and costly.

1.5.3 Methods in development

Due to the limitations with the current methods described above, there is a need to develop rapid, low-volume, nonradioactive, high performance and simpler assay alternatives to replace the RIAs and overcome the limitations associated with ECL and ELISA assays. The ability to detect multiple autoantibodies at once and to fully automate the assay procedure would be advantageous in general population screening and in the research and clinical settings.

Recently, luciferase immunoprecipitation assay systems (LIPs) have been developed to provide an inexpensive, low-volume and nonradioactive alternative to RIAs. By using some of the same assay components and steps as the liquid-phase RIAs (Figure 4), but substituting the radioactive tracer for an antigen coupled with a NanoLuc™ luciferase reporter (NLuc), they can maintain some of the benefits of the liquid-phase RIAs negating the limitations associated with radioisotope use (49).

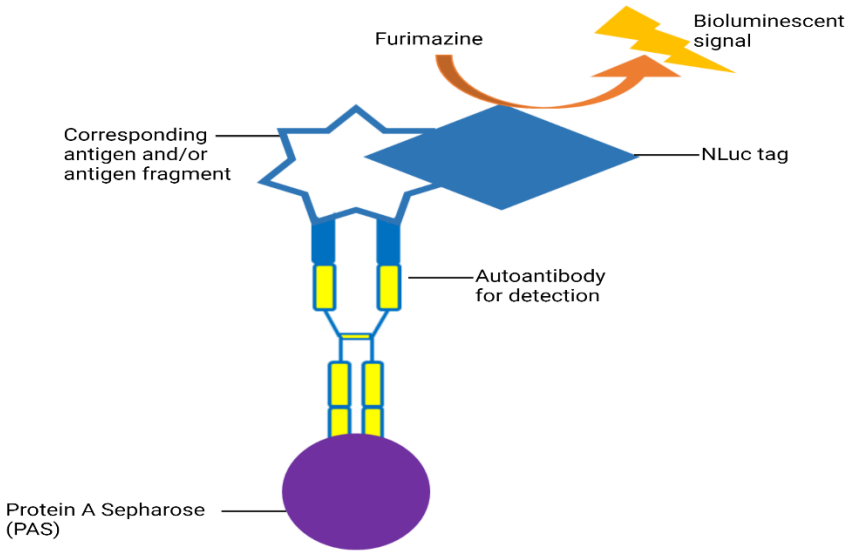

Figure 4: *Schematic diagram of the luciferase immunoprecipitation assay. Autoantibodies in serum specific for the islet autoantigen bind to a Nanoluciferase-tagged (NLuc-) islet autoantigen (GAD, IA-2, ZnT8, insulin). Immune complexes are then precipitated using Protein A Sepharose (PAS) to bind the Fc region of the autoantibody. Serial washes and centrifugation steps exclude unbound excess Nluc-tagged islet autoantigen. After adding the substrate Furimazine (Promega), a bioluminescent signal is produced and detected with a luminometer where the luminescence produced is proportional to the autoantibody level present in serum. (Created with BioRender.com).*

The LIPs assays are advantageous due to their low-volume serum requirement (2µl/per test), their 1-day duration, the nonradioactive tracer, longer shelf life of tracer (months or even years vs. weeks for RIAs) and the use of widely available commercial reagents/equipment, already used by laboratories conducting RIAs (49). However, the placement of the NLuc tag needs to be carefully placed in the antigen sequence so as not to affect antigen conformation and consequently, the autoantibody-antigen binding.

The most recently developed low-volume and nonradioactive immunoassay is antibody detection by agglutination-PCR (ADAP). In brief, antigen-specific

autoantibodies in serum (2µl) are agglutinated by antigen-specific DNA conjugates, which enables DNA ligation and resultant quantification by qPCR. This assay is a simple method that utilises standard PCR consumables, has enhanced sensitivity and a broad dynamic range (88).

Both these methods in development have performed well in IASP workshops over recent years and offer several advantages above the current gold-standard RIAs (33; 89). One key feature of the LIPs, ADAP, ELISA and ECL methods above the current RIAs is that there are ways in which these can be multiplexed for the detection of multiple markers in a single test, making them promising for future use in general population screening, and with further adaptations, could be used in point of care testing.

1.5.4 Islet autoantibody characteristics

Developments in the current methods of islet autoantibody testing have allowed us to further characterise the islet autoantibodies by assessing their epitope specificity, their affinity and we can also tease apart the isotype response. Each characteristic and how it can be assessed is further described below.

1.5.4.1 Islet autoantibody epitope specificity

Epitopes are the 3D peptide domains of an antigen that an antibody “recognises” and binds to. In conventional immunoassays, epitope analysis can be conducted by replacing the radiolabelled antigen with a truncated or mutated version. A nonradioactive GADA epitope analysis is described in detail in Chapter 4.

For GADA and IA-2A specific epitopes have been identified that are associated with risk of progression to T1D, but no convincing high-risk epitope has been characterised for IAA (82; 90). Full-length GAD65 [amino acid (aa) 1-585] is commonly used to detect GADA in T1D. However, through different truncations of the GAD65 antigen, epitope analysis showed that most GADA recognise the middle- and COOH-catalytic domains and bind to the NH_2-terminal poorly (90-92). Truncation of the NH_2-terminal (aa1-95) in the current RIA led to improved assay specificity and discrimination of first-degree relatives with higher risk of progression to diabetes (93; 94). For IA-2A, the antigen is made up of an extracytoplasmic (EC) domain, a transmembrane region and an intracytoplasmic (IC) domain [subdivided into juxtamembrane (JM) and protein tyrosine

phosphatase (PTP) domains]. The IC domain, overall, has been identified as the major region of IA-2 that is recognised by IA-2A in new-onset T1D, but IA-2A also recognise the PTP-region of the homologue IA-2β (82; 90; 95; 96). Whilst reactivity to IA-2 epitopes have varied in studies of T1D risk, reactivity of IA-2A towards multiple epitopes and the discovery of epitope spreading, is associated with the identification of individuals at high-risk of diabetes (24; 90; 97-99).

1.5.4.2 Islet autoantibody affinity

Autoantibody affinity is described as how strong the autoantibody-antigen (epitope) interaction is at the antigen-binding site. Assessment of autoantibody affinity is carried out by competitive displacement immunoassays, where small amounts of unlabelled corresponding antigen is added in increasing concentrations (90). High-affinity autoantibodies require very little unlabelled antigen to be displaced from the radiolabelled antigen (mean CPM reaches assay background at lower unlabelled antigen concentrations). This is because the autoantibody will preferentially bind to the unlabelled antigen. Conversely, low-affinity autoantibodies require higher concentrations of unlabelled antigen to be displaced from the radiolabelled antigen (mean CPM remains high at higher unlabelled antigen concentrations). This method is described in detail in Chapter 4.

Generally, high-affinity autoantibodies have been associated with multiple markers of high-risk of progression to diabetes. For IAA, high-affinity autoantibodies have been associated with positivity to multiple islet autoantibodies, the *HLA-DR4* haplotype and younger age at IAA seroconversion (100-102). High-affinity autoantibodies to GADA were also associated with multiple islet autoantibody positivity, but also with the *HLA-DR3* haplotype (90; 103; 104). In contrast, affinity of IA-2A has not been seen to be associated with progression or risk of T1D (105), but this may be due to the rarity of low affinity IA-2A.

1.5.4.3 Islet autoantibody IgG subclass response

The presence of the different IgG subclasses (IgG1, IgG2, IgG3, IgG4) in the islet autoantibody response can be investigated using the conventional RIAs by replacing the PGS and/or PAS, used to immunoprecipitate the antibody-antigen

complex, with biotinylated IgG subclass-specific mouse anti-human monoclonal antibodies bound to Streptavidin Sepharose beads (24). This method is described in detail in Chapter 4.

The presence of an unrestricted IgG subclass response (presence of IgG2-IgG4, in addition to IgG1) was associated with an increased risk of T1D for IA-2A and IAA, but not for GADA. Additionally, the number of subclasses present was associated with higher autoantibody levels, suggesting that higher levels of IA-2A and IAA can discriminate risk (24; 106).

1.6 Analytical and clinical considerations of islet autoantibody testing

1.6.1 Introduction

There are a number of aspects of islet autoantibody testing to consider when choosing which tests to use and interpreting and using their results to inform diabetes diagnosis and management. For ease, we have split these into analytical considerations (concerning how the assay is run) and clinical considerations (including patient and population characteristics).

1.6.2 Analytical considerations

1.6.2.1 Test threshold and specificity

Islet autoantibodies are a continuous variable, often found at low detectable levels within the general population (approximately 1-2%), and thus interpretation requires well-defined thresholds to be set using a robust control population (107; 108). Without well-defined cut-offs for positivity, low clinical specificity may result in false positive and false negative results and sera may be found to be discrepant between laboratories and assays (107). Whilst standardisation and harmonisation programmes have improved the sensitivity, specificity and concordance of islet autoantibody assays, the process of threshold definition is not standard across all laboratories with various different sized and aged control populations used. Further, in the 2018 IASP program, only 37 laboratories submitted results for their GADA assays (33), therefore there must be a large number of laboratories testing for islet autoantibodies that do not take part in the program. There are also many different variations of autoantibody assays used (9 different assay formats for GADA were assessed in the 2018 IASP program

(33)), and taken with varying positivity thresholds, limits the ability to compare prevalence and levels of islet autoantibodies across national and international studies.

1.6.2.2 False positivity

Detection of islet autoantibodies is not necessarily diagnostic of autoimmune aetiology diabetes, due to detectable islet autoantibodies being observed in the general population without diabetes; the presence of an autoantibody does not necessarily mean that (T-cell mediated) beta cell destruction is ongoing at the islet level. Single positive islet autoantibodies have a very modest predictive ability for the development of autoimmune diabetes (22% 5 year risk of progression to diabetes in TrialNet) (109). Imperfect test specificity resulting from non-robust threshold setting can lead to false positive results that are likely to be due non-disease associated (disease-irrelevant) autoantibodies, this is especially a problem when testing populations where autoimmune diabetes is unlikely (Bayes theorem) (25; 110; 111).

Positivity for multiple islet autoantibodies has high specificity for T1D, which increases with each additional autoantibody positivity (112). There is a greater than 80% risk of developing T1D by age 20 if multiple islet autoantibody positivity is detected in early childhood (28).

The presence of high levels of GADA has been described in several severe autoimmune pathologies of the central nervous system, (for example: stiff man syndrome, cerebellar ataxia and Batten disease), these levels of GADA detected are usually 100-fold higher than those found in T1D (77; 113). In autoimmune polyendocrinopathy (APS), GADA, IA-2A, ZnT8A and IAA can also be detected. GADA in these patients is relatively common and has a strong association with gastrointestinal dysfunction, whereas IA-2A, ZnT8A and IAA in these patients are rare and tend to associate with concurrent or future development of T1D (82; 114; 115).

1.6.2.3 False negativity

If T1D is suspected but there is a negative autoantibody result, this does not exclude the possibility of a diagnosis of T1D. One study reported ~10% of those diagnosed with T1D under the age of 18 years were found to be islet autoantibody

negative with this percentage increasing with age of diagnosis (13% and 26% found to be islet autoantibody negative, for those diagnosed with T1D between 18-30 years and those diagnosed >30 years, respectively) (116). These individuals may have autoantibodies to autoantigens which have not yet been characterised (117), be from ethnicities where the prevalence of islet autoantibodies is reduced or the islet autoantibody level has fallen below detectable limits (118-120), or may have other (non-autoimmune) causes of their diabetes. The lower rate of positive islet autoantibodies in adults diagnosed with T1D may potentially represent misclassification of diabetes type (121; 122).

Negative islet autoantibody results may lead the clinician to suspect an alternative type of diabetes such as maturity-onset diabetes of the young (MODY) or ketosis-prone T2D (123). A negative islet autoantibody result in a minority (<5%) of children in one study were shown to have monogenic diabetes (116), and therefore multiple negative islet autoantibody results may help stratify those who should receive genetic testing in children.

1.6.3 Clinical considerations

1.6.3.1 Prior prevalence

The prior prevalence of autoimmune diabetes within a population should be taken into consideration when interpreting islet autoantibody results as it has a significant effect on the implications of a positive result. The positive predictive value (PPV: the % with disease when the test is positive) of an islet autoantibody assay is highly dependent of the prevalence of autoimmune diabetes and can be calculated using the following equation (124):

$$\text{Positive Predictive Value} = \frac{\text{sensitivity} \times \text{prevalence}}{(\text{sensitivity} \times \text{prevalence}) + ((1 - \text{specificity}) \times (1 - \text{prevalence}))}$$

The PPV of an islet autoantibody assay detecting T1D will be lower in an adult population where the prior prevalence of T1D is low (~5%). Therefore those who test positive in this population are more likely to be a mixture of T1D (true positives) and T2D or disease-irrelevant autoantibodies (false positives) than in a higher prevalence of autoimmune diabetes population such as children and

adolescents with T1D, where the proportion of false positives will be lower (Figure 5). The negative predictive value (NPV: the % without disease when the test is negative) is equally affected by the prior prevalence of autoimmune diabetes within the population and can be calculated using the below equation:

$$\text{Negative Predictive Value} = \frac{\text{specificity x (1 – prevalence)}}{\text{(specificity x (1 - prevalence)) + ((1 – sensitivity) x prevalence)}}$$

As such when the prior prevalence of autoimmune diabetes is low, the NPV of an islet autoantibody assay within the population is higher. Consequently a negative islet autoantibody result in an adult population makes T1D an unlikely diagnosis, whereas a negative result in a higher prevalence population does not necessarily rule out T1D.

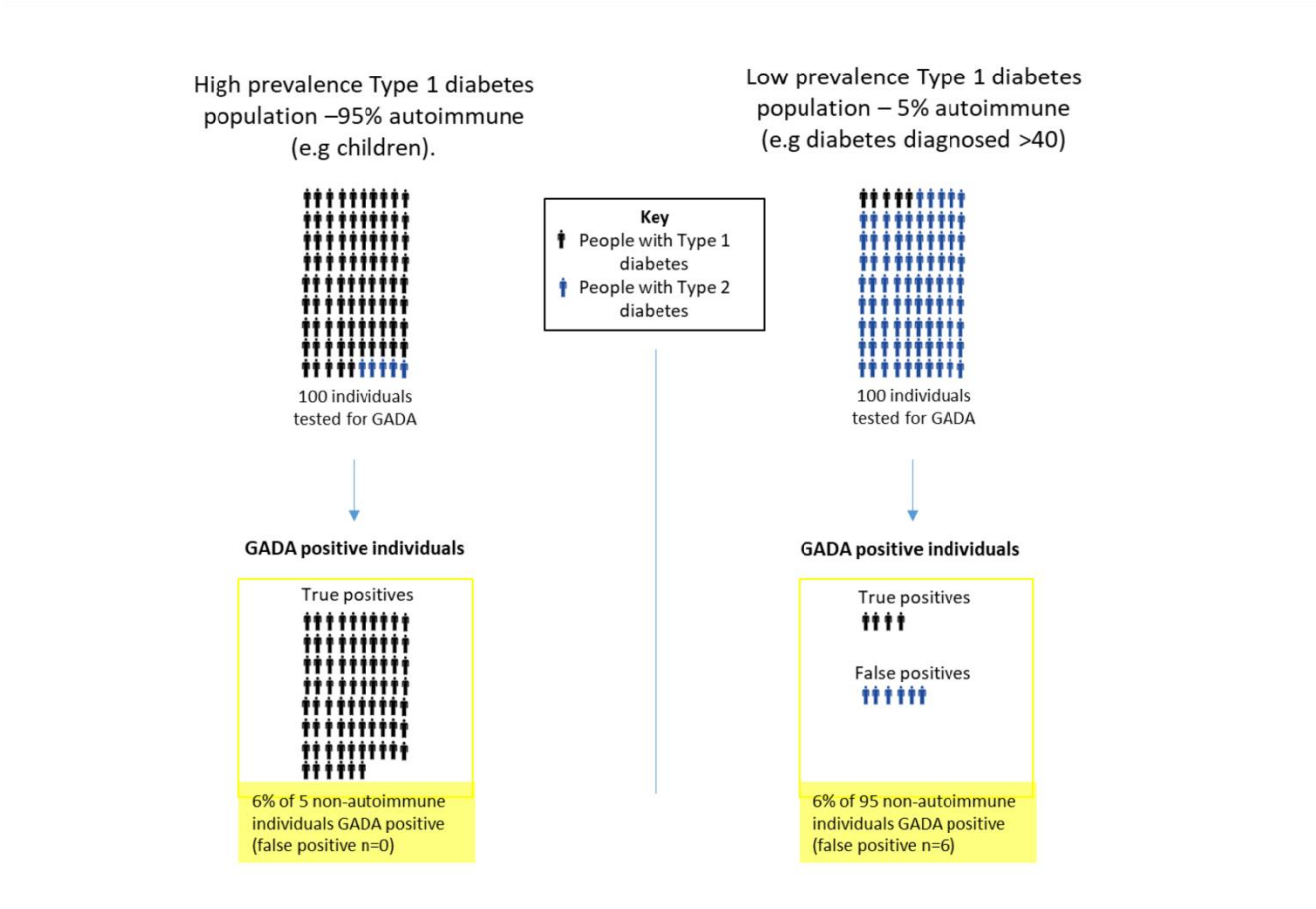

Figure 5: *Proportion of GADA (Glutamate decarboxylase autoantibody) positive individuals who have autoimmune aetiology diabetes in 95% and 5% prevalence population. Expected results from testing 100 participants, using median GADA assay performance from the 2010 IASP Workshop (assay specificity 94%, sensitivity 86%). Adapted with permission from Jones et al (19) using BioRender.com.*

1.6.3.2 Age at testing

Prevalence of islet autoantibodies can vary by age at testing, with a general overall decreasing frequency of islet autoantibodies as age at testing increases. IAA are particularly common in the very young, and rare in adult-hood, therefore testing for IAA in older populations would have less clinical utility (125). GADA are prevalent at any age; some studies suggest GADA and ZnT8A, are less common in those under age 10, but not all studies agree (125-127). In adult-onset diabetes (>20 years) GADA are more common than IA-2A and ZnT8A (126; 128), whereas, IA-2A tend to have high prevalence in those <10 years and in adolescents (120; 125). Age at diagnosis can significantly impact on the persistence of islet autoantibodies over time. Those diagnosed younger tend to have the biggest and quickest decline in islet autoantibody titres over a shorter disease duration (129; 130). Therefore age at testing should influence islet autoantibody testing strategy.

1.6.3.3 Diabetes duration at testing

Duration of T1D should be considered when testing for islet autoantibodies. The longest running diabetes studies (with >50 years diabetes duration), the Golden Years cohort (U.K.) (131) and Joslin Medallist program (USA) (132), both cohorts of survivors, show that autoantibodies can persist for decades after diabetes diagnosis. GADA were the more likely to persist, followed by ZnT8A (only measured in Golden Years Cohort) and then IA-2A. However GADA prevalence did vary between the two studies (48% in Golden Years vs. 18.4% in Joslin Medallists). In a study where diabetes duration was between 10 and 50 years, the Bart's Oxford (BOX) Family Study (U.K.), IA-2A was the most prevalent (52%), followed by GADA (32%) and ZnT8A (14%) (133). Other studies with 10-50 years diabetes duration reported GADA (50% and 21%) as the most prevalent, followed by IA-2A (30% and 20%) and ZnT8A (28 and 7%), with ZnT8A being lost exponentially (134; 135). There are more studies analysing autoantibodies within the first 10 years of diabetes duration. After 5 years duration in one study GADA prevalence was maintained (61% vs. 66%), but IA-2A (54% vs. 40%) and ZnT8A (61% vs. 34%) prevalence was lower (136). In a Danish study, after 3-6 years duration, prevalence of GADA, IA-2A and ZnT8A had all decreased (137). Some studies also suggest that islet autoantibody positivity can be gained post

diagnosis (133-135; 138). To summarize, GADA tends to be the most prevalent post diabetes diagnosis than IA-2A and ZnT8A, but this depends on the prevalence of each islet autoantibody at diagnosis; with ZnT8A levels more likely to decline rapidly from clinical diagnosis (120; 139). The above studies show that autoantibody loss and prevalence can vary with disease duration, with greater variation occurring with diabetes duration >10 years. Therefore, the false negative rate of autoantibodies will increase with diabetes duration. It would be best practice to test for islet autoantibodies as close to onset as practical, and in those with long-standing diabetes (>5-10 years), due to the variability in islet autoantibody levels and prevalence, C-peptide testing could have greater clinical utility in differentiating T1D from other diabetes subtypes in long-standing diabetes than islet autoantibodies (140). C-peptide is reflective of the rate of beta cell failure, and therefore the degree of need for insulin therapy (7).

1.6.3.4 Ethnicity

The majority of studies into islet autoantibodies have been conducted in white European populations where the incidence of T1D is high, such as Finland where the prevalence and incidence of T1D is the highest in the world (141). The characterisation of islet autoantibodies in non-white ethnic groups is understudied (142). There is some evidence to suggest that migrant populations adopt the local risk of T1D in the country they now live (143-145). It is suggested that the prevalence of islet autoantibody positivity in these populations may be similar to that of the white ethnic populations (145-148). Data are increasing but incomplete autoantibody testing predominates and the picture is not complete. However, in a study conducted of T1D by Bravis *et al* in the U.K, the overall prevalence of autoantibody positivity was lower in those of non-white ethnicity (73%) compared with those of white European ethnicity (85%) (128). However, those of non-white ethnicity made up ~9% of this cohort. A North American study reported that GADA were most common in those of non-Hispanic black ethnicity, IA-2A was most common in Hispanics and IA-2A and ZnT8A were less common in those with Asian heritage, compared with other ethnicities (139). Outside of North America and Europe, smaller cross-sectional studies have generally suggested an association between ethnicity and differences in islet autoantibody prevalence (149-151).

Specificity of islet autoantibodies may be markedly reduced in these non-European origin populations due to the widely used islet autoantibody assays (including the RIAs and ELISAs) having been developed and positivity thresholds established in white European control populations. There are very few published control data by ethnicity and positive threshold selection has been shown to have impact on the specificity of islet autoantibody assays (152).

1.7 Clinical uses of islet autoantibody testing

1.7.1 Differentiating type 1 and type 2 diabetes

The main difference in the course of T1D in comparison to T2D is the severe insulin deficiency in T1D versus T2D where insulin resistance is more pronounced. It is critical at the time of diagnosis to correctly classify whether a patient has T1D or T2D, based on a definition that will ensure appropriate treatment. However, this is challenging as the age at onset of T1D and T2D have begun to converge in recent years with the increasing prevalence of obesity at a younger age and recognition of the relatively high proportion of incident cases of T1D in adulthood (17). Islet autoantibodies, present in approximately 90% of cases of T1D at diagnosis have also been found to present in the sera of those clinically diagnosed with T2D but at a lower prevalence both in adults (For example: GADA prevalence can range from less than 3% to greater than 15%) and children (~10% in both) (153-155). The presence of one or more islet autoantibody has high specificity for T1D (107; 123) but comprehensive testing has only recently been indicated in adult-onset patients in clinical practice (30). There are considerations associated with the use of islet autoantibodies to classify diabetes subtype; such as if performed individually their specificity for classification is low, as they are not detectable at diagnosis in all patients with T1D and their presence in the years after diagnosis is variable (as discussed in section **1.6.3.3**). However, if high performance (specificity and sensitivity) tests are used at diagnosis, then there is clinical utility in islet autoantibody assessment. Limitations surrounding GADA testing to distinguish diabetes type in adult-onset patients has also been discussed elsewhere in this introduction.

At diagnosis, there may be more value in the measurement of islet autoantibodies, compared to C-peptide assessment, to distinguish between the two types, as many diagnosed with T1D can experience a “honeymoon” phase in

their insulin production and therefore C-peptide testing would be less robust (7; 156). Due to the decline in autoantibody titres post-diagnosis, C-peptide may be more efficient at differentiating T1D from T2D 5 years post diagnosis and give a better representation of an individual's insulin secretory capacity (140).

1.7.2 Differentiating T1D and MODY

Maturity-onset of the young (MODY) is rare (~1% diabetes), typically presenting in young lean adults and often misdiagnosed as T1D, and is a heterogeneous group of monogenetic disorders with mutations in at least seven different genes leading to alteration in insulin secretion (157). Distinguishing between MODY and T1D is important as those with MODY either require no treatment or are successfully treated with sulphonyleurea tablets in contrast to the education and insulin treatment required by those with a diagnosis of T1D (123).

Islet autoantibodies should be tested for if MODY is suspected before proceeding to more expensive molecular genetic testing. A positive GADA or IA-2A result makes the diagnosis of MODY very unlikely as the prevalence of these autoantibodies in MODY is the same as in healthy control subjects (123). While a false positive is possible, given that the occurrence of MODY is as rare as a false positive result, a positive result would effectively exclude a diagnosis of MODY unless other clinical characteristics strongly suggest MODY rather than T1D.

1.7.3 Identifying rapid insulin requirement in apparent T2D

GADA testing is often used to identify people with apparent T2D who have islet autoimmunity and require early insulin treatment, now termed slowly evolving immune-mediated diabetes. Due to the high prevalence of T2D in older adults (where only ~2% of newly diagnosed individuals with diabetes have T1D) and imperfect test specificity, it is likely that many positive results in those without classical symptoms of T1D will be "false positive results" – these individuals may test positive but will not have autoimmune aetiology diabetes (19). However, in people with antibody positive T2D, progression to insulin therapy is highly variable. Therefore utility of this test is limited in this population. Despite improvements in the specificity of islet autoantibody testing, there is still a need for more specific antibody assays to improve prediction of who will need early

insulin therapy. In a study by the UKPDS, they found that in patients with apparent T2D, <35 years, 94% (ICA positive) and 84% (GADA positive) required insulin therapy within 6 years, compared to 14% of those without islet autoantibody positivity (158). A follow-up study in the same cohort, found that although positivity for IA-2A (2.2%) is rare in this cohort, it was highly predictive of future need for insulin therapy (159). However, a limitation in the use of GADA, and IA-2A, for identifying patients likely to require early insulin therapy, is that not all patients will be tested for islet autoantibodies. By testing for the T1D GRS in GADA positive patients, you can improve upon the clinical utility to predict early insulin requirement. Grubb *et al* (160), found that the presence of GADA with a high T1D genetic risk score increased risk of rapid progression to insulin therapy.

Stratification of treatment response is an area where testing for islet autoantibodies has been researched but results have varied as there is limited data examining a direct relationship between treatment response, glycaemic deterioration and islet autoantibodies. It has been reported that those with T2D and positive for autoantibodies (GADA or IA-2A), had a significantly reduced glycaemic response to GLP-1 receptor agonist therapy (161). However, whilst independently predictive of C-peptide in this study, most of those with positive autoantibodies had low C-peptide levels and severe insulin deficiency as these patients were receiving insulin co-treatment. Thunander *et al* (162), suggested that antibody prevalence and GADA level at baseline did not affect the outcome of their clinical trial; response to early insulin therapy. However, a small four-arm randomised trial suggested that in those with low GADA levels, long-term blood glucose control (mean HbA1c <7%) was maintained for longer in those on rosiglitazone compared with sulfonylureas (163).

1.8 Summary of Introduction

Islet autoantibodies are key biomarkers of autoimmune diabetes, and can be of great use in the prediction and classification of T1D. However, there are many clinical and analytical factors surrounding islet autoantibody testing that should be taken into consideration by researchers and clinicians when interpreting and applying results. Key areas highlighted in this introduction to be improved are:

1) The use of robustly defined thresholds of positivity in order to improve specificity of the assays.

2) There are limited data on whether antibody level at diagnosis of T1D can provide key information to clinicians.
3) The specificity of GADA in adult-onset diabetes, in classification of diabetes type and predict insulin requirement, needs to be improved.

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Chapter 2: Zinc transporter 8 autoantibody testing requires age-related cut-offs

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2.1 Acknowledgments of co-authors and contributions to paper

All authors conceived the idea and designed the study. Angela Cooper and Timothy J. McDonald collected the data. I analysed the data with assistance from Angela Cooper, Angus G. Jones and Timothy J. McDonald. I drafted the manuscript and responded to reviewers' comments with assistance from Angus G. Jones and Timothy J. McDonald. All authors critically revised the manuscript and approved the final version.

2.2 Abstract

Introduction

Zinc transporter 8 autoantibodies (ZnT8A) are biomarkers of beta cell autoimmunity in type 1 diabetes (T1D) that have become more widely available to clinicians in recent years. Robust control population defined thresholds are essential to ensure high clinical specificity in islet autoantibody testing. We aimed to determine optimal cut-offs for ZnT8A testing.

Research Design & Methods

97.5th and 99th centile cut-offs were determined using residual clinical sera from 1559 controls aged between 0-83 years, with no history of diabetes and a HbA1c level of less than 6.0% (<42 mmol/mol). ZnT8A were measured by ELISA (RSR Ltd, Cardiff, UK) on a Dynex DS2ELISA robot (Dynex, Preston, UK). We assessed the impact of age-related cut-offs in comparison to manufacturers recommended threshold in a mixed cohort of young onset (<age 30) diabetes (UNITED study, n=145).

Results

Using the manufacturer's limit of detection, 6 WHO U/ml, 16.2% of people in the control cohort had detectable levels of ZnT8A, those that had detectable ZnT8A were much more likely to be younger ($p<0.0001$). The 97.5th and 99th centile thresholds were substantially higher in younger participants: 18 and 127 WHO U/ml (tested under 30 years) in comparison to 9 and 21 WHO U/ml (tested 30 years and over). In the UNITED cohort some of those found to be ZnT8A positive by the manufacturer's threshold, but negative using the appropriate 99th centile cut off (127 WHO U/ml), displayed characteristics suggestive of type 2 diabetes.

Conclusions

Age-related thresholds are needed for ZnT8A testing. In those aged <30 years, use of manufacturers' recommended cut-offs may result in low test specificity and potentially high rates of false positive test results in patients who do not have autoimmune diabetes.

What is already known about this subject?

ZnT8A are increasingly used in clinical practice for the classification of diabetes and for the prediction of type 1 diabetes in a research setting. However thresholds of positivity have not yet been robustly defined and the impact of age on thresholds has not been examined.

What are the new findings?

- Healthy controls, with no history of diabetes and aged under 30 years, are more likely to have higher levels of detectable zinc transporter 8 autoantibodies;
- The 97.5th and 99th centiles that are traditionally used as thresholds were significantly higher in those tested under 30 years in comparison to those tested over 30 years;
- Applying the new 99th centile threshold of positivity retrospectively to a mixed cohort of young onset diabetes reclassified some participants who exhibited a more type 2 diabetic phenotype;
- When these age-related thresholds were used in an international islet autoantibody programme, they improved the sensitivity and specificity of the assay.

How might these results change the focus of research or clinical practice?

Clinical laboratories need to establish robust age-specific thresholds for ZnT8A testing, as without these, false positive results are likely to be common in young people with diabetes of non-autoimmune aetiology.

2.3 Introduction

Islet autoantibodies, including glutamate decarboxylase (GADA), islet antigen-2 (IA-2A) and insulin (IAA), have traditionally been used as biomarkers of the

autoimmune attack occurring in Type 1 Diabetes (T1D). The presence of islet autoantibodies are useful in differentiating T1D from other forms of diabetes and are used in research to stratify the risk of progression to T1D in cohort studies (1; 2). Zinc Transporter 8 autoantibodies (ZnT8A) are a more recently characterised islet autoantibody target of T1D autoimmunity: found in 66-80% of Caucasian patients at diagnosis and detectable in approximately 26% of individuals with T1D previously categorised as autoantibody negative (3). In those diagnosed young, ZnT8A have been found to be associated with a more acute onset of disease and a greater probability of ketoacidosis at presentation (4). ZnT8A have also been found to help exclude maturity onset diabetes of the young (MODY), identifying an additional 18% of probable T1D (in comparison to glutamate decarboxylase and islet antigen-2 autoantibodies alone) in individuals with significant endogenous insulin secretion (5). ZnT8A testing has become widely available in clinical practice.

The interpretation of islet autoantibodies requires robust and well-defined cut-offs to ensure high clinical specificity. Islet autoantibodies are found on a continuum of concentration and are often detectable at low levels in the non-diabetic population (6; 7). Therefore, islet autoantibody cut-offs are normally based on centiles of a control population. Usually, the 97.5th or 99th centile of a non-diabetic population are used as a positive cut-off (specificity 97.5% or 99%), depending on the specificity required for the clinical scenario (8-10). However, testing of sufficient numbers of non-diabetic controls to accurately determine test threshold is expensive and in practice the use of the limit of detection (LOD), or manufacturers' recommended cut-off, is common (11). The impact of age on optimal thresholds of ZnT8A positivity has not yet been assessed.

We aimed to define robust thresholds of positivity for ZnT8A testing in a European population using the RSR ZnT8A ELISA assay, and assess whether these are affected by age of the participant tested.

2.4 Participants and Methods

Study participants

To set threshold

To establish an antibody titre cut-off for ZnT8A and assess the impact of age, residual sera from 1559 routine clinical samples of patients aged between 0 and 83 years of age without a clinical history of diabetes and an HbA1c level of less than 6.0% (<42 mmol/mol) were analysed at the Academic Department of Blood Sciences, Royal Devon and Exeter Hospital (Exeter, U.K.).

Assessment of threshold validity

Threshold validity and the effects of age-related cut-offs were assessed on 145 patients with young onset diabetes (111 diagnosed with T1D, 31 with T2D and 3 with another diabetes type). Patients were identified from the UNITED cohort (12). An unselected cross sectional cohort of participants diagnosed with diabetes less than 30 years, but recruited before the age of 50 years. All included participants had been tested for ZnT8A (Table 1).

Characteristic	Value
n	145
Sex (%male)	74 (51.0)
Age at Diagnosis (years)	18.9 (13.7-25.5)
Age at Antibody Test (years)	27.5 (20.1-37.2)
Duration of Diabetes At Antibody testing (years)	5.8 (1.6-14.7)
Diabetes Type	
Type 1 Diabetes (%)	111 (76.6)
Type 2 Diabetes (%)	31 (21.4)
Other Diabetes type (%)	3 (2.1)
Autoantibody Positivity	
GADA positive (%)	60 (41.4)
IA-2A positive (%)	50 (34.5)
ZnT8A positive (%)	81 (55.9)

Table 1: *UNITED Cohort Characteristics. Participants were included if they were diagnosed ≤30 years, were ≤50 years at recruitment and had a ZnT8A measurement. Value is median (IQR) unless otherwise stated. ZnT8A considered positive according to RSR original threshold (≥15 U/ml).*

ZnT8A analysis

ZnT8A were analysed using an enzyme linked immunosorbent assay commercial kit (RSR Ltd, Cardiff, U.K.) on a Dynex DS2 ELISA robot (Dynex, Preston, U.K.). The RSR ZnT8A ELISA is capable of detecting and quantifying autoantibodies specific to R325 or to W325 polymorphic variants of ZnT8A. This laboratory

participates in the islet autoantibody standardisation programme (IASP), an external blinded quality assurance programme for islet autoantibodies (13). The assay LOD is 6 U/ml and the manufacturer's recommended cut-off for positivity was ≥15 U/ml.

Data Analysis

The 5-year rolling average percentage with detectable ZnT8A was plotted (Figure 1). The cut-offs were defined by using the 97.5th and 99th centiles. The specificity of the manufacturer's LOD was assessed by applying it to the overall control cohort, conducting a sub-analysis of each age group (less than 30 years and 30 years and over), and evaluating by the proportion positive. Fisher's exact test was used to compare groups above and below the LOD.

To assess the validity of the newly defined age-related cut-offs, the 99th centile cut-offs were applied, to reduce the potential misclassification of MODY patients, retrospectively to the UNITED cohort (5). In those tested under 30, we assessed the characteristics of participants who were positive for ZnT8A using the manufacturer's recommended cut-off (15 U/ml) but negative for ZnT8A using an appropriate (99th centile) cut-off based from the control population of comparable age.

All statistical analysis was carried out using Stata/SE 16.0 (StataCorp, College Station, TX) and Figure 2 was drawn with GraphPad Prism Software, Version 8.

2.5 Results

Detectable ZnT8A levels in the control population are common in those aged under 30 years

Using the manufacturer's reported limit of detection (LOD), 6 U/ml, 16.2% (95% CI 0.14-0.18) of participants in the control cohort had detectable ZnT8A. The 5-year rolling average of the % detectable was plotted, and a marked decline in the % detectable in early adulthood with stable low levels of detectable ZnT8A after age 30 was observed (Figure 1). Therefore we split the control cohort into those tested under and over 30 years. The prevalence of detectable ZnT8A was strongly related to age: in those aged under 30 years, 21% (n = 229; 79% specificity; 95% CI 0.19-0.24) had detectable ZnT8A; in contrast only 5% (n = 24;

95% specificity; 95% CI 0.03-0.07) of those tested 30 years and over were over the LOD ($p < 0.0001$).

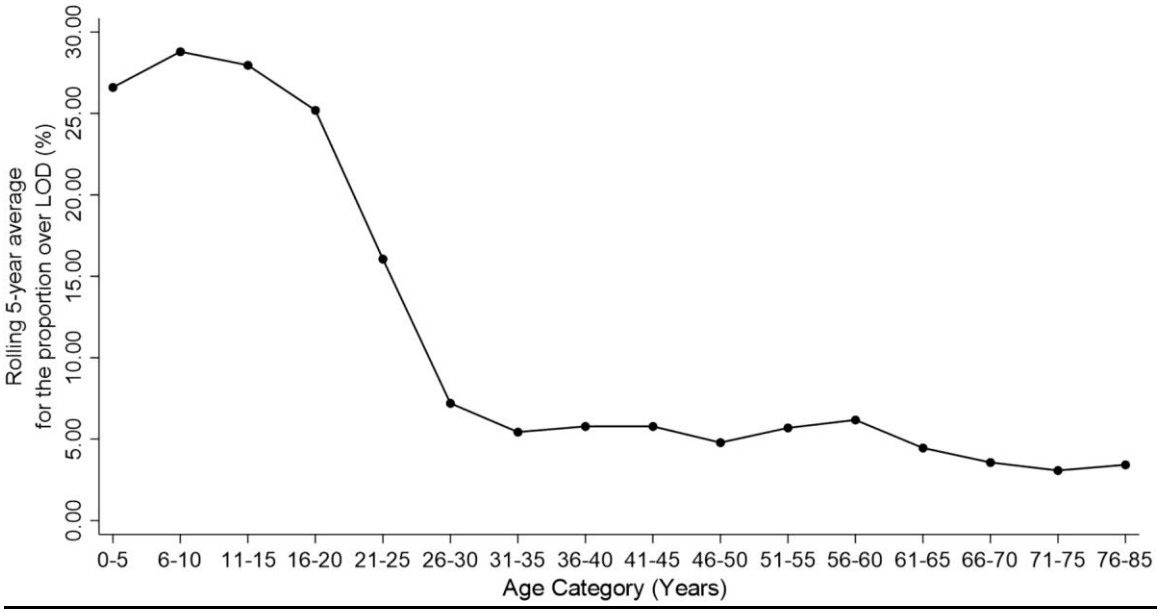

Figure 1: *Line graph of the 5-year rolling average of the percentage of those with detectable ZnT8A. The rolling average shows a drop to consistently <10% with detectable ZnT8A after the age of 30 years. Limit of detection (LOD) ≥6 U/ml.*

Appropriate cut-offs for ZnT8A testing widely vary by age

For those tested under 30 years; the 97.5th and 99th centile cut-offs were 18 and 127 U/ml respectively. However, for those tested aged 30 years and over; the 97.5th and 99th centile cut-offs were 9 and 21 U/ml (Figure 2). Therefore, to maintain assay specificity, those tested under the age of 30 years would need a higher cut-off in comparison to those tested aged 30 and over.

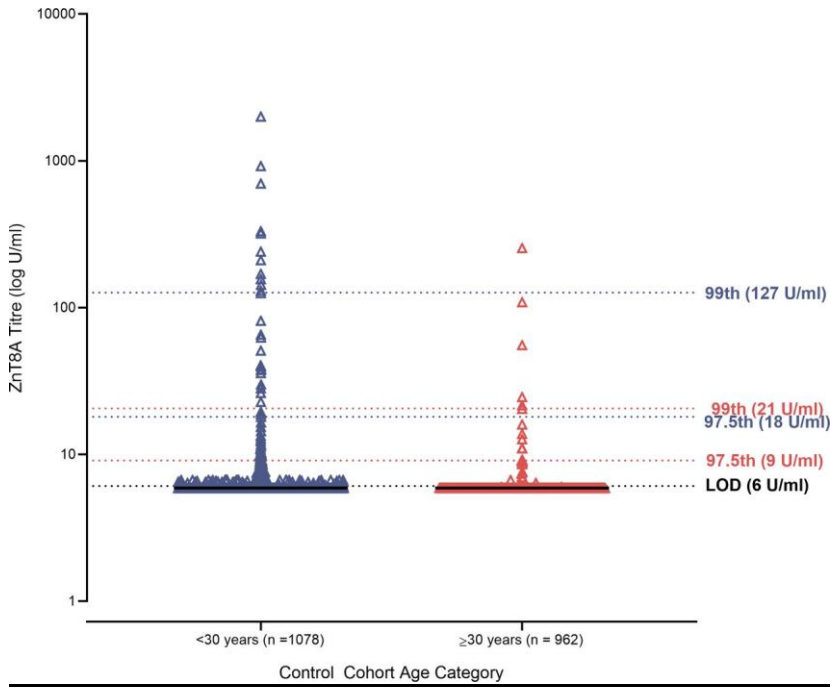

Figure 2: *Plot of Znt8A titres in healthy controls (n = 1559). Dotted lines indicate the 97.5th and 99th percentiles for <30 years (purple; n = 1078) and ≥30 years (red; n = 481). Black dotted line indicates the limit of detection (6 U/ml). Black solid lines indicate the median ZnT8A titre.*

Age-related cut-offs for ZnT8A testing reclassify patients as negative that have a more type 2 diabetic phenotype

When the age-related 99th centile cut-offs of 127 U/ml and 21 U/ml (increased from the manufacturer's cut-off [15 U/ml]) were applied to the UNITED cohort retrospectively, 23 participants tested under 30 years were reclassified as ZnT8A negative. Of these 8 were now classified as autoantibody negative and some exhibited a more type 2 diabetic phenotype (Table 2). 3/8 were non-insulin treated and 3/5 had high urinary C-peptide/creatinine ratio levels (≥0.6nmol/L). Moreover, four of the patients were overweight (BMI ≥25) and three were obese (BMI ≥30). In contrast, in those that remained positive for ZnT8A, above the 99th centile age-related cut-off; 85% were positive for one or more additional islet autoantibodies (GADA or IA-2A) and all those that were single ZnT8A positive above the 99th centile were on insulin treatment.

Patient Number	Age of Diagnosis Bracket (years)[a]	Diabetes Duration (years)	Current Treatment[b]	Adjusted BMI[b]	HbA1C (mmol/mol)[%][b]	UCPCR (nmol/mmol)	Duration at UCPCR (years)	ZnT8A Titre (U/ml)	Duration at ZnT8A (years)
1	10-15	3.8	Insulin	28.3	48 [6.5]	0.79	3.8	56.6	4.2
2	10-15	14.8	Insulin	24.5	48 [6.5]	0.29	14.8	54.1	15.1
3	15-20	0.2	Insulin	46.7	107 [11.9]	2.02	0.2	52.4	0.1
4	15-20	5.9	Insulin	21.0	86 [10]	0.21	5.9	35.9	6.0
5	25-30	1.0	Tablet	47.5	53 [7]	-	-	32.7	1.0
6	20-25	1.2	Insulin	22.0	43 [6.1]	0.98	3.0	26.6	3.1
7	25-30	0.9	Tablet	-	40 [5.8]	-	-	24.9	1.0
8	25-30	0.2	Tablet	32.5	115 [12.7]	-	-	17.2	0.0

Table 2: *Reclassified single ZnT8A positives (with manufacturers LOD) from the UNITED Study (n = 8). Inclusion criteria was now negative for ZnT8A with new 99th centile cut-off and negative for glutamate decarboxylase and islet antigen-2 autoantibodies. [a]For reasons of patient anonymity; [b]At latest follow-up; - indicates missing data. UCPCR: Urinary c-peptide/creatinine ratio, a result ≥0.6 indicates substantial endogenous insulin secretion. ZnT8A titre: level of units of ZnT8A present in the serum.*

Age-specific thresholds for ZnT8A in the islet autoantibody standardisation programme increased specificity without loss of sensitivity

When the 99th centile age-specific cut-offs described here were applied to a separate set of control samples (n=140) as part of the IASP 2020 workshop, specificity was high (98.9%), without loss of sensitivity (74.0%). This is in comparison to a specificity of 97% and a sensitivity of 74% reported, in the assay insert, by the manufacturer when their ≥15 U/ml cut-off was used (14).

2.6 Discussion

This study has shown that detectable ZnT8A levels above the manufacturer's limit of detection are very common in a non-diabetic control population: occurring in 21% of those tested under the age of 30 years and in 5% of those tested aged 30 and over. Where ZnT8A are detectable, titres are higher in younger individuals. Therefore using the same cut-off to define ZnT8A positivity for all ages could result in lower test specificity in young patients, and potentially high rates of false positive test results. In our unselected population with young onset diabetes, some of the participants with positive ZnT8A above the manufacturer's recommended cut-off, but ZnT8A negative using an appropriate population based (99th centile) threshold, had the clinical characteristics of type 2 diabetes.

The 99th age-specific cut-offs improved performance of this assay in IASP, increasing specificity without loss of sensitivity (98.9% and 74.0%, respectively). The average specificity and sensitivity were 94.4% and 64.1% for this assay across the other laboratories submitting results (15), therefore increasing confidence in the use of age-restricted thresholds for this assay.

The mechanisms that result in increased background prevalence and titre of ZnT8A in the young healthy control cohort are unknown. In healthy people we may speculate that it could be due to differences in immune system maturity or cross reactivity to an infection mode commonly seen in the young; for example, cross reactivity between ZnT8A and an antigen expressed by *Mycobacterium avium subsp. Paratuberculosis,* that has been described (16). Another possibility for the higher titre and prevalence of ZnT8A in those tested <30 years in the control cohort is that there is a potential enrichment of individuals in the prodrome stage of type 1 diabetes. However, the overall life prevalence of type 1 diabetes

is less than 0.3% (with approximately half of cases occurring after age 30) (17), and therefore will be contributing only a very small amount to the difference between <30 and >30 cohorts (~0.15%). To our knowledge, this is one of the first studies to show age effects of ZnT8A on thresholds of positivity. A previous study by Vermeulen *et al* (2011) reported using age-restricted cut-offs for positivity for their liquid-phase radiobinding assay to detect ZnT8A. Their age cut-offs differed from ours (0-14 years and 15-39 years) and their control population was considerably smaller (n=761), with only those aged up to 39 assessed (7).

Due to the nature of the control cohort, the findings of this study are limited to one population which is predominantly of European descent. In addition, this study only used one assay type for ZnT8A, however the RSR ELISA is used by many clinical laboratories since it was distributed and validated in 2013 (18-20). Differences in ZnT8A prevalence have been reported in non-white ethnic patients with diabetes: defining appropriate reference ranges for different ethnicities and for other assays will be important areas for future research (21; 22). A further limitation is that although this is a large cohort, far in excess of what has been previously reported (9; 19; 23), the sample size is still insufficient to do more than visually assess an optimal age cut-off. Therefore, an even larger sample size would be needed to give greater detail on optimal test cut-offs for different age groups.

High clinical specificity of islet autoantibody tests is of particular importance in the setting of low prior prevalence, such as the case of prediction of T1D in the general population or the diagnosis of autoimmune diabetes in older adults (17). By setting robust and well-defined cut-offs for each assay based on appropriate control populations, this will ensure high assay specificity and reduce potentially high rates of false positives in those with non-autoimmune diabetes. This is of increasing importance for studies into the prediction of T1D, due to the recent progressions in intervention therapy research (24), and in differentiating autoimmune from other forms of diabetes (25). We have shown potential misclassification of diabetes types due to the use of manufacturer's recommended cut-off. This is likely to affect previous work where reported prevalence of ZnT8A has varied and manufacturers' guidelines of cut-offs have been used instead of a robust population-defined threshold.

2.7 Conclusion

In those aged <30 years, a higher age-related threshold is likely to be needed for ZnT8A testing to prevent low test specificity and potentially high rates of false positive test results in patients who do not have autoimmune diabetes.

2.8 Acknowledgements

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Chapter 3: Islet autoantibody level distribution in type 1 diabetes and their association with genetic and clinical characteristics

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3.1 Acknowledgement of co-authors and contributions to paper

I assayed the ADDRESS-2 autoantibody samples during a technical role at Bristol, subsequently during my PhD I researched and analysed the data as well as writing the manuscript. Kashyap A. Patel and Jack Bowden helped analyse the data and write the manuscript. Angus G. Jones and Timothy J. McDonald helped plan the analysis, contributed to the discussion and reviewed/edited the manuscript. Beverley M. Shields and Trevelyan J. McKinley contributed to data analysis, provided statistical support and reviewed/edited the manuscript. Angus G. Jones set up the study from which the replication cohort was selected. Helen C. Walkey, Akaal Kaur, Shivani Misra, Nick S. Oliver and Desmond G. Johnston set up the ADDRESS-2 study from which the original cohort was selected, collected the clinical data and reviewed the final manuscript. Kashyap A. Patel and I responded to reviewers' comments with support from Angus G. Jones.

3.2 Abstract

Context

The importance of the autoantibody level at diagnosis of type 1 diabetes is not clear.

Objective

We aimed to assess the association of glutamate decarboxylase (GADA), islet antigen-2 (IA-2A) and zinc transporter 8 (ZnT8A) autoantibody levels with clinical and genetic characteristics at diagnosis of type 1 diabetes.

Design, Setting and Patients

We conducted a prospective cross-sectional study. GADA, IA-2A and ZnT8A were measured in 1644 individuals with type 1 diabetes at diagnosis using radiobinding assays. Associations between autoantibody levels and the clinical and genetic characteristics for individuals were assessed in those positive for these autoantibodies. We performed replication in an independent cohort of 449 people with type 1 diabetes

Results

GADA and IA-2A levels exhibited a bimodal distribution at diagnosis. High GADA level was associated with older age at diagnosis (median 27 years vs. 19 years, $P=9\times10^{-17}$), female sex (52% vs. 37%, $P=1\times10^{-8}$), other autoimmune diseases

(13% vs. 6%, P=3x10^{-6}) and *HLA-DR3-DQ2* (58% vs. 51%, P=0.006). High IA-2A level was associated with younger age of diagnosis (median 17 years vs. 23 years, P=3x10^{-7}), *HLA-DR4-DQ8* (66% vs. 50%, P=1x10^{-6}) and ZnT8A positivity (77% vs. 52%, P=1x10^{-15}). We replicated our findings in an independent cohort of 449 people with type 1 diabetes where autoantibodies were measured using enzyme-linked immunosorbent assays (ELISA).

Conclusions

Islet autoantibody levels provide additional information over positivity in type 1 diabetes at diagnosis. Bimodality of GADA and IA-2A autoantibody levels highlights the novel aspect of heterogeneity of type 1 diabetes. This may have implications on type 1 diabetes prediction, treatment and pathogenesis.

3.3 Introduction

Islet autoantibodies are commonly used in the diagnosis and prediction of type 1 diabetes. They are well established as the biomarkers of the underlying autoimmune pathogenesis (1). Autoantibodies to islet cell antigen (ICA), glutamate decarboxylase (GADA), islet antigen-2 (IA-2A), insulin (IAA) and zinc transporter 8 (ZnT8A) are the most commonly used islet autoantibodies at diagnosis (2). As detectable islet autoantibodies overlap between health and disease, a test is usually considered positive for a given islet autoantibody when the antibody level is higher than a 97.5–99th centile of a control population (3; 4). In routine clinical practice, quantitative islet autoantibody results are usually interpreted as positive or negative, and the level of the islet autoantibody, is not thought to be clinically meaningful.

Islet autoantibody levels may provide additional information over positivity in type 1 diabetes at diagnosis. Similar to type 1 diabetes, autoantibodies to a specific antigen are commonly used for diagnosis in many other autoimmune diseases (such as TSH receptor autoantibodies in Graves' disease and tissue transglutaminase in coeliac disease). For Graves' disease and coeliac disease,

along with autoantibody positivity for these antigens, autoantibody level at diagnosis is associated with disease severity, prognosis and treatment success (5; 6). Multiple studies have shown a role for islet autoantibody level in the prediction of onset of type 1 diabetes, those with a higher levels of IA-2A, IAA and ICA have an increased risk of developing type 1 diabetes in at-risk populations (1; 7-9). However, it is not clear if the islet autoantibody level at diagnosis of type 1 diabetes, in addition to its interpretation as 'positive', is associated with the clinical phenotype similar to other autoimmune diseases.

In this study, we undertook an analysis of GADA, IA-2A and ZnT8A levels at diagnosis in a large cohort of participants with type 1 diabetes, assessing the association of islet autoantibody levels on genetic and clinical characteristics at diagnosis in people with type 1 diabetes.

3.4 Materials and Methods

Study cohorts

We recruited 1644 participants with a clinician-assigned diagnosis of type 1 diabetes (age at diagnosis range 4–75 years) who were positive for any of GADA, IA-2A or ZnT8A at diagnosis. These participants were recruited as part of the UK-wide ADDRESS-2 study. The detailed protocol for the study has been published previously (10). The included participants were recruited at diagnosis (<6 months), were >4y of age and insulin-treated from diagnosis. DNA and serum samples, clinical data (including the characteristics of diabetes at diagnosis) were collected at recruitment. The overall cohort characteristics are provided in Table 1 and distribution of age at diabetes diagnosis is provided in Figure 1A.

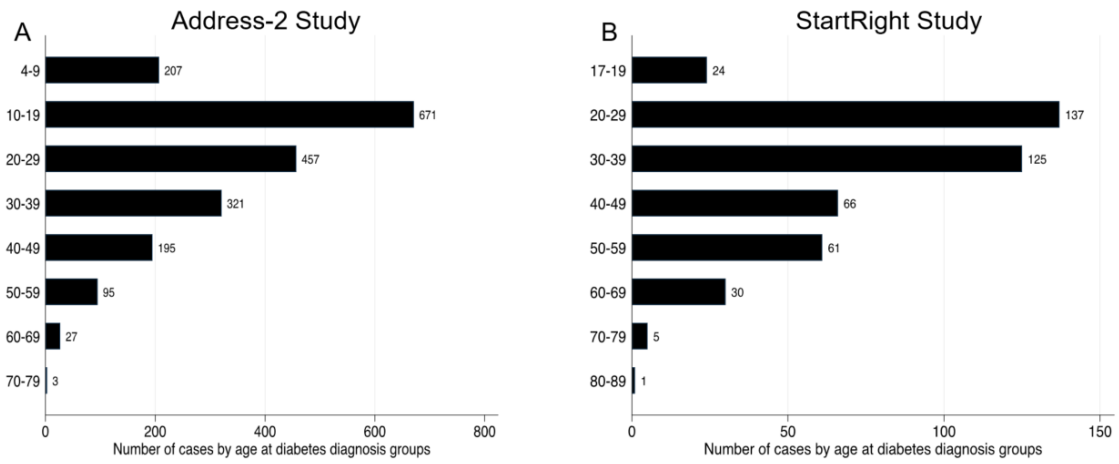

Figure 1: *Distribution of age at diabetes diagnosis. A) In ADDRESS-2 cohort. B) In StartRight cohort.*

Characteristic	ADDRESS-2	StartRight
n	1,644	449
Female (%)	704 (43)	221 (49)
Non-European descent (%)	108 (6.6)	22 (5)
Age of Diagnosis (years)	21 (13, 31)	34 (26, 46)
Duration of Diabetes at Antibody testing (weeks)	11 (6, 18)	15 (6, 32)
Hospital Admission (%)	1,251 (76)	267 (60)
DKA (%)	687 (43)	156 (35)
Polyuria (%)	1,562 (96)	411 (92)
Weight Loss (%)	1,393 (87)	386 (86)
HbA1c (mmol/mol)	80 (59, 107)	64 (50, 87)
BMI (kg/m^2)	23.4 (21.1, 26.2)	24.5 (21.8, 27.2)
C-Peptide (picomol/L)	-	464 (278, 684)
Parent with Diabetes (%)	260 (16)	88 (20)
Other autoimmune condition (%)	125 (8)	68 (15)
T1D-GRS	0.274 (0.255, 0.292)	-
***HLA-DR3-DQ2* (%)**	861 (52)	-
***HLA-DR4-DQ8* (%)**	880 (54)	-
Number of positive autoantibodies		
One (%)	475 (29)	160 (36)
Two (%)	565 (34)	126 (28)
Three (%)	604 (37)	163 (36)
GADA positive (%)	1,364 (83)	404 (90)
IA-2A positive (%)	1,099 (67)	237 (53)
ZnT8A positive (%)	954 (58)	260 (58)

Table 1: *Clinical characteristics for the study cohorts. '-' indicates unavailable data for this cohort. Values expressed as median (interquartile range) unless stated.*

We used a second independent replication cohort of 449 participants with clinically diagnosed type 1 diabetes (age at diagnosis range 17–81 years) and positivity to any of the three islet autoantibodies (GADA, IA-2A and ZnT8A). The participants were part of the UK-wide StartRight study (11). All were recruited <12 months from diagnosis, and insulin-treated from diagnosis. They had random non-fasting serum c-peptide at baseline. They also had post-meal urine sample for Urinary C-Peptide /Creatinine Ratio (UCPCR) at baseline, 1 year and 2 years

from the diabetes diagnosis. The overall cohort characteristics is provided in Table 1 and distribution of age at diabetes diagnosis is provided in Figure 1B.

Islet autoantibody measurement

ADDRESS-2 study: The islet autoantibodies (GADA, IA-2A, ZnT8RA and ZnT8WA) were measured using established radiobinding assays by the Diabetes and Metabolism group at the University of Bristol (Bristol, U.K.) (12; 13) at a median of 11 weeks diabetes duration. Results for GADA and IA-2A are expressed in digestive and kidney units/mL (DK units/mL) or arbitrary units (AU/mL) for ZnT8A (the ZnT8A level represents the highest value of either ZnT8RA or ZnT8WA) calculated from standard curves consisting of diluted patient sera in antibody-negative sera from healthy donors. Positive thresholds were set at 97.5th percentile of 974 control samples for GADA (≥33 DK U/ml), the 98th percentile of 500 control samples for IA-2A (≥1.4 DK U/ml) and the 97.5th percentile of 523 healthy school children for ZnT8A (≥1.8 AU/ml) (14). The laboratory participates in the Islet Autoantibody Standardisation Program (IASP) (Table 2) (15).

Method	Antibody	%Sensitivity	%Specificity	IASP Workshop
RBA	GADA	74	96.7	2015
RBA	IA-2A	72	100	2015
RBA	ZnT8RA	60	100	2015
RBA	ZnT8WA	46	100	2015
RSR ELISA	GADA	74.0	98.9	2020
RSR ELISA	IA-2A	72.0	98.9	2020
RSR ELISA	ZnT8A	74.0	98.9	2020

Table 2: *IASP Workshop performance for each islet autoantibody assay. The radiobinding assays were all conducted centrally in Bristol (U.K.) by the Diabetes and Metabolism Group. The RSR ELISA assays were all conducted centrally by the Academic Department of Clinical Biochemistry (Royal Devon and Exeter NHS Trust) in Exeter (U.K.). RBA: Radiobinding immunoassay; ELISA: Enzyme-linked immunosorbent assay.*

StartRight study: ELISA assays (RSR Limited, Cardiff, U.K.) were used to measure GAD (RRID: AB_2910239, https://scicrunch.org/resolver/AB_2910239), IA-2 (RRID: AB_2910240, https://scicrunch.org/resolver/AB_2910240) and ZnT8 (RRID: AB_2910241, https://scicrunch.org/resolver/AB_2910241) islet autoantibodies on a Dynex DS2 automated ELISA system (Launch Diagnostics, Longfield, U.K.), at a median of 15 weeks diabetes duration by the Academic Department of Blood Sciences, Royal Devon and Exeter Hospital (Exeter, U.K.) (16). Positive thresholds were set at the 97.5th percentile of 1559 non-diabetic control subjects (GAD ≥ 11 WHO (World Health Organization) U/mL, IA-2 ≥ 7.5 WHO U/mL, ZnT8 age ≥ 30 years ≥ 10 U/mL, ZnT8 age < 30 years ≥ 65 U/mL). Upper reporting limits for GADA, IA-2A and ZnT8A were 2000 WHO U/mL, 4000 WHO U/mL and 2000 U/mL respectively. The laboratory also participates in IASP (Table 2) (17). The analysis of samples from the 2018 IASP workshop showed that islet autoantibodies levels

measured by this assay were highly correlated to the radiobinding assay used in the ADDRESS-2 cohort for all three islet autoantibodies (Figure 2).

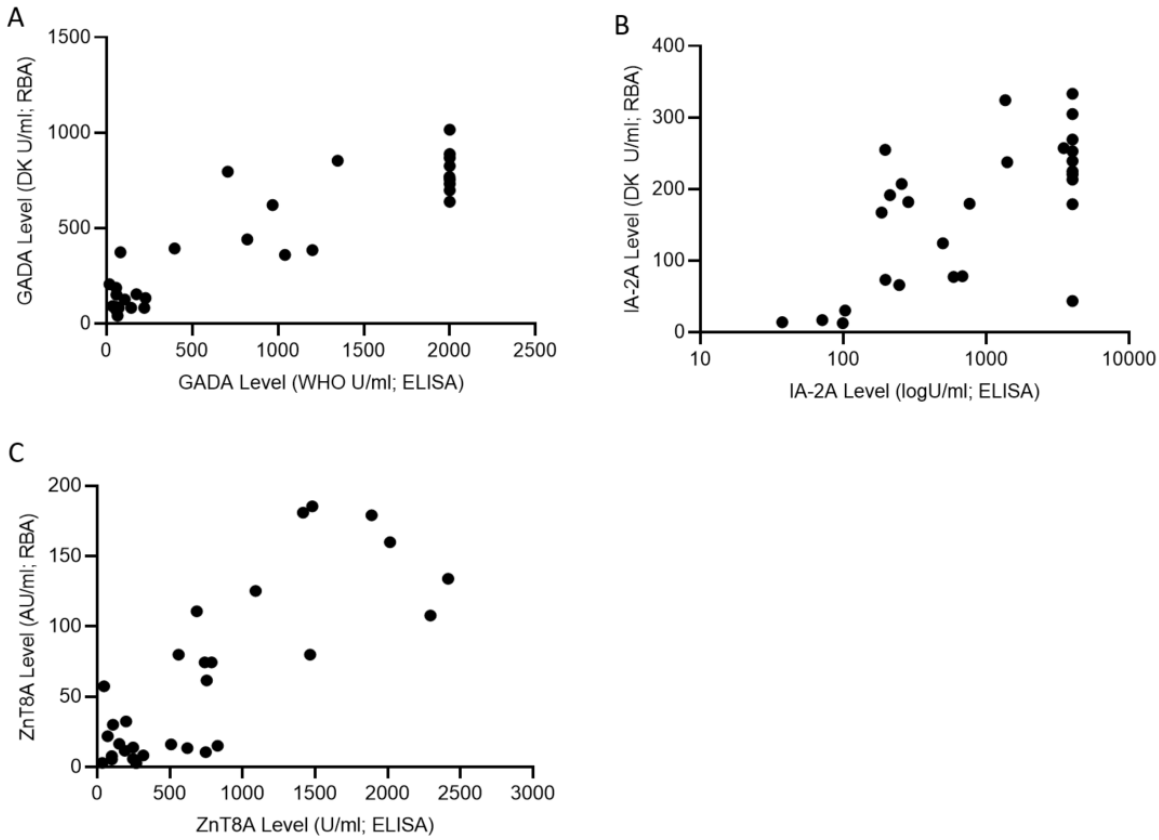

Figure 2: *Scatter plots of islet autoantibody levels measured by radiobinding assay and ELISA assay for each autoantibody on the same samples from the IASP 2018 Workshop. The data is presented for the samples which were positive on the both assays. All three autoantibodies show high level of correlation between radiobinding assay and ELISA assays with Pearson's correlation coefficient of 0.91 (n=32, 95%CI 0.82-0.96) for GADA (A), 0.81(n=30, 95% CI 0.63-0.91) for ZnT8A (C) and slightly lower 0.56 (n=28 95%CI 0.24-0.77) for IA-2A (B).*

Type 1 diabetes genetic risk score (T1D-GRS) and HLA genotypes

We generated weighted T1D-GRS from 30 common type 1 diabetes genetic variants (single nucleotide polymorphisms [SNPs]) for HLA and non-HLA loci as described in our previous paper (18). *HLA DR3-DQ2* and *HLA-DR4-DQ8* were imputed from two SNPs as described in Barker *et al* and our previous paper (18; 19).

Statistical Analysis

We used histograms to assess the distribution of islet autoantibody levels in those positive for that autoantibody. Autoantibody levels with bimodal distributions were split into high or low level categories using the nadir (lowest point between distributions). We also used a normal mixture model analysis and likelihood ratio tests to assess whether uni-modal, bi-modal or multi-modal distributions were best supported by the data. This analysis was performed on log-transformed autoantibody level data so that it was better approximated by a mixture of continuous, symmetric normal distributions. This was implemented using the mixtools package in R (20). We performed Mann-Whitney tests to compare the continuous variables and Pearson chi-square tests were used to compare categorical variables between autoantibody level categories.

For modelling annual UCPCR, the intercept and slopes were determined using mixed effect models as described previously (21), with random effects at the individual level to allow each individual to contribute multiple C-peptide values at different time points. The benefit of this random-intercept, random-slope model is that it allows for variability between individuals in terms of both C-peptide level at diagnosis (the intercept) and in the percentage change in C-peptide over time (the slope). Groups categorised by GADA and IA-2A level were separately assessed using an interaction term within the mixed effects model. Due to the slope being on a log scale, they were interpreted in terms of the percentage change per year (Calculated from the exponential of the ß-coefficient-1). The variability of individual slopes in the longitudinal models was determined using the SD range (calculated by back transforming the ß-coefficient ± 1 SD of the

slope). All statistical analysis was carried out using Stata/SE 16.0 (StataCorp, College Station, TX) unless otherwise stated.

3.5 Results

GAD and IA-2 but not ZnT8 autoantibody levels exhibit a bimodal distribution at diagnosis in type 1 diabetes.

We first assessed the distribution of GADA level in GADA positive type 1 diabetes people (n=1364), IA-2A level in IA-2A positive type 1 diabetes people (n=1099), and ZnT8A level in ZnT8A positive type 1 diabetes people (n=954). The distribution of the GADA and IA-2A levels showed two peaks consistent with a bimodal distribution (Figure 3A & 3B). ZnT8A level showed a single peak with right-skewed distribution (Figure 3C).

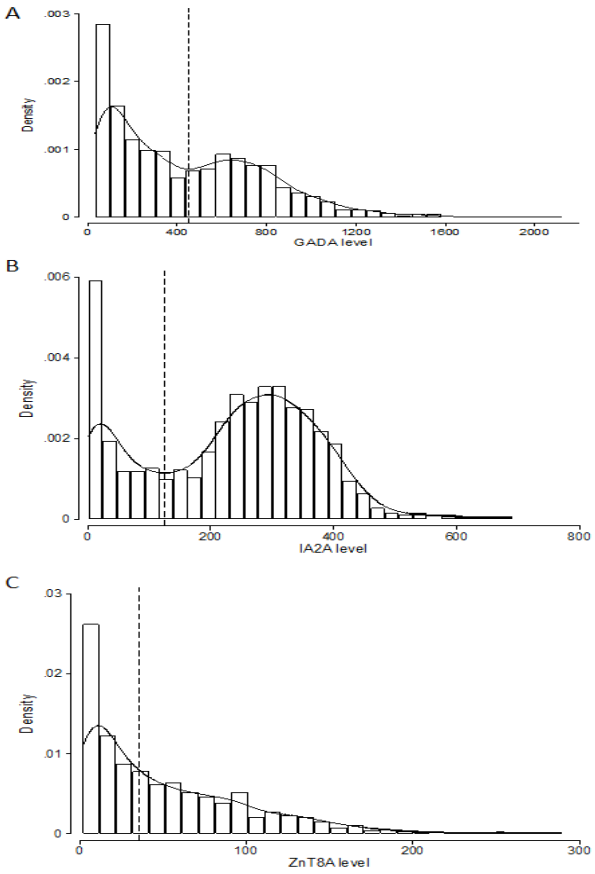

Figure 3: *Histograms with kernel density curves showing the distribution of islet autoantibody levels in patients with type 1 diabetes at diagnosis. A) Histogram of GADA level at diagnosis measured using radiobinding assay for type 1 diabetes cases who were positive for GADA (n=1,364). GADA level exhibits a bimodal distribution. The nadir value of 452 DK U/ml between the two modes is highlighted with black dashed line and used to defined high GADA level group (≥450 DK U/ml) and low level group (<450 DK U/ml). B) Histogram of IA-2A level at diagnosis measured using radiobinding assay for type 1 diabetes cases who were positive for IA-2A (n=1,099). IA-2A level exhibits a bimodal distribution. The nadir value of 125 DK U/ml between the two modes is highlighted with black dashed line and used to defined high IA-2A level group (≥125 DK U/ml) and low level group (<125 DK U/ml). C) Histogram of ZnT8A levels at diagnosis measured using radiobinding assay for type 1 diabetes cases who were positive for ZnT8 (n=954) show a right skewed distribution. Median value of the distribution (35.6 AU/ml) is highlighted with black dashed lines and used to define high level ZnT8A (≥35.6 AU/ml) and low level (<35.6 AU/ml) groups.*

Bimodality of GADA and IA-2A levels was also confirmed using a mixture model analysis (Supplementary Figure 1A & 1B) (15). Specifically, we analysed IA-2A and GADA levels on the log scale and used a likelihood ratio test (LRT) on 3 degrees of freedom to compare the log-likelihood of a one-component normal distribution with two parameters (one mean and one variance) versus that of a two-component, five parameter normal mixture (two means, two variances and a

weight determining the relative proportion of each component). These analyses yielded overwhelming evidence in favour of the two-component model ($LRT_{IA\text{-}2A}$ = 1219, $p<5x10^{-264}$, LRT_{GADA}= 352, $p<5x10^{-76}$). For the subsequent analysis, we used the nadir value between the two peaks to divide the bimodal distribution of the autoantibody levels into two groups (low vs. high levels) (Figure 3A & 3B). The nadir value for GADA levels was 450 DK U/ml. All participants with a GADA level lower than this value were grouped into a low level GADA group (mean level 180, SD +/-118, 760/1364 (56%)) and the participants with a GADA level above or equal this value were grouped into a high level GADA group (770, +/-245, 604/1364 (44%)). Similarly, the nadir value of 125 DK U/ml between the two peaks of IA-2A levels divided people into a low level IA-2A group (mean level 38, SD+/-35, 296/1099 (27%)) and a high level IA-2A group (299, +/-89, 803/1099 (73%)). Bimodality of GADA level remained after excluding individuals with autoimmune thyroid disease which is reported to associate with higher GADA level (Supplementary Figure 2) (15; 22).

Higher GADA levels were associated with later age at diagnosis of type 1 diabetes, female sex, and *HLA-DR3-DQ2*.

To assess the association of bimodal GADA levels to clinical features at diagnosis, we compared the clinical features between the people with low level (lower mode) and high level (higher mode) GADA as defined above. Those in the high level GADA group were diagnosed later compared to the low GADA level group (median 27 years [IQR 17-38] vs. 19 years [13-29], $P=9x10^{-17}$) (Figure 4).

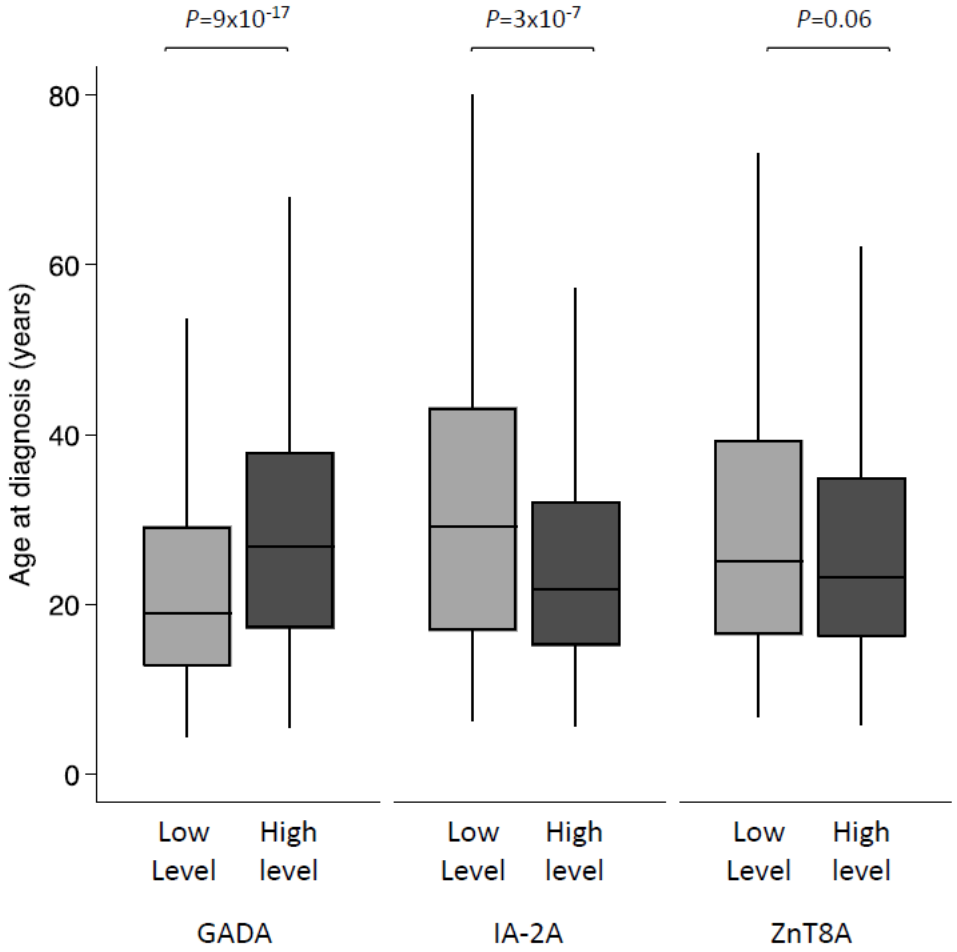

Figure 4: *Box plot showing age of diagnosis of type 1 diabetes in high and low level groups for GADA, IA-2A and ZnT8A. The nadir value between the two modes of GADA level (450 DK U/mL) and IA-2A level (125 DK U/mL) distribution at diagnosis of type 1 diabetes who were positive for respective autoantibodies was used to define high and low level categories. There were 604/1,364 and 760/1364 cases in low and high level GADA groups and 803/1099 and 296/1099 cases in high and low level IA-2A groups. The median value of ZnT8A level (35.6 AU/ml) was used for defining low and high level groups (n=477 each). Median age of diagnosis was higher for the high level GADA group ($P=9x10^{-17}$), lower for the higher level IA-2A ($P=3x10^{-7}$) and similar between ZnT8A level categories (P=0.06).*

They were more likely to be female (52% vs. 37%, $P=1x10^{-8}$), have a parent with diabetes (20% vs. 14%, $P=0.002$) and have other autoimmune diseases (13% vs. 6%, $P=3x10^{-6}$) compared to low level GADA group (Table 3). They had modest enrichment for *HLA-DR3-DQ2* (58% vs. 51%, $P=0.006$) but had similar T1D-GRS (median 0.273 [IQR 0.256-0.292] vs. 0.275 [0.255-0.292], $P=0.48$) based on 30 type 1 diabetes associated common variants (23). The presentation characteristics (DKA, weight loss, polyuria, HbA1c and BMI), the number of other islet autoantibodies and other islet autoantibody levels were similar between the

two GADA level groups. In line with this result, more people with adult-onset T1D were in high level GADA group compared to childhood-onset T1D (53% v 33%, P=1x10^{-13}) (Table 4; Supplementary Figure 3A & 3B) (15).

Characteristic	High Level GADA	Low Level GADA	High vs Low *P* value
n (% of GADA positives)	604 (44)	760 (56)	
GADA level (DK U/ml)	723 (594, 877)	149 (76, 274)	
Female (%)	317 (52)	282 (37)	$1x10^{-8}$*
Non-European descent (%)	45 (7)	51 (7)	0.60
Age of Diagnosis (years)	27 (17, 38)	19 (13, 29)	$9x10^{-17}$*
Duration of Diabetes (weeks)	10 (6, 17)	11 (6, 18)	0.29
Hospital Admission (%)	434 (72)	577 (76)	0.10
DKA (%)	250 (42)	323 (43)	0.74
Polyuria (%)	580 (97)	712 (95)	0.21
Weight Loss (%)	522 (88)	641 (86)	0.23
HbA1c (mmol/mol)	87 (64, 110)	81 (58, 107)	0.01
BMI, kg/m^2	23.7 (21.4, 26.7)	23.2 (20.9, 26.2)	0.04
Parent with Diabetes (%)	121 (20)	104 (14)	0.002*
Other autoimmune condition (%)	77 (13)	42 (6)	$3x10^{-6}$*
T1D-GRS	0.273 (0.256, 0.292)	0.275 (0.255, 0.292)	0.48
HLA-DR3-DQ2 (%)	353 (58)	387 (51)	0.006
HLA-DR4-DQ8 (%)	291 (48)	401 (53)	0.09
Number of positive autoantibodies			0.45
One (%)	165 (27)	195 (26)	
Two (%)	183 (30)	217 (29)	
Three (%)	256 (42)	348 (46)	
IA-2A (%)	372 (62)	484 (64)	0.43
IA-2A level (DK U/ml)	254 (91, 340)	255 (124, 338)	0.66
High level IA-2A (%)	265 (44)	362 (48)	0.17
ZnT8A (%)	323 (53)	429 (56)	0.27
ZnT8A level (AU/ml)	43 (13, 83)	36 (12, 76)	0.13
High level ZnT8A (%)	180 (30)	215 (28)	0.54

Table 3: *Comparison of clinical characteristics at diagnosis between high and low GADA level groups for GADA positive type 1 diabetes cases. Bimodal GADA level distribution was divided into two groups using the nadir between the two modes at 450 DK U/ml. Values expressed as median (interquartile range) unless stated. Autoantibody levels were assessed in those people who were positive for that antibody. * indicates a p value lower than threshold the P value for multiple comparisons (0.05/22 =0.0023).*

	Whole cohort	Childhood-onset type 1 diabetes (diagnosed <20y of age)	Adult-onset type 1 diabetes (diagnosed ≥20y of age)	*P* value
Low GADA level	760/1364 (56%)	401/599 (67%)	359/765 (47%)	$1x10^{-13}$
High GADA level	604/1364 (44%)	198/599 (33%)	406/765 (53%)	
Low IA-2A level	296/1099 (27%)	133/624 (21%)	163/475 (34%)	$2x10^{-6}$
High IA-2A level	803/1099 (73%)	491/624 (79%)	312/475 (66%)	

Table 4: *Distribution of childhood-onset and adult-onset type 1 diabetes by high and low GADA or IA-2A categories.*

Higher IA-2A levels were associated with earlier age at diagnosis of type 1 diabetes and *HLA-DR4-DQ8* and ZnT8A positivity.

We next compared the clinical features of low and high IA-2A level groups to assess association of bimodal IA-2A level distribution to clinical features at diagnosis (Table 5). Contrary to GADA, those in the higher level IA-2A group were diagnosed earlier compared to the lower IA-2A level group (median 17 years [IQR 12-25] vs. 23 years [13-34], $P=3x10^{-7}$) (Figure 4). They were more likely to be multiple autoantibody positive (60% vs. 42% with three autoantibodies, $P=9x10^{-9}$), positive for ZnT8A (77% vs. 52%, $P=1x10^{-15}$) and more likely to have higher ZnT8A levels (median level 44 AU/ml [IQR 16-84] vs. 26 [7-54]) ($P=3x10^{-6}$). Those with higher IA-2A levels were more likely to have *HLA-DR4-DQ8* (66% vs. 50%, $P=1x10^{-6}$). The presentation characteristics were similar between IA-2A level groups (Sex, DKA, weight loss, polyuria, HbA1c and BMI, parent with diabetes) (Table 5). In line with this result, more people with childhood-onset T1D were in high level IA-2A group compared to adult-onset T1D (79% v 66%, $P=2x10^{-6}$)(Table 4; Supplementary Figure 3C & 3D) (15).

Characteristic	High Level IA-2A	Low Level IA-2A	P value
n (% of IA-2A positives)	803 (73)	296 (27)	
IA-2A level (DK U/ml)	295 (237, 359)	24 (8, 65)	
Female (%)	355 (44)	117 (40)	0.16
Non-European descent (%)	48 (6)	17 (6)	0.88
Age of Diagnosis (years)	17 (12, 25)	23 (13, 34)	$3x10^{-7}$*
Duration of Diabetes (weeks)	12 (7, 18)	11 (6, 18)	0.20
Hospital Admission (%)	650 (81)	217 (74)	0.01
DKA (%)	350 (44)	131 (45)	0.88
Polyuria (%)	767 (97)	279 (96)	0.21
Weight Loss (%)	679 (87)	244 (86)	0.20
HbA1c (mmol/mol)	76 (56, 101)	83 (60, 114)	0.01
BMI	23.2 (21.2, 25.9)	23.7 (21.5, 26.6)	0.07
Parent with Diabetes (%)	110 (14)	47 (16)	0.37
Other autoimmune condition (%)	45 (6)	24 (8)	0.13
T1D-GRS	0.275 (0.257, 0.293)	0.275 (0.254, 0.294)	0.73
HLA-DR3-DQ2 (%)	354 (44)	165 (56)	$6x10^{-4}$*
HLA-DR4-DQ8 (%)	533 (66)	149 (50)	$1x10^{-6}$*
Number of positive autoantibodies			$9x10^{-9}$*
One (%)	40 (5)	38 (13)	
Two (%)	283 (35)	134 (45)	
Three (%)	480 (60)	124 (42)	
GADA (%)	627 (78)	229 (77)	0.80
GADA level (DK U/ml)	323 (117, 698)	374 (149, 671)	0.19
High level GADA (%)	265 (33)	107 (36)	0.33
ZnT8A (%)	616 (77)	153 (52)	$1x10^{-15}$*
ZnT8A level (AU/ml)	44 (16, 84)	26 (7, 54)	$3x10^{-6}$*
High level ZnT8A (%)	348 (43)	60 (20)	$2x10^{-12}$*

Table 5: *Comparison of clinical characteristics at diagnosis between high and low IA-2A level groups for positive IA-2A type 1 diabetes cases. Bimodal IA-2A level distribution was divided into low and high level groups using the nadir between the modes at 130 DK U/ml. Autoantibody levels were assessed in those people who were positive for that antibody. * indicates a P value lower than threshold the P value for multiple comparisons (0.05/22 =0.0023)*

ZnT8A level at diagnosis was not associated with age at diagnosis of type 1 diabetes.

To assess the association of ZnT8A level to clinical features at diagnosis, we divided 954 type 1 diabetes cases who were positive for ZnT8A by the median value of the distribution (35.6 AU/ml) due to lack of clear bimodal distribution. No statistically or clinically significant relationship was found between ZnT8A level and age at diagnosis (high level: 17.0 years [IQR12-26] vs. low level: 19 years [12, 30], *P*=0.06) (Figure 4) or *HLA-DR3-DQ2* or *DR4-DQ8*. Both groups also exhibited similar presentation characteristics (DKA, weight loss, polyuria, HbA1c and BMI). However, those with higher level ZnT8A were more likely to be multiple autoantibody positive (71% vs. 56% with three autoantibodies $P=5x10^{-6}$) and more likely to be positive for IA-2A (86% vs. 76% $P=1x10^{-4}$) at higher levels (median level 290 DK U/ml [IQR 215-359] vs. 245 DK U/ml [IQR 117-326] $P=1x10^{-5}$) (Table 6). Similar results for the lack of association of ZnT8A level to age at diagnosis were observed with linear regression analysis of log ZnT8A level to age at diagnosis (β=-0.55, 95% CI -1.2,0.08, *P*=0.09).

Characteristic	High Level ZnT8A	Low Level ZnT8A	High vs Low Level P value	ZnT8A Negative‡	Low vs ZnT8A Negative P value
n (%)	477 (50)	477 (50)		630 (57)	
ZnT8A level (AU/ml)	78 (54, 110)	11 (5, 21)		0.23 (0.16, 0.43)	
Female (%)	207 (43)	196 (41)	0.47	270 (43)	0.56
Non-European descent (%)	27 (6)	21 (4)	0.37	0 (0)	$1x10^{-7}$*
Age of Diagnosis (years)	17 (12, 26)	19 (12, 30)	0.06	25 (15, 37)	$6x10^{-10}$*
Duration of Diabetes (weeks)	11 (6, 18)	11 (6, 19)	0.74	11 (6, 18)	0.25
Hospital Admission (%)	372 (78)	385 (81)	0.3	439 (70)	2x10-5
DKA (%)	196 (42)	218 (46)	0.16	242 (39)	0.02
Polyuria (%)	462 (98)	457 (97)	0.29	587 (94)	0.04
Weight Loss (%)	400 (85)	410 (87)	0.38	530 (86)	0.6
HbA1c (mmol/mol)	78 (59, 101)	79 (58, 112)	0.35	85 (61, 109)	0.4
BMI	23.4 (21.3, 26.5)	23.3 (20.9, 25.5)	0.11	23.5 (21.3, 26.4)	0.06
Parent with Diabetes (%)	61 (13)	69 (15)	0.49	116 (19)	0.08
Other autoimmune condition (%)	37 (8)	32 (7)	0.54	50 (8)	0.45
T1D-GRS	0.276 (0.257, 0.293)	0.273 (0.255, 0.292)	0.54	0.274 (0.256, 0.293)	0.98
HLA-DR3-DQ2 (%)	256 (54)	233 (49)	0.14	335 (53)	0.15
HLA-DR4-DQ8 (%)	264 (55)	283 (59)	0.21	314 (50)	0.002
Number of positive autoantibodies			$5x10^{-6}$*		$2x10^{-129}$*
One (%)	12 (3)	25 (5)		401 (64)	
Two (%)	127 (27)	186 (39)		229 (36)	
Three (%)	338 (71)	266 (56)		0 (0)	
GADA (%)	395 (83)	357 (75)	0.003	556 (88)	$6x10^{-9}$*

GADA level (DK U/ml)	371 (146, 728)	298 (111, 675)	0.03	378 (141, 666)	0.06
High level GADA (%)	180 (38)	143 (30)	0.01	255 (40)	$3x10^{-4}$*
IA-2A (%)	408 (86)	361 (76)	$1x10^{-4}$*	303 (48)	$2x10^{-20}$*
IA-2A level (DK U/ml)	290 (215, 359)	245 (117, 326)	$1x10^{-5}$*	177 (23, 301)	$1x10^{-6}$*
High level IA-2A (%)	348 (73)	268 (56)	$6x10^{-8}$*	169 (27)	$4x10^{-23}$*

Table 6: *High vs. Low ZnT8A level comparison and Low ZnT8A level vs. Negative ZnT8A but positive for GADA and/or IA-2A. ZnT8A level distribution was divided into two groups using the median of the distribution at 35.6 AU/ml. Values expressed as median (interquartile range) unless stated. Autoantibody levels were assessed in those people who were positive for that antibody.* ‡*Other islet autoantibody positive. *indicates a p value lower than threshold the p value for multiple comparisons (0.05/22 =0.0023).*

GADA negative individuals were younger at diagnosis in comparison to IA-2A/ZnT8A negative individuals who were older at diagnosis.

The comparison of people with low level of GADA to negative GADA (positive for IA-2A and/or ZnT8A) showed that the people GADA negative were diagnosed younger (median 14 years [IQR 10, 21] vs. 19 years [13, 29], P=$2x10^{-12}$) and had higher DR4-DQ8 (53% vs 69%, P=$6x10^{-6}$) (Supplementary Table 1) (15). Whereas people negative for IA-2A (positive for GADA and/or ZnT8A) were diagnosed older compared to low level of IA-2A (median 28 years [IQR 18, 38] vs. 23 years [13, 34], P=$6x10^{-6}$) (Supplementary Table 2) (15). Similar results were also observed for ZnT8A (median 25 years [IQR 15, 37] for those ZnT8A negative vs. 19 years [12, 30] with low level ZnT8A, P=$6x10^{-10}$) (Table 6). Both IA-2A and ZnT8A negative groups were highly enriched for GADA positive people (93% and 88% respectively).

Bimodal distributions of GAD and IA-2 islet autoantibody levels were also observed in second independent cohort

To replicate our results with different assay and different cohort, we analysed GADA, IA-2A and ZnT8A levels in 449 patients with type 1 diabetes at diagnosis from the StartRight study where islet autoantibody levels were measured using ELISA assays; another commonly used assay for islet autoantibody measurement.

Similar to our primary cohort, GADA and IA-2A levels showed a bimodal distribution in this replication cohort, with ZnT8A showing one peak with right skewed distribution (Figure 5). The shape of the distribution is different in this cohort due to the clinical laboratory conducting the ELISAs not reporting results that are outside the standard curve leading to truncation at higher levels.

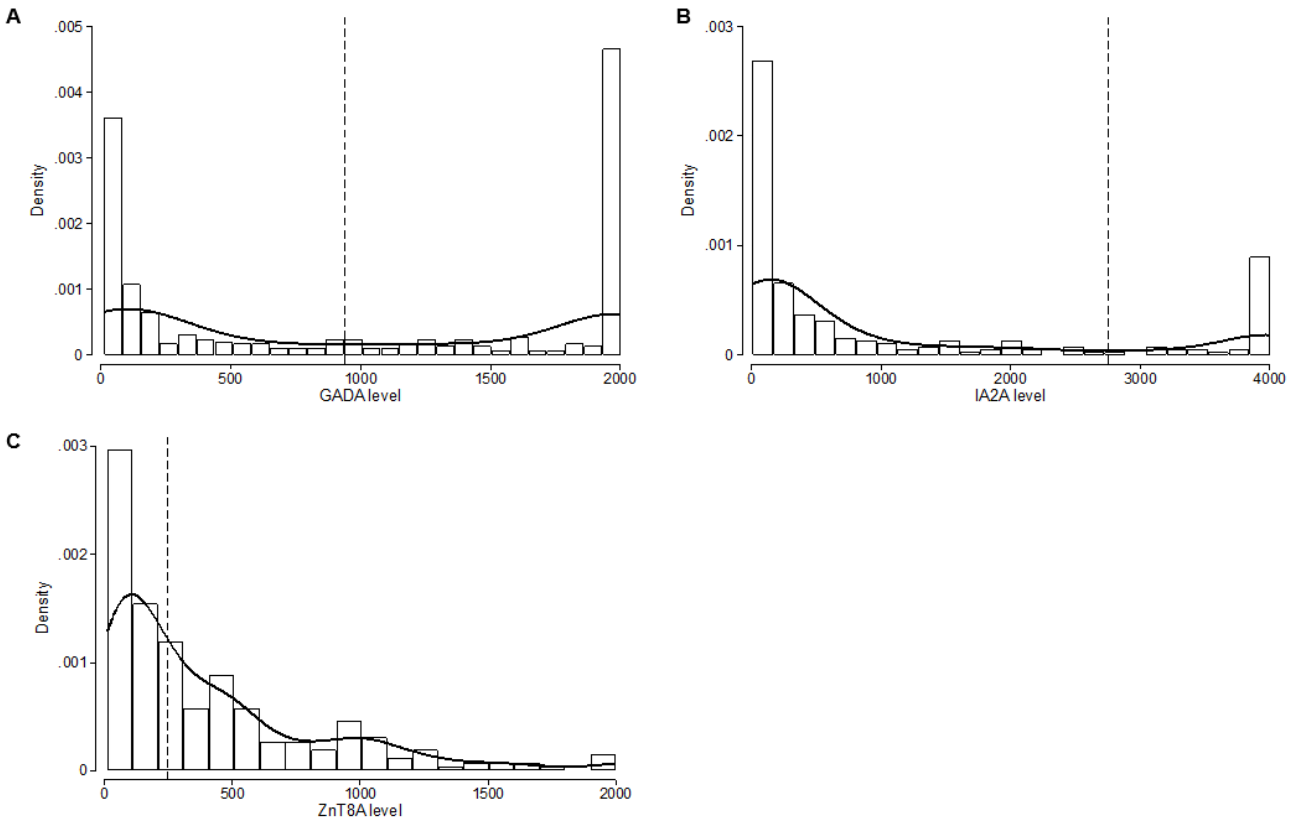

Figure 5: *Histograms with kernel density curves showing the distribution of islet autoantibody levels at diagnosis measured using ELISA assay in patients with type 1 diabetes in the replication cohort (StartRight cohort). A) Histogram of GADA level for type 1 diabetes cases who were positive for GADA (n=404) at diagnosis. GADA levels exhibit a bimodal distribution. The ELISA assay was calibrated to maximum value of 2000 WHO U/ml. The nadir value of 937 WHO U/ml between the two modes is highlighted with black dashed line and used to defined high level group (≥937 WHO U/ml) and low level group (<937 WHO U/ml). B) Histogram of IA-2A level for type 1 diabetes cases who were positive for IA-2A (n=237) at diagnosis. IA-2A levels exhibit a bimodal distribution. The ELISA assay was calibrated to maximum value of 4000 WHO U/ml. The nadir value of 2756 WHO U/ml between the two modes is highlighted with black dashed line and used to defined high level group (≥2756 WHO U/ml) and low level group (<2756 WHO U/ml). C) Histogram of ZnT8A level for type 1 diabetes cases positive for ZnT8A (n=260) at diagnosis show a right skewed distribution. The ELISA assay was calibrated to maximum value of 2000 WHO U/ml. Median value of the distribution (247 AU/ml) is highlighted with black dashed lines.*

Using the same method as our primary cohort, we divided the GADA and IA-2A bimodal distributions into high and low autoantibody level groups using the nadir between the peaks (Figure 5). Similarly to our primary analysis, those in the high level GADA group were diagnosed later compared to the low GADA level group (40 years [IQR 31-53] vs. 30 years [I24-38], P=6x10^{-13}) and were more likely to be female (60% vs. 43%, P=5x10^{-4}). There were no differences in presentation characteristics, parent with diabetes, or random non-fasting C-peptide at

diagnosis (Supplementary Table 7) (15). However, people with high level GADA showed trend towards faster decline of c-peptide in first 2 years compared to low level of GADA (annual decline in UCPCR -48% [95% CI -41%, -55] vs. -42% [95% CI -33%, -50%], *P*=0.258) (Figure 6A).

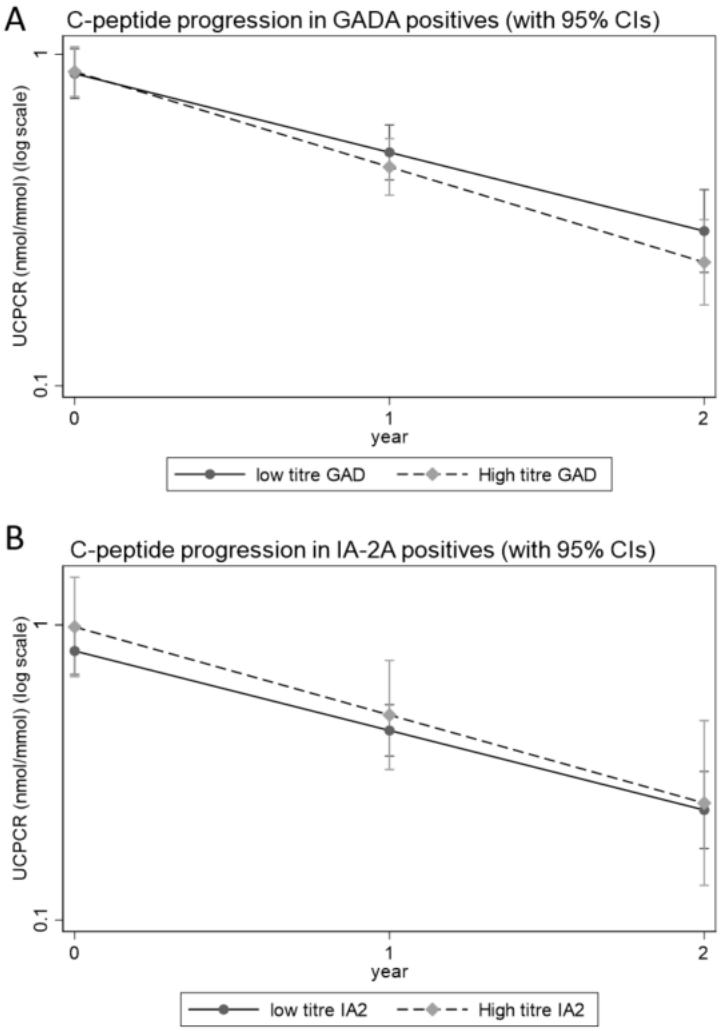

Figure 6: *Urinary C-Peptide /Creatinine Ratio (UCPCR) decline in the first 2 years following diagnosis of type 1 diabetes in StartRight Study. [A] Log-linear c-peptide decline in those with high and low GADA levels. [B] Log-linear c-peptide decline in those with high and low IA-2A levels. High level GADA/IA-2A: dotted lines. Low level GADA/IA-2A: solid line.*

The participants with higher IA-2A levels were younger (median 28 years [IQR 22, 48] vs. 33 years [25, 46]) compared to the ones with lower IA-2A levels, as our primary cohort, but this difference was not statistically significant

(Supplementary Table 4) (15). The baseline and annual decline in C-peptide was also similar between the two IA-2A level groups (-50%, 95%CI -31%, -63%; vs. -46%, 95% CI -38%,-54%, *P*=0.708) (Figure 6B).

3.6 Discussion

Our study shows that GADA and IA-2A level at diagnosis of type 1 diabetes show clear bimodal distributions. Dichotomising levels into high or low groups according to the observed modes exhibits strong associations with age at diagnosis of type 1 diabetes but not with severity of type 1 diabetes at diagnosis.

Bimodality of GADA and IA-2A is a novel finding in type 1 diabetes and may point towards a type 1 diabetes pathogenesis. There have been multiple studies of islet autoantibody levels in at-risk populations for type 1 diabetes (1; 7-9) and within type 1 diabetes populations but none of the studies to our knowledge have reported that GADA and IA-2A levels have a bimodal distribution. However, the bimodality of GADA levels but not IA-2A levels has been reported in people with latent autoimmune diabetes (LADA) (24; 25). The older-onset type 1 diabetes have a higher proportion of GADA whereas childhood-onset diabetes has a higher proportion of IA-2A, IAA and ZnT8A (26). We found that levels of GADA and IA-2A also follow the same pattern with higher GADA levels in older individuals and higher IA-2A levels in younger individuals. Although this does not confirm but may suggest that a higher level of autoantibody in an individual point towards the triggering (first) autoantibody and a lower level of autoantibody in an individual points towards spreading autoantibodies. This can also be supported by the observation that those positive for *HLA-DR3-DQ2* had higher levels of the associated triggering GADA and lower levels of spreading IA-2A and the reverse observed in those positive for *HLA-DR4-DQ8* (higher levels of the associated

triggering IA-2A but lower GADA levels) (Supplementary Figure 4)(15). ZnT8A levels were not seen to differ between positivity for either *HLA-DR4-DQ8* or *HLA-DR3-DQ2*.

The additional factors including genetic predisposition underlies the observed bimodality. There was an enrichment of *HLA DR3-DQ2* and *HLA DR4-DQ8* in people with higher GADA levels and IA-2A levels respectively. Both these associations are well-described with the positivity of the respective autoantibodies but not with the autoantibody levels (27; 28). The difference in HLA susceptibility suggests a role for humoral immunity and antigen recognition as one of the factors underlying bimodality. However, the association with HLA was modest in our study and the overall genetic risk score was similar with high and low autoantibody level groups suggesting that there are additional factors which are responsible for the observed bimodality. Previous studies have shown that high GADA levels are correlated with higher affinity autoantibodies, the central and c-terminal epitopes, and multiple autoantibodies positivity (29). We did not observe the association of high GADA level with multiple autoantibodies, but we did observe the association of higher IA-2A level with multiple autoantibodies. These data suggest that affinity, the difference in epitopes, and the presence of other autoantibodies may also contribute towards bimodality.

GADA and IA-2A levels are associated with age at onset but in opposite directions. The participants who had high GADA levels were nearly 7 years older at diagnosis compared to the ones with low level GADA. This was replicated in a second independent cohort of adults with type 1 diabetes. Contrary to GADA, participants with high IA-2A levels were 5.4 years younger at diagnosis compared to those with low IA-2A levels. We did not observe a statistically significant

reduction in age at diagnosis in people with the high level of IA-2A in our second independent cohort. This may be due to the combination of the overall older age of onset of diabetes (median 34 years), longer time to islet autoantibody measurement from the diagnosis (25% were measured at >31 weeks) and use of ELISA assay compared to our primary cohort. Interestingly, we note that both GADA and IA-2A levels but not ZnT8A level follows the same association with age as positivity of these autoantibodies (14; 30; 31). In contrast to our findings in type 1 diabetes, studies of LADA have shown that higher GADA level is associated with early age of onset. This may relate to the very different populations studied and the relationship between age and prior prevalence of autoimmune diabetes which, as recently suggested, may markedly alter antibody false positive rates in populations of apparent type 2 diabetes (25; 31; 32).

The observed association of high level of GADA with concurrent other autoimmune disease was mainly due to thyroid/celiac autoimmunity (68/77 in high level GADA and 34/42 in low level GADA). This association is likely due to the shared HLA risk alleles *DR3-DQ8* for thyroid and celiac autoimmunity and high level of GADA in our study (33; 34).

Autoantibody level at diagnosis was not strongly associated with severity of presentation of type 1 diabetes but may associate with beta cell function at follow up. We did not observe association of autoantibody levels with symptoms of hyperglycaemia, BMI, HBA1c and C-peptide at diagnosis. These results contrast previous studies in LADA where high levels of GADA were associated with lower BMI, C-peptide and higher HbA1c (25). Interestingly, we did observe a trend towards a lower c-peptide at follow up in our second cohort. The small sample size and shorter follow-up means this finding was not statistically significant.

Similar trend has been reported with GADA level by a recent cross-sectional study of patients with type 1 diabetes (35). This observed association if replicated in the larger cohort, provides an exciting opportunity to identify type 1 diabetes subtypes which has important clinical significance for the prediction of islet function and early intervention to prevent severe metabolic complication. Our findings have important implications for the prediction, treatment and prognosis of type 1 diabetes. It is well known that type 1 diabetes is a heterogeneous disease with heterogeneity in islet autoantibodies, beta-cell function, genetics as well as response to immunomodulatory therapy (36). The research to date mainly focuses on the positivity of autoantibodies rather than autoantibody levels in type 1 diabetes. We believe that autoantibody levels showing a bimodality for GADA and IA-2A is an important consideration in understanding the heterogeneity and pathogenesis of type 1 diabetes. It is well known that immunomodulatory therapy has a variable response on beta-cell function in clinical trials (37). Currently, the reason for this variable response is not entirely known but is proposed to be due to variation in T cell response (38; 39). The bimodality of the levels may represent a surrogate marker of a specific immune response and identify the subgroup of individuals with differential response to immunomodulatory therapy but this needs testing in further studies. This, along with the association of GADA level with c-peptide in a recent study, provides an exciting possibility of a stratified approach to type 1 diabetes treatment and prognosis which is currently lacking (35). Our findings also make a strong case to assess the usefulness of the bimodality of GADA and IA-2A levels in the prediction models of progression of diabetes in at-risk population in addition to autoantibody positivity.

Our islet autoantibody levels were assessed using radiobinding assays in our primary cohort. We validated our findings using a replication cohort and a second method of islet autoantibody assessment (ELISA) which is more-commonly used in routine clinical laboratories. However, both assays showed high level of correlation for all three autoantibodies in the same samples during the IASP 2018 workshop (Figure 2). This was also in line with the previous studies (40; 41). This suggests that our findings are applicable to levels measured by both methods. Although a bimodal distribution of GADA and IA-2A was observed using both assay methods, the shape of the distributions does look different between assays. We believe this is due to the clinical laboratory conducting the ELISAs not reporting results that are outside the standard curve causing truncation at both ends in comparison the RBA laboratory which reports extrapolated results.

Our study was limited by the use of 97.5th -98th centiles of the controls to define autoantibody positivity in our study. Although this cut-off is widely used in clinical practice, the use of these cut-offs, despite the higher prior probability of our cohort would have led to the inclusion of a small number of people with low levels as positive for each autoantibody (up to 2.5% for each autoantibody). This may have a small effect on the distribution at the lower levels but is unlikely to change the bimodality and overall conclusion of our study due to the large sample size of our cohort. Also, we only had c-peptide information at diagnosis and follow up in one of the study cohorts, therefore we are limited in our ability to assess the impact of autoantibody level distribution on beta cell function. We did not study insulin autoantibodies (IAA) as this would not be appropriate due to the nature of our cohorts being recruited in the weeks after diagnosis of type 1 diabetes and commencement of insulin therapy.

In conclusion, we show that GADA and IA-2A levels exhibit a bimodal distribution at diagnosis of type 1 diabetes, which is biologically important to the understanding of the heterogeneity of type 1 diabetes and opens the exciting possibility of further research to assess its implication on prediction, treatment and prognosis of type 1 diabetes.

3.7 Acknowledgements

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3.8 Prior Data Presentation

This work, in part, has been presented as a scientific poster at the nPOD conference (2021) and the Diabetes UK Professional Conference (2021).

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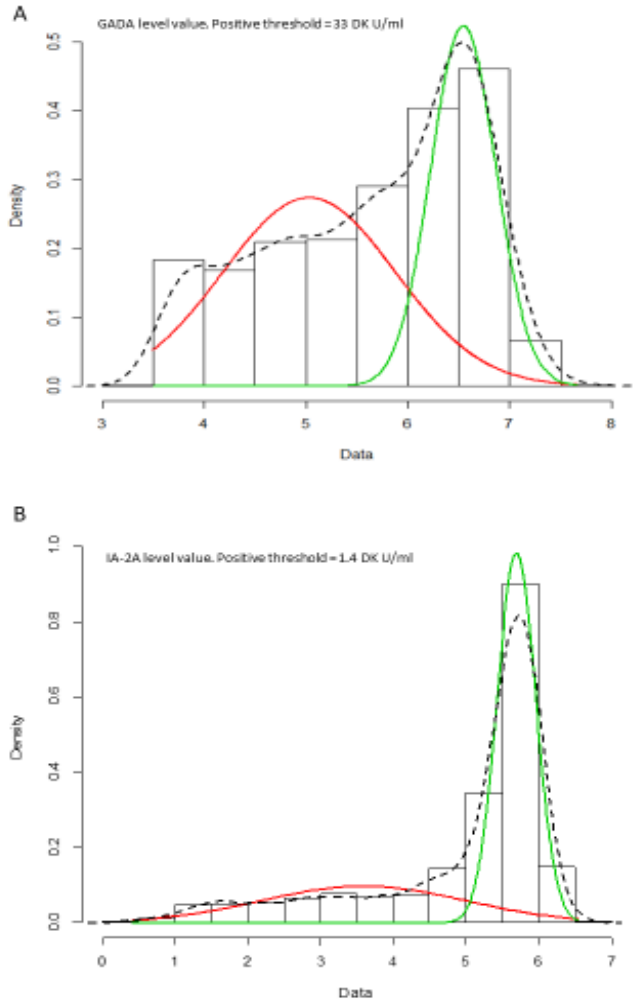
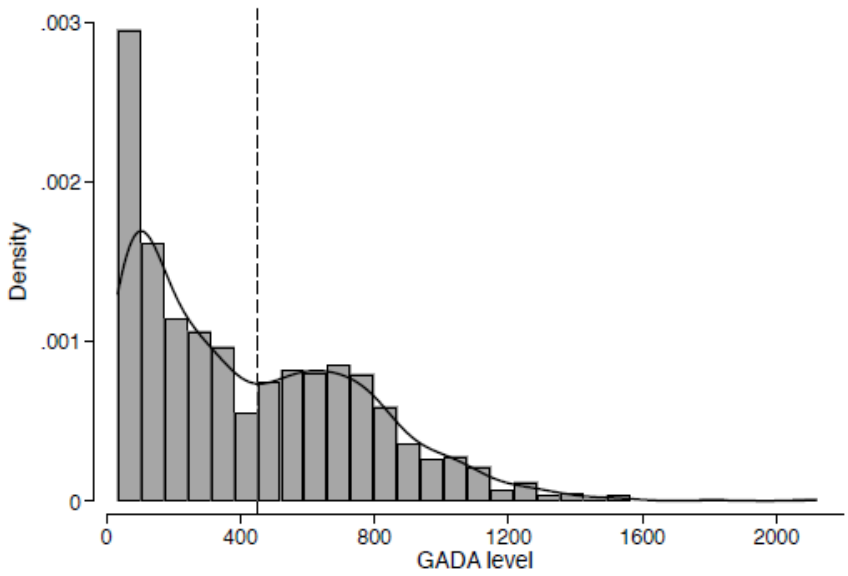
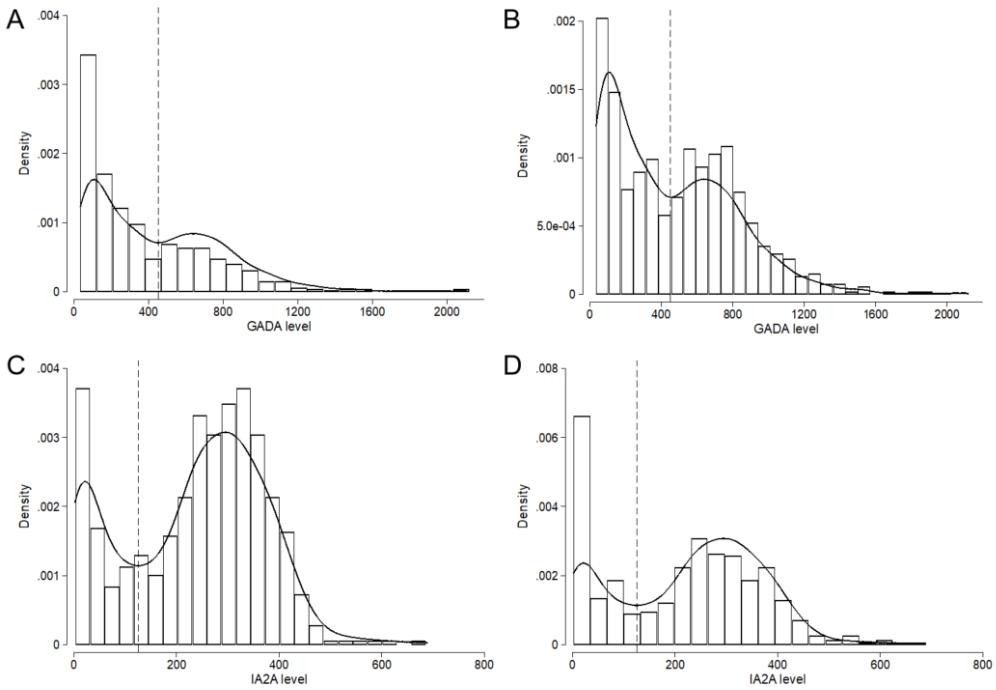
3.10 Supplementary Material



Supplementary Figure 1: *Log autoantibody level density plots showing the two best fitting normal densities for GADA and IA-2A level distributions. A) Log GADA level density plot. Histogram and black dashed line show observed density. Red and green lines show the two best fitting normal densities which are used in the calculation of the likelihood ratio test. Likelihood ratio test: normal distribution versus two component mixture distribution: Log(GADA) ~ $N(\theta, \sigma^2)$ vs. Log(GADA) ~ $w_1N(\theta_1, \sigma_1^2)+w_2N(\theta_2, \sigma_2^2)$. LRT = 352 on 3 degrees of freedom, $p<5x10^{-76}$. B) Log IA-2A level density plot. Histogram and black dashed line show observed density. Red and green lines show the two best fitting normal densities which are used in the calculation of the likelihood ratio test. Likelihood ratio test: univariate normal distribution versus two component mixture distribution: Log(IA-2A) ~ $N(\theta, \sigma^2)$ vs. Log(IA-2A) ~ $w_1N(\theta_1, \sigma^2)+w_2N(\theta_2, \sigma^2)$. LRT = 1219 on 3 degrees of freedom, p-value $<5x10^{-264}$.*



Supplementary Figure 2: *Histogram with kernel density curve showing the distribution of glutamate decarboxylase autoantibody levels in patients with type 1 diabetes at diagnosis and no autoimmune thyroid disease. Histogram of GADA level at diagnosis measured using radiobinding assay for type 1 diabetes cases who were positive for GADA. GADA level exhibits a bimodal distribution when those with autoimmune thyroid disease was removed. The nadir value of 450 DK U/ml between the two modes is highlighted with the black dashed line*



Supplementary Figure 3: *Distribution of GADA and IA-2A levels in patients with childhood-onset type 1 diabetes and adult-onset type 1 diabetes at diagnosis. Histogram of GADA level at diagnosis measured using radiobinding assay for A) 599 childhood onset type 1 diabetes (diagnosed <20y of age) and B) 765 adult onset type 1 diabetes (diagnosed ≥20y) who were positive for GADA. The nadir value between the two GADA modes based on the whole cohort is highlighted with black dashed line. Histogram of IA-2A level at diagnosis measured using radiobinding assay for C) 624 childhood onset type 1 diabetes (diagnosed <20y of age) and D) 475 adult onset type 1 diabetes (diagnosed ≥20y) who were positive for IA-2A. The nadir value between the two IA-2A modes based on the whole cohort is highlighted with black dashed line.*

Characteristic	High Level GADA	Low Level GADA	High vs Low Level P value	GADA Negative‡	Low vs GADA Negative P value
n (% of GADA positives)	604 (44)	760 (56)		268 (26)	
GADA level (DK U/ml)	723 (594, 877)	149 (76, 274)		7 (0, 18)	
Female (%)	317 (52)	282 (37)	$1x10^{-8}$*	97 (36)	0.79
Non-European descent (%)	45 (7)	51 (7)	0.6	0 (0)	$1x10^{-5}$*
Age of Diagnosis (years)	27 (17, 38)	19 (13, 29)	$9x10^{-17}$*	14 (10, 21)	$2x10^{-12}$*
Duration of Diabetes (weeks)	10 (6, 17)	11 (6, 18)	0.29	14 (8, 21)	$2x10^{-4}$*
Hospital Admission (%)	434 (72)	577 (76)	0.1	231 (87)	$3x10^{-4}$*
DKA (%)	250 (42)	323 (43)	0.74	110 (41)	0.58
Polyuria (%)	580 (97)	712 (95)	0.21	258 (98)	0.09
Weight Loss (%)	522 (88)	641 (86)	0.23	220 (85)	0.7
HbA1c (mmol/mol)	87 (64, 110)	81 (58, 107)	0.01	72 (54, 104)	0.08
BMI, kg/m2	23.7 (21.4, 26.7)	23.2 (20.9, 26.2)	0.04	23.0 (21.0, 25.1)	0.25
Parent with Diabetes (%)	121 (20)	104 (14)	0.002*	35 (13)	0.83
Other autoimmune condition (%)	77 (13)	42 (6)	$3x10^{-6}$*	6 (2)	0.03
T1D-GRS	0.273 (0.256, 0.292)	0.275 (0.255, 0.292)	0.48	0.275 (0.258, 0.294)	0.57
HLA-DR3-DQ2 (%)	353 (58)	387 (51)	0.006	113 (42)	0.01
HLA-DR4-DQ8 (%)	291 (48)	401 (53)	0.09	184 (69)	$6x10^{-6}$*
Number of positive autoantibodies			0.45		$6x10^{-42}$*
One (%)	165 (27)	195 (26)		107 (40)	
Two (%)	183 (30)	217 (29)		161 (60)	
Three (%)	256 (42)	348 (46)		0 (0)	
IA-2A (%)	372 (62)	484 (64)	0.43	235 (88)	$2x10^{-13}$*
IA-2A level (DK U/ml)#	254 (91, 340)	255 (124, 338)	0.66	246 (95, 316)	0.1

High level IA-2A (%)	265 (44)	362 (48)	0.17	168 (63)	$2x10^{-5}$*
ZnT8A (%)	323 (53)	429 (56)	0.27	194 (72)	$4x10^{-6}$*
ZnT8A level (AU/ml) #	43 (13, 83)	36 (12, 76)	0.13	24 (8, 75)	0.08
High level ZnT8A (%)	180 (30)	215 (28)	0.54	78 (29)	0.8

Supplementary Table 1: *High vs. Low GADA level comparison and Low GADA level vs. negative for GADA but positive for IA-2A and/or ZnT8A comparison. Bimodal GADA level distribution was divided into two groups using the nadir between the two modes at 450 DK U/ml (High vs Low level GADA). Values expressed as median (interquartile range) unless stated. Autoantibody levels were assessed in those people who were positive for that antibody. ǂOther islet autoantibody positive. *indicates a p value lower than threshold the p value for multiple comparisons (0.05/22 =0.0023).*

Characteristic	High Level IA-2A	Low Level IA-2A	High vs Low Level P value	IA-2A Negative‡	Low vs IA-2A Negative P value
n (%)	803 (73)	296 (27)		502 (63)	
IA-2A level (DK U/ml)	295 (237, 359)	24 (8, 65)		0 (0, 0)	
Female (%)	355 (44)	117 (40)	0.16	210 (42)	0.52
Non-European descent (%)	48 (6)	17 (6)	0.88	0 (0)	$6x10^{-8}$*
Age of Diagnosis (years)	17 (12, 25)	23 (13, 34)	$3x10^{-7}$*	28 (18, 38)	$6x10^{-6}$*
Duration of Diabetes (weeks)	12 (7, 18)	11 (6, 18)	0.2	10 (6, 18)	0.77
Hospital Admission (%)	650 (81)	217 (74)	0.01	346 (69)	0.19
DKA (%)	350 (44)	131 (45)	0.88	190 (39)	0.11
Polyuria (%)	767 (97)	279 (96)	0.21	476 (95)	0.92
Weight Loss (%)	679 (87)	244 (86)	0.2	431 (88)	0.11
HbA1c (mmol/mol)	76 (56, 101)	83 (60, 114)	0.01	87 (63, 111)	0.46
BMI	23.2 (21.2, 25.9)	23.7 (21.5, 26.6)	0.07	23.4 (20.9, 26.2)	0.13
Parent with Diabetes (%)	110 (14)	47 (16)	0.37	93 (19)	0.33
Other autoimmune condition (%)	45 (6)	24 (8)	0.13	51 (10)	0.33
T1D-GRS	0.275 (0.257, 0.293)	0.275 (0.254, 0.294)	0.73	0.273 (0.256, 0.292)	0.8
HLA-DR3-DQ2 (%)	354 (44)	165 (56)	$6x10^{-4}$*	316 (63)	0.05
HLA-DR4-DQ8 (%)	533 (66)	149 (50)	$1x10^{-6}$*	187 (37)	$3x10^{-4}$*
Number of positive autoantibodies			$9x10^{-9}$*		$7x10^{-78}$*
One (%)	40 (5)	38 (13)		360 (72)	
Two (%)	283 (35)	134 (45)		142 (28)	
Three (%)	480 (60)	124 (42)		0 (0)	
GADA (%)	627 (78)	229 (77)	0.8	469 (93)	$4x10^{-11}$*
GADA level (DK U/ml)	323 (117, 698)	374 (149, 671)	0.19	382 (136, 679)	0.74

High level GADA (%)	265 (33)	107 (36)	0.33	212 (42)	0.09
ZnT8A (%)	616 (77)	153 (52)	$1x10^{-15}$*	175 (35)	$3x10^{-6}$*
ZnT8A level (AU/ml)	44 (16, 84)	26 (7, 54)	$3x10^{-6}$*	18 (6, 65)	0.65
High level ZnT8A (%)	348 (43)	60 (20)	$2x10^{-12}$*	65 (13)	0.01

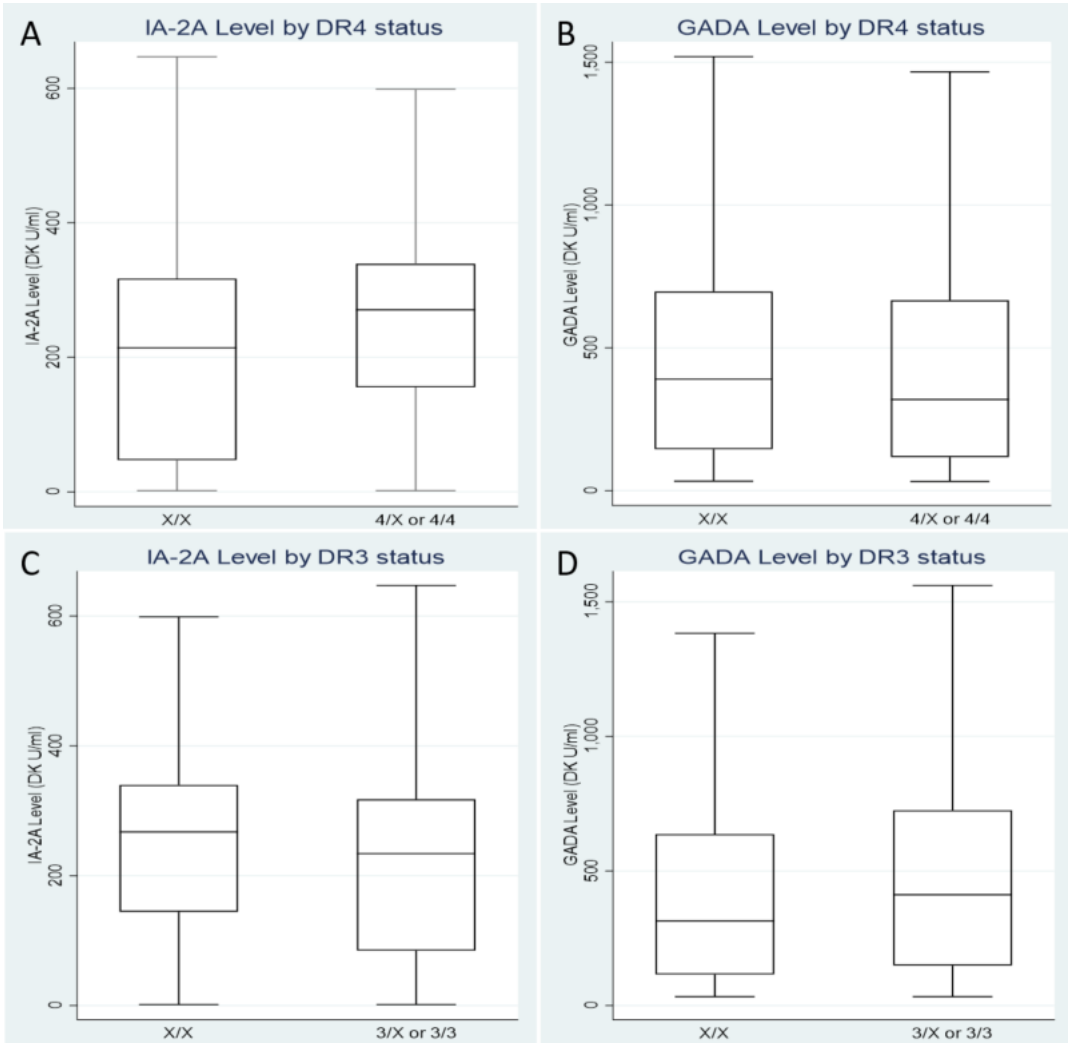
Supplementary Table 2: *High vs. Low IA-2A level comparison and Low IA-2A level vs. negative for IA-2A but positive for GADA and/or ZnT8A comparison. Bimodal IA-2A level distribution was divided into low and high level groups using the nadir between the two modes at 125 DK U/ml. Values expressed as median (interquartile range) unless stated. Autoantibody levels were assessed in those people who were positive for that antibody.* ‡*Other islet autoantibody positive. *indicates a p value lower than threshold the p value for multiple comparisons (0.05/22 =0.0023).*

Characteristic	Low Level GADA	High Level GADA	P value
n (%)	207 (51)	197 (49)	
GADA level (U/ml)	94 (46, 309)	2001 (1830, 2001)	
Female (%)	88 (43)	118 (60)	$5x10^{-4}$*
Non-European descent (%)	10 (5)	11 (6)	0.73
Age of Diagnosis (years)	30 (24, 38)	40 (31, 53)	$6x10^{-13}$*
Duration of Diabetes (weeks)	15 (6, 32)	15 (7, 29)	0.85
Hospital Admission (%)	129 (63)	113 (57)	0.28
DKA (%)	70 (34)	71 (36)	0.67
Polyuria (%)	192 (93)	178 (90)	0.39
Weight Loss (%)	169 (82)	178 (90)	0.01
HbA1c (mmol/mol)	65 (51, 87)	64 (50, 89)	0.94
BMI (kg/m2)	24.2 (21.4, 27.0)	24.7 (21.9, 27.5)	0.25
C-Peptide (picomol/L)	480 (273, 684)	457 (275, 683)	0.94
Parent with Diabetes (%)	38 (18)	42 (21)	0.46
Other autoimmune condition (%)	23 (11)	42 (21)	0.005
Number of positive autoantibodies			0.95
One (%)	65 (31)	61 (31)	
Two (%)	60 (29)	55 (28)	
Three (%)	82 (40)	81 (41)	
IA-2A (%)	106 (51)	99 (50)	0.85
IA-2A level (U/ml)	360 (86, 1950)	210 (40, 1575)	0.11
High level IA-2A (%)	22 (21)	17 (17)	0.51
ZnT8A (%)	118 (57)	118 (60)	0.56
ZnT8A level (AU/ml)	233 (93, 542)	279 (97, 592)	0.62
High level ZnT8A (%)	57 (48)	62 (53)	0.52

Supplementary Table 3: *Comparison of clinical characteristics between high and low level GADA groups for positive GADA type 1 diabetes cases in StartRight study. Bimodal GADA level distribution was divided into two groups using the nadir between the two modes at 937 WHO U/ml. Values expressed as median (interquartile range) unless stated. Autoantibody levels were assessed in those people who were positive for that antibody. * indicates a p value lower than threshold the p value for multiple comparisons (0.05/20 =0.0025).*

Characteristic	Low Level IA-2A	High Level IA-2A	P value
n (%)	192 (81)	45 (19)	
IA-2A level (U/ml)	141 (38, 553)	4001 (3999, 4001)	
Female (%)	87 (45)	25 (56)	0.22
Non-European descent (%)	7 (4)	3 (7)	0.36
Age of Diagnosis (years)	33 (25, 46)	28 (22, 48)	0.27
Duration of Diabetes (weeks)	15 (7, 35)	11 (7, 17)	0.04
Hospital Admission (%)	119 (62)	30 (67)	0.59
DKA (%)	72 (38)	16 (36)	0.79
Polyuria (%)	176 (92)	43 (96)	0.38
Weight Loss (%)	166 (86)	33 (74)	0.03
HbA1c (mmol/mol)	63 (49, 86)	66 (57, 91)	0.36
BMI (kg/m2)	24.6 (22.3, 27.0)	25.3 (21.2, 29.2)	0.55
C-Peptide (picomol/L)	482 (296, 637)	443 (291, 756)	0.75
Parent with Diabetes (%)	30 (16)	8 (18)	0.72
Other autoimmune condition (%)	31 (16)	6 (13)	0.64
Number of positive autoantibodies			0.91
One (%)	17 (9)	4 (9)	
Two (%)	44 (23)	9 (20)	
Three (%)	131 (68)	32 (71)	
GADA (%)	166 (86)	39 (87)	0.97
GADA level (U/ml)	910 (91, 2001)	439 (76, 2001)	0.97
High level GADA (%)	82 (49)	17 (44)	0.51
ZnT8A (%)	140 (73)	34 (76)	0.72
ZnT8A level (AU/ml)	279 (114, 606)	533 (335, 1088)	0.002
High level ZnT8A (%)	74 (53)	27 (79)	0.005

Supplementary Table 4: *Comparison of clinical characteristics between high and low level IA-2A groups for positive for IA-2A type 1 diabetes cases in StartRight study. Bimodal IA-2A level distribution was divided into low and high level groups using the nadir between the modes at 2756 WHO U/ml. Autoantibody levels were assessed in those people who were positive for that antibody. Values expressed as median (interquartile range) unless stated. *Indicates a p value lower than threshold the p value for multiple comparisons (0.05/20=0.0025).*



Supplementary Figure 4: *Box plots of GADA and IA-2A levels by HLA-DR4-DQ8 and HLA-DR3-DQ2. A-B) Higher IA-2A levels (371 DK U/ml [155, 339]) and lower GADA levels (319 DK U/ml [118, 667]) were observed in those with HLA-DR4-DQ8 compared to those without HLA-DR4-DQ8 (IA-2A: 214 DK U/ml [47, 317], $p=2x10^{-7}$ and GADA: 391 [146, 697], p=0.011). C-D) Those with HLA-DR3-DQ2 had higher GADA levels (412 DK U/ml [149, 725] vs. 314 DK U/ml [116, 63], p=0.0002) and lower IA-2A levels (234 DK U/ml [85, 318] vs. 267 DK U/ml [144, 340], p=0.0006) compared to those without HLA-DR3-DQ2.*

Chapter 4: Autoantibodies to truncated GAD stratify risk of early insulin requirement in adult-onset type 2 diabetes

Sian L. Grace, Kathleen M. Gillespie, Claire L. Williams, Vito Lampasona, Peter Achenbach, Ewan R. Pearson, Alistair J.K. Williams, Anna E. Long, Timothy J. McDonald, Angus G. Jones

In Preparation

4.1 Acknowledgement of co-authors and contributions to paper

I conducted the GADA characterisation and IA-2A LIPS assays, with guidance from Anna E. Long and Claire L. Williams, I also researched and analysed the data as well as writing the manuscript. The specialist assays were developed by Alistair J.K. Williams, Vito Lampasona and Peter Achenbach. Kathleen Gillespie, Angus G. Jones, Timothy J. McDonald, Anna E. Long and Claire L. Williams helped plan the analysis, contributed to the discussion and reviewed/edited the manuscript. Angus G. Jones and Ewan R. Pearson set up the studies from which samples and participants were selected.

4.2 Abstract

Objective

Progression to insulin therapy is variable in individuals diagnosed with type 2 diabetes positive for full length GAD antibodies (f-GADA). We investigated whether a GAD epitope, IgG subclasses and affinity could identify early insulin requirement in adult-onset cases.

Research Design and Methods

We assessed truncated (t-)GADA positivity, f-GADA IgG subclasses, and f-GADA IgG affinity in 179 f-GADA positive participants diagnosed with type 2 diabetes and assessed association of GADA characteristics with progression to insulin treatment, type 1 diabetes genetic risk score (T1DGRS) and C-peptide. We compared these characteristics to GADA positive type 1 diabetes (n=144) and antibody negative type 2 diabetes (n=6420).

Results

t-GADA positivity was lower in individuals with type 2 diabetes without early insulin requirement (72%) in comparison to those who progressed rapidly (97%, $p=7x10^{-5}$). t-GADA positivity was similar between those with type 2 diabetes and early insulin requirement and those with f-GADA positive type 1 diabetes (95%, p=0.565). t-GADA positivity (in those f-GADA positive) identified a group with a higher type 1 diabetes genetic susceptibility [mean T1DGRS 0.248 vs. 0.225, p=0.003 (type 1 diabetes 0.274, f-GADA negative type 2 diabetes 0.228)], lower c-peptide (1155 pmol/L vs. 4289 pmol/L, $p=1x10^{-7}$) and increased IA-2A positivity

(23% vs. 6%, p=0.02). In survival analysis, t-GADA positivity was associated with higher risk of progression to early insulin therapy in comparison to those f-GADA positive but t-GADA negative [HR 8.4 (95% CI 2.05, 34.4) p=0.003]. The presence of an IgG1 restricted f-GADA subclass response was not associated with early insulin requirement compared with IgG unrestricted subclass responses [HR 1.07 (95% CI 0.62, 1.9), p=0.813]. High f-GADA affinity was less common in those with f-GADA positive type 2 diabetes than in the type 1 reference cohort but did not stratify those with early insulin requirement [HR 1.04 (95% CI 0.62, 1.73), p=0.886].

Conclusions

t-GADA testing of f-GADA positive individuals with type 2 diabetes identifies those who have genetic and clinical characteristics similar to type 1 diabetes and stratifies those at higher risk of early progression to insulin therapy. These data suggest that implementation of t-GADA as part of clinical testing has the potential to predict early insulin requirement.

4.3 Introduction

Autoantibodies to GAD are common in adults initially diagnosed and treated as having type 2 diabetes, with prevalence varying from 2 to >10% depending on population and assay. This patient group, often described as having latent autoimmune diabetes (LADA) and recently defined by the WHO as slowly evolving immune-mediated diabetes (1; 2), is highly heterogeneous, varying from those with very rapid progression to insulin therapy and a type 1 diabetes like phenotype, to those with the clinical course and characteristics of type 2 diabetes.

Whether this heterogeneity is best explained by a heterogeneous intermediate form of autoimmune diabetes, or a mixture of autoimmune and non-autoimmune diabetes, due to the combination of imperfect islet antibody specificity and low prior likelihood of autoimmune diabetes (Bayes Theorem), or both, is a matter of debate (3). Approaches that improve specificity of GAD testing for identifying patients with the clinical course of type 1 diabetes would allow targeting of intensive monitoring, advice and early insulin initiation to those most likely to benefit.

Developments in assay technology have meant that we are now able to measure additional GAD characteristics beyond full length GAD titre, including epitope specificity, affinity and IgG subclass (4-6). The clinical utility of these GADA characteristics is unclear. Previous research in type 1 diabetes prediction has shown that GADA reactive to the n-terminally truncated GAD antigen (GAD96-585) are more disease-specific in first degree relatives of patients with diabetes, whilst maintaining sensitivity and specificity in those diagnosed with type 1 diabetes (5). Reactivity to t-GADA (7) and high f-GADA affinity (8) have been found to be associated with risk of early insulin treatment in those with adult-onset diabetes, and increased IgG3 and IgG4 IgG subclasses have been reported to be associated with a slower rate of beta cell destruction in LADA (9).

We aimed to determine whether assessment of GADA truncated epitope specificity, affinity and subclass within those with f-GADA positive type 2 diabetes (LADA), post diagnosis, could improve the identification of patients with early insulin requirement and the C-peptide and genetic characteristics of type 1 diabetes.

4.4 Research Design and Methods

Study Cohorts

Participants were included in this study if they had a clinical diagnosis of type 2 diabetes, were greater than 18 years of age at diagnosis and were treated without insulin for the first 6 months from diagnosis. They were identified from the Genetics of Diabetes Audit and Research Tayside Study (GoDarts) (10), Diabetes Alliance for Research in England (DARE) (11), Predicting Response to Incretin Based Agents in Type 2 Diabetes (PRIBA) (12), MRC MASTERMIND Progressors (13) and StartRight Studies (14) in the U.K and described together previously (15). Participants with type 1 diabetes were also identified from DARE. These studies recruited participants from primary and secondary care and are population based, with the exception of PRIBA and MRC MASTERMIND Progressors, which account for <12% of participants. Participants from the GoDarts study were excluded if their diabetes diagnosis date was before January 1st 1994, due to insufficient prescribing information as we were unable to define time to insulin prior to this date. In the DARE cohort, only those with saved serum recruited in the Exeter centre were included.

We assessed f-GADA characteristics in 179 f-GADA positive participants with a clinical diagnosis of type 2 diabetes after age 18, and no insulin requirement within 6 months of diabetes (Supplementary table 1). We compared islet autoantibody, genetic and C-peptide characteristics with 6,420 participants with f-GADA negative type 2 diabetes (clinical diagnosis and >6 months to insulin), and 144 participants with type 1 diabetes (f-GADA positive, on insulin therapy from diagnosis, clinical diagnosis of type 1 diabetes). Detailed characteristics including age at diagnosis and duration of diabetes are shown in Table 1, with the type 2 diabetes cohorts split by recruiting study in Supplementary table 2.

Variable	f-GADA Positive T1D Reference Cohort	T2D (All)	T2D with f-GADA positivity	T2D with f-GADA negativity
n	144	6,599	179 (2.7%)	6,420 (97.3%)
Male (%; 95% CI)	68 (47%; 39, 56)	3,761 (57%; 56, 58)	79 (56%; 48, 63)	2,758 (57%; 56, 58)
Ethnicity (%non-Caucasian; 95% CI)	5 (3.5%; 1.14, 1.79)	2 (0.03%; 0.004, 0.11)	2 (1.1%)	0 (0%)
Age at Diagnosis (Years)	22 (16, 35.5)	61 (52, 68)	56.7 (46, 65.9)	60.8 (52, 68)
Duration of diabetes at latest follow up (years)	15 (5, 28)	5.6 (3.0, 9.6)	12 (8.8, 18.2)	11 (7, 14.7)
BMI (at first visit; kg/m^2)	24.5 (22.5, 27.6)	30.8 (27.3, 35.1)	29.0 (25.4, 33.3)	30.8 (27.4, 35.1)
Duration of diabetes at BMI (months)	15.8 (7.6, 27.5)	5.4 (0.89, 58.5)	6 (0.5, 80)	5.3 (0.9, 58.2)
f-GADA titre (WHO U/ml)	107 (43, 1244)	5 (4.9, 5)	114 (28, 1510)	5 (4.9, 5)
Duration at f-GADA (years)	16 (6, 29)	5.6 (3.0, 9.6)	4.9 (1.5, 10.0)	5.6 (3, 9.5)
Insulin treated within 5 years (%, 95% CI)	144 (100%)	492 (7.5%; 6.9, 8.2)	63 (35%; 28, 43)	429 (6.7%, 6.1, 7.3)
HbA1c (mmol/mol)*	68 (59, 81)	57 (48, 75)	61 (50, 81)	57 (48, 74)
HbA1c (%)*	8 (8, 10)	7 (7, 9)	8 (7, 10)	7 (7, 9)
Duration at HbA1c (years)	16 (7, 27)	1.2 (0.08, 10.2)	2.9 (0.13, 10.7)	1.2 (0.08, 10.2)
T1D GRS	0.275 (0.257, 0.295)	0.231 (0.206, 0.254)	0.250 (0.218, 0.270)	0.230 (0.205, 0.253)
C-peptide (pmol/l)	6 (2.9, 39)	2090 (1290, 3075)	1100 (577, 1750)	2146 (1350, 3125)

Table 1: *Overall cohort characteristics. Data displayed as n (%; 95% CI) or median (IQR). *At latest follow-up*

Assessment of HbA1c and Diabetes Progression (Time to Insulin)

Available HbA_{1c} at latest follow-up [median diabetes duration 11 years (range 7-15)] was obtained from electronic health care records for the GoDarts study (n=3,893) or was measured on a research sample in recruitment centres' local laboratories (all are accredited NHS blood science laboratories) for the Exeter cohorts (PRIBA, MRC Progressors, StartRight, DARE; n=2,706).

For GoDarts, time to insulin was defined from electronic prescription records. For Exeter cohorts (DARE, PRIBA, and MRC MASTERMIND Progressors), insulin treatment, date of commencing insulin and date of diagnosis were self-reported at a single visit. For StartRight, insulin treatment, date of commencing insulin and date of diagnosis were self-reported at three visits.

Laboratory Measurement of autoantibodies to full-length GAD65(1-585)

f-GADA analysis was conducted for all participants at The Academic Department of Blood Sciences, Royal Devon and Exeter Hospital using the RSR Limited ELISA (RSR Limited, Cardiff, U.K.) on the Dynex DS2 ELISA Robot (Dynex Technologics, Worthing, U.K.). The cut-off for positivity was ≥11 units/mL, based on the 97.5th centile of 1,559 control participants without diabetes (16). The lowest reportable value (lowest calibrant) was 5.0 units/mL. The laboratory participates in the International Islet Autoantibody Standardization Program (IASP) with assay specificity and AS95 of 98.9% and 86%, respectively, in the 2020 IASP program.

Assessment of GADA characteristics

Of 6,618 participants with a diagnosis of type 2 diabetes screened for f-GADA, 198 (3%) were found to be f-GADA positive, 179 (2.7%) had sera available for further characterisation. These, and the 144 f-GADA positive patients with type 1 diabetes, underwent further analysis to explore autoantibody characteristics; truncated GAD65(96-585) (t-GADA) epitope specificity, f-GADA affinity, and f-GADA IgG subclasses were assessed using specially adapted assays as follows:

Measurement of GADA to truncated GAD65(96-585)

t-GADA epitope specificity was determined by a luciferase immunoprecipitation (LIPS) assay system using nanoluciferase-tagged GAD65/67 kDa isoform of GAD antigen, with the n-terminal amino acids 1-95 truncated (Nluc-GAD65(96-585)). 1 microgram of Nluc-GAD65(96-585) antigen in a pCMVTNT vector was incubated for 1.5 hrs at 30°C with reagents from the SP6 *in vitro* transcription/translation coupled kit (Promega); 40µl reticulocyte master mix and 2µl 1mM methionine. After incubation the Nluc-GAD65(96-585) antigen was purified using a NAP5™ desalting column (GE Healthcare) and Tris buffered saline with Tween-20 buffer (TBST; 20 mM Tris, 150 mM NaCl, pH 7.4 + 0.5%

Tween-20). Luminescence activity (Light units, LU) was detected by a LB 960 microplate luminometer Centro XS3 (Berthold Technologies, GmbH & Co. KG, Bad Wilbad, Germany) using 2µl of the reaction mix and 40µl of furimazine substrate diluted 1:50 in NanoGlo® LIPS assay buffer supplied in the NanoGlo® coupled kit (Promega) [Reagent for luminescence detection: RLD]. Nluc-GAD65(96-585) antigen was diluted in TBST to a concentration of $4.0x10^6$ LU/25µl (± 200,000 LU). Sera (1µl, 2 replicates) were pipetted into a 96-well plate and incubated with 25µl of diluted Nluc-GAD65(96-585) for 2.5 hours at room temperature protected from light. Immune complexes were precipitated using a Protein A Sepharose (PAS) suspension washed four times in TBST buffer (GE Healthcare Life Sciences, Chicago, IL USA; 6.25µl/well) for 1 hour with orbital shaking (~700 rpm) at 4°C. After incubation, excess Nluc-GAD65(96-585) was excluded by centrifugation (503xg at 4°C for 3 minutes) and five serial washes with TBST and then transferred into a 96-well OptiPlate™ (Perkin Elmer). Optiplates™ were centrifuged (503xg at 4°C for 3 minutes) and aspirated to remove excess buffer for a final volume of 30µl. To detect residual luminescence, 40µl of RLD (further diluted 1:3 with TBST) was added to each well immediately prior to LU determination using a standardised protocol on the Centro XS3 (inject, shake 5 seconds/well, detect 2 seconds/well). Diabetic kidney (DK) units/ml were calculated using a logarithmic standard curve and the threshold of positivity was ≥10.7 DK units/ml (based on the 97.5th centile of 221 school children). In the IASP 2020 workshop, the specificity and AS95 for this assay were 100% and 86%, respectively.

Measurement of GADA IgG subclasses to full-length GAD65(1-585)

Determination of IgG subclasses to f-GADA was based upon the approach previously described (6; 17), using [35S]-methionine-labelled GAD65 and biotinylated IgG subclass-specific mouse anti-human monoclonal antibodies [*BD Biosciences, San Diego, USA; ** Invitrogen, Thermo Fisher, CA, USA] bound by Streptavidin-Sepharose beads (Sigma Aldrich, Dorset, UK). Mouse anti-human IgG subclass antibodies used were IgG1 (clone G17-1*), IgG2 (clone G18-21*), IgG3 (clone HP6047**), and IgG4 (clone JDC-14*). A 50% suspension of Ethanolamine-blocked Protein G Sepharose (EB-PGS; 25µl in a 50µl volume per well) in TBST was used to detect all IgG subclasses as a total IgG control. All

IgG-specific results were expressed in mean CPM and non-specific binding was determined by subtraction of mean CPM of a mouse anti-rat IgM monoclonal antibody (clone G53-238*) termed delta (Δ) CPM. Two composite IgG subclass positivity quality controls (QCs; IgG1/IgG2 and IgG1/IgG3/IgG4) were previously developed for the assay by Dr Claire Williams (Bristol, U.K.), and were run in all assays alongside samples (27 and 21 CV% and 5, 39 and 40 CV% respectively). A negative control from a healthy adult was also run in all assays to assess the assay background. Due to serum availability, a sub-cohort of those f-GADA positive were selected for subclass analysis. Equal proportions of all three cohorts (type 2 diabetes with and without early insulin requirement and the type 1 diabetes reference cohort) were selected. Where possible, samples were matched for f-GADA titre quartile and f-GADA affinity result, and samples from each cohort studied were run in each assay run. Results were expressed as mean delta cpm (IgG specific subclass cpm – anti-rat IgM cpm) and converted to a SD score (SDS) calculated as: [(IgG Subclass-specific counts delta cpm – mean delta cpm of control subjects/SD delta cpm of control subjects] and considered positive for that subclass if the SDS ≥3.

Measurement of GADA Affinity to full-length GAD65(1-585)

Affinity of f-GADA was measured by competitive binding experiments based on the approach developed by Mayr *et al* (4). Briefly, serum (2µl) was plated in duplicate and incubated for 24 hours at 4°C with 25µl/well of [^{125}I]-recombinant human GAD65 ($1.88x10^{-10}$ mol/l; RSR Limited, Cardiff, U.K.) at five increasing quantities of unlabelled human GAD65 diluted in TBST [(0.15% Tween-20); $1.5x10^{-15}$, $1.5x10^{-14}$, $1.5x10^{-13}$, $1.5x10^{-12}$, and $1x10^{-11}$ mol/well] (RSR Limited, Cardiff, U.K.) or TBST only. Immune complexes were precipitated for 1hr (orbital shaking ~700rpm at 4°C) with 12.5µl/well PAS (PAS suspension washed four times in TBST buffer). After incubation, excess unbound [^{125}I]-recombinant human GAD65 was excluded by centrifugation (503xg at 4°C for 3 minutes) and five serial washes with TBST, transferred to microtubes (STARLABS, Milton Keynes, U.K.), and measured using a TopCount gamma counter (Perkin Elmer), where the results were expressed as mean counts per minute (cpm).

IC_{50} and K_d values were calculated by non-linear regression analysis using a one-site model (R^2 >0.90), assuming equal antibody binding by labelled and unlabelled GAD65, on GraphPad Prism3 (GraphPad Software, San Diego, CA, USA) (18). The f-GADA affinity of each sample was expressed as the reciprocal K_d value (l/mol). Samples that were not fully competed were diluted 1:5 or 1:10 in TBST buffer for accurate f-GADA affinity assessment. The calculation of K_d values was limited to samples with IC_{50} values greater than the concentration of labelled GAD65 ($1.88x10^{-10}$ mol/l). For samples with an IC_{50} <$1.88x10^{-10}$ mol/L, the f-GADA affinity of the sample was set at K_d >$8x10^{11}$ l/mol. A negative quality control sample from a healthy adult was run in each assay to control for non-specific binding. A positive control sample [f-GADA positive relative without diabetes, with sufficient volume for multiple testing (38% CV)] was run alongside samples in each assay.

Assessment of IA-2A Positivity

IA-2A positivity was determined using a LIPS assay specific to the intracytoplasmic (aa606-979) region of islet antigen-2 (IA-2ic) in those f-GADA positive. The NLuc-tagged antigen was expressed in Expi293F™ cells using Expi293™ expression system (Thermo Fisher Scientific, Waltham, MA, USA) and was kindly provided by Vito Lampasona (Milan, Italy). The antigen was then diluted 1:1000, filtered through a 0.45µm sterile syringe filter (Fisher Scientific), aliquoted, and stored at -80°C. This assay then followed the same protocol as the t-GADA assay described above. Digestive kidney (DK) U/ml were calculated using a logarithmic standard curve and the threshold of positivity was ≥0.3 DK U/ml (based on the 98th centile of 112 school children). In the IASP2020 workshop, the specificity and AS95 for this assay was 100% and 78%, respectively.

In those f-GADA negative (n=2,607), study IA-2A results were used. These were tested on the same serum sample as the f-GADA at The Academic Department of Blood Sciences, Royal Devon and Exeter Hospital using the RSR Limited ELISA (RSR Limited, Cardiff, U.K.) on the Dynex DS2 ELISA Robot (Dynex, Preston, U.K.). The cut-off for positivity was ≥7.5 units/mL, based on the 97.5^{th} centile of 1,559 control participants without diabetes (16). The laboratory

participates in the International Islet Autoantibody Standardization Program (IASP) with assay specificity and AS95 of 98.9% and 72% in the 2020 IASP program.

Additional laboratory analysis (C-peptide and type 1 diabetes genetic risk score)

Urinary C-peptide and plasma C-peptide were measured by electrochemiluminescence immunoassay (intra-assay CV, 3.3%; inter-assay CV, 4.5%) on a Roche Diagnostics (Mannheim, Germany) E170 analyser by the Blood Sciences Department at the Royal Devon and Exeter NHS Foundation Trust (Exeter, U.K.)

We generated weighted T1D-GRS from 30 common type 1 diabetes genetic variants [single nucleotide polymorphisms (SNPs)] for HLA and non-HLA loci as we previously described (15; 19).

Statistical Analysis

We assessed f-GADA positivity in 6,618 patients diagnosed with type 2 diabetes, those that tested f-GADA positive but did not have sera available for further analysis were removed from the study. In the f-GADA positives with type 2 diabetes, with sera available for further analysis, we assessed the following GADA characteristics (dichotomised for categorical analysis): t-GADA status (positive vs. negative), IgG Subclass response (IgG1 restricted vs. IgG unrestricted) and affinity category (higher vs. lower affinity). We assessed the proportions in each GADA characteristic category for each cohort and compared the proportions (using Pearson chi-squared tests) in each category between those with f-GADA positive type 2 diabetes with and without insulin therapy within 5 years and to those with f-GADA positive type 1 diabetes. We then compared clinical and biochemical patient characteristics (C-peptide, T1D GRS, IA-2A positivity, f-GADA titre, age at diagnosis and insulin therapy within 5 years) between characteristic categories using Pearson chi-squared tests for proportions of categorical variables (Graphs drawn using GraphPad Prizm) and t-tests for continuous variables. We then assessed the relationship between GADA characteristics and progression to insulin (censored at 5 years or the latest

available time point not on insulin if earlier) using cox proportional hazard models in univariable and multivariable models (adjusting for co-variates including f-GADA titre, duration of diabetes at f-GADA test and age at diagnosis). For f-GADA affinity and IgG subclass response, we also assessed whether there would be an association between higher affinity and IgG1 restricted responses and progression to insulin therapy independent of t-GADA specificity in addition to the above co-variates. All statistical analysis was carried out using Stata/SE 16.0 (StataCorp, College Station, TX) unless otherwise stated.

4.5 Results

In total, of 6,599 participants initially diagnosed with type 2 diabetes, that had f-GADA assessed, 198 (3%) were positive for f-GADA (measured at a median of 5.6 years diabetes duration), of whom 179 had sera stored for further analysis. Characteristics of these participants, by GADA status (f-GADA positives restricted to those with available sera for assessment of GAD characteristics) are shown in Table 1. 99.5% of participants had had been followed for at least 5 years; median follow-up time, calculated as the median time to censoring (insulin treatment or latest follow-up), was 11 years (IQR 7, 15). A total of 7.5% (n=492) of those participants had progressed to insulin ≤5 years. For comparison, we studied 144 f-GADA positive patients (measured at a median of 16 years diabetes duration) with type 1 diabetes. Their characteristics are also displayed in Table 1.

Participants with positive GADA for a truncated epitope have enrichment for genetic and clinical characteristics associated with type 1 diabetes

Positivity for t-GADA was similar between individuals with type 1 diabetes and those with f-GADA positive type 2 diabetes requiring early insulin (≤5 years) 95% (95% CI 90, 98) vs. 97% (95% CI 89, 100) respectively, p=0.565). In contrast, the proportion of those with t-GADA positivity in those without early insulin requirement was significantly lower [72% (95% CI 63, 80)] than those with early insulin requirement ($p=7x10^{-5}$) and the type 1 diabetes cohort ($p=4x10^{-7}$)) (Figure 1). t-GADA positivity identified a group diagnosed younger [mean 55 years (95% CI 52, 57) vs. 62 years (95% CI 58, 66), p=0.002], with a higher T1D-GRS [mean 0.248 (95% CI 0.241, 0.254) vs. 0.225 (95% CI 0.213, 0.237), p=0.003], lower c-

peptide levels [mean 1155 pmol/L (95% CI 918, 1393) vs. 4289 pmol/L (95% CI 845, 7732), $p=1x10^{-7}$ at a median duration of 12 years at C-peptide testing] and increased positivity for IA-2A [23% (95% CI 17, 31) vs. 6% (95% CI 0.7, 19.7), p=0.022] than those positive for f-GADA but t-GADA negative (Table 2).

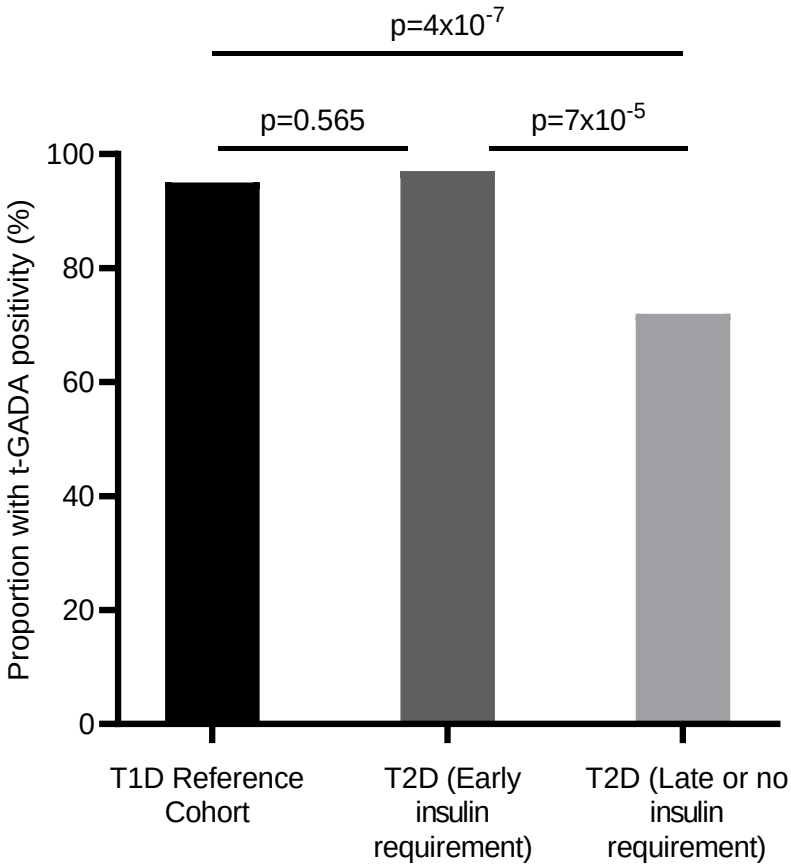


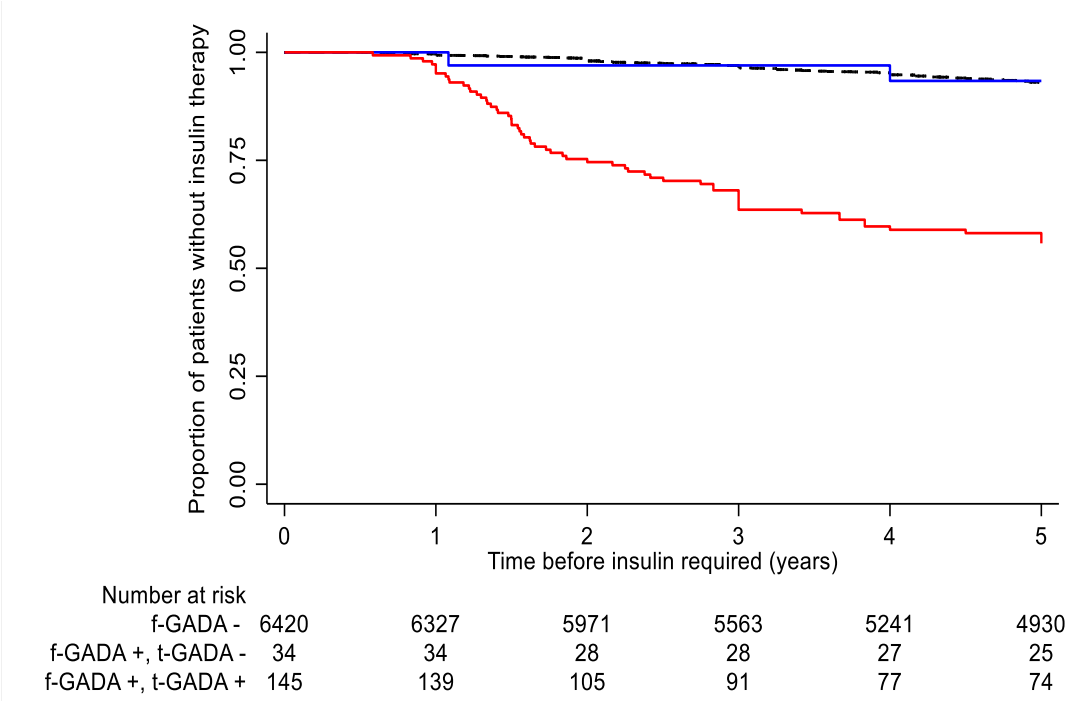
Figure 1: *Bar chart showing the proportions t-GADA positive in each cohort. T1D; Type 1 diabetes. T2D; type 2 diabetes. t-GADA; truncated GAD(96-585) autoantibody.*

	T2D f-GADA & t-GADA positive	T2D f-GADA positive & t-GADA negative	T2D t-GADA positive vs. T2D t-GADA negative *p* value	T1D Reference cohort	T2D with f-GADA negativity
n	141	34		144	6,420
Age at Diagnosis (Years)	56 (45, 64)	63 (55, 69)	0.002	22 (16, 35.5)	61 (52, 68)
f-GADA Titre (WHO U/ml)	209 (36, 2000)	28 (18, 95)	0.0004	107 (43, 1244)	5 (4.9, 5)
T1D Genetic Risk Score	0.256 (0.230, 0.272)	0.224 (0.204, 0.252)	0.003	0.275 (0.257, 00.295)	0.230 (0.205, 0.253)
C-Peptide (pmol/L)	1030 (575, 1490)	3870 (2740, 5838)	$1x10^{-7}$	6 (2.9, 39)	214.6 (1350, 3125)
IA-2A Positive (%)	33 (23%)	2 (5.9%)	0.022	70 (50%)	15 (0.6%)*
Insulin treated within 5 years (%)	61 (42%)	2 (5.9%)	0.00008	144 (100%)	429 (6.7%)

Table 2: *Diabetes characteristics comparison between those positive and negative for t-GADA in those f-GADA positive. Data displayed as n (%) or median (IQR). *Out of 2,607 tested. T1D; Type 1 Diabetes. T2D; Type 2 Diabetes. t-GADA; truncated GAD(96-585) autoantibody. f-GADA; full length GAD(1-585) autoantibody.*

Truncated GADA epitope positivity is associated with increased risk of early insulin therapy

Using cox proportions hazard model for survival analysis, t-GADA positivity (in those f-GADA positive) identified participants at markedly higher risk of early progression to insulin compared to those f-GADA positive & t-GADA negative [HR 8.4 (95% CI 2.1, 34.4)) p=0.003] (Figure 2). The association between t-GADA positivity (in those f-GADA positive) and early insulin requirement persisted after adjustment for age of diagnosis, f-GADA titre and duration of diabetes [adjusted HR 5.7 (95% CI 1.4, 23.5) p=0.017] compared to those f-GADA positive and t-GADA negative. Those positive for f-GADA but negative for t-GADA had similar risk of progression to early insulin requirement compared to those with f-GADA negative type 2 diabetes [HR 0.93 (95% CI 0.23, 3.72), p=0.9], this was similar after adjustment for age at diagnosis, f-GADA titre and duration of diabetes [adjusted HR 0.98 (95% CI 0.24, 3.95), p=0.978]. Table 3 shows the cox proportional hazards regression models for time to insulin (censored at 5 years) unadjusted and controlling for age at diagnosis, duration of diabetes and f-GADA titre. These hazard ratios where greater than those obtained when risk of early insulin requirement was stratified by f-GADA positivity (Supplemental Table 3).



Number at risk						
f-GADA -	6420	6327	5971	5563	5241	4930
f-GADA +, t-GADA -	34	34	28	28	27	25
f-GADA +, t-GADA +	145	139	105	91	77	74

Figure 2: *Kaplan-Meier plot of probability of requiring insulin therapy during 5-year follow-up, in those clinically diagnosed with type 2 diabetes, by risk group of f-GADA and t-GADA positivity. Solid lines represent f-GADA positive groups and dashed lines represent f-GADA negative group. Blue indicates t-GADA negative and red is t-GADA positive. +, positive. -, negative.*

	Unadjusted Model		Adjusted Model	
Overall survival with f-GADA negatives	**HR(95% CI)**	***p* value**	**HR(95% CI)**	***p* value**
f-GADA Negative	1		1	
f-GADA Positive, t-GADA Negative	0.93 (0.23, 3.72)	0.916	0.98 (0.24, 3.95)	0.978
f-GADA Positive, t-GADA Positive	8.4 (6.4, 11.0)	<0.001	7.06 (5.0, 10.1)	<0.001
Age of Diagnosis (per 1 year increase)			0.96 (0.95, 0.96)	<0.001
f-GADA Titre (per 1 unit increase)			1 (1, 1)	0.744
Duration of Diabetes at f-GADA testing (per 1 year increase)			0.98 (0.97, 1.0)	0.03
Survival analysis t-GADA pos vs t-GADA neg (In those f-GADA positive)	**Unadjusted Model**		**Adjusted Model**	
t-GADA negative	1		1	
t-GADA positive	8.4 (2.05, 34.4)	0.003	5.7 (1.4, 23.5)	0.017
Age of Diagnosis (per 1 year increase)			0.94 (0.92, 0.96)	<0.001
f-GADA Titre (per 1 unit increase)			1 (1, 1)	0.536
Duration of Diabetes at f-GADA testing (per 1 year increase)			0.88 (0.83, 0.94)	<0.001

Table 3*: Hazard Ratios from Cox proportional regression model (unadjusted and adjusted) for time to insulin censored at 5 years (t-GADA positivity). t-GADA; truncated GAD(96-585) autoantibody. f-GADA; full length GAD(1-585) autoantibody.*

Full-length GADA IgG subclasses do not identify those at risk of early insulin therapy

The prevalence of each f-GADA IgG subclass was similar between f-GADA positive type 2 diabetes participants with and without early insulin requirement and those in the f-GADA positive type 1 diabetes (Supplemental Table 4). The rank order of frequencies of IgG subclass was the same between those with type 2 diabetes and early insulin requirement and those without early insulin requirement (IgG1>IgG3>IgG2>IgG4). In the type 1 diabetes reference cohort the rank order of frequencies of IgG subclasses was IgG1>IgG3>IgG4>IgG2. f-GADA IgG subclasses were unable to be detected in 13 (6%) of the subset tested. IgG1 was the most common IgG subclass in all three cohorts, we therefore split the cohort into groups based on the subclass responses being

IgG1 only (restricted) response vs. multiple IgG subclass (unrestricted) responses for further analysis. The proportion of those with an IgG1 restricted response was similar between those with type 2 diabetes and early insulin requirement vs. those without early insulin requirement [42% (95% CI 29, 57) vs. 39% (95% CI 28, 52), p=0.7). The proportion of those with an IgG1 restricted response in the type 1 diabetes reference cohort was also similar [40% (95% CI 29, 53), p vs. other subgroups >0.8) (Supplemental Figure 1). IgG subclass responses did not show differences in clinical characteristics (age at diagnosis, type 1 diabetes genetic susceptibility, c-peptide levels and IA-2A positivity) but those with an IgG1 restricted response were more likely to have lower levels of f-GADA than those with an IgG unrestricted response (mean 468 WHO U/ml (95% CI 283, 652) vs. 1130 WHO U/ml (95% CI 918, 1342), p<0.0001 (Supplemental Table 5).

In survival analysis, an IgG1 restricted response did not identify those at risk of early insulin requirement in those that were f-GADA positive [HR 1.07 (95% CI 0.62, 1.9), p=0.8] (Supplemental Figure 2). This was still the case when the model was adjusted for age of diagnosis and duration of diabetes [HR 1.02 (95% CI 0.58, 1.8) p=0.9] (Supplemental Table 6). f-GADA titre was omitted as a co-variate as there was an increased likelihood of subclasses being undetectable in samples with lower f-GADA titres. The presence of each individual IgG subclass was not associated with progression to insulin in survival analysis (Supplemental Table 7).

The proportion of higher affinity full-length GADA was lower in those with type 2 diabetes

The affinities of f-GADA detected ranged from $7.57x10^6$ - >8 $x10^{11}$ l/mol across all groups (type 2 diabetes with early insulin requirement 3.94 $x10^7$ - >8 $x10^{11}$ l/mol, type 2 diabetes with no/later insulin requirement 7.57 $x10^6$ - >8 $x10^{11}$ l/mol, type 1 diabetes reference cohort $3.76x10^7$ - >8 $x10^{11}$ l/mol). Affinities exhibited a clear and marked bimodal distribution (Supplemental Figure 3), therefore, they were split into lower and higher affinity categories for further analysis: <=$2x10^{11}$ l/mol affinity (Range 7.57 $x10^6$ – $1.99x10^{11}$ l/mol for low affinity, 2 $x10^{11}$ - >8 $x10^{11}$ l/mol for high affinity). The proportion of those with higher affinity f-GADA was similar

between those with type 2 diabetes with and without early insulin requirement [41% (95% CI 28, 54) vs. 38% (95% CI 28, 48), p=0.7]. Those with type 1 diabetes had a higher proportion of those with higher affinity f-GADA [59% (95% CI 50, 67)] compared to those with early insulin requirement (p=0.02) and without (p=0.002) (Supplemental Figure 4). Analysis by affinity category showed no differences in age at diagnosis, type 1 diabetes genetic susceptibility, c-peptide levels and IA-2A positivity (Supplemental Table 8). However, those with higher affinity f-GADA had lower f-GADA titres [mean 363 WHO U/ml (95% CI 202, 524)] than those with lower affinity f-GADA [mean 976 WHO U/ml (95% CI 798, 1153), $p=4x10^{-6}$].

Stratification by f-GADA affinity category in those f-GADA positive did not stratify risk of progression to insulin therapy [HR 1.04 (95% CI 0.62, 1.73) p=0.886] (Supplemental Figure 5). Again, this was still the case when the model was adjusted for age at diagnosis, f-GADA titre and duration of diabetes at f-GADA testing [HR 0.87 (95% CI 0.49, 1.5) p=0.645]. f-GADA affinity did not further stratify early insulin requirement in those found to be t-GADA positive (Supplemental Figure 6).

4.6 Conclusions

Our study shows that in individuals with f-GADA positive type 2 diabetes, testing for t-GADA identified those with a more type 1 diabetes like phenotype (diagnosed younger, increased proportion positive for IA-2A, increased type 1 diabetes genetic susceptibility and lower c-peptide levels). To our knowledge this is the first study to show that t-GADA identified those that are at risk of early insulin requirement independently of f-GADA titre, duration of diabetes at GADA assessment and age of diagnosis. In contrast, testing f-GADA affinity and IgG subclass did not improve identification of those with early insulin requirement or a type 1 diabetes like phenotype.

A strength of our study is the size and detailed follow-up data of the initial adult-onset cohort with type 2 diabetes (>6,000) screened for f-GADA. This is a highly unique cohort as we had follow-up C-peptide data from diagnosis as well as T1D GRS data. We identified 2.7% of this cohort as f-GADA positive, using a highly robust and specific assay (RSR ELISA, obtaining 98.9% specificity on the latest IASP workshop). We were also able to apply a series of well-developed strategies

and high quality tests to examine in detail the characteristics of GADA in this well-defined cohort, and compare them to a cohort with f-GADA positive type 1 diabetes. The t-GADA LIPS assay used within our study is non-radioactive and requires very low serum volumes, which could be easier to implement in the clinical setting than the RIA used in the previous study by Achenbach *et al* (7).

A caveat of our research is that t-GADA testing was not applied to the whole cohort, due to time, sample availability and cost constraints. Therefore our results can only currently be applied to those that have previously tested f-GADA positive. We may find that a significant number of false positives in the >6000 of our cohort not tested for t-GADA which could blunt the diagnostic accuracy and hazard ratios of t-GADA testing reported in this study. Previously, Williams *et al*, identified 1% of those that previously tested f-GADA negative to be t-GADA positive (5). These low rates of t-GADA positivity in those who are negative for f-GADA negative reported by Williams *et al*, could lend support to the assumption that t-GADA is likely to have high specificity when applied to a whole population.

Another limitation of our study is that the f-GADA characterisation assays were conducted in different assay formats to the initial screening assay. Our original f-GADA screen was conducted using the RSR ELISA assay, whilst the f-GADA characteristics were conducted using liquid-phase RIA and LIPS assays. Differences between the ELISA and liquid-phase assays have been, reported previously, to impact on specificity and sensitivity (20; 21). Whilst, subclass, epitope and affinity characteristics are unable to be assessed via RSR bridging ELISA assays at this time, the characterisation assays used are of high quality. We were unable to rescreen all of our initial screening cohort by either RIA or LIPS f-GADA assays. However, as the RSR bridging ELISA is the most commercially and clinically used f-GADA assay, this allows us to compare the t-GADA assay with to a highly specific and currently used assay in the clinical setting.

Our study supports previous studies into GADA epitope characterisation in adult-onset diabetes, where those positive for t-GADA, in particular to the n-terminally truncated GAD65 antigen (96-585), identify those at higher risk of progressing to insulin therapy within 5 years and with a more type 1 diabetes-like phenotype (7). Thus providing more support to the argument that t-GADA testing can replace or

add to f-GADA testing in a clinical setting. Our study goes beyond this study by including survival analysis to assess the performance of t-GADA in predicting risk of early insulin requirement. Achenbach *et al*'s study also had a shorter follow up compared to this study and did not include C-peptide or T1D GRS data.

Like Hillman *et al* (9) we found the presence of all IgG subclasses of f-GADA present in our adult-onset type 2 diabetes cohort, with similar proportions observed for IgG1, 2 and 4. However, we also observed a higher proportion of IgG3 in our type 2 diabetes cohort (50% vs. 10% observed by Hillman *et al*), which could be due to improvements in the clone used for the anti-human IgG3 antibody and the previous one used by Hillman *et al* being discontinued. We observed the presence of IgG4 subclass in our type 1 diabetes reference cohort (21% had f-GADA IgG4 subclass) which Hillman *et al* did not previously observe. The difference in the presence of IgG4 subclass may be due to the differences in duration of diabetes at analysis, with Hillman *et al* testing samples within days of diagnosis and ours at a median of 16 years post diagnosis. Suggesting, the presence of IgG4 subclasses present in our study could be due to prolonged stimulation and maturation of B cells.

Unlike previous studies, we have shown that higher affinity GADA do not identify those at a higher risk of early insulin requirement (8). Our study has much larger numbers compared to the previous study (n=193 vs n=47), resulting in higher statistical power.

Diagnosing autoimmune diabetes in later life is an important and challenging clinical problem, and full length GAD assays are unlikely to be sufficiently specific to confirm autoimmune diabetes in the setting of those diagnosed initially as type 2 diabetes. Therefore, approaches that improve islet autoantibody test specificity are needed to improve identification of autoimmune diabetes in adults. (3). Our findings suggest that assays using t-GADA may have improved performance for identification of patients with early progression and the phenotype of type 1 diabetes, potentially improving identification of these patients in clinical practice and research. However assays for GADA affinity or IgG subclass are unlikely to improve identification of this group.

In order to improve upon the clinical ELISA assay, future work could consider trying to incorporate the n-terminally truncated assay into the plate format, as whilst the t-GADA LIPs assay can be used to screen in a research setting it's not set up on an automated platform. Our study looked at the isotype and affinity of the GADA, reactive to the f-GAD antigen. Further research into the characteristics of the GADA reactive to the n-terminally truncated antigen, could allow us to further investigate the underlying pathology in autoimmune adult-onset diabetes. As our study was not a prospective assessment from diagnosis, further studies using at diagnosis samples are required to gather supporting evidence of the use of t-GADA in predicting early insulin requirement.

4.7 References

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4.8 Supplemental Material

Supplemental Table 1*: Overall type 2 diabetes cohort characteristics split by study. Data displayed as n (%; 95% CI) or median (IQR). *One-sided, 97.5% confidence interval.*

Variable	DARE	GoDarts	MRC Progressors	PRIBA	StartRight
n	1,906	3,893	212	558	30
Male (%; 95% CI)	1,148 (60%; 58, 62)	2,134 (55%(; 53, 56)	133 (63%; 56, 70)	326 (58%; 54, 63)	20 (67%; 47, 83)
Ethnicity (%non-Caucasian; 95% CI)	1,906 (100%; 100, 100*)	3,893 (100%; 100, 100*)	212 (100%; 98, 100*)	558 (100%; 99, 100*)	28 (93%; 78, 99)
Age at Diagnosis (Years)	62 (52, 70)	62 (54, 69)	57 (51, 63)	51 (45, 57)	41 (34, 50)
Duration of diabetes at latest follow up (years)	5 (2, 11)	12 (10, 15)	14 (12, 17)	8 (5, 13)	2.5 (2, 2.7)
f-GADA titre (WHO U/ml)	4.9 (4.9, 4.9)	5 (5, 5)	4.9 (4.9, 4.9)	10.9 (4.9, 10.9)	1194 (239, 2001)
Duration of diabetes at f-GADA assessment (years)	6 (2, 11)	5 (3, 8)	13 (11, 16)	8 (5, 13)	0.5 (0.3, 0.8)
f-GADA Positive (& sera available for further analysis) (%; 95% CI)	54 (2.8%; 2.1, 3.7)	84 (2.2%; 1.7, 2.7)	11 (5.2%; 2.6, 9)	0 (0%; 0, 0.7*)	30 (100%; 88, 100*)

Supplemental Table 2: *full-length GADA positive type 2 diabetes cohort split by study. Data displayed as n (%; 95% CI) or median (IQR). *One-sided, 97.5% confidence interval.*

Variable	DARE	GoDarts	MRC Progressors	StartRight
n	54	84	11	30
Male (%; 95% CI)	32 (59%; 45, 72)	40 (48%; 37, 59)	8 (73%; 39, 94)	20 (67%; 47, 83)
Ethnicity (%non-Caucasian; 95% CI)	54 (100%; 93, 100*)	84 (100%; 96, 100*)	11 (100%; 72, 100*)	28 (93%; 78, 99)
Age at Diagnosis (Years)	55 (47, 67)	62 (56, 70)	53 (49, 58)	41 (34, 50)
Duration of diabetes at latest follow up (years)	7 (3, 12)	11 (9, 15)	12 (11, 15)	2.6 (2, 2.7)
f-GADA titre (WHO U/ml)	63 (29, 1755)	93 (21, 969)	27 (15, 82)	1194 (239, 2001)
Duration of diabetes at f-GADA assessment (years)	7 (3, 12)	5 (2, 8)	11 (10, 14)	0.5 (0.3, 0.7)

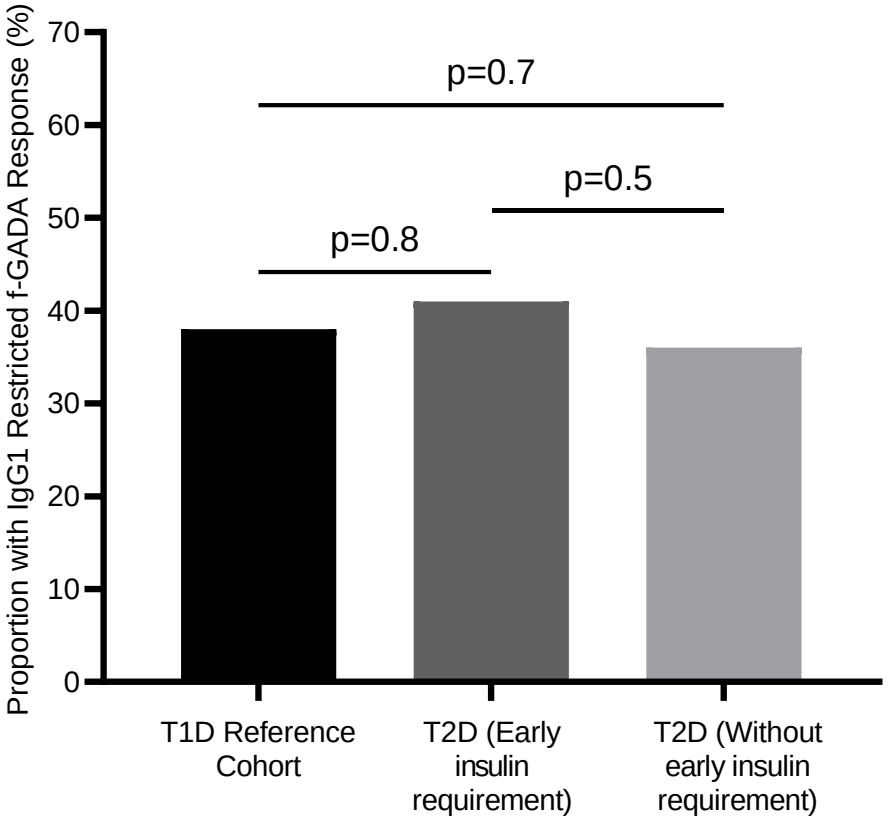
Supplemental Table 3*: Hazard Ratios from Cox proportional regression model (unadjusted and adjusted) for time to insulin censored at 5 years (f-GADA positivity). f-GADA; full length GAD(1-585) autoantibody.*

	Unadjusted Model		Adjusted Model	
	HR(95% CI)	*p* value	HR(95% CI)	*p* value
f-GADA Negative	1		1	
f-GADA Positive	6.7 (5.1, 8.7)	<0.001	5.4 (3.8, 7.7)	<0.001
Age of Diagnosis (per 1 year increase)			0.96 (0.95, 0.96)	<0.001
f-GADA Titre (per 1 unit increase)			1 (1, 1)	0.288
Duration of Diabetes at f-GADA testing (per 1 year increase)			0.98 (0.97, 1.0)	0.03

Supplemental Table 4: *Individual IgG Subclass prevalence. Data shown as n (%). RC; Reference cohort. f-GADA; full length GAD(1-585) autoantibody. T1D; Type 1 Diabetes. T2D; Type 2 Diabetes*

f-GADA IgG Isotype	T2D without early insulin requirement	T2D with early insulin requirement	T2D without vs. with early insulin requirement *p* value	T1D Reference Cohort	T1D RC vs T2D with early insulin requirement *p* value	T1D RC vs T2D without early insulin requirement *p* value
IgG1	66 (87)	52 (96)	0.085	72 (95)	0.676	0.092
IgG2	20 (26)	10 (19)	0.298	14 (18)	0.989	0.243
IgG3	39 (51)	26 (48)	0.722	36 (47)	0.93	0.626
IgG4	15 (20)	7 (13)	0.31	16 (21)	0.234	0.84
IgG Isotype Combination						
IgG1 Restricted	27 (39)	22 (42)	0.724	29 (38)	0.766	0.889

Supplemental Figure 1: *Bar chart showing the proportions with an IgG1 restricted in each cohort. T1D; Type 1 diabetes. T2D; type 2 diabetes. f-GADA; full-length GAD(1-585) autoantibodies. T1D RC 38% (95% CI 27, 50). T2D & Early insulin requirement 41% (28, 55). T2D without early insulin requirement 36% (95% CI 25, 47)*



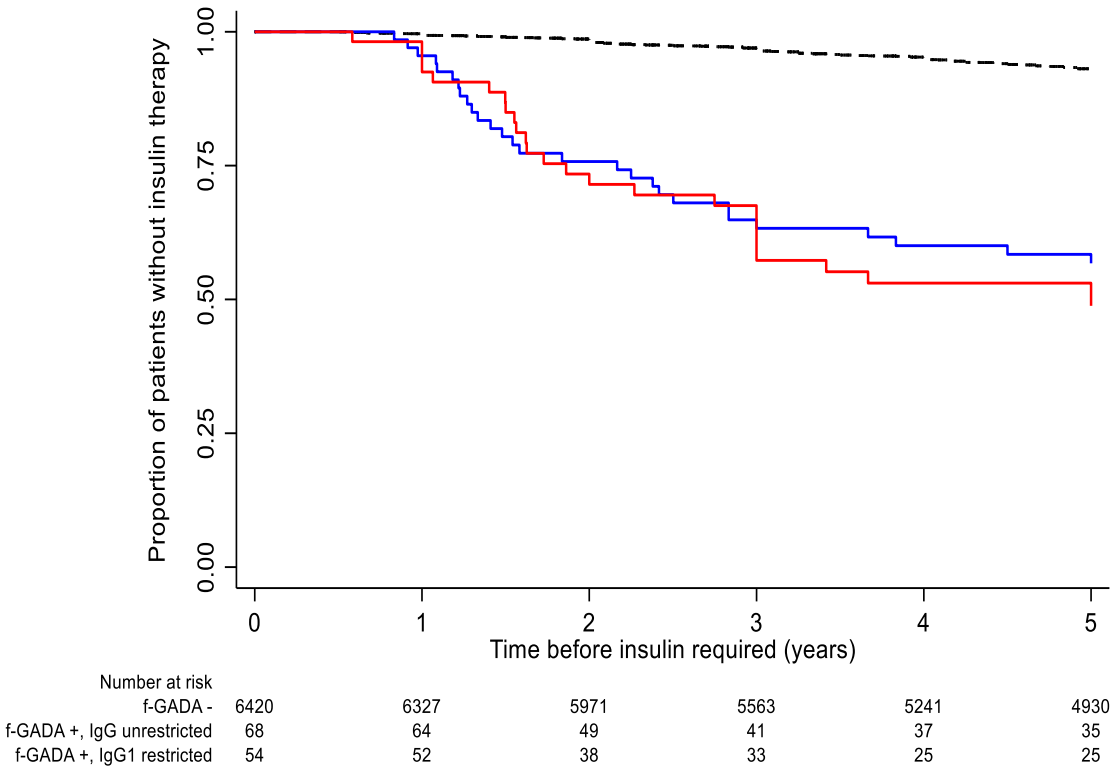
Supplemental Table 5: *Diabetes characteristics comparison between those with an IgG1 restricted and an IgG unrestricted response in those f-GADA positive. Data displayed as n (%) or median (IQR) *n=2,607 tested for IA-2A. F-GADA; full length GAD(1-585) autoantibody. T1D; Type 1 Diabetes. T2D; Type 2 Diabetes.*

Characteristic	T2D IgG1 Restricted	T2D IgG Unrestricted	IgG1 Restricted vs. IgG Unrestricted *p* value	T1D Reference cohort	T2D f-GADA Negative
n	53 (41)	76 (59)		144	6,420
Age at Diagnosis (Years)	57 (47, 65)	53 (44, 64)	0.422	22 (16, 35.5)	61 (52, 68)
f-GADA Titre (WHO U/ml)	138 (41, 694)	1271 (71, 2001)	p<0.0001	107 (43, 1244)	5 (4.9, 5)
T1D Genetic Risk Score *	0.251 (0.230, 0.272)	0.258 (0.230, 0.274)	0.955	0.275 (0.257, 0.295)	0.230 (0.205, 0.253)
C-Peptide (pmol/L)	1100 (363, 1740)	945 (655, 1400)	0.931	6 (2.9, 39)	214.6 (1350, 3125)
IA-2A Positive (%)	9 (18)	22 (31)	0.132	70 (50%)	15 (0.6%)*
Insulin treated within 5 years (%)	22 (45)	30 (42)	0.724	144 (100%)	429 (6.7%)

Supplemental Table 6: *Hazard Ratios from Cox proportional regression model (unadjusted and adjusted) for time to insulin censored at 5 years (IgG subclass response). f-GADA; full length GAD(1-585) autoantibody.*

	Unadjusted Model		**Adjusted Model**	
Survival analysis with f-GADA negatives	**HR(95% CI)**	***p* value**	**HR(95% CI)**	***p* value**
f-GADA Negative	1		1	
f-GADA Positive, IgG1 Restricted	9.3 (6.0, 14.2)	<0.001	9.1 (5.9, 14.1)	<0.001
f-GADA Positive, IgG Unrestricted	8.4 (5.8, 12.2)	<0.001	7.1 (4.9, 10.4)	<0.001
Age of Diagnosis (per 1 year increase)			0.96 (0.95, 0.97)	<0.001
Duration of Diabetes at f-GADA testing (per 1 year increase)			0.99 (0.97, 1.0)	0.082
Survival analysis IgG1 Restricted vs. IgG Unrestricted (in those f-GADA positive)	**Unadjusted Model**		**Adjusted Model**	
IgG Unrestricted	1		1	
IgG1 Restricted	1.07 (0.62, 1.9)	0.813	1.02 (0.58, 1.80)	0.937
Age of Diagnosis (per 1 year increase)			0.94 (0.92, 0.96)	<0.001
Duration of Diabetes at f-GADA testing (per 1 year increase)			0.89 (0.83, 0.95)	<0.001

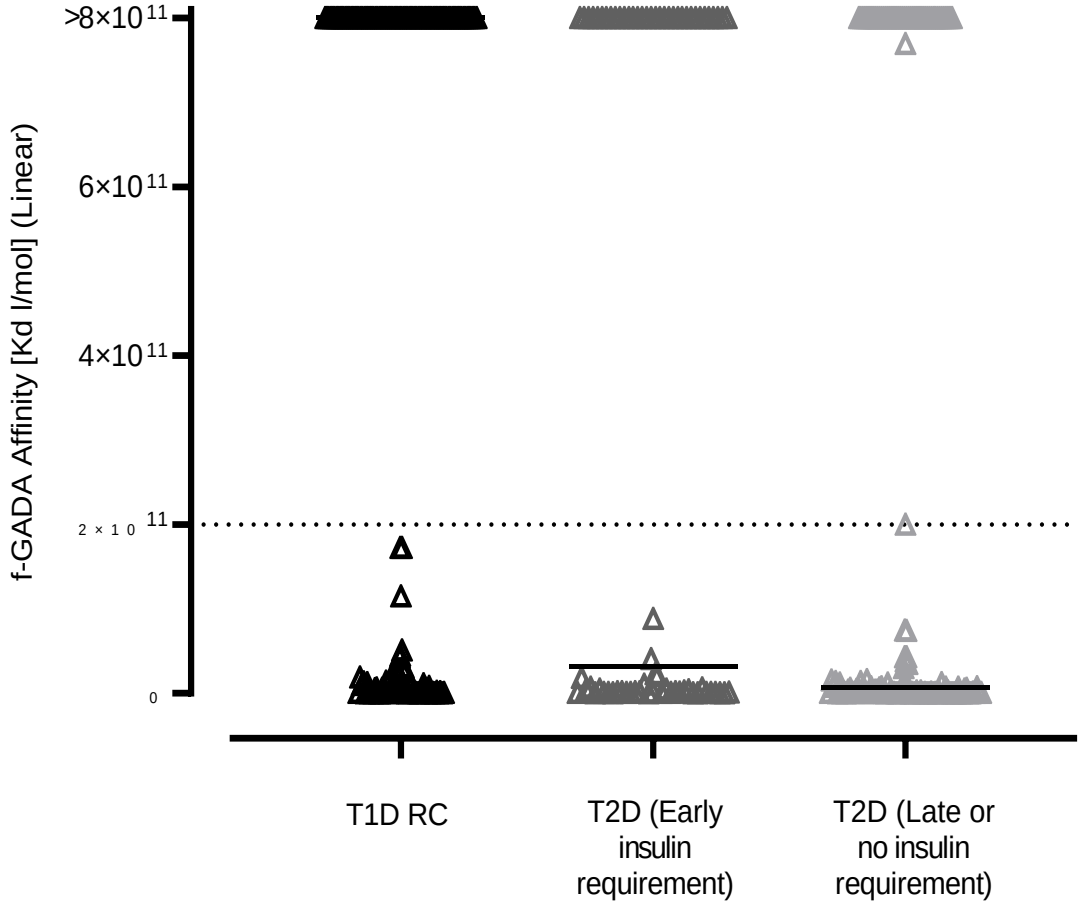
Supplemental Figure 2: *Kaplan-Meier plot of probability of requiring insulin therapy during 5-year follow-up, in those clinically diagnosed with type 2 diabetes, by risk group of f-GADA positivity and subclass. Solid lines represent f-GADA positive groups and dashed line represent f-GADA negative group. Blue indicates IgG unrestricted response and red is IgG1 restricted response. +, positive. -, negative*



Supplemental Table 7: *Hazard Ratios from Cox proportional regression model (unadjusted) for time to insulin censored at 5 years (IgG subclass present).*

	Unadjusted Model	
	HR (95% CI)	***p* value**
Respective Subclass not present	1	
IgG Subclass present		
IgG1	3.0 (0.7, 12.1)	0.133
IgG2	0.7 (0.4, 1.4)	0.31
IgG3	0.9 (0.6, 1.6)	0.845
IgG4	0.7 (0.3, 1.5)	0.357

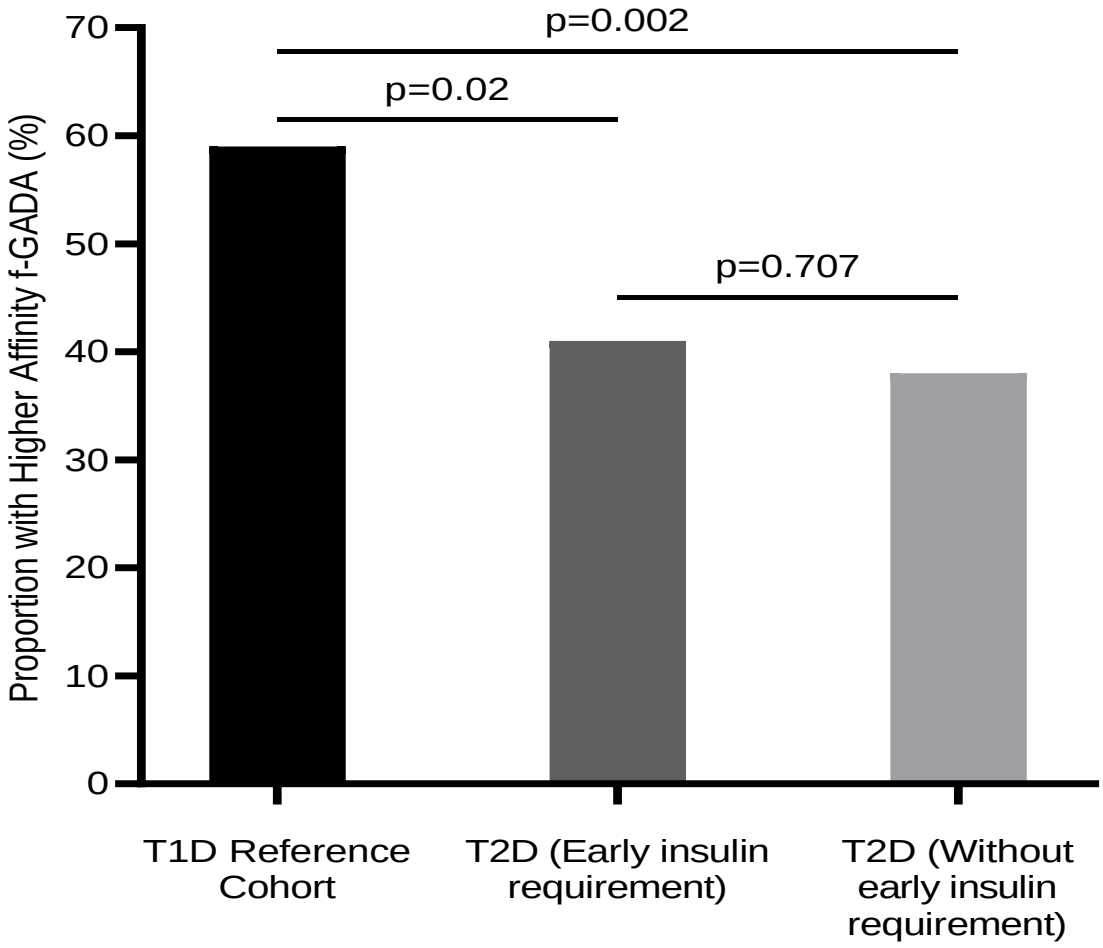
Supplemental Figure 3*: Distribution of f-GADA affinities across each cohort. Dashed black line indicates where affinities were categorised into high and low. Solid black lines indicate medians. T1D; type 1 diabetes. T2D; type 2 diabetes. f-GADA; full-length GAD(1-585) autoantibody.*



Supplemental Table 8: *Diabetes characteristics comparison between those with higher and lower affinity f-GADA. Data displayed as n (%) or median (IQR). *Out of 2,607 tested. T1D; Type 1 Diabetes. T2D; Type 2 Diabetes. f-GADA; full length GAD(1-585) autoantibody.*

	T2D with higher affinity f-GADA	T2D with lower affinity f-GADA	T2D higher vs. lower Affinity *p* value	T1D Reference cohort	T2D with f-GADA negativity
n	63	98		144	6,420
Age at Diagnosis (Years)	57 (47, 64)	56 (46, 65)	0.88	22 (16, 35.5)	61 (52, 68)
f-GADA Titre (WHO U/ml)	57 (21, 307)	898 (46, 2000)	$3x10^{-6}$	107 (43, 1244)	5 (4.9, 5)
T1D Genetic Risk Score	0.256 (0.230, 0.270)	0.250 (0.219, 0.273)	0.97	0.275 (0.257, 00.295)	0.230 (0.205, 0.253)
C-Peptide (pmol/L)	1130 (579, 1808)	912 (539, 1470)	0.72	6 (2.9, 39)	214.6 (1350, 3125)
IA-2A Positive (%)	10 (16%)	24 (24%)	0.19	70 (50%)	15 (0.6%)
Insulin treated within 5 years (%)	25 (40%)	36 (37%)	0.71	144 (100%)	429 (6.7%)

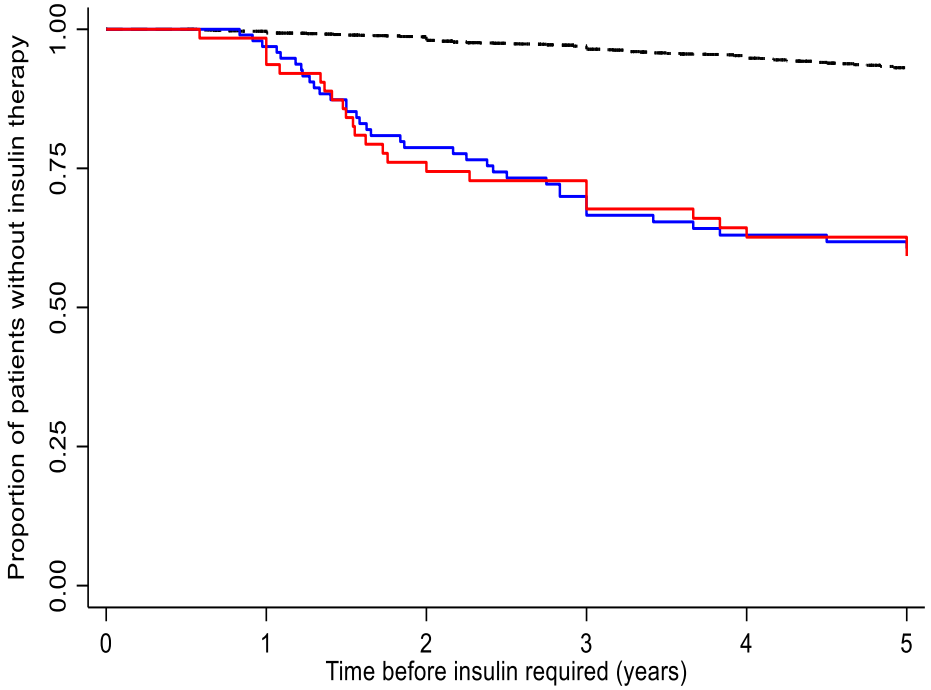
Supplemental Figure 4*: Bar chart showing the proportions with higher affinity f-GADA in each cohort. T1D; Type 1 diabetes. T2D; type 2 diabetes. f-GADA; full-length GAD(1-585) autoantibodies.*



Supplemental Table 9: *Hazard Ratios from Cox proportional regression model (unadjusted and adjusted) for time to insulin censored at 5 years (f-GADA affinity category). f-GADA; full length GAD(1-585) autoantibody.*

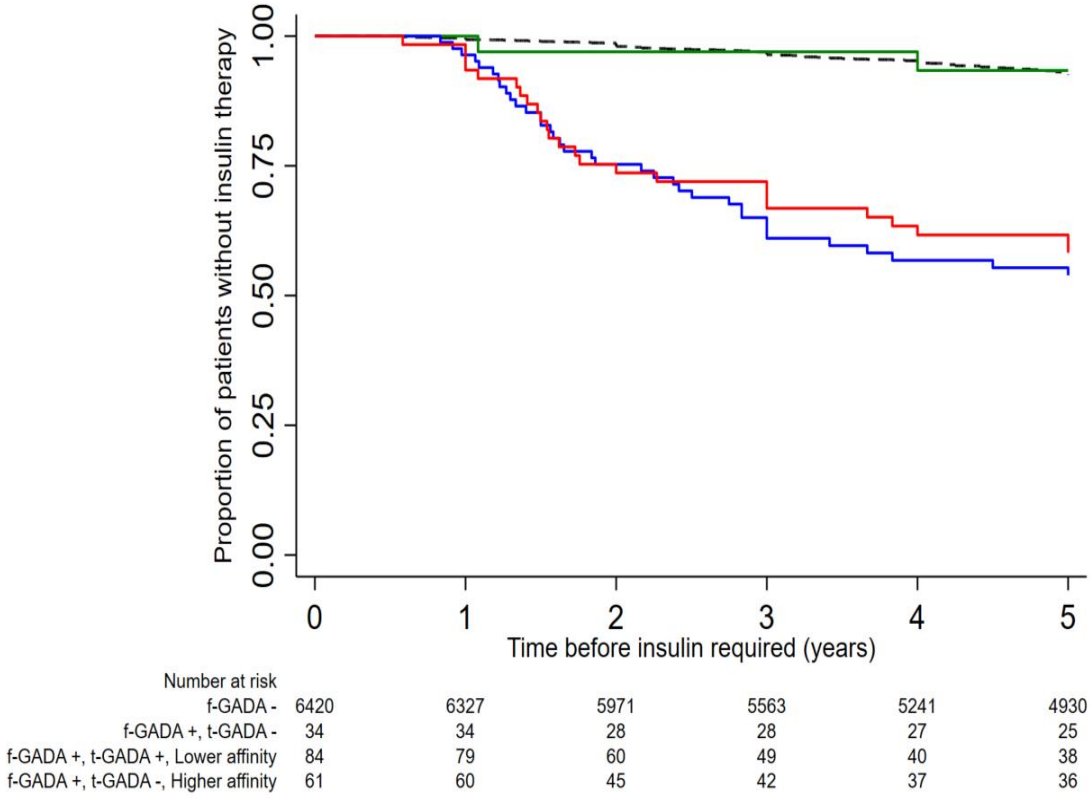
	Unadjusted Model		Adjusted Model	
Survival with f-GADA negatives	**HR(95% CI)**	***p* value**	**HR(95% CI)**	***p* value**
f-GADA Negative	1		1	
f-GADA Positive, Lower Affinity f-GADA	7.22 (5.14, 10.15)	<0.001	5.51 (3.40, 8.92)	<0.001
f-GADA Positive, Higher Affinity f-GADA	7.34 (4.94, 11.08)	<0.001	6.56 (4.29, 10.02)	<0.001
Age of Diagnosis (per 1 year increase)			0.96 (0.95, 0.96)	<0.001
f-GADA Titre (per 1 unit increase)			1 (1, 1)	0.398
Duration of Diabetes at f-GADA testing (per 1 year increase)			0.98 (0.97, 1)	0.036
Higher vs. Lower Affinity in those f-GADA positive	**Unadjusted Model**		**Adjusted Model**	
Lower Affinity f-GADA	1		1	
Higher Affinity f-GADA	1.04 (0.62, 1.73)	0.886	0.873 (0.49, 1.55)	0.645
Age of Diagnosis (per 1 year increase)			0.94 (0.92, 0.96)	<0.001
f-GADA Titre (per 1 unit increase)			1 (1, 1)	0.594
Duration of Diabetes at f-GADA testing (per 1 year increase)			0.88 (0.82, 0.93)	<0.001

Supplemental Figure 5: *Kaplan-Meier plot of probability of requiring insulin therapy during 5-year follow-up, in those clinically diagnosed with type 2 diabetes, by risk group of f-GADA positivity and affinity. Solid lines represent f-GADA positive groups and dashed line represent f-GADA negative group. Blue indicates low affinity f-GADA and red is high affinity f-GADA. +, positive. -, negative. Ab, antibodies.*



Number at risk						
f-GADA -	6420	6327	5971	5563	5241	4930
f-GADA +, Low Affinity Ab	98	93	73	62	53	51
f-GADA +, High Affinity Ab	63	62	46	43	38	37

Supplemental Figure 6*: Kaplan-Meier plot of probability of requiring insulin therapy during 5-year follow-up, in those clinically diagnosed with type 2 diabetes, by risk group of t-GADA positivity and affinity. Solid red and blue lines represent t-GADA & f-GADA positive groups, solid green indicates t-GADA negative but f-GADA positive and dashed black line represents f-GADA negative group. Blue indicates low affinity f-GADA and red is high affinity f-GADA. +, positive. -, negative. Ab, antibodies.*



Chapter 5:

Discussion

5.1 Discussion

Islet autoantibody tests have transformed the prediction, classification and understanding of the pathogenesis of diabetes. Before their use in clinical practice and research, there are many aspects of islet autoantibody testing which should be considered, both practically and clinically. Studies presented in this thesis on positivity threshold setting, levels at diagnosis of type 1 diabetes and GADA characteristics in adult-onset diabetes show the importance of these considerations. Islet autoantibodies provide deeper insights into the immunopathology of diabetes and new more specific islet autoantibody tests can be used clinically to improve outcomes for patients with diabetes. These discoveries can lead to improved clinical utility of islet autoantibody testing and understanding of autoimmune diabetes biology.

This section of the thesis will summarise the findings of each data chapter, discuss the strengths and limitations of each project, and consider the impact of these studies, along with future research that may give further insights.

5.2 Chapter 2- Zinc transporter 8 autoantibody testing requires age-related cut-offs

Main Conclusions

In this chapter, I aimed to define robust thresholds of positivity for ZnT8A testing in a European population using clinically available RSR ZnT8A ELISA data because interpretation of islet autoantibodies requires well-defined positivity cut-offs. The hypothesis tested was that age-related cut-offs would improve the specificity of the RSR ELISA assay for ZnT8A assessment. I studied the prevalence and level of ZnT8A in 1559 control samples from the general population without diabetes (0-83 years old), using the most commonly clinically available test for ZnT8A, to determine whether age-related positivity cut-offs are required for ZnT8A testing.

I found that, by using the manufacturer's LOD (10 U/ml), ~16% of the control population had detectable ZnT8A, with the proportion of those with detectable ZnT8A declining in early adulthood. This decline hit a plateau after the age of 30 years. When I split the controls into those aged below and above 30 years, the prevalence of detectable ZnT8A was 21% in those under 30 years compared to

5% in those over 30 years. Using these age groups, I looked at the 97.5th and 99th centile cut-offs (commonly used thresholds) and the differences in these between the age groups. The 97.5th and 99th centiles, and thus the detectable level of ZnT8A, were higher for those under 30 years (18 and 127 U/ml, respectively) than those over 30 years (9 and 21 U/ml, respectively).

Application of these age-related cut-offs to the UNITED cohort (n=145, median age of diagnosis 18.9 years), retrospectively, identified 23 previously ZnT8A positives who became negative; of these 8 were only positive for ZnT8A and therefore become islet autoantibody negative. These 8 participants exhibited a more T2D like phenotype, where they were less likely to be on insulin therapy, had higher levels of c-peptide and were more likely to have an overweight or obese BMI. In contrast, all those that remained positive for ZnT8A alone (n=14) were on insulin therapy. When these age-related cut-offs were used during the IASP 2020 workshop, specificity remained high, without loss of sensitivity, in comparison to the use of the manufacturer's own threshold.

Strengths and Limitations

To our knowledge this is the largest study to assess ZnT8A positivity thresholds, in controls with the largest age range, with the only previous study to look at this having half the controls, assessing a smaller age range and using a different assay format (RIA) (1). Not only did this study show differences in prevalence and levels of ZnT8A in the general population and define age-related cut-offs but we also had access to data from multiple IASP workshops that allowed assessment of whether these new cut-offs would improve the sensitivity and specificity or the assay. Our >30 years threshold (21 U/ml) is not too dissimilar to the manufacturer's recommended positive threshold (≥15 U/ml), showing that this threshold is quite robust, even across different laboratories, for testing over 30s in different populations. However, the assay information provided by RSR did not state how this threshold was validated so we are unable to compare control populations. We were also able to retrospectively apply these cut-offs to a study of young-onset mixed diabetes type where those that were reclassified as ZnT8A negative exhibited a more type 2 diabetic phenotype. Another strength of this study is that the assay used in this study was already a highly specific, sensitive

and clinically available assessment of ZnT8A and we have been able to improve the specificity through the use of age-specific cut-offs.

The control cohort used in this study was primarily of European descent, and thus the results can only be generalised to this population. Further, whilst we saw a decline around the 30 years mark, the numbers are not large enough to provide greater detail where the optimal age cut-off should be set. With general population studies targeting those under the age of 13 years (for practical reasons around tying screening in with vaccinations), it would be prudent to look at optimal age cut-offs in those in the 3-13 years age bracket (2). We only studied the effects of age-related cut-offs on one type of ZnT8A assessment, and as such the use of age-related cut-offs is only justified for the RSR ELISA assay. Laboratories using other assay formats should consider these results when selecting their own thresholds.

Implication of findings

The RSR ELISA for ZnT8A assessment used in this study is the most common clinically available ZnT8A test and the company recommends that each laboratory evaluates its' own thresholds. Many would set this in an adult or child population and differences related to age would not be seen. Whilst this data has been published, we will be discussing this data with RSR, with the suggestion that they recommend that laboratories set thresholds in both adult and child populations.

In the most recent NICE recommended guidelines regarding the diagnosis and classification of diabetes, it was noted that testing for islet autoantibodies at diagnosis of diabetes to avoid misclassification should not be discouraged, particularly when type 1 diabetes is suspected. Age-related cut-offs will improve the use of ZnT8A testing at diagnosis and avoid misclassification of diabetes. Overall this research will improve outcomes for patients. Therefore, for ZnT8A, age-related thresholds can be used to reduce false positive results in those with non-autoimmune diabetes. It is of importance to only include those with high risk of true autoimmune diabetes in studies into the prediction of T1D, particularly where those participants with true autoimmune diabetes benefitting the most from being invited into intervention trials. Studies such as these, showing the

importance of robustly defined positivity thresholds are important in minimising the potential ethical implications of giving out a misleading or inaccurate islet autoantibody result. Studies such as T1 Early and EarLy Surveillance for Autoimmune diabetes (ELSA) are ongoing, to examine the feasibility of integrating antibody positive children into primary care and screening into the NHS clinical pathways (3).

Future Research

As our control cohort was predominantly of European descent, future studies are needed to establish whether age-related cut-offs would be needed to improve the specificity of this assay in other ethnic populations. This is of importance as studies have shown differences in prevalence of ZnT8A and its importance in understanding islet autoimmunity in these populations (4-7). It would also be of value to explore whether different assay formats for ZnT8A assessment also require age-related cut-offs to determine positivity. Future work is also required to give greater detail on optimal test cut-offs for different ages, as although the number used in this study far exceeds what has reported previously (8; 9), it is insufficient to do more than visually assess the optimal age-cut off.

This study focused on the assessment of ZnT8A positivity in the general population, future work is required to assess whether age-related cut-offs are required for each of the other characterised autoantibodies, GADA, IA-2A, IAA and TSPAN7A, by all detection methods.

5.3 Chapter 3- Islet autoantibody level distribution in type 1 diabetes and their association with genetic and clinical characteristics

Main Conclusions

In this chapter, we aimed to assess whether the level of an islet autoantibody is important at the diagnosis of type 1 diabetes. We hypothesised that the level of islet autoantibodies may have clinical importance and would provide greater insight into the heterogeneity of type 1 diabetes presentation. This study has shown that in 1,644 patients at diagnosis of type 1 diabetes (median age of diagnosis 21 years (IQR 13, 31), that both GADA and IA-2A levels have a bimodal distribution that is associated with age of diagnosis, albeit in opposite directions. I also showed that these bimodal distributions were present in a second

replication cohort (StartRight) [n=449; median age of diagnosis 34 (IQR 26, 46)] with the islet autoantibodies assessed using a different method. For GADA, those with high levels are more likely to be older at diagnosis, female, *HLA-DR3-DQ2* positive and to be diagnosed with another autoimmune disease (88% had concurrent thyroid or celiac autoimmune disease. In contrast, those with high level IA-2A are more likely to be younger at diagnosis, have the *HLA-DR4-DQ8* risk allele and are more likely to have ZnT8A as an additional islet autoantibody. I also showed that ZnT8A levels did not exhibit a bimodal distribution in the ADDRESS-2 cohort, whilst numbers were too small to assess in the replication cohort. Positivity for ZnT8A in the StartRight cohort was assessed using the age-related cut-offs described in Chapter 2.

Strengths and Limitations

A strength of this study is that we were able to replicate the bimodal distribution in StartRight cohort, using RSR ELISAs to detect GADA and IA-2A. This validated our assumption that this is a true biological finding rather than an artefact of the assay used. This study included participants of all ethnicities, and therefore our findings are not limited to populations of European descent as many other islet autoantibody studies are. Both the RBA and ELISA assays used in this study perform well in the international standardisation workshops (IASP) and exhibit high specificity and sensitivity. We compared performance of the same sample set in both assay types, and high level of correlation of islet autoantibody levels was observed for all three islet autoantibodies tested (GADA, IA-2A, ZnT8A), strengthening the results of this study.

C-peptide and longitudinal follow up was available for 2 years in our replication cohort (StartRight), but not in our larger initial cohort (ADDRESS-2). We report a slight difference in C-peptide decline in the first two years post diagnosis, between those with high and low level GADA, but not for IA-2A where available data were significantly less.

We were not able to study IAA in either cohort, due to recruitment of participants more than two weeks after commencement of insulin therapy. As IAA are another key islet autoantibody, particularly in children, it would be of interest to see if a similar bimodal distribution is observed.

In our data we have shown a bimodal distribution and were able to split our cohort into groups of high and low level antibodies based on the nadir of the distribution. However, an important point to consider in the application of this data is that the nadir, for any particular test in different laboratories, is likely to vary so this would need to be assessed for each test. Therefore, whilst we can say that identifying "high" levels of islet autoantibodies is important, we cannot specifically state what "high" is.

Implication of findings

In this study, we have shown bimodal distributions of GADA and IA-2A levels at the diagnosis of type 1 diabetes, which could be biologically important to the understanding of the heterogeneity of type 1 diabetes at presentation.

Diabetes-risk prediction studies have long used positivity for single and multiple islet autoantibodies, along with genetic susceptibility to stratify risk of progression to diabetes (10-12). However, studies into the use of islet autoantibody level to help stratify risk of progression to diabetes have been variable (13-21). Ng *et al*'s study suggested that level does influence stratification of risk of progression, with GADA and IA-2A islet autoantibody level exhibiting a bimodal risk distribution (22). Combined with our data, islet autoantibody level could be used to better predict diabetes and could be used to improve prediction models.

Future Research

Future work will assess whether the bimodal distribution of GADA and IA-2A levels at diagnosis is associated with post diagnosis beta cell function for both GADA and IA-2A. A study published by Williams *et al*, observed in a small cohort, evidence of ongoing autoimmunity and beta cell function (assessed by UCPCR) in those with long-standing diabetes (median 22 years at follow-up). Whilst they assessed the relationship between GADA, IA-2A, IAA, ZnT8RA and ZnT8WA at baseline and follow-up against UCPCR levels, they did not observe a significant effect (23). A larger study, with more frequent islet autoantibody and UCPCR sampling is necessary.

We did not assess IAA levels at diagnosis in either of our cohorts due to the effect of exogenous insulin on IAA production. A future study using at diagnosis samples, taken within two weeks of commencement of insulin therapy, would be

necessary to explore the distribution of IAA levels at diagnosis of type 1 diabetes. This is an important area of future research due to evidence suggesting that IAA are most prevalent in young children where presentation is more clinically severe (24), high levels of IAA are a major determinant of age of diabetes diagnosis (20) and those with high IAA levels may benefit from intervention trials (25).

5.4 Chapter 4- Autoantibodies to truncated GAD stratify risk of early insulin requirement in adult-onset type 2 diabetes

Main Conclusions

This chapter aimed to investigate whether testing for further GAD autoantibody characteristics, in those f-GADA positive, would have clinical utility in predicting early insulin requirement. I have shown that positivity for t-GADA was similar between those diagnosed with type 2 diabetes requiring early insulin requirement (within 5 years) and those with type 1 diabetes, but lower in those with f-GADA positive type 2 diabetes, without early insulin requirement. Those with t-GADA positivity had a more type 1 like phenotype; lower C-peptide, higher T1D GRS and positivity for IA-2A. A novel finding observed was that t-GADA stratified risk of progression to insulin therapy in those found to be f-GADA positive by a highly specific assay. In contrast, assessing f-GADA affinity and IgG subclass responses did not identify those with a more type 1 diabetes like phenotype or stratify risk of progression to early insulin requirement

Strengths and Limitations

Our initial f-GADA screening cohort of adult-onset patients with type 2 diabetes was large (>6000), predominantly population-based and had detailed follow-up data available (including C-peptide and T1D GRS), which made it unique. Further, the initial f-GADA screening by RSR GADA ELISA was conducted in one laboratory and across all cohorts. The assay used is highly specific (98.9% specificity in the latest IASP workshop) due to a very robustly defined positivity threshold (based on a large predominant adult control population), thus reducing the risk of including false f-GADA positives. The assays to measure GADA affinity and isotype used in this study were well-developed and high quality and we were able to compare our results of these to a reference cohort of patients with f-GADA positive type 1 diabetes.

This study builds upon an initial study by Achenbach *et al*, who also showed that the presence of t-GADA was associated with a clinical phenotype of autoimmune T1D and assisted in the prediction of insulin therapy, as we had access to C-peptide and T1D GRS data, as well as patients with a longer follow-up allowing us to use survival analysis to assess the performance of t-GADA, from post diagnosis samples, in predicting risk of early insulin requirement (26).

In our cox proportional hazards model, a linear relationship was assumed between the continuous co-variates and time to insulin progression. Future research may consider this an invalid assumption.

We had to combine participants from multiple cohorts, collected under differing study protocols, in order to obtain sufficient numbers of f-GADA positive samples for further characterisation. This meant for T1D GRS and f-GADA (as mentioned above), the same tests were used to assess these across all cohorts. However, in the Exeter cohorts, time to insulin progression was mainly self-reported which may have introduced imprecision.

Further, optimisation of the current t-GADA and f-GADA RBA and LIPS assays was conducted in a cohort of new onset diabetes where the median age of diagnosis was 11.6 years, range 1.3 – 21 years); not in individuals diagnosed in adulthood.

Implications of findings

There is clinical utility in identifying patients that are at high risk of early insulin requirement in both the clinical and research setting. In research, patients who are likely to require early insulin therapy could be targeted for intervention clinical trials that aim to slow diabetes progression. Whereas in the clinical setting, individualised treatment pathways can be developed based on the patient's risk of early insulin requirement, which can optimise their treatment and priorities for monitoring.

Future Research

Previous studies have shown that the prevalence of f-GADA increases with age at diabetes onset, even in childhood (27), future studies need to establish whether this pattern is the same for specificity to the n-terminally truncated GADA epitope

used in this study. Subsequent studies are needed to confirm positivity thresholds in samples from non-diabetic adults from the general population. It would also be of interest to examine the prevalence of t-GADA in other ethnic cohorts.

This study showed the potential clinical utility of t-GADA in older populations to predict early insulin requirement and possible misclassification of diabetes type, using post diagnosis samples. Future work will need to involve establishment of the clinical utility of testing for t-GADA in those f-GADA positive at diagnosis to predict early insulin requirement, and also as the primary test.

Characterisation of the n-terminal t-GAD(96-585) epitope specific autoantibodies, such as IgG subclass response, further isotype responses (IgM and IgA) and affinity is necessary to understand more about the pathology of these autoantibodies and whether they can contribute towards the explanation of the heterogeneity of diabetes presentation and progression. Further, other antigen truncations and fragments of GAD65 could be explored to further our understanding of the epitope specificity of these GADA.

The t-GADA and IA-2A LIPs assays used in this study are non-radioactive and low volume. Non-radioactive assays for islet autoantibody assessment are sought to replace the current gold-standard RIAs in research and would be easier to implement in the clinical setting. Although the LIPS assays in their current format are not set up for the high throughput experienced by clinical laboratories, the t-GAD antigen could be utilised in a bridging assay plate format to increase output.

5.5 Final remarks

The studies presented in this thesis were designed to improve the clinical utility of islet autoantibodies in adult-onset diabetes so that their use in classification, diagnosis and prediction could be refined. To this aim, it has identified that age-related cut-offs are necessary when using the commercially available RSR assay for the assessment of ZnT8A (Chapter 2). It has also unveiled a greater understanding of islet autoantibody levels of three of the key islet autoantibodies (GADA, IA-2A and ZnT8A) at diagnosis of both childhood and adult-onset type 1 diabetes (Chapter 3). It has provided more evidence to support the use of testing

for t-GADA epitope specificity in adult-onset diabetes to identify those with a T1D-like phenotype and predict early insulin requirement (Chapter 4).

The majority of this thesis has been completed in a time which the world came up against the global COVID-19 pandemic. This presented new challenges when it came to conducting the research and then its dissemination. Initially, there had been other laboratory work planned to explore the feasibility of using dried blood spot sample collection instead of venous or capillary sample collection to obtain samples for islet autoantibody testing. Instead, a data-based chapter, where I had analysed the samples prior to the commencement of this thesis, was included due to the work from home mandate in the UK. Unfortunately, due to reallocation of resources and availability of laboratory equipment and space, there were delays in collecting the data for Chapter 4. This resulted in only a subset of samples being tested for the IgG subclass analysis and ZnT8A analysis being omitted from this thesis but will be included in the subsequent publication for this work. Additionally, the COVID-19 pandemic and working from home mandate resulted in myself facing personal challenges, which included less organic and spontaneous discussions with colleagues that may have improved upon this research and its dissemination.

The practical achievements of this thesis are laid out in Appendix 1. In summary, the work published in this thesis has led to two publications so far, with Chapters 2 and 3 published as journal articles. Work from this thesis has also been presented at international conferences, including both online and in-person events, and in poster and oral presentation formats. I was fortunate to present data from Chapters 2, 3 and 4 at the national T1D Consortium meetings, where diabetes researchers across the UK attend. I have also presented at departmental seminars, where both clinicians and academics were included in the audience, who would be using these islet autoantibody tests in clinics and research respectively.

There are challenges in taking the key findings of this thesis from research through to clinical practice. To disseminate the findings from Chapters 2 and 3, it would be a relatively straightforward case of presenting the data at conferences, publishing the study results, and collecting enough evidence for including them in best practice documentation. For Chapter 3 however, even if clinical

laboratories are aware of limitations of their current islet autoantibody tests, their ability to make changes are restricted by accreditation regulations and often large and expensive service contracts, as are the large commercial companies that supply the commercial tests. If we were to replace f-GADA testing with the more disease-specific t-GADA in commercially available GADA assays, either the current test would have to be changed by the large commercial companies supplying the test or a new test created. This would incur large research and development costs and new validation, verification and accreditation would need to occur. These are time-consuming and expensive procedures for both the clinical laboratories and larger biotechnology companies. However clinical testing should ensure the best possible outcomes for patients and this should not be delayed by commercial considerations. Publishing updated recommendations and guidelines can help drive change.

Improving islet autoantibody testing is a challenging and ever-changing area of research, as evidenced by the fact that since ICA were identified in 1974, five other islet autoantibodies have been identified and characterised with multiple ways of testing for these established. Detection methods have been designed, refined and even replaced through international standardisation programs and collaborations. This has included the development of novel methods to further characterise islet autoantibodies; this thesis just scrapes the tip of a research iceberg. However, the research in this thesis, does begin to bridge the gap between the basic science research into the biology of these islet autoantibodies and their use in clinical practice.

5.6 References

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6.1 Appendix - Achievements

Conference Presentations

Oral Presentation: Title: **Age-related thresholds in zinc transporter 8 autoantibody analysis.** T1D UK Immunotherapy Consortium. March 2020. Online.

Oral Presentation: Title: **Autoantibody characterization in glutamate decarboxylase autoantibody positive adult-onset diabetes**. T1D UK Immunotherapy Consortium. November 2021. Online

Conference Poster Presentation: Title: **Glutamate Decarboxylase and Islet Antigen-2 Autoantibody titres at Diagnosis in People with Type 1 Diabetes showed Bimodality and an Age of Diagnosis Association.** Network for Pancreatic Organ Donors with Diabetes Conference. February 2021. Online

Conference Poster Presentation: Title: **Autoantibodies specific to the n-terminally truncated GAD(96-585) can stratify those that rapidly progress to insulin therapy in adult-onset diabetes**. Immunology of Diabetes Society Conference. October 2021. Online

Conference Poster Presentation. Title: **Rapid insulin progression in glutamate decarboxylase autoantibody positive adult-onset diabetes can be better predicted by detecting autoantibodies to n-terminally truncated GAD(96-585).** Diabetes UK Professional Conference. March 2022. Online. *Shortlisted for Diabetes UK Clinical Science Poster Award.*

Conference Oral Presentation. Title: **Glutamate decarboxylase autoantibody characteristics can stratify those at risk of rapid progression to insulin therapy in adult-onset type 2 diabetes.** EASD. September 2022. Stockholm, Sweden (In Person).

Published Conference Abstracts

(Presented as online poster presentations)

S.L. Grace, A. Cooper, A.G. Jones and T.J. McDonald. **Zinc transporter 8 autoantibody testing requires age-specific cut-offs (2020), Basic and**

clinical science posters: Immunology/autoimmunity. Diabet. Med., 37: 49-51. https://doi.org/10.1111/dme.14_14245

Sian L. Grace, Helen C. Walkey, Akaal Kaur, Shivani Misra, Nick S. Oliver, Tim J. McDonald, Desmond G. Johnston, Angus G. Jones and Kashyap A Patel. **Glutamate Decarboxylase and Islet Antigen-2 autoantibody titres at diagnosis in people with Type 1 Diabetes showed bimodal distribution and an association with age at diagnosis and HLA genotype (2021), Basic and clinical science posters: Immunology/autoimmunity.** Diabet. Med., 38: e14_14556. https://doi.org/10.1111/dme.14_14556

SL Grace, AE Long, KM Gillespie, AJK Williams, V Lampasona, P Achenbach, ER Pearson, TJ McDonald, AG Jones. **Rapid insulin progression in glutamate decarboxylase autoantibody positive adult-onset diabetes can be better predicted by detecting autoantibodies to n-terminally truncated GAD(96-585)** (2022), Poster abstracts. Diabet. Med., 39: e14810. https://doi.org/10.1111/dme.14810