New insights into autoimmune mediated neonatal diabetes

M B J Johnson

Doctor of Philosophy in Medical Studies

September 2017

New insights into autoimmune mediated neonatal diabetes

Submitted by Matthew Johnson to the University of Exeter

as a thesis for the degree of

Doctor of Philosophy in Medical Studies

In September 2017

This thesis is available for Library use on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

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

Signature: ……………………………………………………………

Acknowledgments and dedication

Firstly, I would like to thank my supervisors Sarah Flanagan, Andrew Hattersley and Sian Ellard for their guidance, patience and advice for the last three years. I have been extremely fortunate to have had the opportunity to work with such brilliant minds and kind hearted people during my postgraduate studies. I have learnt far more than the scope of this thesis and I will be eternally grateful for the opportunities they have afforded me.

I would like to thank my family for their support over the years and for showing me the benefit of hard work. I would also like to thank my best friends, Beau and Lara, for their support through the tough times as well as the good, and for sharing in my achievements with me – even those that must have seemed obscure. To Pepper - thank you for keeping me calm and for giving me a reason to get outside for a walk and some well-needed fresh air.

Finally, I would like to dedicate this work to my soon-to-be wife, Anna-Marie. Without you, none of this would make sense.

Abstract

Monogenic autoimmune diseases are highly variable syndromes that usually have onset in the first year of life and are often fatal in early childhood. Identifying monogenic autoimmune diabetes is important as it can have implications for medical management of patients, informs families and clinicians of prognosis and recurrence risk, and gives insights into beta-cell autoimmunity and immune tolerance.

The first section of this thesis introduces monogenic autoimmune disease, with focus on the conditions that have autoimmune endocrine disorders as part of their clinical phenotype. The following section details the methodologies used throughout this thesis.

In chapter 1, we used a type 1 diabetes genetic risk score (T1D-GRS) based on the top 10 risk alleles for T1D to identify patients with monogenic autoimmunity from patients with early-onset polygenic diabetes and additional autoimmunity. We showed that the T1D-GRS was highly discriminatory of monogenic autoimmunity, especially when combined with age of onset (ROC-AUC 0.88). We also identified 16 families for gene discovery studies. Furthermore, this work shows that polygenic risk for the development of T1D does not affect the development of diabetes in monogenic autoimmunity.

Chapter 2 describes the genetic and phenotypic information for the largest cohort of patients with IPEX syndrome, caused by hemizygous mutations in *FOXP3*, reported to date (n=48). We analysed this data to determine if there were any genotypic or clinical characteristics of IPEX syndrome that could predict prognosis. We did not find evidence of phenotype-genotype relationships and

showed that presenting feature did not predict prognosis. Medical management of IPEX syndrome cannot, therefore, be based on genotype or presentation.

In chapter 3 we employed whole exome sequencing to look for causal variant(s) in a patient with diabetes (diagnosed aged 7 weeks) and autoimmune lymphoproliferative disease. This identified recessively inherited causative variants in *LRBA.* We then used targeted next generation sequencing (NGS) to screen a large cohort of patients (n=169) and identified an additional 8 probands and an affected family member. This confirms the role of *LRBA* as a neonatal diabetes gene*,* bringing the total number of genes to 25.

In chapter 4, we assessed if immunoglobulin E (IgE) could be useful to identify patients with early-onset multisystem autoimmune disease caused by gain of function (GOF) *STAT3* mutations. We showed that serum IgE was below the lower limit of the normal reference range (2KU/L) in all patients with *STAT3* GOF (n=6), giving this threshold a sensitivity of 100% (95% CI: 54.1 – 100) and specificity 97.2% (95% CI: 96.2-97.9). We also found that IgE in patients with IPEX (n=16) was significantly higher than those with *STAT3* GOF (p=0.002) suggesting it could be useful to identify IPEX from *STAT3* GOF in non-consanguineous males with early-onset autoimmunity.

The final concluding section summarises the key findings of each chapter, the impact of these findings and suggests future avenues for research.

Identifying monogenic autoimmunity has enabled prenatal diagnoses, given families and clinicians knowledge on recurrence risk, and could enable targeted therapies to be employed. This body of work will enable better discrimination of monogenic autoimmunity from polygenic clustering of early-onset autoimmunity, and gives insights into the factors that determine disease phenotype and clinical

course in monogenic autoimmunity. Gene discovery on the remaining patients will give new insights into the mechanisms of beta-cell autoimmunity and the regulation of the adaptive immune system and maintenance of immune tolerance.

Table of contents

Organisation of thesis 9
Abbreviations 12
Introduction Part 1 14
Supplementary data: Monogenic Autoimmune Diseases of the Endocrine System 59
Introduction Part 1 – post script 69
Introduction Part 2 75
Methods 111
Immunoglobulin testing 112
DNA extraction and quantification 113
Polymerase Chain Reaction (PCR) 114
Droplet digital PCR (ddPCR) 115
Dideoxy chain terminator sequencing (Sanger sequencing) 117
Next Generation Sequencing 117
Custom Targeted Next Generation Sequencing 118
Exome Capture 121
Massively parallel high throughput sequencing (Illumina) 121
Bioinformatics analysis 122
Targeted NGS pipeline 125
Exome pipeline 125
Type 1 Diabetes Genetic Risk Score (T1D-GRS) 126
Statistical analysis 127
Ethical approval 127
Patient recruitment and follow up 127
Chapter 1 130
Supplementary data 153
Chapter 2 156
Chapter 3 188
Supplementary data 213
Chapter 4 Part A 221
Chapter 4 Part B 231
Conclusions 239
Final remarks 254
APPENDIX 1 266
Reference sequence transcripts and genes in targeted pancreatic panel v5.1 266

APPENDIX 2 268
Genetic testing for early-onset multiple autoimmune disease... 268

Organisation of thesis

As each data chapter within this thesis is presented as a publication, each begins with acknowledgments to co-authors and personal contributions of the first author.

Introduction

The introduction is split into two parts. The first, part 1, is a review article published in the Lancet Diabetes and Endocrinology detailing monogenic disorders that include diabetes and autoimmune thyroid disease as part of the disease spectrum. This is followed by a post-script discussing heterozygous *TNFAIP3* mutations and new insights into the phenotype associated with *AIRE* and *STAT1* mutations which were published after the publication of the review. Part 2 of the introduction details other monogenic autoimmune disorders that do not include diabetes as a feature, neonatal diabetes, maturity-onset diabetes of the young (MODY), type 1 diabetes, the use of biomarkers in monogenic disease and contains information to introduce genetic risk scores to the reader.

Methods

This section details the methods used in this thesis, ranging from immunoglobulin testing to next generation sequencing. It references the chapters in which each method was used and contains information on bioinformatic and statistical methods employed, as well as wet-lab techniques.

Chapter 1

Chapter 1 is an article currently under review in Diabetologia titled 'A Type 1 diabetes genetic risk score can discriminate monogenic autoimmunity with

diabetes from early onset clustering of polygenic autoimmunity with diabetes'. In this we used a genetic risk score based on risk alleles for type 1 diabetes to identify patients with monogenic autoimmunity and showed that it performed better than clinical features.

Chapter 2

Chapter 2 is a manuscript prepared for submission to the Journal of Clinical Immunology titled 'Genotype and clinical phenotype do not predict prognosis in IPEX syndrome'. In this chapter, we studied a large cohort of patients with hemizygous *FOXP3* mutations (n=48) to identify genotype-phenotype relationships in IPEX disorder and determine if clinical features could predict prognosis. We also discuss in detail atypical cases that are worthy of note.

Chapter 3

This chapter is a manuscript published in Diabetes entitled 'Recessively inherited *LRBA* mutations cause autoimmunity presenting as neonatal diabetes'. In this chapter, we identified *LRBA* as a novel cause of neonatal diabetes in 9 probands and one affected family member, and go on to show that it has a minimum prevalence of 0.6% of patients diagnosed with diabetes under 12 months.

Chapter 4

This chapter is split into two sections. The first, part a, gives background on immunoglobulin E (IgE) in health and disease and provides data on serum immunoglobulin in patients with gain-of-function STAT3 mutations and hemizygous FOXP3 mutations. It also discusses a recent publication which is related to Chapter 4b. Part b is a letter published in Clinical Chemistry which

describes the use of low IgE to identify patients with STAT3 gain-of-function mutations and its specificity and sensitivity.

Conclusions

This chapter summarises the 4 data chapters, discusses the impact of the key findings in each chapter and discusses future directions for research. At the end of the thesis, the final remarks section places the research into context (including the broader contribution to knowledge and how this may translate to type 1 diabetes research), highlights the issues faced during this the research that is contained with this thesis and how these were overcome, and suggests the future avenues of research for the cohorts studied.

Abbreviations

ACMG – American College of Medical Genetics and Genomics

AIRE – Autoimmune regulator

APS1 – Autoimmune polyendocrine syndrome type 1

CVID – Common variable immunodeficiency

ddPCR – Digital droplet polymerase chain reaction

FOXP3 – Forkhead box containing protein 3

GAD – Glutamic acid decarboxylase

GOF – Gain of function

GRS – Genetic risk score

GWAS – Genome wide association study

HGMD – Human gene mutation database

HLA – Human leukocyte antigen

HSCT – Haematopoietic stem cell transplant

IA2 - Islet antigen 2

IAA - Insulin autoantibody

IgE – Immunoglobulin E

IL2RA – Interleukin-2 receptor subunit Alpha

IPEX – Immunodysregulation, Polyendocrinopathy, Enteropathy, X-linked syndrome

IQR – Inter-quartile range

IQR – Inter-quartile range

ITCH - Itchy E3 ubiquitin ligase

KU/L – Kilo units per litre (equivalent to International Units per Litre)

LOF – Loss of function

LRBA – Lipopolysaccharide responsive beige-like anchor protein

MHC – Major histocompatibility complex

MODY – Maturity onset diabetes of the young

NA – Not applicable

ND – No data

NDM – Neonatal diabetes

NF-kappa β – Nuclear factor kappa-light-chain-enhancer of activated B cells

NGS – Next generation sequencing

OMIM – Online Mendelian inheritance in man

PCR – Polymerase chain reaction

PTV – Protein truncating variant

STAT – Signal transducer and activator of transcription

T1D – Type 1 diabetes mellitus

T2D – Type 2 diabetes mellitus

TNFAIP3 - Tumour necrosis factor, alpha-induced protein 3

Treg – Regulatory T cell

WTCCC – Wellcome Trust case control consortium

ZnT8 - Zinc transporter 8

Introduction Part 1

Monogenic Autoimmune Diseases of the Endocrine System

M. B. Johnson, A. T. Hattersley, S. E. Flanagan

Lancet Diabetes & Endocrinology 2016 4(10):862-872

Search strategy and selection criteria:

We searched PubMed and Google scholar for articles published in English between January 1970 and July 2015 and examined original articles, case reports, large series write-ups and review articles. The following terms were used in various combinations; “monogenic”, “autoimmune”, “endocrinopathy”, “single gene”, “mutation”, “mendelian”, “inherited”, “congenital”, “neonatal diabetes”, “type-1 diabetes”, “autoimmune thyroid disease”, “hypothyroidism”, “hyperthyroidism”, “Graves’ disease”, “Hashimoto’s”, “AIRE”, “APS1”, “Autoimmune Polyendocrinopathy Syndrome Type 1”, “FOXP3”, “IPEX”, “Immunodysregulation, Polyendocrinopathy, Enteropathy, X-Linked”, “IL2RA”, “CD25”, “Immunodeficiency 41 with lymphoproliferation and autoimmunity”, “ITCH”, “Ubiquitin ligase E3”, “Autoimmune disease, multisystem, with facial dysmorphism”, “LRBA”, “Common Variable Immunodeficiency -8”, “CVID-8”, “STAT1”, “Gain of function”, “Immunodeficiency 31C”, “STAT3”, “Infantile-onset multisystem autoimmune disease”. Where less than 50 individuals are reported with a specific disorder we used all cases to inform our article, otherwise large series were used. We used our judgement to select articles in order to provide a summary of the monogenic causes of autoimmune endocrine disease rather than aiming to provide an exhaustive list of all research into any particular subtype.

Summary

The most common endocrine diseases, type-1 diabetes and hyper/hypo thyroid disease, are the result of autoimmunity. Clustering of autoimmune endocrinopathies can result from polygenic predisposition or more rarely may present as part of wider syndrome due to a mutation within one of 7 genes. These monogenic autoimmune diseases show highly variable phenotypes both within and between families with the same mutations. The average age of onset of the monogenic forms of autoimmune endocrine disease is younger than that of the common polygenic forms and this, combined with the manifestation of other autoimmune disease and/or specific hallmark features can inform clinicians as to the relevance of genetic testing. A genetic diagnosis can guide medical management, give an insight into prognosis, inform families of recurrence risk and can facilitate prenatal diagnoses.

The major disorders of the endocrine system result from autoimmune destruction of cells within the endocrine glands. The autoimmune thyroid diseases, hypo- or hyperthyroidism, are the most commonly diagnosed endocrinopathy with a European prevalence of 3% and 0.75% respectively.[2] Type 1 diabetes which results from autoimmune infiltration and destruction of the insulin-producing pancreatic beta cells is the second most commonly diagnosed endocrine disorder with a UK prevalence of 0.34%.[3]

Clustering of Autoimmune Endocrine Disease

Due to the high prevalence of autoimmune endocrinopathies some individuals will develop more than one disease over a lifetime. The number of patients with multiple autoimmune endocrine disease is however higher than expected by chance (table 1).[2, 3] Clustering of autoimmune endocrinopathies that is not the result of an underlying monogenic aetiology is due to a polygenic predisposition.[4-6] Common variants identified by genome-wide association studies account for approximately 9.3% of the heritability of autoimmune thyroid disease.[7] For type 1 diabetes over 50% of the λ_s (sibling relative-risk) is explained by common genetic variants,[8] with the greatest contributor being variation in the human leukocyte antigen (HLA) genes (see box 1). This compares to estimates of up to 88% (type 1 diabetes) and 39-75% (autoimmune thyroid disease) heritability calculated from disease concordance studies in twins. [5, 9] Whilst there are known flaws in twin studies (i.e. the effects of shared environment and possible epistasis) this missing heritability suggests that there are additional rare variants to be discovered.

Perhaps the most striking example of polygenic clustering of autoimmune endocrinopathies is observed in polyendocrine syndrome type II (APS2, or Schmidt’s syndrome). APS2 has a prevalence of 1.4-4.5 cases per 100,000 and

most commonly affects females during middle age. The syndrome is characterised by autoimmune adrenal insufficiency and either autoimmune thyroid disease (69%) type 1 diabetes (52%), or both. The DR3 and DR4 HLA haplotypes are strongly associated with autoimmune adrenal insufficiency, autoimmune thyroid disease (DR3) and type 1 diabetes (DR3, DR4 and DR3/DR4). [10-12] This shared polygenic predisposition may offer some explanation for the clustering of autoimmune features reported in this syndrome.

Box 1: Genome-wide Association studies

Genome-wide association studies (GWAS) have investigated polygenic susceptibility to the major autoimmune diseases. Large meta-analyses have identified 53 different loci for type 1 diabetes and 22 loci for autoimmune hyperthyroidism (supplementary table 1).[1] 43% of the genes identified are involved in immune function (23/53 diabetes loci, 9/22 hyperthyroidism loci). Furthermore, 18 associations have been identified in three of the genes described within this article (1 within *AIRE*, 11 in *IL2RA* and 6 in *STAT3*) as contributing to the development of diverse immunological diseases and traits, ranging from type 1 diabetes to anti-retroviral drug response (supplementary table 2).[1] In at least two cases the same association increases risk to two different autoimmune diseases suggesting a shared mechanism is driving pathogenesis. Whilst these findings highlight the importance of these genes in normal immune function, many GWAS 'hits' are in genes or non-coding regions of unknown function and such studies have so far yielded limited new information on the underlying mechanisms of disease.

When to Suspect a Monogenic Aetiology

Highly penetrant mutations in a single gene can give rise to multiple autoimmune endocrine diseases. To date pathogenic mutations in seven genes have been reported to cause multi-organ autoimmunity which includes type 1 diabetes and autoimmune thyroid disease (table 2). In these individuals monogenic autoimmunity is often suspected when multiple autoimmune conditions present in early childhood or when specific features are present (table 2). There may also be a family history suggesting Mendelian inheritance.

Diagnosing Monogenic Autoimmune Endocrinopathy

Genetic testing must be performed to accurately diagnose monogenic autoimmune endocrine disease. Specific patterns of disease both in terms of the organs affected and the age at diagnosis are observed in some monogenic subtypes (table 2). Whilst this clinical phenotype may provide a guide as to the most likely underlying genetic aetiology, the variability in penetrance associated with each condition means they are not pathognomonic.

The Importance of a Diagnosis

The underlying pathophysiological mechanism of autoimmune disease is likely to differ in monogenic disorders compared to the polygenic counterpart. This has implications for medical management as personalised therapy may be possible for individuals with autoimmunity resulting from a single gene defect. Identifying these individuals also provides a unique opportunity to investigate the role of the gene in health, development and disease *in vivo.*

Of the seven known causes of monogenic autoimmune polyendocrinopathy, four have been discovered since 2010 reflecting advances in genetic technology and recognition of the importance of a genetic diagnosis for these individuals. Identifying novel genes has improved understanding of the disease mechanism which has led to some progress in the development of targeted therapies for these disorders.[13, 14] This review will describe the established and newly identified genes in which mutations are known to cause multiple autoimmune disease including type 1 diabetes and autoimmune thyroid disease. For the purpose of this review these syndromes will be referred to as monogenic autoimmune polyendocrinopathies. We will explore the specific clinical manifestations, underlying mechanisms of disease and treatment options associated with each genetic subtype and provide a comparison to common polygenic autoimmunity.

Autoimmune Polyendocrinopathy Syndrome type 1 - *AIRE*

Disease Phenotype

Autoimmune Polyendocrinopathy syndrome type 1 (APS1, also known as APECED - Autoimmune Polyendocrinopathy Candidiasis Ectodermal Dystrophy) is the most commonly reported monogenic autoimmune syndrome with 107 disease causing mutations listed in the Human Gene Mutation Database (accessed 04/02/2016).[15] The majority of knowledge on the disease course of APS1 has come from Finnish and Sardinian longitudinal studies where there is increased research activity on this disorder as a result of founder mutations within these populations. [16-18] APS1 presents early in childhood (median age: 3.3 years) and is characterised by three major components; chronic mucocutaneous candidiasis is usually the presenting feature which is followed by the development of hypoparathyroidism and later autoimmune adrenal insufficiency (see table 2).[16, 17] Rarer disease components often dominate the clinical picture. These include type 1 diabetes which is present in 13% of individuals at 30 years, and autoimmune hepatitis which affects 20% of individuals at 18 years[16, 17]. Gonadal insufficiency occurs in approximately 50% of individuals with APS1 and is usually primary. Alopecia is present in 39% of adults and ectodermal dystrophies are common with dental enamel hypoplasia identified in 77% of individuals in one large series.[19]

Genetics

APS1 is most commonly caused by recessively inherited loss-of-function mutations in the autoimmune regulator gene, *AIRE*.[20, 21] Increased rates of APS1 are observed in the Finnish (1:25,000) and Sardinian (1:14,000) populations as a result of founder mutations and also in the Jewish Iranians (1:9000) which is

likely to reflect the high rate on consanguineous unions within this population. [17, 22-24] Dominant negative missense mutations have also been reported in six families with APS1. These mutations are located within specific domains of the protein and act to reduce the function of the AIRE tetramer.[25-27] Patients with dominantly acting mutations often show a less severe phenotype compared to those with recessive mutations. [26, 27]

Disease mechanism

AIRE encodes a protein by the same name which forms a homotetramer that activates the ectopic transcription of tissue-specific self-antigens within the thymus (box 2).[28] The self-antigens under the control of AIRE are subsequently presented to naïve T cells via the major histocompatibility complex resulting in the negative selection of self-reactive T-cells. Individuals with loss-of-function *AIRE* mutations are unable to express these specific ectopic transcripts within the thymus and consequently there is no negative selection of self-reactive T-cells. Failure of the immune system to recognise specific tissues as 'self' ultimately results in the cells of the immune system attacking the target organs. AIRE also has a role in the development of regulatory T-cells which are important for maintaining peripheral tolerance (box 2).[29] Dominant mutations reported in *AIRE* exert their effects via a dominant negative mechanism resulting in reduced function of the multimeric protein. Residual protein function may explain the reduced phenotypic severity associated with these mutations when compared to recessively inherited mutations.[27]

Medical management

Treatment for APS1 focusses on the management of the individual components of the syndrome. The prognosis depends predominantly on the effective

treatment of endocrine deficiencies, the management of which is particularly challenging in some individuals due to the co-existence of type 1 diabetes, adrenal insufficiency and/or autoimmune thyroid disease. Dietetic management to maintain electrolyte balance is often necessary and parenteral feeding may be required for patients with severe malabsorption due to enteropathy. Combined systemic and topical treatments are used to combat chronic mucocutaneous candidiasis. For some patients with severe enteropathy or autoimmune hepatitis immune suppressive therapy is required.[16, 17] Early detection of autoimmune disease can assist in treatment optimisation, therefore educating patients on the chronic features of APS-1 is important.[16]

When should genetic testing be considered?

Genetic testing of AIRE should be considered in patients with any two of the most common features of APS1 (autoimmune adrenal insufficiency, hypoparathyroidism and/or chronic mucocutaneous candidiasis), or one feature in those with a family history of APS1.

Immunodysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX) Syndrome - *FOXP3*

Disease Phenotype

IPEX syndrome is an X-linked disorder affecting males. There are currently 77 mutations recorded in the HGMD database (accessed 31/05/2016) and a recent international study confirmed a genetic diagnosis of IPEX syndrome in 0.7% of individuals diagnosed with diabetes before the age of six months (14/1020).[30] This however is likely to be underestimation of the true prevalence of the disease as the high mortality rate will mean that some patients will not receive a genetic diagnosis.

The most common feature of IPEX syndrome is enteropathy which presents as severe protein losing diarrhoea and can be life threatening. Type 1 diabetes usually follows and is often diagnosed in the first six months of life, additional features include dermatitis.[31] Whilst individuals have a demonstrable ability to mount an effective immune response, a break down in the skin and gut barrier function due to the severe eczema, autoimmune enteropathy and intensive immunosuppressive therapy increases the risk and severity of infections. [32, 33] The majority of individuals present extremely early with aggressive autoimmunity however a less severe phenotype has been reported in some families (table 2).[34] Recent studies suggest that IPEX syndrome may begin to manifest before birth and that miscarriage of male foetuses can occur due to IPEX syndrome.[35]

Genetics

Recessively acting loss-of-function mutations in *FOXP3* cause IPEX syndrome. As *FOXP3* is located on the X chromosome a single copy of a mutation (hemizygosity) is sufficient to cause disease in males. The variability in phenotype observed with this syndrome can be explained by the effect of the specific mutation with some functionally milder (hypomorphic) mutations that result in residual protein function described in a few individuals with a milder phenotype.[31-34] Additional genetic modifiers and environmental factors may also influence the severity of the disease. There is often no family history of IPEX syndrome as the patient will have inherited their X chromosome, and hence *FOXP3* mutation, from their unaffected mother who has both a normal and mutated copy of the gene.[36, 37]

Box 2: Loss of tolerance

The immune system strikes a careful balance between a continued ability to mount effective responses to pathogens whilst remaining inert to self-antigens. This is achieved by several mechanisms of tolerance including central tolerance, the deletion of self-reactive cells in the thymus before their release into the periphery, and on-going peripheral tolerance mediated by regulatory T cells.

Central tolerance is achieved by exposing naïve lymphocytes to self-antigens. The autoimmune regulator (encoded by *AIRE*) promotes the ectopic expression of tissue specific proteins in medullary thymic epithelial cells (mTECs). The mTECs display these proteins to the developing T-cells and promote apoptosis in those that are self-reactive, preventing their release into the lymphatic system. A similar, less rigorous, process occurs in the bone marrow in order to negatively select self-reactive B cells.

Peripheral tolerance is an on-going process mediated by regulatory T-cells. Developing both within the thymus and in the periphery, the regulatory T cells account for 1-10% of $CD4^{+}$ cells in healthy individuals. These cells constitutively express the IL-2 receptor, which regulates immune activity, and high levels of CTLA-4 - a potent inhibitor of effector T cells.

Regulatory T cells are essential for immune homeostasis and the regulation of immune responses; they both stimulate reactions to pathogens and suppress inappropriate autoimmune responses. As such the balance between self-reactive effector cells and their suppression by regulatory T cells maintains tolerance and prevents autoimmune disease. Six of the genes in which defects cause autoimmune endocrinopathies have a role in the differentiation and/or function of regulatory T-cells (figure 1).

Disease mechanism

FOXP3 encodes a master transcription factor which controls the differentiation of lymphocytes into regulatory T cells (box 2).[38] The absolute deficiency of regulatory T cells, due to a *FOXP3* mutation, results in a loss of peripheral tolerance leading to unregulated responses by self-reactive T cells and consequently autoimmune destruction of endocrine and non-endocrine tissues.[38] There is growing evidence that functional FOXP3 is also required for the on-going suppressive function of regulatory T cells.

Medical management

Diabetes can be difficult to manage in patients with IPEX syndrome due to the co-existence of severe enteropathy. Whilst parenteral nutrition may combat the effects of severe protein-losing diarrhoea it generally has limited success.[31] Immunosuppressive therapy is often required to manage the enteropathy, with Sirolimus offering a targeted approach. Sirolimus blocks IL-2 dependent signal-

transduction via interaction with mTOR (mammalian target of rapamycin), thus preventing T and B cell activation.[39] It preferentially targets effector T cells over regulatory T cells, which are inherently compromised in patients with *FOXP3* mutations, thus giving increased benefit over other immunosuppressive agents.[40] Haematopoietic stem cell transplant (HSCT) can be curative for IPEX; an large international study showed that for 56% of IPEX patients receieving HSCT (n=58) it was fully curative, with an estimated overall survival rate of 73% at 15 years post-transplant.[41] In order to improve outcomes and prevent non-reversible damage to the endocrine system, particularly the pancreatic islets, early pre-emptive HSCT is necessary.[42] Dermatological manifestations are managed by topical treatment with steroids and pain management.

When should genetic testing be considered?

Genetic testing for IPEX syndrome should be considered in any male presenting with infancy-onset severe enteropathy (manifesting as protein losing diarrhoea) and any patient diagnosed with diabetes before the age of six months.[30]

Immunodeficiency 41 with lymphoproliferation and autoimmunity *- IL2RA*

Disease Phenotype

Reports of females with features very similar to IPEX syndrome (an X-linked disorder affecting males) led to the search for a novel aetiological gene. [43, 44] The disease was termed Immunodeficiency 41 with lymphoproliferation and autoimmunity and is characterised by extremely early presentation (median age 5 weeks, see table 2) with severe enteropathy leading to malabsorption and failure to thrive, and chronic and recurring cytomegalovirus (CMV) infection.[43-47] In the four reported cases additional early onset autoimmunity including type 1

diabetes, hypothyroid disease, dermatological manifestations and alopecia were reported .[43, 47]

Disease mechanism and genetics

Immunodeficiency 41 with lymphoproliferation and autoimmunity results from recessively inherited loss-of-function mutations in *IL2RA* which encodes the interleukin-2 receptor alpha chain (also known as CD25). The IL2RA subunit forms part of a receptor that is essential for maintaining immune homeostasis following the binding of its ligand interleukin-2 (IL-2). IL-2 signalling is involved in immunoregulatory pathways and also promotes the transcription of *FOXP3*,[48] stimulating the differentiation of regulatory T cells (box 2). *IL2RA* is constitutively and highly expressed in regulatory T cells allowing for both their rapid recruitment in immune responses to pathogens and suppressive function.[44] Loss of IL2RA reduces the suppressive function of regulatory T cells leading to loss of peripheral tolerance, unregulated responses by self-reactive T cells and ultimately autoimmunity.

Medical management

Prophylactic treatment with a combination of antibiotics is prudent as these individuals have a reduced ability to fight infections and to mount an adaptive response to previously encountered pathogens.[43-47] Immunosuppressive therapy is required to treat severe and chronic autoimmunity and may have some effect in improving symptoms particularly those resulting from enteropathy.[43] Sirolimus is the immunosuppressive of choice as this drug preferentially targets effector T cells over regulatory T cells which have reduced suppressive function in these individuals.[40] Haematopoietic stem cell transplantation has proved successful in

one case with a complete remission of autoimmune symptoms achieved following treatment.[46]

When should genetic testing be considered?

Screening of IL2RA should be performed in infants with immunodysregulatory features including recurring infections, particularly cytomegalovirus, and/or enteropathy. This is particularly true for individuals from populations with high rates of consanguineous unions where there is an increased likelihood of a recessive genetic aetiology.

Multisystem Autoimmune Disease with Facial Dysmorphism *- ITCH*

Disease Phenotype

This complex and multifaceted disease has been described in a single large 'Old-Order Amish' pedigree with 10 affected individuals.[49] This disorder combines significant craniofacial abnormalities, macrocephaly, growth failure with decreased muscle development and autoimmunity. Autoimmune endocrine disease was present in 5 individuals with 4 having autoimmune thyroid disease and one patient having type 1 diabetes diagnosed before 23 years (table 2).[49] Autoimmune hepatitis was reported in one case. Nine individuals had chronic lung disease, three died of respiratory failure in early childhood with pathology studies revealing nonspecific interstitial pneumonitis.[49]

Disease mechanism and genetics

Multisystem autoimmune disease with facial dysmorphism results from recessive mutations in the ubiquitin ligase gene *ITCH.*[49] ITCH is involved in the addition of ubiquitin to proteins, which often targets these molecules for degradation, a process that is essential for the maintenance of normal immune function.[50]

Deficiency of functional ITCH is proposed to lead to uncontrolled activation of effector T cells leading to autoimmune attack. The non-immune phenotype of patients may relate to the absence of ubiquitination in multiple protein networks. *Itch* knockout mice show fatal autoimmune disease with multiple organ infiltration by lymphocytes, the dysmorphic features that are observed in humans are not detected.[50]

Medical management

Sirolimus, an immunosuppressive, was reported to improve severe enteropathy in one individual and combined treatment with bronchodilators, antibiotics and corticosteroids has been used to treat lung disease with some response.[49] Although ITCH replacement drugs are not currently available new therapies targeting the ubiquitination pathway are under investigation which may be relevant for patients diagnosed with this condition.[51]

When should genetic testing be considered?

Although ITCH mutations are extremely rare screening of this gene should be considered in patients presenting with macrocephaly and craniofacial dysmorphisms with or without additional autoimmunity, especially when other forms of monogenic autoimmune polyendocrinopathy have been excluded.

Common Variable Immunodeficiency-8 with Autoimmunity (CVID-8) - *LRBA*

Disease Phenotype

The major presenting feature of Common Variable Immunodeficiency-8 with Autoimmunity (CVID-8) is autoimmune haematological disease which is observed in 79% of reported cases (23 of 29 reported individuals, table 2).[13, 52-58] Inflammatory bowel disease was observed in 69% of cases with type 1 diabetes

and autoimmune hypothyroid disease present in 17% and 14% of cases respectively (table 2). Immunodeficiency is a common feature of this disorder with recurrent infections resulting from hypogammaglobulinaemia.[13, 52-58] Clinically CVID-8 is a heterogeneous disease with the phenotype ranging from severe multiple early-onset autoimmunity through to isolated irritable bowel disease.[54, 57]

Disease mechanism and genetics

CVID-8 results from recessively inherited loss-of-function mutations in *LRBA*.[53] This gene encodes an intracellular protein which is highly expressed in T and B lymphocytes.[53, 59] Recently LRBA has been shown to have an essential role in the post-translational regulation and trafficking of CTLA-4, a receptor expressed on T regulatory cells with potent inhibitory function (see Box 2). Deficiency of LRBA prevents the CTLA-4 receptor from reaching the cell surface of regulatory T cells in response to T cell receptor activation. This abolishes the inhibitory function of CTLA-4 which decreases the suppressive action of the regulatory T cells leading to an unregulated immune response and autoimmune attack (box 2).[13]

Medical management

Blood transfusions can treat prolonged cytopenic episodes with intravenous immunoglobulin therapy administered to reduce infections associated with hypogammaglobulinaemia. Two patients have been treated with haematopoietic stem cell transplantation and showed an improved clinical picture.[52] Personalised medicine is also available for these individuals in the form of Abatacept™, a CTLA4-immunoglobulin fusion drug which mimics suppressive CTLA-4 signalling. Six patients with *LRBA* mutations have been effectively treated with Abatacept™, which resulted in a dramatic improvement in inflammatory and

autoimmune symptoms with long term treatment (5-8 years duration).[13] Abatacept prevents co-stimulation of T-cells by CD80 and CD86 and thus reduces the level of T-cell activation.[60]

When should genetic testing be considered?

Genetic testing of LRBA is warranted in patients presenting with autoimmune haematological disorders (e.g. haemolytic anaemia, thrombocytopenia) and inflammatory bowel disease in early childhood.

Immunodeficiency 31C - *STAT1*

Disease Phenotype

Over 50 patients have been reported with Immunodeficiency 31C syndrome.[61, 62] The hallmark feature of this disorder is chronic mucocutaneous candidiasis, an intractable fungal infection of the mucous membranes, nails and groin. There is also an increased incidence of autoimmune hypothyroid disease having been reported in 19% of cases (table 2).[61] Five individuals have developed severe multi-organ autoimmunity which included type 1 diabetes, enteropathy and dermatitis.[62] Short stature, delayed puberty and overt cardiac or vascular defects of unknown aetiology were also observed.

Disease mechanism and genetics

Dominantly acting gain-of-function mutations in the Signal Transducer and Activator of Transcription 1 (*STAT1*) gene cause Immunodeficiency 31C. STAT proteins are phosphorylated by Janus Kinases (JAK), which leads to their activation as a transcription factor. Activated STAT1 protein is involved in converting extracellular signals, via cytokines such as interferon alpha (IFNα), into transcriptional responses which in turn activate multiple cellular processes.[63]

STAT1 mutations prevent de-phosphorylation of STAT1, leaving it in a state of constitutive activation. Recent studies suggest that increased IFNα signalling can cause autoimmunity as patients with various cancers who have been treated with IFNα therapy have an increased risk of developing hypothyroidism, autoimmune hepatitis and type 1 diabetes.[64, 65] The chronic mucocutaneous candidiasis that dominates the clinical picture in this disorder results from impaired IL-17-driven immunity caused by increased STAT1-dependent cellular responses to IL-17 inhibitors which include IFNα.[66]

Medical management

Systemic steroid treatment, intravenous immunoglobulin therapy and immunosuppressive agents can be used to treat acute autoimmune disease (e.g. cytopenic episodes). Combined therapy with systemic and topical antifungal agents is required to manage *Candida* infection, whilst treatment with antibiotics may worsen or initiate episodes of chronic mucocutaneous candidiasis.

When should genetic testing be considered?

STAT1 screening should be considered in patients with chronic mucocutaneous candidiasis in whom mutations in AIRE (causing APS1) have been excluded especially if additional autoimmune features are present.

Infancy-Onset Multi-system Autoimmune Disease *- STAT3*

Disease Phenotype

Early-onset multiple autoimmune disease is the most recent of the monogenic autoimmune disorders to be described.[67] In the first report 4 of 5 individuals were diagnosed with neonatal diabetes (median age: 2.5 weeks, range 0-43 weeks) with autoimmune hypothyroid disease observed in 2 of the 5 cases (table 2). All

4 individuals with neonatal diabetes had intrauterine growth retardation (median birth weight: -1.59 SDS) suggesting that insulin secretion was impaired during foetal development and that autoimmune destruction of pancreatic beta-cells was occurring *in utero*.[67] Subsequent reports have identified a further 15 individuals with this disorder, and have noted a lower incidence of diabetes (2/15) although autoimmune lymphoproliferation has since emerged as a common feature (14/20 reported cases). Additional features include short stature (< 5th centile), enteropathy (in 50% of individuals) and recurring infections (60%).[14, 68, 69]

Disease mechanism and genetics

Dominantly acting germline gain-of-function mutations in *STAT3* cause Infantile-onset multisystem autoimmune disease. The STAT3 protein couples intra- and extracellular signals to multiple cellular functions including cell growth, differentiation and proliferation. The underlying mechanism of disease is yet to be fully elucidated, however it was recently shown that Th17 cell numbers were increased in an individual harbouring a *STAT3* gain-of-function mutation.[69] Th17 cells, which develop in a *STAT3*-dependent manner, contribute to pro-inflammatory responses in autoimmune disease by releasing IL-17.[70] A reduction in the number of regulatory T cells has been reported in these patients; therefore reduced peripheral tolerance may also have a role in the development of autoimmunity.[14] Loss of-function *STAT3* mutations cause the opposing phenotype of Hyper-IgE (Job) syndrome which is characterised by recurrent bacterial and fungal infections, increased serum IgE and facial dysmorphism. Autoimmune endocrinopathies have not been reported in patients with Hyper IgE syndrome.[71]

Medical management

Immune suppression may be useful to treat the severe autoimmune disease.[69] Haematopoietic stem cell transplantation has been performed in two patients; one patient survived the procedure and had complete resolution of autoimmune enteropathy, autoimmune hypothyroidism and recurrent cytopenias.[14] There has been some progress in the development of targeted therapies for these individuals specifically in the form of a monoclonal antibody against the cytokine, IL-6.[14] IL-6 is a pleitropic inflammatory cytokine that exerts its effects via janus kinases.[72] The antibody binds to the IL-6 receptor preventing IL-6 from exerting its pro-inflammatory effects. [72, 73] For one patient with severe polyarthritis and scleroderma, treatment with this antibody resulted in a marked improvement in acute arthritic and dermatological symptoms.[14]

When should genetic testing be considered?

Screening of STAT3 should be performed in individuals presenting with early-onset autoimmune lymphoproliferation and recurrent infections. In older children, short stature combined with autoimmunity should direct the clinician to a genetic test.

MAKING A DIAGNOSIS OF MONOGENIC POLYENDOCRINOPATHY

Identifying individuals for testing

Genetic testing is essential for the accurate diagnosis of monogenic autoimmunity. Whilst a specific combination of autoimmune features, together with laboratory biomarkers (table 3), can help guide the clinical diagnosis, the variability in disease progression and phenotype, together with the overlap in clinical features observed with each monogenic subtype can make a clinical diagnosis challenging. Although family history of multiple early-onset autoimmune disease suggests a monogenic aetiology the absence of

autoimmune disease in other family members should not preclude testing as dominant, recessive and X-linked conditions will often be sporadic.[31, 53, 62, 67]

Genetic testing should be considered when autoimmune disease is diagnosed atypically early, for example when diabetes is diagnosed before the age of 6 months,[30] or when two or more autoimmune conditions present in early childhood. For example the median age at presentation of autoimmunity in individuals with monogenic disease ranges from 2 weeks to 3.3 years (table 2). In contrast the median age at diagnosis of polygenic type 1 diabetes is 10 years whilst autoimmune thyroid disease generally presents between the ages of 20 and 50 years (table 1).

Genetic technology and access to testing

Until recently genetic testing has predominantly relied on Sanger sequencing. Although robust and accurate, this analysis is relatively slow and expensive as single genes are tested in sections (by exon) and sequentially. This approach is problematic for disorders such as those described in this article where extensive overlap in phenotype exists both within and between genetic aetiologies. The recent adoption of massively parallel next-generation sequencing (NGS) by diagnostic laboratories has revolutionised the way in which we can now screen for these disorders.[74] NGS allows for the targeted analysis of a panel of genes within a single reaction. This means that for genetically and phenotypically heterogeneous disorders such as monogenic autoimmune disease, detailed prior knowledge of the patients phenotype is no longer required to guide the order of genetic testing as all genes will be analysed in each patient who meets the criteria for genetic testing.

NGS technologies have reduced the cost of genetic testing by a factor of between 100 and 200 in the last 5 years; the cost of sequencing an entire human genome is now comparable to the cost of a single gene test.[75] As genetic testing continues to decrease in price and analysis methods improve, genetic testing will become more accessible to larger numbers of individuals with suspected monogenic autoimmunity. Moreover, current research efforts are focussing on characterising the genetics and phenotypic features of individuals with mutations in the known genes as well as searching for novel aetiologies. Consequently opportunities exist for individuals with suspected monogenic autoimmunity to enrol in research funded studies (supplementary table 3). This is particularly important for individuals from developing countries where there is limited affordable access to genetic testing.

Disparities in genetic testing worldwide will have resulted in a gross underestimation of the true incidence of monogenic autoimmune endocrine disease. For example many countries with reduced access to genetic testing have an increased incidence of recessively inherited disease due to the high prevalence of consanguineous unions. As 4 of the known monogenic multiple autoimmune diseases are recessively inherited it seems likely that these conditions will be genetically undiagnosed in many individuals from these regions of the world. Moreover, the severity and complexity of disease observed in many of these conditions means that in countries without an adequate healthcare system many individuals are likely to die before a diagnosis is made which will also result in an underreporting of these conditions.

IMPLICATIONS OF A GENETIC DIAGNOSIS

A genetic diagnosis provides important knowledge of recurrence risk which will inform family planning decisions, facilitate pre-implantation genetic testing and allow accurate prenatal screening. Crucially, identifying the underlying genetic aetiology also allows for the monitoring of disease progression, the introduction of timely treatment regimens to minimise complications, and provides important knowledge of the underlying disease mechanism(s) which is key for the initiation of personalised and targeted therapies.

FUTURE PROSPECTS

For Patients

Recognising individuals with monogenic autoimmunity has implications for many areas of genetic and health research. The development of novel therapies and optimisation of existing treatment for both 'rare' monogenic and 'common' polygenic autoimmune endocrine disease relies on understanding the underlying mechanism(s) of disease. The fledgling field of gene therapy, where the genome is edited in a targeted manner, has shown some successes in monogenic disease.[76] Whilst there is much work to be done to prove efficacy and assess potential side effects, the replacement of faulty genes with wild-type versions could lead to a cure for monogenic autoimmune endocrinopathies.

Knowledge of the molecular basis of disease in monogenic disorders often yields limited results for clinical care. For example, the role of mutant AIRE protein in the development of APS1 is relatively well understood but personalised treatment for this disorder remains elusive. Understanding the polygenic factors contributing to both the variability of disease and treatment response observed in patients with monogenic autoimmune endocrinopathies will be a key factor in the introduction of personalised therapies.

For individuals with monogenic autoimmune endocrinopathies resulting from *FOXP3*, *IL2RA*, *LRBA* or *STAT3* mutations haematopoietic stem cell transplantation has been undertaken successfully to treat the disease. Whilst it is acknowledged that earlier transplantation can result in improved outcomes, the risks associated with this procedure often means that the decision to transplant is made once the disease has become life-threatening. Genotype-phenotype studies are therefore extremely important as information on the likely prognosis may inform clinicians on the decision to transplant early before further symptoms develop.

For Science

Academic led research teams and population level initiatives such as the United Kingdom's 100,000 genomes project are likely to identify new genetic aetiologies for multiple diseases including monogenic polyendocrinopathies.[77] As further genes are identified novel pathways of autoimmunity may be revealed which will be important for furthering understanding into both polygenic and monogenic autoimmune disease.

Individuals with monogenic autoimmunity provide a unique opportunity to investigate how the targeted knock out of a single gene leads to severe autoimmune disease, providing valuable insight into the complex world of the adaptive immune system. Furthermore, the accuracy of data generated by clinical trials and genome wide association studies depends on the correct phenotypic classification of the participants. Identifying those with monogenic disease will prevent these individuals from enrolling in studies designed for their polygenic counterparts where the mechanism of pathogenesis is distinct.

CONCLUSIONS

Monogenic autoimmune endocrinopathies are a clinically and genetically heterogeneous group of disorders which rely on genetic testing for an accurate diagnosis. A monogenic aetiology should be considered in all individuals with two or more early onset autoimmune conditions diagnosed before the age of 5 years as a genetic diagnosis is crucial for informing treatment strategies as well as providing important information on prognosis and recurrence risk.

Acknowledgments

The authors would like to thank Dr Elisa De Franco, Dr Thomas Laver, Dr Pia Leete, Dr Timothy McDonald, Dr Mark Russell and Dr Bijay Vaidya for their useful comments and suggestions. ATH is supported by a Wellcome Trust Senior Investigator award and SEF has a Sir Henry Dale Fellowship jointly funded by the Wellcome Trust and the Royal Society (Grant Number: 105636/Z/14/Z).

Contributors

ATH conceived the review and all authors planned the manuscript. MJ completed the literature search and created the figures. MJ compiled the data, supervised by SF. MJ drafted and, with SF, finalised the manuscript. ATH provided clinical input. All authors reviewed the figures and text.

Declaration of interest

We declare no competing interests.

Role of funding source

N/A

Ethics committee approval

N/A

FIGURE LEGENDS

Figure 1

Stylised representation of the mechanisms that underlie monogenic autoimmune endocrine disease pathogenesis. Mutations in genes known to cause monogenic autoimmunity, which includes type 1 diabetes and/or autoimmune thyroid disease, exert their effects by 1) breaking down immune tolerance related to the development or on-going suppressive function of regulatory T cells or 2) promoting the activation and proliferation of self-reactive effector T cells. (–) Denotes a reduction in normal function, (+) denotes an increase in normal physiological function. Bracketed genes have a putative/hypothesised role without direct evidence.

Figure 2

Infogram showing the major manifestations of four monogenic causes of multiple autoimmune endocrine disease. Whilst there is considerable overlap in phenotype associated with each genetic subgroup the prevalence of each disease varies and hallmark features exist for each of the disorders. The size of organs/systems in this image reflects the prevalence of the feature in the specific disorder. [A]Refers to the phenotype at age 30 years.

References

1. Burdett T HP, Hastings E, Hindorff LA, Junkins HA, Klemm AK, MacArthur J, Manolio TA, Morales J, Parkinson H and Welter D. The NHGRI-EBI Catalog of published genome-wide association studies 2016 [cited 2016 14/02/2016]. The NHGRI-EBI Catalog of published genome-wide association studies]. Available from: www.ebi.ac.uk/gwas.

2. Garmendia Madariaga A, Santos Palacios S, Guillen-Grima F, Galofre JC. The incidence and prevalence of thyroid dysfunction in Europe: a meta-analysis. J Clin Endocrinol Metab. 2014;99(3):923-31.

3. Boelaert K, Newby PR, Simmonds MJ, Holder RL, Carr-Smith JD, Heward JM, et al. Prevalence and relative risk of other autoimmune diseases in subjects with autoimmune thyroid disease. The American journal of medicine. 2010;123(2):183.e1-9.

4. Brix TH, Kyvik KO, Christensen K, Hegedus L. Evidence for a major role of heredity in Graves' disease: a population-based study of two Danish twin cohorts. J Clin Endocrinol Metab. 2001;86(2):930-4.

5. Redondo MJ, Jeffrey J, Fain PR, Eisenbarth GS, Orban T. Concordance for Islet Autoimmunity among Monozygotic Twins. New England Journal of Medicine. 2008;359(26):2849-50.

6. Thomsen SF, Duffy DL, Kyvik KO, Skytthe A, Backer V. Relationship between type 1 diabetes and atopic diseases in a twin population. Allergy. 2011;66(5):645-7.

7. Chu X, Pan CM, Zhao SX, Liang J, Gao GQ, Zhang XM, et al. A genome-wide association study identifies two new risk loci for Graves' disease. Nat Genet. 2011;43(9):897-901.

8. Polychronakos C, Li Q. Understanding type 1 diabetes through genetics: advances and prospects. Nat Rev Genet. 2011;12(11):781-92.

9. Hansen PS, Brix TH, Iachine I, Kyvik KO, Hegedus L. The relative importance of genetic and environmental effects for the early stages of thyroid autoimmunity: a study of healthy Danish twins. Eur J Endocrinol. 2006;154(1):29-38.

10. Lee HJ, Li CW, Hammerstad SS, Stefan M, Tomer Y. Immunogenetics of autoimmune thyroid diseases: A comprehensive review. Journal of Autoimmunity. 2012.

11. Morran MP, Vonberg A, Khadra A, Pietropaolo M. Immunogenetics of type 1 diabetes mellitus. Mol Aspects Med. 2015;42:42-60.

12. Betterle C, Lazzarotto F, Presotto F. Autoimmune polyglandular syndrome Type 2: the tip of an iceberg? Clinical & Experimental Immunology. 2004;137(2):225-33.

13. Lo B, Zhang K, Lu W, Zheng L, Zhang Q, Kanellopoulou C, et al. Patients with LRBA deficiency show CTLA4 loss and immune dysregulation responsive to abatacept therapy. Science. 2015;349(6246):436-40.

14. Milner JD, Vogel TP, Forbes L, Ma CA, Stray-Pedersen A, Niemela JE, et al. Early-onset lymphoproliferation and autoimmunity caused by germline STAT3 gain-of-function mutations. Blood. 2015;125(4):591-9.

15. Stenson PD, Mort M, Ball EV, Shaw K, Phillips AD, Cooper DN. The Human Gene Mutation Database: building a comprehensive mutation repository for clinical and molecular genetics, diagnostic testing and personalized genomic medicine. Human Genetics. 2014;133(1):1-9.

16. Husebye ES, Perheentupa J, Rautemaa R, Kampe O. Clinical manifestations and management of patients with autoimmune polyendocrine syndrome type I. J Intern Med. 2009;265(5):514-29.

17. Perheentupa J. Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy. J Clin Endocrinol Metab. 2006;91(8):2843-50.

18. Valenzise M, Fierabracci A, Cappa M, Porcelli P, Barcellona R, De Luca F, et al. Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy: Report of Seven Additional Sicilian Patients and Overview of the Overall Series from Sicily. Hormone Research in Paediatrics. 2014;82(2):127-32.

19. Pavlic A, Waltimo-Siren J. Clinical and microstructural aberrations of enamel of deciduous and permanent teeth in patients with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy. Archives of oral biology. 2009;54(7):658-65.

20. Aaltonen J, Bjorses P, Perheentupa J, Horelli-Kuitunen N, Palotie A, Peltonen L, et al. An autoimmune disease, APECED, caused by mutations in a novel gene featuring two PHD-type zinc-finger domains. Nat Genet. 1997;17(4):399-403.

21. Nagamine K, Peterson P, Scott HS, Kudoh J, Minoshima S, Heino M, et al. Positional cloning of the APECED gene. Nat Genet. 1997;17(4):393-8.

22. Björses P, Halonen M, Palvimo JJ, Kolmer M, Aaltonen J, Ellonen P, et al. Mutations in the AIRE Gene: Effects on Subcellular Location and Transactivation Function of the Autoimmune Polyendocrinopathy-Candidiasis–Ectodermal Dystrophy Protein. The American Journal of Human Genetics. 2000;66(2):378-92.

23. Meloni A, Perniola R, Faa V, Corvaglia E, Cao A, Rosatelli MC. Delineation of the molecular defects in the AIRE gene in autoimmune

polyendocrinopathy-candidiasis-ectodermal dystrophy patients from Southern Italy. J Clin Endocrinol Metab. 2002;87(2):841-6.

24. Valenzise M, Wasniewska M, Mirabelli S, De Luca F, Cervato S, Betterle C. Identification of two novel mutations in the first Sicilian APECED patient with no R203X mutation in AIRE gene and review of Italian APECED genotypes. Gene. 2012;499(2):343-6.

25. Ilmarinen T, Eskelin P, Halonen M, Rüppell T, Kilpikari R, Torres GD, et al. Functional analysis of SAND mutations in AIRE supports dominant inheritance of the G228W mutation. Human Mutation. 2005;26(4):322-31.

26. Cetani F, Barbesino G, Borsari S, Pardi E, Cianferotti L, Pinchera A, et al. A Novel Mutation of the Autoimmune Regulator Gene in an Italian Kindred with Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy, Acting in a Dominant Fashion and Strongly Cosegregating with Hypothyroid Autoimmune Thyroiditis. The Journal of Clinical Endocrinology & Metabolism. 2001;86(10):4747-52.

27. Oftedal BE, Hellesen A, Erichsen MM, Bratland E, Vardi A, Perheentupa J, et al. Dominant Mutations in the Autoimmune Regulator AIRE Are Associated with Common Organ-Specific Autoimmune Diseases. Immunity. 2015;42(6):1185-96.

28. Anderson MS, Venanzi ES, Klein L, Chen Z, Berzins SP, Turley SJ, et al. Projection of an immunological self shadow within the thymus by the aire protein. Science. 2002;298(5597):1395-401.

29. Aschenbrenner K, D'Cruz LM, Vollmann EH, Hinterberger M, Emmerich J, Swee LK, et al. Selection of Foxp3+ regulatory T cells specific for self antigen expressed and presented by Aire+ medullary thymic epithelial cells. Nat Immunol. 2007;8(4):351-8.

30. De Franco E, Flanagan SE, Houghton JAL, Allen HL, Mackay DJG, Temple IK, et al. The effect of early, comprehensive genomic testing on clinical care in neonatal diabetes: an international cohort study. The Lancet. 2015;386(9997):957-63.

31. Rubio-Cabezas O, Minton JA, Caswell R, Shield JP, Deiss D, Sumnik Z, et al. Clinical heterogeneity in patients with FOXP3 mutations presenting with permanent neonatal diabetes. Diabetes Care. 2009;32(1):111-6.

32. McMurchy AN, Gillies J, Allan SE, Passerini L, Gambineri E, Roncarolo MG, et al. Point mutants of forkhead box P3 that cause immune dysregulation, polyendocrinopathy, enteropathy, X-linked have diverse abilities to reprogram T cells into regulatory T cells. J Allergy Clin Immunol. 2010;126(6):1242-51.

33. Wildin RS, Smyk-Pearson S, Filipovich AH. Clinical and molecular features of the immunodysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome. J Med Genet. 2002;39(8):537-45.

34. Gambineri E, Perroni L, Passerini L, Bianchi L, Doglioni C, Meschi F, et al. Clinical and molecular profile of a new series of patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome: Inconsistent correlation between forkhead box protein 3 expression and disease severity. Journal of Allergy and Clinical Immunology. 2008;122(6):1105-12.e1.

35. Xavier-da-Silva MM, Moreira-Filho CA, Suzuki E, Patricio F, Coutinho A, Carneiro-Sampaio M. Fetal-onset IPEX: report of two families and review of literature. Clin Immunol. 2015;156(2):131-40.

36. Powell BR, Buist NRM, Stenzel P. An X-linked syndrome of diarrhea, polyendocrinopathy, and fatal infection in infancy. The Journal of Pediatrics. 1982;100(5):731-7.

37. Chatila TA, Blaeser F, Ho N, Lederman HM, Voulgaropoulos C, Helms C, et al. JM2, encoding a fork head–related protein, is mutated in X-linked autoimmunity–allergic disregulation syndrome. The Journal of Clinical Investigation. 2000;106(12):R75-R81.

38. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol. 2003;4(4):330-6.

39. Sehgal SN. Rapamune® (RAPA, rapamycin, sirolimus): mechanism of action immunosuppressive effect results from blockade of signal transduction and inhibition of cell cycle progression. Clinical Biochemistry. 1998;31(5):335-40.

40. Yong PL, Russo P, Sullivan KE. Use of sirolimus in IPEX and IPEX-like children. Journal of clinical immunology. 2008;28(5):581-7.

41. Barzaghi F, Amaya Hernandez LC, Neven B, Ricci S, Kucuk ZY, Bleesing J, et al. Long-term follow up of IPEX syndrome patients after different therapeutic strategies: an international multicenter retrospective study. J Allergy Clin Immunol. 2017.

42. Kucuk ZY, Bleesing JJ, Marsh R, Zhang K, Davies S, Filipovich AH. A challenging undertaking: Stem cell transplantation for immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome. J Allergy Clin Immunol. 2016;137(3):953-5.e4.

43. Bezrodnik L, Caldirola MS, Seminario AG, Moreira I, Gaillard MI. Follicular bronchiolitis as phenotype associated with CD25 deficiency. Clin Exp Immunol. 2014;175(2):227-34.

44. Goudy K, Aydin D, Barzaghi F, Gambineri E, Vignoli M, Ciullini Mannurita S, et al. Human IL2RA null mutation mediates immunodeficiency with lymphoproliferation and autoimmunity. Clin Immunol. 2013;146(3):248-61.

45. Sharfe N, Dadi HK, Shahar M, Roifman CM. Human immune disorder arising from mutation of the α chain of the interleukin-2 receptor. Proceedings of the National Academy of Sciences. 1997;94(7):3168-71.

46. Roifman CM. Human IL-2 receptor alpha chain deficiency. Pediatric research. 2000;48(1):6-11.

47. Caudy AA, Reddy ST, Chatila T, Atkinson JP, Verbsky JW. CD25 deficiency causes an immune dysregulation, polyendocrinopathy, enteropathy, X-linked-like syndrome, and defective IL-10 expression from CD4 lymphocytes. J Allergy Clin Immunol. 2007;119(2):482-7.

48. Zorn E, Nelson EA, Mohseni M, Porcheray F, Kim H, Litsa D, et al. IL-2 regulates FOXP3 expression in human CD4+CD25+ regulatory T cells through a STAT-dependent mechanism and induces the expansion of these cells in vivo. Blood. 2006;108(5):1571-9.

49. Lohr NJ, Molleston JP, Strauss KA, Torres-Martinez W, Sherman EA, Squires RH, et al. Human ITCH E3 Ubiquitin Ligase Deficiency Causes Syndromic Multisystem Autoimmune Disease. American Journal of Human Genetics. 2010;86(3):447-53.

50. Matesic LE, Copeland NG, Jenkins NA. Itchy mice: the identification of a new pathway for the development of autoimmunity. Current topics in microbiology and immunology. 2008;321:185-200.

51. Masumoto K, Kitagawa M. E3 ubiquitin ligases as molecular targets in human oral cancers. Current cancer drug targets. 2015.

52. Seidel MG, Hirschmugl T, Gamez-Diaz L, Schwinger W, Serwas N, Deutschmann A, et al. Long-term remission after allogeneic hematopoietic stem cell transplantation in LPS-responsive beige-like anchor (LRBA) deficiency. Journal of Allergy and Clinical Immunology. 2014;135(5):1384-90.e8.
53. Lopez-Herrera G, Tampella G, Pan-Hammarstrom Q, Herholz P, Trujillo-Vargas CM, Phadwal K, et al. Deleterious mutations in LRBA are associated with a syndrome of immune deficiency and autoimmunity. Am J Hum Genet. 2012;90(6):986-1001.
54. Alangari A, Alsultan A, Adly N, Massaad MJ, Kiani IS, Aljebreen A, et al. LPS-responsive beige-like anchor (LRBA) gene mutation in a family with inflammatory bowel disease and combined immunodeficiency. J Allergy Clin Immunol. 2012;130(2):481-8 e2.
55. Burns SO, Zenner HL, Plagnol V, Curtis J, Mok K, Eisenhut M, et al. LRBA gene deletion in a patient presenting with autoimmunity without hypogammaglobulinemia. J Allergy Clin Immunol. 2012;130(6):1428-32.
56. Revel-Vilk S, Fischer U, Keller B, Nabhani S, Gámez-Díaz L, Rensing-Ehl A, et al. Autoimmune lymphoproliferative syndrome-like disease in patients with LRBA mutation. Clinical Immunology. 2015;159(1):84-92.
57. Serwas NK, Kansu A, Santos-Valente E, Kuloglu Z, Demir A, Yaman A, et al. Atypical manifestation of LRBA deficiency with predominant IBD-like phenotype. Inflammatory bowel diseases. 2015;21(1):40-7.
58. Charbonnier LM, Janssen E, Chou J, Ohsumi TK, Keles S, Hsu JT, et al. Regulatory T-cell deficiency and immune dysregulation, polyendocrinopathy, enteropathy, X-linked-like disorder caused by loss-of-function mutations in LRBA. J Allergy Clin Immunol. 2015;135(1):217-27.

59. Wu C, Orozco C, Boyer J, Leglise M, Goodale J, Batalov S, et al. BioGPS: an extensible and customizable portal for querying and organizing gene annotation resources. Genome biology. 2009;10(11):R130.

60. Rochman Y, Yukawa M, Kartashov AV, Barski A. Functional characterization of human T cell hyporesponsiveness induced by CTLA4-Ig. PLoS One. 2015;10(4):e0122198.

61. Liu L, Okada S, Kong XF, Kreins AY, Cypowyj S, Abhyankar A, et al. Gain-of-function human STAT1 mutations impair IL-17 immunity and underlie chronic mucocutaneous candidiasis. J Exp Med. 2011;208(8):1635-48.

62. Uzel G, Sampaio EP, Lawrence MG, Hsu AP, Hackett M, Dorsey MJ, et al. Dominant gain-of-function STAT1 mutations in FOXP3 wild-type immune dysregulation-polyendocrinopathy-enteropathy-X-linked-like syndrome. J Allergy Clin Immunol. 2013;131(6):1611-23.

63. Darnell JE, Jr., Kerr IM, Stark GR. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science. 1994;264(5164):1415-21.

64. Ioannou Y, Isenberg DA. Current evidence for the induction of autoimmune rheumatic manifestations by cytokine therapy. Arthritis and rheumatism. 2000;43(7):1431-42.

65. Al-Ansari M, Weilert F, Dickson G. Interferon-induced haemolytic anaemia in hepatitis C. The New Zealand medical journal. 2008;121(1278):78-81.

66. Zheng J, van de Veerdonk FL, Crossland KL, Smeekens SP, Chan CM, Al Shehri T, et al. Gain-of-function STAT1 mutations impair STAT3 activity in patients with chronic mucocutaneous candidiasis (CMC). European journal of immunology. 2015:n/a-n/a.

67. Flanagan SE, Haapaniemi E, Russell MA, Caswell R, Lango Allen H, De Franco E, et al. Activating germline mutations in STAT3 cause early-onset multi-organ autoimmune disease. Nat Genet. 2014;46(8):812-4.

68. Haapaniemi EM, Kaustio M, Rajala HL, van Adrichem AJ, Kainulainen L, Glumoff V, et al. Autoimmunity, hypogammaglobulinemia, lymphoproliferation, and mycobacterial disease in patients with activating mutations in STAT3. Blood. 2015;125(4):639-48.

69. Wienke J, Janssen W, Scholman R, Spits H, van Gijn M, Boes M, et al. A novel human STAT3 mutation presents with autoimmunity involving Th17 hyperactivation. Oncotarget. 2015;6(24):20037-42.

70. Yang XO, Panopoulos AD, Nurieva R, Chang SH, Wang D, Watowich SS, et al. STAT3 Regulates Cytokine-mediated Generation of Inflammatory Helper T Cells. Journal of Biological Chemistry. 2007;282(13):9358-63.

71. Holland SM, DeLeo FR, Elloumi HZ, Hsu AP, Uzel G, Brodsky N, et al. STAT3 mutations in the hyper-IgE syndrome. The New England journal of medicine. 2007;357(16):1608-19.

72. Hodge DR, Hurt EM, Farrar WL. The role of IL-6 and STAT3 in inflammation and cancer. Eur J Cancer. 2005;41(16):2502-12.

73. Wake MS, Watson CJ. STAT3 the oncogene - still eluding therapy? FEBS J. 2015;282(14):2600-11.

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

75. Siva N. UK gears up to decode 100,000 genomes from NHS patients. Lancet. 2015;385(9963):103-4.

76. Prakash V, Moore M, Yanez-Munoz RJ. Current progress in therapeutic gene editing for monogenic diseases. Mol Ther. 2016.

77. England G. The 100,000 Genomes Project 2016 [cited 2016 01/02/2016]. Available from: http://www.genomicsengland.co.uk/the-100000-genomes-project/.

Tables

Autoimmune disorder	General population	Type 1 diabetes	Autoimmune Hyperthyroidism	Autoimmune Hypothyroidism
Median age of onset (Y)	-	10	20 – 40	30-50
F:M ratio	-	-	6:1	7:1
Type 1 Diabetes	0.34%	-	1.11%	1.01%
Autoimmune Hypothyroidism	3%	10.5%	-	-
Autoimmune Hyperthyroidism	0.75%	1%	-	-
Autoimmune adrenal disease	0.01%	0.5%	0.11%	1.41%
Autoimmune Enteropathies	0.31%	3.4%	1.2%	1.2%

Table 1: A summary of coexisting conditions in patients with polygenic type 1 diabetes and autoimmune thyroid diseases. *F:M – female:male. Enteropathies include inflammatory bowel disease and coeliac disease.*[2, 3]

GENE	*AIRE*		*FOXP3*	*IL2RA*	*ITCH*	*LRBA*	*STAT1*	*STAT3*
Disorder (OMIM number)	**Autoimmune Polyendocrinopathy Syndrome type 1[A] (#240300)**		**Immunodysregulation Polyendocrinopathy, Enteropathy, X-linked (#304790)**	**Immunodeficiency 41 with lymphoproliferation and autoimmunity (#606367)**	**Multisystem Autoimmune Disease with Facial Dysmorphism (#613385)**	**Common variable immunodeficiency 8 with autoimmunity (#614700)**	**Immunodeficiency 31C (#614162)**	**Infancy-Onset Multisystem Autoimmune Disease (#615952)**
	At 5 years	At 30 years						
Mode of Inheritance	Recessive		X-Linked	Recessive	Recessive	Recessive	Dominant	Dominant
Median age of onset	3.3 years		2 weeks	5 weeks	2 years	2 years	1 year	2 years
Endocrine disorders								
Type 1 diabetes	2/91 (2%)	12/91 (13%)	39/55 (71%)	1/4 (25%)	1/10 (10%)	5/29 (17%)	3/52 (6%)	6/20 (30%)
Autoimmune Hypothyroidism	1/91 (1%)	13/91 (14%)	19/55 (35%)	1/4 (25%)	4/10 (40%)	4/29 (14%)	10/52 (19%)	4/20 (20%)
Autoimmune Hyperthyroidism	1/91 (1%)	1/91 (1%)	–	–	–	–	–	–
Hypoparathyroidism	31/91 (34%)	77/91 (85%)	–	–	–	–	–	–
Pituitary	1-3%	1-3%	–	–	–	1/29 (3%)	Short stature (4/52, 8%)	Short stature (12/20, 65%)
Adrenal	8/91 (9%)	71/91 (78%)	–	–	–	–	–	–
Non-endocrine disorders								
Enteropathies	7/91 (8%)	20/91 (22%)	54/55 (98%)	4/4 (100%)	2/10 (20%)	20/29 (69%)	4/52 (8%)	10/20 (50%)
Haematological	–	–	–	2/4 (50%)	–	23/29 (79%)	2/52 (4%)	14/20 (70%)
Dermatological	2/91 (2%)	83/91 (27%)	38/55 (69%)	4/4 (100%)	–	3/29 (10%)	5/52 (10%)	10/20 (50%)
Chronic Mucocutaneous Candidiasis	45/91 (48%)	89/91 (98%)	–	1/4 (25%)	–	–	51/52 (99%)	–
Recurrent Infections	–	–	Secondary to treatment	4/4 (100%)	–	12/29 (41%)	4/52 (8%)	12/20 (60%)
Other notable features	Primary gonadal insufficiency (~50%) Enamel hypoplasia (~75%) Autoimmune hepatitis, Nephritis, Splenic hypoplasia		Only present in males		Develop-mental delay and macrocephaly (10/10, 100%)		Cardiovascular malformations (3/52, 6%)	
References	16-18		41	43-47	349	13, 52-58	61, 62	14, 67-69

Table 2: Summary of the main features present in monogenic autoimmune syndromes where diabetes and autoimmune thyroid disease is reported*. Where <50 patients are reported in the literature, all individuals' data is included. Where >50 patients are reported, large series were used.* [A] *Data is split into patient phenotype at 5 years and 30 years to reflect prognosis.*

Gene	Syndrome name	Specific diagnostic test/criteria	Notes
AIRE	Autoimmune Polyendocrine Syndrome type 1	Two of the following: Addison's disease, hypoparathyroidism, CMC, urticarial eruption, intestinal dysfunction and enamel hypoplasia; anti-interferon-w antibody testing	Only one required for diagnosis if positive family history; Anti-IFN-w antibodies highly specific for APS1;
FOXP3	Immunodysregulation, Polyendocrinopathy, Enteropathy, X-linked	FACS analysis of $CD4^{+}CD25^{+}FOXP3^{+}$ lymphocytes; Measurement of IgE and eosiniphils; Presence of autoimmune enteropathy with eczema	Usually decreased, but may be normal; Raised IgE and/or eosinophilia common
IL2RA	Immunodeficiency 41 with lymphoproliferation and autoimmunity	FACS analysis of $CD4^{+}CD25^{+}FOXP3^{+}$ lymphocytes	Decreased/absent expression of CD25
ITCH	Autoimmune Disease, Multisystem, with Facial Dysmorphism	Dysmorphic features and developmental delay	Relative macrocephaly and facial dysmorphism
LRBA	Common Variable Immunodeficiency-8 with autoimmunity	Immunoglobulin profiling	Hypogammaglobulinaemia common
STAT1	Immunodeficiency 31A	Mucosal and disseminated fungal infections	_
STAT3	Infantile-Onset Multisystem Autoimmune Disease	Immunoglobulin profiling; enumeration of CD4+CD25+FOXP3+ lymphocytes	Low IgE; Often low

Table 3: Specific diagnostic tests or clinical criteria associated with the genetic subtypes of autoimmune endocrinopathies. *Significant overlap exits between subtypes (i.e.* LRBA *and* STAT3*,* AIRE *and* STAT1*) reducing the specificity. FACS – fluorescence-activated cell-sorting. CMC – chronic mucocutaneous candidiasis.*

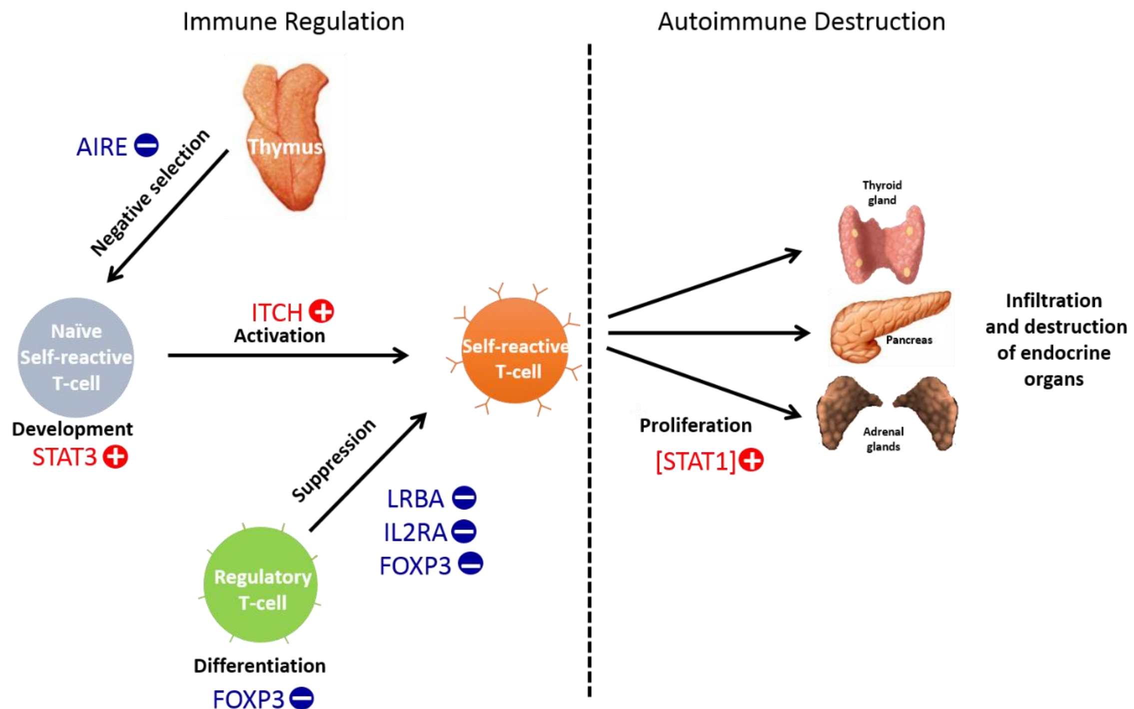
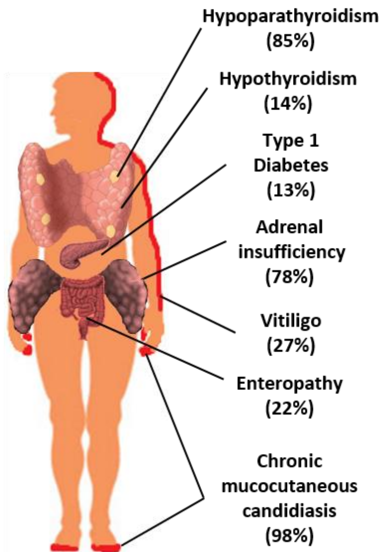


Figure 1: Stylised representation of the mechanisms that underlie monogenic autoimmune endocrine disease pathogenesis. Mutations in genes known to cause monogenic autoimmunity, which includes type 1 diabetes and/or autoimmune thyroid disease, exert their effects by 1) breaking down immune tolerance related to the development or on-going suppressive function of regulatory T cells or 2) promoting the activation and proliferation of self-reactive effector T cells. (–) Denotes a reduction in normal function, (+) denotes an increase in normal physiological function. Bracketed genes have a putative/hypothesised role without direct evidence.

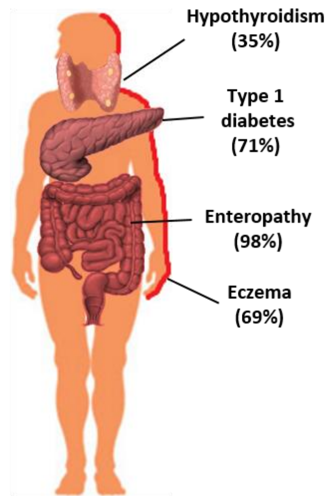
AIRE

Autoimmune Polyendocrinopathy Syndrome Type 1[a]



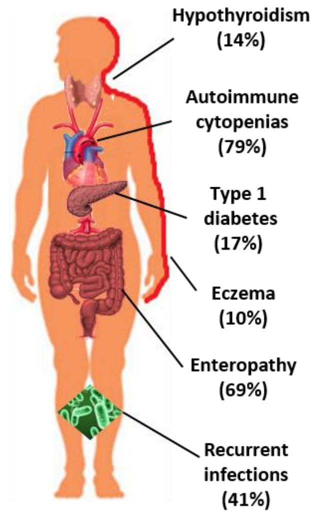
FOXP3

Immunodysregulation, Polyendocrinopathy, Enteropathy, X-linked



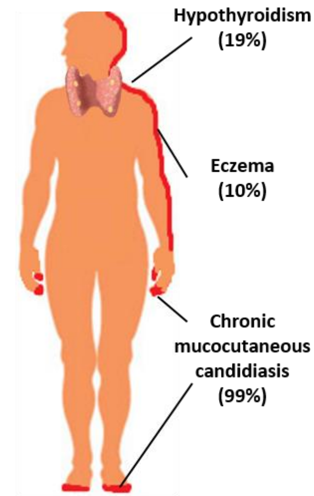
LRBA

Common Variable Immunodeficiency-8 with Autoimmunity



STAT1

Immunodeficiency 31C



STAT3

Infancy-Onset Multisystem Autoimmune Disease

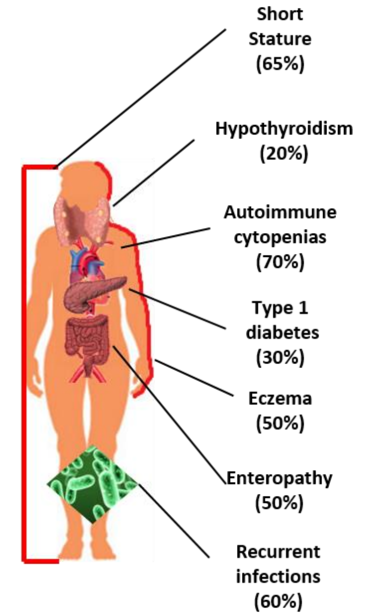


Figure 2: Infogram showing the major manifestations of four monogenic causes of multiple autoimmune endocrine disease. Whilst there is considerable overlap in phenotype associated with each genetic subgroup the prevalence of each disease varies and hallmark features exist for each of the disorders. The size of organs/systems in this image reflects the prevalence of the feature in the specific disorder. [A]Refers to the phenotype at age 30 years.

Supplementary data: Monogenic Autoimmune Diseases of the Endocrine System

M. B. Johnson, A. T. Hattersley, S. E. Flanagan

Lancet Diabetes & Endocrinology 2016 4(10):862-872

Gene(s)	dbSNP rsID (Strongest risk allele)	Risk Allele Frequency	p-Value	Odds Ratio
Type 1 diabetes				
VN1R55P - RNLS	rs10509540 (?)	0.71	1×10^{-28}	1.33
SMIM20 - RBPJ	rs10517086 (A)	0.3	5×10^{-10}	1.09
UBASH3A*	rs11203203 (?)	NR	2×10^{-9}	NR
BACH2*	rs11755527 (?)	NR	5×10^{-8}	NR
RPL32P23 - RBM17	rs12251307 (?)	NR	1×10^{-13}	NR
CLEC16A	rs12708716 (?)	NR	2×10^{-16}	NR
ZFP36L1	rs1465788 (?)	0.71	2×10^{-12}	1.16
PTPN2*	rs1893217 (?)	NR	4×10^{-15}	NR
IFIH1*	rs1990760 (?)	NR	7×10^{-9}	NR
SIRPG*	rs2281808 (?)	0.64	1×10^{-11}	1.11
GSDMB	rs2290400 (?)	0.5	6×10^{-13}	1.15
ERBB3	rs2292239 (?)	NR	2×10^{-25}	NR
PTPN22*	rs2476601 (?)	NR	9×10^{-85}	NR
GAB3*	rs2664170 (G)	0.32	8×10^{-9}	1.16
RPS14P1 - IL10*	rs3024505 (?)	0.83	2×10^{-9}	1.19
CTLA4*	rs3087243 (?)	NR	1×10^{-15}	NR
SH2B3*	rs3184504 (?)	NR	3×10^{-27}	NR
PRKD2	rs425105 (?)	0.84	3×10^{-11}	1.16
KIAA1109	rs4505848 (?)	NR	5×10^{-13}	NR
CD69*	rs4763879 (A)	0.37	2×10^{-11}	1.09
IL27* - NUPR1	rs4788084 (G)	0.42	3×10^{-13}	1.09
LINC01550 - C14orf177	rs4900384 (G)	0.29	4×10^{-9}	1.09
RPL39P23 - COBL	rs4948088 (?)	0.95	4×10^{-8}	1.3
RPS3AP51 - LIF*	rs5753037 (T)	0.39	3×10^{-16}	1.1
GLIS3	rs7020673 (?)	0.5	5×10^{-12}	1.14
MIR4686 - ASCL2	rs7111341 (?)	NR	4×10^{-48}	NR
CTRB2 - CTRB1	rs7202877 (G)	0.1	3×10^{-15}	1.28
CCR7* - SMARCE1	rs7221109 (?)	0.65	1×10^{-9}	1.05
SKAP2	rs7804356 (?)	0.76	5×10^{-9}	1.14
HLA-DRA*	rs9268645 (?)	NR	1×10^{-100}	NR
CENPW	rs9388489 (G)	0.45	4×10^{-14}	1.17
EFR3B	rs478222 (?)	0.59	4×10^{-9}	1.22
LMO7	rs539514 (?)	0.5	6×10^{-11}	1.43

LINC00574 - RPL12P23	rs924043 (?)	0.85	8×10^{-9}	1.35
NAA25	rs17696736 (G)	NR	6×10^{-18}	NR
C1QTNF6 - SSTR3	rs229541 (T)	0.43	2×10^{-8}	1.11
CTSH	rs3825932 (T)	0.68	3×10^{15}	1.16
PHTF1	rs6679677 (A)	NR	1×10^{-40}	NR
HLA-DQA1*	rs9272346 (G)	NR	6×10^{-129}	NR
LOC399716	rs947474 (G)	0.19	4×10^{-9}	1.1
UBASH3A*	rs9976767 (C)	NR	2×10^{-8}	1.16
IGF2, IGF2-AS, INS-IGF2	rs1004446 (C)	0.65	4×10^{-9}	1.61
IKZF4*	rs1701704 (C)	0.35	9×10^{-10}	1.25
HLA-DQB1* - MTCO3P1	rs2647044 (A)	0.13	1×10^{-16}	8.3
CLEC16A	rs2903692 (G)	0.62	7×10^{-11}	1.54
CUX2	rs1265564 (?)	NR	1×10^{-16}	1.45
IL2RA*	rs61839660 (?)	NR	5×10^{-9}	1.6
CEP76 - PTPN2*	rs2542151 (C)	0.16	1×10^{-14}	1.3
CD226*	rs763361 (A)	0.47	1×10^{-8}	1.16
TYK2*	rs2304256 (C)	0.71	4×10^{-9}	1.16
MEG3	rs941576 (A)	0.57	1×10^{-10}	1.11
RPS26 - ERBB3	rs11171739 (C)	0.42	1×10^{-11}	1.34
CLEC2D*	rs3764021 (C)	0.47	5×10^{-8}	1.57
Autoimmune hyperthyroidism				
FCRL3*	rs3761959 (A)	0.4	2×10^{-13}	1.23
Un	rs6832151 (G)	0.35	1×10^{-13}	1.24
HLA-DPA2*	rs2281388 (T)	0.32	2×10^{-65}	1.64
TSHR	rs12101261 (T)	0.64	7×10^{-24}	1.35
NPM1P33 - CTLA4*	rs1024161 (T)	0.69	2×10^{-17}	1.3
HCG22 - C6orf15	rs4947296 (C)	0.14	4×10^{-51}	1.77
HLA-S* - MICA*	rs1521 (T)	0.79	2×10^{-65}	1.92
HLA-DQB1* - MTCO3P1	rs6457617 (T)	0.45	7×10^{-33}	1.4
RNASET2 - MIR3939	rs9355610 (G)	0.47	7×10^{-10}	1.19
C6orf10	rs2273017 (A)	0.51	2×10^{-22}	1.53
HLA-W* - MICD	rs3893464 (G)	0.36	2×10^{-20}	1.53
HLA-J*, ZNRD1-AS1	rs4313034 (T)	0.83	2×10^{-15}	1.67
ABCF1*	rs3132613 (C)	0.25	2×10^{-13}	1.43
MUC22	rs4248154 (C)	0.54	1×10^{-13}	1.38
ITPR3	rs9394159 (T)	0.53	4×10^{-12}	1.36

MLN - LINC01016	rs4713693 (T)	0.65	7×10^{-13}	1.4
SLAMF6*	rs1265883 (C)	0.1	2×10^{-18}	1.34
TG	rs2294025 (T)	0.19	8×10^{-9}	1.16
LINC01550 - C14orf177	rs1456988 (?)	0.53	5×10^{-9}	1.12
C1QTNF6	rs229527 (?)	0.71	5×10^{-20}	1.23
GPR174 - KIF4CP	rs5912838 (?)	0.58	2×10^{-33}	1.32
ABO	rs505922 (?)	0.53	2×10^{-10}	1.14

Supplementary table 1: Regions associated with type 1 diabetes and autoimmune hyperthyroidism identified by genome wide association studies. Only those reaching genome wide significance are recorded (i.e. p value < 5×10^{-8}). NR - not recorded (data not provided by study).[1]

Gene	Disease/trait(s)	dbSNP ID (strongest risk allele)	Odds Ratio	p-Value	Reference
AIRE	Rheumatoid arthritis	rs2075876 (A)	1.18	4×10^{-9}	[2]
IL2RA	Multiple sclerosis; Crohn's disease	rs12722489 (C)	1.23; 1.11	4×10^{-8}; 3×10^{-9}	[3, 4]
IL2RA	Inflammatory biomarkers	rs7911500 (NR)	NR	5×10^{-9}	[5]
IL2RA	Inflammatory bowel disease	rs12722515 (C)	1.102	4×10^{-10}	[6]
IL2RA	Type 1 diabetes	rs61839660 (NR)	1.6	5×10^{-9}	[7]
IL2RA	Type 1 diabetes autoantibodies	rs12722495 (A)	1.61	1×10^{-38}	[8]
IL2RA	Vitiligo	rs706779 (A)	1.27	3×10^{-9}	[9]
IL2RA	Rheumatoid arthritis	rs706778 (T)	1.1	5×10^{-14}	[10]
IL2RA	Alopecia areata; Multiple sclerosis	rs3118470 (G)	1.41; 1.12	2×10^{-12}; 3×10^{-11}	[11, 12]
IL2RA	Response to anti-retroviral therapy	rs12722486 (NR)	38.2	2×10^{-9}	[13]
IL2RA	Multiple sclerosis	rs7090512 (G)	1.19	5×10^{-20}	[12]
IL2RA	Type 1 diabetes	rs12251307 (NR)	NR	1×10^{-13}	[14]
STAT3	Multiple sclerosis	rs2293152 (C)	1.22	4×10^{-8}	[3]
STAT3	Multiple sclerosis	rs744166 (G)	1.15	3×10^{-10}	[15]
STAT3	Crohn's disease	rs744166 (A)	1.18	7×10^{-12}	[16]
STAT3	Multiple sclerosis	rs9891119 (C)	1.11	2×10^{-10}	[12]
STAT3	Crohn's disease	rs9891119-(A)	1.37	2×10^{-15}	[17]
STAT3	Inflammatory bowel disease	rs12942547 (A)	1.103	6×10^{-22}	[6]

Supplementary table 2: Genome wide associations identified within the seven genes causing autoimmune polyendocrinopathy. All reach genome wide significance ($<5 \times 10^{-8}$). Where multiple associations were found for the same SNP all data is listed in the same order. Genes mapped by National Center for Biotechnology information. NR – not recorded (data not provided by study).

Trial name	Brief description	ClinicalTrials.gov Identifier
Immune Disorder HSCT Protocol	Assessing the possibility reduced intensity immunosuppression in patients with disorders of immune function including IPEX syndrome	NCT01821781
Reduced Intensity Conditioning for Hemophagocytic Syndromes or Selected Primary Immune Deficiencies	Testing the efficacy of intermediate conditioning in patients with primary immunodeficiencies including IPEX syndrome	NCT01998633
Treosulfan and Fludarabine Phosphate Before Donor Stem Cell Transplant in Treating Patients with Non-malignant Inherited Disorders	Testing whether a new conditioning regimen with reduced intensity can result in favourable outcomes in patients with non-malignant diseases requiring bone marrow transplant, specifically using Treosulfan and Fludarabine.	NCT00919503
Reduced Intensity Conditioning in Patients Aged ≤35 With Non-Malignant Disorders Undergoing UCBT, BMT, or PBSCT (RIC HSCT NMD)	Aim to demonstrate the efficacy of reduced intensity conditioning regimen for bone marrow transplant long term.	NCT01962415
CAMB/MAT2203 in Patients with Mucocutaneous Candidiasis (CAMB)	Dose-titration trial to study the efficacy, safety and pharmacokinetics of oral cochleate amphotericin B in patients with chronic mucocutaneous candidiasis.	NCT02629419
Genetic Basis of Primary Immunodeficiencies	The evaluation of patients with primary immunodeficiency disorders to identify patients with mutations in certain genes, including *STAT1*.	NCT00001788
Detection and Characterization of Infections and Infection Susceptibility	Screening study to identify patients with immune disorders for further evaluation.	NCT00404560
Natural History of Individuals with Immune System Problems That Lead to Fungal Infections	Long term study of people with immune system problems that lead to fungal infections	NCT01386437

Studies of Disorders with Increased Susceptibility to Fungal Infections	The collection of biological samples to study immune system disorders that make people susceptible to fungal infections	NCT01222741

Supplementary table 3: Studies/clinical trials currently recruiting patients with monogenic autoimmune and immunodysregulatory disorders. Taken from www.ClinicalTrials.gov (date accessed: 25/05/2016). Search terms; AIRE, APECED, APS1, IL2RA, CD25, FOXP3, IPEX, LRBA, ITCH, Chronic Variable Immunodeficiency, Primary Immunodeficiency Autoimmune, STAT1, STAT3, Monogenic Autoimmune, Congenital Autoimmune.

Supplementary references:

1. Welter D, MacArthur J, Morales J, Burdett T, Hall P, Junkins H, et al. The NHGRI GWAS Catalog, a curated resource of SNP-trait associations. Nucleic Acids Res. 2014 Jan;42(Database issue):D1001-6.
2. Terao C, Yamada R, Ohmura K, Takahashi M, Kawaguchi T, Kochi Y, et al. The human AIRE gene at chromosome 21q22 is a genetic determinant for the predisposition to rheumatoid arthritis in Japanese population. Hum Mol Genet. 2011 Jul 1;20(13):2680-5.
3. Patsopoulos NA, Esposito F, Reischl J, Lehr S, Bauer D, Heubach J, et al. Genome-wide meta-analysis identifies novel multiple sclerosis susceptibility loci. Annals of neurology. 2011 Dec;70(6):897-912.
4. Franke A, McGovern DP, Barrett JC, Wang K, Radford-Smith GL, Ahmad T, et al. Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. Nat Genet. 2010 Dec;42(12):1118-25.
5. Aslibekyan S, Kabagambe EK, Irvin MR, Straka RJ, Borecki IB, Tiwari HK, et al. A genome-wide association study of inflammatory biomarker changes in response to fenofibrate treatment in the Genetics of Lipid Lowering Drug and Diet Network. Pharmacogenetics and genomics. 2012 Mar;22(3):191-7.
6. Jostins L, Ripke S, Weersma RK, Duerr RH, McGovern DP, Hui KY, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. Nature. 2012 Nov 1;491(7422):119-24.
7. Huang J, Ellinghaus D, Franke A, Howie B, Li Y. 1000 Genomes-based imputation identifies novel and refined associations for the Wellcome Trust Case Control Consortium phase 1 Data. European journal of human genetics : EJHG. 2012 Jul;20(7):801-5.

8. Plagnol V, Howson JM, Smyth DJ, Walker N, Hafler JP, Wallace C, et al. Genome-wide association analysis of autoantibody positivity in type 1 diabetes cases. PLoS Genet. 2011 Aug;7(8):e1002216.

9. Jin Y, Birlea SA, Fain PR, Gowan K, Riccardi SL, Holland PJ, et al. Variant of TYR and autoimmunity susceptibility loci in generalized vitiligo. The New England journal of medicine. 2010 May 6;362(18):1686-97.

10. Okada Y, Wu D, Trynka G, Raj T, Terao C, Ikari K, et al. Genetics of rheumatoid arthritis contributes to biology and drug discovery. Nature. 2014 Feb 20;506(7488):376-81.

11. Petukhova L, Duvic M, Hordinsky M, Norris D, Price V, Shimomura Y, et al. Genome-wide association study in alopecia areata implicates both innate and adaptive immunity. Nature. 2010 Jul 1;466(7302):113-7.

12. Sawcer S, Hellenthal G, Pirinen M, Spencer CC, Patsopoulos NA, Moutsianas L, et al. Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. Nature. 2011 Aug 11;476(7359):214-9.

13. Leger PD, Johnson DH, Robbins GK, Shafer RW, Clifford DB, Li J, et al. Genome-wide association study of peripheral neuropathy with D-drug-containing regimens in AIDS Clinical Trials Group protocol 384. Journal of neurovirology. 2014 Jun;20(3):304-8.

14. Barrett JC, Clayton DG, Concannon P, Akolkar B, Cooper JD, Erlich HA, et al. Genome-wide association study and meta-analysis find that over 40 loci affect risk of type 1 diabetes. Nat Genet. 2009 Jun;41(6):703-7.

15. Jakkula E, Leppa V, Sulonen AM, Varilo T, Kallio S, Kemppinen A, et al. Genome-wide association study in a high-risk isolate for multiple sclerosis reveals associated variants in STAT3 gene. Am J Hum Genet. 2010 Feb 12;86(2):285-91.

16. Barrett JC, Hansoul S, Nicolae DL, Cho JH, Duerr RH, Rioux JD, et al. Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. Nat Genet. 2008 Aug;40(8):955-62.

17. Yamazaki K, Umeno J, Takahashi A, Hirano A, Johnson TA, Kumasaka N, et al. A genome-wide association study identifies 2 susceptibility Loci for Crohn's disease in a Japanese population. Gastroenterology. 2013 Apr;144(4):781-8.

Introduction Part 1 – post script

Since the publication of the review in this chapter (part 1) an additional genetic syndrome of autoimmunity that includes diabetes has been reported (caused by heterozygous mutations in *TNFAIP3*), and new knowledge on the clinical manifestations associated with pathogenic variants in *AIRE* and *STAT1* has been identified by studies of large series. This post script summarises this new knowledge.

TNFAIP3

Behcet-like familial autoinflammatory syndrome (OMIM #616744 (1)) is caused by autosomal dominant mutations in *TNFAIP3 (2)*. In the original report from Zhou *et al* 11 affected individuals from 6 unrelated pedigrees with variable degrees of autoinflammatory disorders were found to harbour loss of function mutations in *TNFAIP3*. Whole exome sequencing was used to search for causative mutations in two unrelated pedigrees and screening of an additional 150 phenotypically similar patients identified a further 4 families. None of the mutation carriers in the original report had endocrinopathy. Since the original report an additional 9 affected individuals from 4 families have been identified (3-6). Most families reported have had protein truncating variants (9/10) in *TNFAIP3*. The mutation was inherited from affected parents in 5 families.

The median age of onset of Behcet-like familial autoinflammatory syndrome is 8.5 years, later than most other monogenic syndromes than include autoimmune diabetes. The most common feature in the patients with *TNFAIP3* mutations so far reported is oral and/or genital ulcers that are refractory to treatment (17/20 individuals, 85%). Gastrointestinal symptoms, including gastritis and ulcers were present in 7 individuals (35%) and inflammatory arthritis was also present in 7 affected patients (35%). As with other forms of monogenic disease, as new patients are identified the phenotypic

spectrum observed has widened. One patient reported has autoimmune lymphoproliferative disease and transicent hepatic dysfunction, and another had diabetes and intrerstitial lung disease. It seems likely that *TNFAIP3* mutations are underrecognized due to this wide phenotypic variability, and the clinical similarity to Behcets syndrome in many cases. Identifying additional patients will therefore improve the understanding of this disorder and the disease manifestations associated with it. The observed phenotype in these patients resembles Behcets disease (OMIM %109650), a polygenic autoinflammatory disorder associated with the HLA B51 haplotype, as well as non-HLA loci (7, 8).

TNFAIP3 limits inflammatory processes by inhibiting NF-κβ, which regulates immune responses to infection (9, 10). Loss of inhibition results in unchecked NF-κβ signalling in the immune system and therefore uncontrolled inflammation (11). This is the first monogenic autoinflammatory disorder to include diabetes as part of the disease spectrum. The innate immune system can activate the adaptive immune system via cytokine signalling and this may underlie the organ specific autoimmunity observed in one individual (12).

APS1

Autoimmune polyendocrine syndrome type 1 (APS1, also known as Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy [APECED]; see introduction part 1) commonly features chronic mucocutaneous candidiasis, hypoparathyroidism and adrenal insufficiency as part of a syndrome of autoimmunity and immundysregulation. Presentation with 2/3 of this classic triad is clinically diagnostic. The majority of knowledge of this rare disorder comes from studies of European

patients, most of whom (>80%) are homozygous for the founder mutations p.Arg257Ter or p.Tyr82Cys.

Ferre *et al*. studied a large (n=35) series of North American patients longitudinally to define the clinical features in this population, who harboured a more diverse set of mutations in *AIRE* (13). They found that the classic diagnostic diad took longer to present, typically appearing 7.4 years after the first symptom. The most common non-classical feature in this population was urticarial eruption (in 23/35, 66%), contrasting to previous reports where it was present only sporadically. Non-endocrine manifestations were more common in this cohort compared to previous reports, including urticarial eruption (66%), hepatitis (43%%) and intestinal dysfunction (80%). Ferre *et al*. therefore suggest new diagnostic criteria including urticarial eruption, intestinal dysfunction and enamel hypoplasia are used; the incorporation of these would have enabled diagnosis ~4 years earlier in these American individuals. The reasons for the different phenotype observed are not clear, but may include the different genotypes observed in this cohort compared to Europeans or environmental factors. Alternatively, the systematic evaluation employed may have allowed for recognition of manifestations that otherwise go unnoticed/unreported.

STAT1

Toubiana *et al*. followed a large international case series (274 individuals from 167 families) with gain of function (GOF) variants in STAT1 (see introduction part 1)(14). Their study widened the clinical manifestations associated with GOF STAT1 and identified the aspects of disease associated with higher mortality. 98% of the patients studied had chronic mucocutaneous candidiasis (CMC), in keeping with previous reports, and the authors also suggested that GOF STAT1 variants were the most

common cause of CMC as it was present in ~50% of 400 individuals referred to the corresponding authors clinical laboratory for testing. Almost three quarters (74%) of the individuals studied had bacterial infections and 38% had viral infections, which was not previously recognised as a major component of the disease. Cerebral aneurisms and malignancies were both reported in 6% of individuals, resulting in a high burden of mortality in these individuals. The combined susceptibility to fungal, bacterial and viral infections led one commentator to suggest this disease is classified as a combined immunodeficiency rather than a candida-alone related disease (15).

References

1. Amberger JS, Bocchini CA, Schiettecatte F, Scott AF, Hamosh A. OMIM.org: Online Mendelian Inheritance in Man (OMIM(R)), an online catalog of human genes and genetic disorders. Nucleic acids research. 2015;43(Database issue):D789-98.
2. Zhou Q, Wang H, Schwartz DM, Stoffels M, Park YH, Zhang Y, et al. Loss-of-function mutations in TNFAIP3 leading to A20 haploinsufficiency cause an early-onset autoinflammatory disease. Nature genetics. 2016;48(1):67-73.
3. Duncan CJA, Dinnigan E, Theobald R, Grainger A, Skelton AJ, Hussain R, et al. Early-onset autoimmune disease due to a heterozygous loss-of-function mutation in TNFAIP3 (A20). Annals of the rheumatic diseases. 2017.
4. Ohnishi H, Kawamoto N, Seishima M, Ohara O, Fukao T. A Japanese family case with juvenile onset Behcet's disease caused by TNFAIP3 mutation. Allergology international : official journal of the Japanese Society of Allergology. 2017;66(1):146-8.
5. Shigemura T, Kaneko N, Kobayashi N, Kobayashi K, Takeuchi Y, Nakano N, et al. Novel heterozygous C243Y A20/TNFAIP3 gene mutation is responsible for chronic inflammation in autosomal-dominant Behcet's disease. RMD open. 2016;2(1):e000223.
6. Takagi M, Ogata S, Ueno H, Yoshida K, Yeh T, Hoshino A, et al. Haploinsufficiency of TNFAIP3 (A20) by germline mutation is involved in autoimmune lymphoproliferative syndrome. The Journal of allergy and clinical immunology. 2017;139(6):1914-22.
7. de Menthon M, Lavalley MP, Maldini C, Guillevin L, Mahr A. HLA–B51/B5 and the Risk of Behçet’s Disease: A Systematic Review and Meta-Analysis of Case–

Control Genetic Association Studies. Arthritis and rheumatism. 2009;61(10):10.1002/art.24642.

8. MacArthur J, Bowler E, Cerezo M, Gil L, Hall P, Hastings E, et al. The new NHGRI-EBI Catalog of published genome-wide association studies (GWAS Catalog). Nucleic acids research. 2017;45(D1):D896-d901.

9. Cooper JT, Stroka DM, Brostjan C, Palmetshofer A, Bach FH, Ferran C. A20 blocks endothelial cell activation through a NF-kappaB-dependent mechanism. The Journal of biological chemistry. 1996;271(30):18068-73.

10. Song HY, Rothe M, Goeddel DV. The tumor necrosis factor-inducible zinc finger protein A20 interacts with TRAF1/TRAF2 and inhibits NF-kappaB activation. Proceedings of the National Academy of Sciences of the United States of America. 1996;93(13):6721-5.

11. Zhang Q, Davis JC, Lamborn IT, Freeman AF, Jing H, Favreau AJ, et al. Combined immunodeficiency associated with DOCK8 mutations. The New England journal of medicine. 2009;361(21):2046-55.

12. Doria A, Zen M, Bettio S, Gatto M, Bassi N, Nalotto L, et al. Autoinflammation and autoimmunity: bridging the divide. Autoimmunity reviews. 2012;12(1):22-30.

13. Ferre EM, Rose SR, Rosenzweig SD, Burbelo PD, Romito KR, Niemela JE, et al. Redefined clinical features and diagnostic criteria in autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy. JCI insight. 2016;1(13).

14. Toubiana J, Okada S, Hiller J, Oleastro M, Lagos Gomez M, Aldave Becerra JC, et al. Heterozygous STAT1 gain-of-function mutations underlie an unexpectedly broad clinical phenotype. Blood. 2016;127(25):3154-64.

15. Hambleton S. When the STATs are against you. Blood. 2016;127(25):3109-10.

Introduction Part 2

ADDITIONAL CAUSES OF MONOGENIC AUTOIMMUNITY WITHOUT DIABETES AS A REPORTED FEATURE;

Eight other monogenic disorders which can include autoimmune diseases have been identified, as classified by the International Union of Immunological Societies (in section IV, diseases of immune dysregulation, syndromes with autoimmunity) (1). None of these disorders have been reported to include diabetes as a feature, however as additional patients are discovered and the associated phenotype is further characterised diabetes may become part of the spectrum of phenotypes associated with these disorders. The individual disorders are discussed below.

CTLA4 (CD152) - ALPS type V (OMIM 616100)

Heterozygous mutations in *CTLA4* (cytotoxic T-lymphocyte-associated protein 4) were simultaneously reported by two separate groups looking for novel causes of familial autoimmune lymphoproliferative syndrome in 2014 (2-4). The variable syndrome of autoimmunity in the 21 affected individuals (from 10 families) most commonly included autoimmune enteropathy (15/21, 71%), lymphocytic infiltration of tissues (13/21, 62%) and autoimmune haematological diseases (12/21, 57%). Organ specific autoimmunity was also reported, with autoimmune thyroid disease in 2 individuals and type 1 diabetes in an otherwise unaffected carrier. The immunophenotype is variable but was reported to include hypogammaglobulinaemia, CD4+ lymphopenia, low levels of CD45+ naïve T-cells and progressive loss of circulating B cells in some individuals. The regulatory T cells of affected individuals showed reduced expression of FOXP3 and IL2RA (3).

CTLA4 is constitutively expressed by Tregs and can be expressed by $CD4^+$ and $CD8^+$ effector T cells and functions as a potent suppressive receptor molecule (5-7). It

competes with CD28 to bind with CD80/86 and prevent co-activation of T effector cells(8). Upon binding of CD28, the receptor ligand complex undergoes transendocytosis whereby CD28 molecules are stripped from the effector T cell and internalised within the regulatory T cell (9). Suppression is therefore maintained after cell:cell contact. Expression of CTLA4 is induced by binding of the T cell receptor to CD28, resulting in progressive suppression over time with peak expression seen at 48-72hr after activation (5). LRBA is essential for post-translational regulation of CTLA4 whereby it prevents CTLA4 containing vesicles from trafficking to lysosomes for degradation (see introduction part 1, section on CVID-8)(10).

Immunosuppression results in clinical improvement. One patient in the literature was responsive to therapy with abatacept (11), a CTLA4 mimetic which improved symptoms and increased the number and function of $FOXP3^+$ regulatory T cells. Other individuals may therefore benefit from therapy with abatacept. HSCT has been reported in 8 individuals (12), 6 are currently alive and well, 1 died due to transplant related mortality and 1 individual died from diabetic keto-acidosis 2.5 years after HSCT. Both deceased individuals had full donor engraftment.

The age of onset of patients reported in the two large original reports ranged from 2 to 40 years however subsequent reports have identified individuals with onset in the neonatal period (11). Multiple unaffected carriers of pathogenic *CTLA4* variants have been identified, with the oldest being 77 years (3), though some of these had autoimmune disease (e.g. type 1 diabetes) which may be related to impaired CTLA4 function. Taken together this suggests that the penetrance of CTLA4 deficiency is more variable compared to other forms of monogenic autoimmunity. It may be due to the proposed mechanism of haploinsufficiency, whereby some functional CTLA4 exists, as well as undefined genetic modifiers, environmental factors or the stochastic

nature of T cell receptor generation by V(D)J recombination. The true clinical picture of CTLA4 deficiency is still emerging and as more individuals are identified may broaden, as seen in other monogenic autoimmune disorders.

Autoimmune lymphoproliferative syndromes (OMIM: #601859)

First clinically recognised by Canale and Smith in 1967 (13), autoimmune lymphoproliferative syndrome (ALPS) is characterised by childhood presentation of non-malignant lymphadenopathy which may have associated hepatosplenomegaly and autoimmunity (14). The symptoms may wax and wane and can spontaneously improve in the second decade of life. The autoimmunity in these patients is usually directed at blood cells (e.g. haemolytic anaemia and autoimmune cytopenias) and individuals have an increased risk of lymphoma (both Hodgkin and non-Hodgkin). ALPS is a genetically heterogeneous condition; mutations in 6 genes are known to cause ALPS, two of which (KRAS and NRAS) are caused by somatic mutations. The below section summarises the four known forms of inherited ALPS. Approximately 20% of patients with ALPS do not have a mutation in the known genes (15).

FAS – type IA (OMIM #601859)

Genetically characterised by Fisher et al Cell 1995 (16), ALPS type 1A is dominant disorder caused by mutations in the *FAS* gene and is an example of a RASopathy. Haploinsufficiency of *FAS* leads to defective apoptosis and expansion of antigen-specific lymphocyte populations and lymphoproliferation. Heterozygous mutations in *FAS* are identified in 65-75% of individuals with ALPS, however it can also be autosomal recessive or somatic (17-19). The only curative treatment is haematopoietic stem cell transplantation, usually only undertaken in the most severe cases(20). *FAS* encodes a death receptor on the surface of cells and leads to apoptosis. Upon binding

its ligand (FASL), FAS forms the death-inducing signalling complex (DISC) which activates caspase-8 leading to DNA degradation, membrane blebbing and eventual cell death (21).

FASL – type IB (OMIM #601859)

FASL encodes the FAS ligand, a transmembrane protein expressed on cytotoxic lymphocytes. It binds to the FAS receptor (FASR) inducing it's trimerisation. This results in a signalling cascade resulting in apoptosis of the target cell. There have been three reported cases of ALPS caused by dominant or recessive mutations in the FASL gene, with recessively inherited mutations seemingly resulting in a more severe phenotype with earlier onset (22-24).

CASP10 – ALPS type II (OMIM #603909)

Four individuals with ALPS but without mutations in *FAS* or *FASL* have been identified who harboured heterozygous mutations in the *CASP10* gene encoding caspase 10 (25, 26). This encodes cysteine-aspartic acid protease 10 which is important for cleaving numerous protein targets during apoptosis and is essential for FAS induced apoptosis. It normally exists as inactive proenzyme that is itself cleaved by the FAS induced death domain to become active.

PRKCD – ALPS type III (OMIM #615559)

ALPS type III, caused by autosomal recessive mutations in *PRKCD*, has been identified in five individuals from three families (27-30). It is characterised by significant lymphadenopathy, recurrent infections and variable autoimmune manifestations that can include membranous glomerulonephritis and hypothyroidism. The 3 siblings identified by *Belot et al.* also had systemic lupus erythematous (SLE), likely due to

defective B cell apoptosis leading to unchecked production of pathogenic antibodies associated with SLE.

PRKCD encodes protein kinase C-δ, a member of the serine-threonine-specific protein kinases (31). It is ubiquitously expressed and phosphorylates a range of proteins involved in numerous cell signalling networks. Mouse studies showed protein kinase C-δ is essential for peripheral B cell development and a critical regulator of immune homeostasis, negatively regulating B cell proliferation (32). It also has role in self-antigen induced B cell tolerance induction and upon cellular DNA damage, mediates apoptosis(33). Lymphocyte accumulation results from a combination of impaired apoptosis and excessive proliferation.

OTHER 'ORPHAN' DISORDERS

There are also a further 3 genetic disorders that cause syndromes of autoimmunity but that have only been identified in a single family and therefore do not meet the established criteria for a Mendelian disease (34). These are briefly discussed below.

TPP2 (OMIM *190470)

Stepensky *et al*. identified a homozygous frameshift mutation in *TPP2* in two siblings who were the result of consanguineous union (35). Both siblings presented with early-onset Evans syndrome (co-occurrence of autoimmune thrombocytopenia and autoimmune haemolytic anaemia) at 21 and 18 months and had lymphadenopathy. One developed intermittent splenomegaly and had recurrent viral infections (cytomegalovirus and varicella zoster) and was successfully treated with haematopoietic stem cell transplantation. The other sibling died from acute haemolytic crisis at the age of 3 years. The full function of TPP2 is under investigation, but the functional studies presented by Stpensky and colleagues and murine studies indicate

that it is essential for the survival of lymphocytes and prevents them reaching senescence (36), allowing them to continue to fight infection and create diverse ranges of antigen receptors.

FADD (OMIM #613759)

In 4 affected members of a large consanguineous family with ALPS, severe recurrent infections (both bacterial and viral), liver disease, encephalopathy and cardiac malformations Bolze *et al.* identified a homozygous missense variant in *FADD* (encoding Fas-associated death domain protein) that co-segregated with the disease (37). FADD is an adaptor protein that mediates signalling for tumor necrosis factor receptors containing death-domains (38). The mechanism underlying the ALPS in these individuals is thought to be similar to FAS and FASL ALPS, whereby defective apoptosis leads to unchecked proliferation of lymphocytes. The recurrent and severe infections in these patients is likely due to the loss of FADD signaling in type 1 interferon antiviral immunity (39, 40). The developmental defects were suggested to be due to FADD having a role in embryonic development, although no direct evidence for this is available.

CASP8 (OMIM #607271)

Chun *et al.* identified homozygous CASP8 mutations in two siblings from a consanguineous pedigree with ALPS and immunodeficiency (leading to recurrent infections and poor response to vaccination) (41). CASP8 (caspase-8) is a member of the cysteine-aspartic acid protease family, all of which are essential for apoptosis. The finding that individuals homozygous for a mutation in CASP-8 not only have ALPS but also have a defect in T-, B- and natural killer cell activation leading to

immunodeficiency was the first observation that CASP8 has additional functions outside apoptosis.

NEONATAL DIABETES

Neonatal diabetes (NDM) is defined as diabetes with onset before the age of 6 months and has an incidence of approximately 1/100,000 live births (42). It is a highly heterogeneous disorder, with 24 distinct genetic causes identified to date (table 1) (43-45). The mechanisms underlying these genetic causes broadly fit into three categories; defects in glucose sensing/metabolism by the β-cell; defects in the development of the pancreas and defects in the immune system leading to β-cell autoimmunity. Notable exceptions are autosomal recessive mutations in *EIF2AK3* and *IER3IP1* which cause aberrant protein trafficking by the endoplasmic reticulum (ER) and dominant *INS* mutations that cause missfolding of the insulin protein. For all three this results in ER stress and β-cell death (46). Clinically, NDM can be divided into transient and permanent forms. Transient NDM is most commonly caused by methylation defects in the 6q24 locus (table 1 legend) and remits in the first months of life, however may relapse as the child grows and insulin requirement increases (47).

Screening of all known genes by targeted next generation sequencing has meant that a genetic diagnosis is possible in >82% of individuals (43). More than 45% of individuals with neonatal diabetes have an activating mutation in the potassium channel subunits Sur1 and Kir6.2 (encoded by the *ABCC8* and *KCNJ11* genes, respectively). A rapid genetic diagnosis is crucial for these individuals as it can enable personalised therapy; treatment with sulphonylurea tablets improves glycaemic control and removes the need for insulin injections (48). Furthermore, patients with recessively inherited mutations in *SLC19A2* can be treated with thiamine which

improves their anaemia and results in improved glycaemic control and/or lower insulin requirement (De Franco, Diabetes, in press). A genetic diagnosis can also explain additional non-pancreatic features and ameliorate the need for further diagnostic testing and may anticipate the onset of additional features allowing for early-intervention and monitoring. Mutations in 3 genes result in neonatal diabetes as part of a variable syndrome of autoimmunity (*IL2RA*, *FOXP3*, & *STAT3*). These are further discussed in the introduction part 1.

Gene	Additional features	Inheritance pattern
6q24 locus	IUGR, macroglossia, umbilical hernia, neurological features	Varied*
ABCC8	Developmental delay +/- epilepsy	Autosomal dominant/ recessive
EIF2AK3	Skeletal dysplasia, liver dysfunction	Autosomal recessive
FOXP3	Autoimmune enteropathy, eczema, autoimmune thyroiditis, other autoimmune manifestations	X-linked recessive
GATA4	Exocrine insufficiency, congenital heart malformations	Autosomal dominant
GATA6	Exocrine insufficiency, congenital heart malformations, neurological defects, hypothyroidism, gut and hepatobiliary malformation	Autosomal dominant
GCK	-	Autosomal recessive
GLIS3	Hypothyroidism	Autosomal recessive
HNF1B	Exocrine insufficiency, renal cysts	Autosomal dominant
IER3IP1	Microcephaly, epilepsy	Autosomal recessive
IL2RA	Eczema, autoimmune enteropathy, recurrent infections, other autoimmune manifestations	Autosomal recessive
INS	-	Autosomal dominant/ recessive
KCNJ11	Developmental delay +/- epilepsy	Autosomal dominant
MNX1	Sacral agenesis, neurologic defects	Autosomal recessive
NEUROD1	Cerebellar hypoplasia, sensorineural deafness, visual impairment	Autosomal recessive
NEUROG3	Congenital malabsorptive diarrhoea	Autosomal recessive
NKX2-2	Corpus callosum agenesis	Autosomal recessive
PDX1	Exocrine insufficiency	Autosomal recessive
PTF1A	Exocrine insufficiency, cerebellar agenesis (coding mutations)	Autosomal recessive
RFX6	Intestinal atresia and/or malrotation, gall bladder agenesis	Autosomal recessive
SLC19A2	Thiamine-responsive megaloblastic anaemia, sensorineural deafness	Autosomal recessive
SLC2A2	Hepatorenal glycogen accumulation, renal dysfunction, impaired utilization of glucose and galactose	Autosomal recessive
STAT3	Autoimmune enteropathy, thyroid dysfunction, pulmonary disease, juvenile-onset arthritis, short stature	Autosomal dominant
ZFP57	IUGR	Autosomal recessive

Table 1: Genetic causes of neonatal diabetes. IUGR – intrauterine growth retardation. * - NDM associated with the 6q24 locus is caused by overexpression of imprinted genes at this locus. Three distinct inheritance mechanisms are associated; 1. Paternal uniparental disomy of chromosome 6; 2. Duplication of the paternal 6q24 locus; 3. Methylation defect of the maternal region resulting in overexpression. Adapted from De Franco & Ellard 2015 (43).

MATURITY-ONSET DIABETES OF THE YOUNG (MODY)

Maturity-onset diabetes of the young (MODY) is a monogenic disorder accounting for 1.1-4.2% of diabetes that is diagnosed before the age of 25 years (49-52) and has a population minimum prevalence of 1.08/10,000 in the UK (53). MODY is classically defined as a non-syndromic beta cell defect with a monogenic aetiology, is pancreatic autoantibody negative and has young onset in slim individuals (54). Patients are usually non-insulin dependent and generally have a family history suggesting dominant disease. To date, 11 causative genes have been identified as causing this classical description of MODY (*GCK, HNF1A, HNF1B, HNF4A, CEL, ABCC8, KCNJ11, RFX6, INS, NEUROD1 & PAX6*) (54). Heterozygous mutations in *HNF1A* are the most common aetiology, accounting for ~52% of MODY in white Europeans (53).

A genetic diagnosis can improve treatment in these individuals; patients with *HNF1A* or *HNF4A* MODY are highly sensitive to low dosage sulphonylureas and often have better control of their diabetes when switching for insulin injections to tablets (55). Patients with MODY caused by activating mutations in *ABCC8* or *KCNJ11* are likely to respond to high-dose sulphonylurea treatment as seen in NDM, though as this form of MODY is rare empirical data is not available and further study is warranted. Patients with heterozygous mutations in glucokinase (encoded by *GCK*) can usually come off treatment altogether as their blood glucose is only slightly raised above normal, remains stable over their lifetime and does not cause micro/macro vascular complications associated with other forms of diabetes (55).

TYPE 1 DIABETES

Type 1 diabetes results from specific autoimmune destruction of the pancreatic β-cells (56). This leads rapidly and progressively to total insulin deficiency and patients require life-long insulin injections to maintain normal blood sugar levels. Type 1 diabetes is a polygenic disease - to date 61 loci have been identified by GWAS as associated with type 1 diabetes (57). The presence of these genetic risk factors is necessary, but not sufficient, to cause disease. More than half of the genes so far identified have a role in the function of the immune system (introduction part 1 and supplementary material) (58). Variation in the HLA region at chromosome 6p21 infers the greatest risk to the development of type 1 diabetes. In particular, the HLA class II DR and DQ encoding loci infer high risk, with the strongest predisposing haplotypes being DR3 and DR4 (58). HLA class II molecules present antigens at the surface of cells. Compound heterozygosity for DR3/DR4 has a synergistic effect, likely due to the presence of heterodimers of the DQ subunits, meaning four different DQ molecules are present at the cell surface.

In the classic model of T1D pathogenesis (figure 1), genetically predisposed individuals have immunological infiltration of the islets (insulitis) bought on by a precipitating environmental event. This leads to a preclinical asymptomatic stage in which β-cell mass declines and then to the onset of symptoms (polydipsia, polyuria, increased huger and fatigue) as insulin production becomes insufficient to maintain normal blood glucose levels (59). In 13-80% of cases, the initial symptoms are not recognised and patients present with diabetic ketoacidosis, a medical emergency which requires immediate treatment (59, 60).

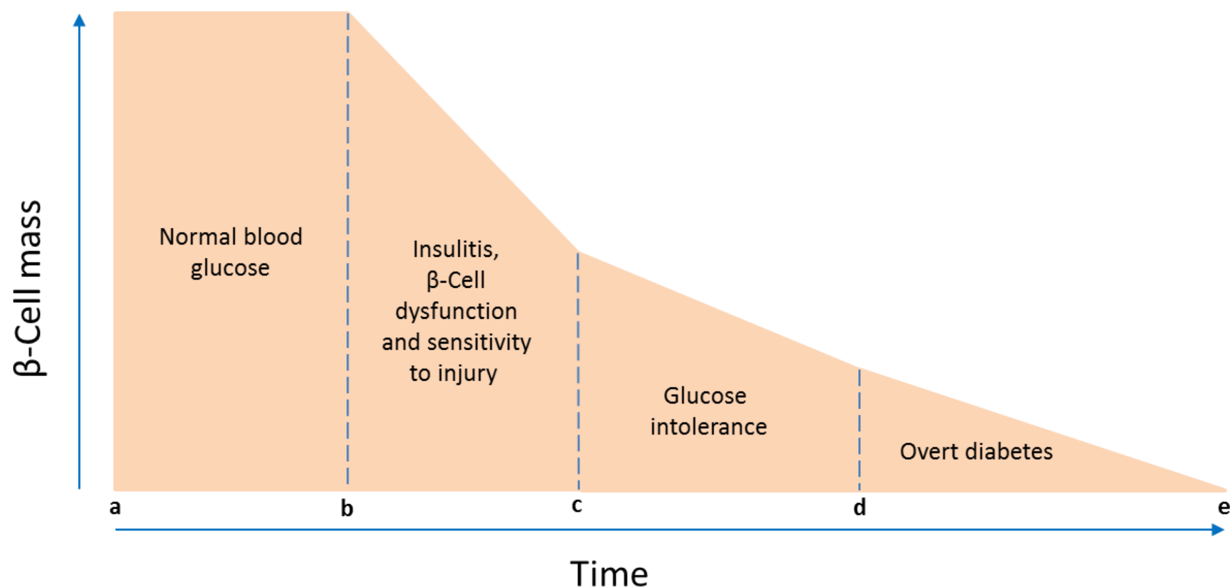


Figure 1: the classical model of type 1 diabetes pathogenesis. Adapted from Atkinson 2001. **a** – A genetically predisposed individual with normal β-cell mass. **b** – A precipitating environmental event/trigger leads to the induction of autoimmunity and infiltration of the pancreatic islets by leukocytes. β-cells are sensitive to injury. **c** – As β-cell mass declines, first phase insulin response is lost and glucose intolerance begins to develop, though individuals are usually asymptomatic. **d** – Further decline in β-cell mass leads to overt symptoms of diabetes. **e** - β-cells are drastically reduced and may be completely absent. Patients require lifelong total replacement of endogenous insulin.

The incidence of T1D varies greatly, from 0.1/100,000 per year in China to 37/100,000 in Finland (61) and there is evidence that some second-generation immigrant populations moving from low incidence to high incidence countries have an increased risk of T1D than their original country's population, which increases with more time spent in the new country (62). This provides strong evidence that environmental factors have a role in the pathogenesis of T1D (56).

A variety of environmental factors/exposures have been implicated in the pathogenesis of type 1 diabetes with perhaps the strongest evidence for a role of viral infection in the induction of islet autoimmunity. A large population-based study of approximately 300,000 infants in Germany showed that risk of developing type 1 diabetes by age 8 years was increased in infants developing respiratory tract infections in the first 6 months of life, especially when caused by viruses (63). Insights from the TEDDY study have also temporally linked respiratory infection to the age of initiation of islet autoimmunity, defined as autoantibody positivity (64). Furthermore, immunohistochemical staining of pancreata from individuals with new onset diabetes has shown that the enteroviral capsid protein vp1 is significantly more common in cases than age matched controls (65). Other environmental factors implicated in the aetiology of type 1 diabetes include adverse life events in childhood (66), the duration of breastfeeding and timing of weaning and nutrition (67).

Since its first application in 1922 (68), the only treatment for type 1 diabetes has been life-long insulin injections and careful monitoring of blood sugar levels. Recent advances in treatment have focussed on the introduction of insulin pumps and continuous glucose monitoring, which automatically monitor blood glucose and administer insulin automatically (69, 70), as well as flash monitoring devices which use a Bluetooth enabled implant and small device to easily monitor glucose levels without the need to draw blood. There are also on-going trials and research programs which are focussing on intervening in the pre-symptomatic stage of diabetes development in order to reduce or reverse β-cell loss (71). The 'holy-grail' of treatment for patients with type 1 diabetes and complete loss of β-cell mass is islet transplantation, whereby healthy islets containing functional β-cells are implanted into patients with long-standing diabetes. More than 1500 individuals have undergone this procedure

worldwide, and the procedure is becoming more viable for increasing numbers of patients (72). Problems around immunological rejection of the transplanted islets and complications arising due to continued immunosuppression have limited its adoption, although it has passed a phase 3 trial (73). Work is underway to derive functional β-cells from patient derived stem cells, removing the prospect of rejection and need for immunosuppression (74). There is hope that in the future implantation of patient-derived β-cells will be possible and type 1 diabetes will no longer be a life-long disease.

Study of the cellular pathology of type 1 diabetes in humans has been limited as very few samples (approximately 200) from recent onset patients exist (75). All come from cases where recently diagnosed patients died, as biopsy of the pancreas is not feasible; a recent study where researchers resected part of the pancreas of individuals with new-onset type 1 diabetes was terminated prematurely due to severe complications bought on by the procedure (76). Animal models have therefore been extensively employed to study the pathogenesis of type 1 diabetes.

The NOD mouse model

The Non-Obese Diabetic (NOD) mouse (also known as the NOD/ShiLtJ mouse) has been widely used as a model organism to study the pathology of type 1 diabetes. Approximately 90% of female mice and 52% of males develop insulin requiring diabetes by the age of 30 weeks, with the median age of onset in females 18 weeks (77). There are broad similarities between human T1D and NOD mice; both develop diabetes early in life, both produce autoantibodies against insulin and the disease has strong association with the HLA class II molecules in both murine and human disease. Histologically, infiltration of mouse β-cells by leukocytes is evident from around 3 weeks of age and shows some similarities to that observed in human disease (78).

The NOD mouse model has been invaluable to furthering understanding of type 1 diabetes, however key differences in the physiology of the immune system (79) pancreas (both macro- and microscopically) (80) and the immunopathology of insulitis exist (81). Furthermore, disease prevention/delay in NOD mice is possible by the application of over 125 therapies, none of which have been translated to treating human type 1 diabetes (59).

BIOMARKERS

Biomarkers are quantifiable and objective molecular signatures which are indicative of a biological state or disease and can be measured robustly and in a reproducible manner (82, 83). In human healthcare, biomarkers are broadly categorised as either prognostic or predictive. Prognostic biomarkers are associated with outcome regardless of treatment and indicate the incidence or progression of disease, for example the measurement of serum triglyceride and LDL cholesterol to predict the likelihood of cardiovascular disease (84). They may also be useful as a diagnostic aid, for example thyroid peroxidase antibody can be used to determine the aetiology of thyroid disease (85). Predictive biomarkers provide information on the likelihood of treatment response for example in the testing of the activating *BRAF* V600E mutation in melanoma. The identification of the mutation predicts treatment response to selective BRAF inhibitors which give improved survival and reduced disease progression (86). Biomarkers are also commonly used as a surrogate endpoint in clinical trials of novel therapeutics.

An ideal biomarker will be easily obtained from the patient/study participant and be minimally invasive (i.e. presence in urine/blood is preferable to presence in a tissue biopsy). It should also be cost effective to assay, with high sensitivity/specificity for the

disease or condition being tested and a high level of reproducibility across centres. A rapid turnaround time from testing to result is beneficial, and therefore if a novel biomarker is amenable to automation and can be tested in common widely-adopted systems this will desirable. The biomarker should also perform consistently between different genders and ethnicities (87).

Biomarkers in autoimmune type 1 diabetes

Previous studies have shown that biomarkers are useful to distinguish monogenic diabetes and type 2 diabetes (T2D) from autoimmune type 1 diabetes (T1D) (88, 89). For example, the presence of either glutamate decarboxylase (GAD) and islet antigen-2 (IA2) autoantibodies are sensitive and specific (>0.57 and >0.99, respectively) for differentiating T1D from non-autoimmune monogenic diabetes, although their utility as a biomarker reduces relative to increasing time post-diagnosis. This is probably due to the reduce concentration of the antibodies in serum which reflects the cessation of the autoimmune process as all beta-cells are destroyed with time (90). Islet autoantibodies also have some use in predicting progression to diabetes in relatives of patients with T1D; positivity for at least two islet autoantibodies infers a 61% risk of developing T1D over 10 years (91).

The production of C-peptide can be used to measure endogenous insulin secretion in patients with diabetes after the honeymoon period, differentiating between T1D and T2D or monogenic diabetes and also monitoring progression to insulin deficiency in T1D (89). This is important as treatment strategy relies on correct classification – T2D and maturity onset diabetes of the young (MODY) can be treated with oral hypoglycaemic agents in the majority of cases, whereas autoimmune diabetes requires insulin treatment.

Whilst pancreatic autoantibodies and/or c-peptide may be useful in differentiating monogenic autoimmune diabetes from MODY or T2D, their utility in distinguishing between T1D and monogenic autoimmune diabetes is less useful. As diabetes in monogenic autoimmunity is likely to result from the lymphocytic infiltration and destruction of the pancreatic islets, islet auto-antibodies are often present in individuals with monogenic autoimmune disease (45, 92, 93). Similarly to T1D, C-peptide is also unlikely to be detectable in these individuals as the disease progresses. Furthermore, most patients require insulin in full replacement doses suggesting complete loss of pancreatic beta cells.

Biomarkers in monogenic autoimmunity

Some subtypes of monogenic autoimmunity have biomarkers which can be used to indicate the underlying genetic aetiology. The majority of individuals with IPEX syndrome, caused by hemizygous mutations in *FOXP3*, can be identified by flow cytometry by the absence of FOXP3+CD25+CD4+ T cells (regulatory T cells) (94). However, in a small number of cases with IPEX syndrome a normal number of regulatory T cells is observed (95). The reason for this difference in the immunological profile of patients with IPEX is not fully understood, and further studies assessing the sensitivity and specificity of this measure as a biomarker are warranted.

A further example is seen with the absence of LRBA expression in individuals with common variable immunodeficiency-8 (CVID-8; caused by biallelic *LRBA* mutations). This approach was recently successfully employed to identify patients for genetic testing (96). In a cohort of 84 patients with a phenotype suggesting CVID-8, 24 had no expression of LRBA and in 14 of those recessive mutations in *LRBA* were identified.

Autoantibodies against harmonin and villin have been shown to be useful biomarkers for identifying patients with IPEX syndrome. Of 13 IPEX patients tested, 12 were positive for anti-harmonin antibodies and 6 for anti-villin antibodies. None of the age matched controls (n=321) or patients with an IPEX-like phenotype but no mutation in *FOXP3* (n=14) were above the reference range (97). Non-specific Immunoglobulin E (IgE) can also aid in the identification of individuals with IPEX syndrome, with >90% of patients having raised serum IgE concentrations, some with levels 100x the upper limit of the normal reference range (98). In addition, autoantibodies to type 1 interferons have been shown to be highly specific (>99.5%) and sensitive (86%) to identify patients with Autoimmune Polyendocrinopathy Syndrome type 1 (APS1) caused by biallelic *AIRE* mutations (93, 99, 100).

Why are they needed?

Identifying affordable and specific biomarkers for further types of monogenic autoimmunity could enable selective genetic testing, reducing the time to diagnosis and cost. Furthermore, the introduction of gene panel tests has increased the identification of variants of uncertain significance and as such information provided by the analysis of biomarkers which supports a clinical diagnosis will aid in variant interpretation. This is especially true for variants identified in individuals from ethnic groups which are under-represented in large sequence variant databases.

GENETIC RISK SCORES

Common diseases often have a complex aetiology involving multiple genetic risk factors which, in combination with environmental factors, lead to the onset of disease. These polygenic diseases have varying heritability, with the total heritability of T1D estimated to be up to 88% based on twin concordance studies (101).

Individual ‘hits’ identified by genome-wide association studies (GWAS) each have a small contribution to disease risk but in combination can be used to derive a genetic risk score (also known as a polygenic risk score, polygenic score or genome-wide score) for a patient. This can provide an estimate of the risk of disease development or can be used to stratify patients with overlapping phenotypes. This is particularly true for diabetes, where patients with similar clinical features at presentation (i.e. raised blood glucose, increased thirst and urination) can have distinct aetiologies and are responsive to specific treatments.

Polygenic risk of type 1 diabetes

GWAS have identified >50 loci that contribute to the risk of developing T1D (57). The HLA DR locus confers the strongest risk with compound heterozygotes for the DR3/DR4 haplotype having the greatest odds ratio of 48.18, meaning those carrying this combination of HLA haplotypes are >48x more likely to develop type 1 diabetes than those with neither (table 1) (102). Of the loci outside of the HLA region, the PTPN22 loci has the largest effect, with an odds ratio of 1.96 (102).

HLA DR allele(s)	Odds ratio	Weight (ln(OR))
DR3/DR4	48.18	3.87
DR3/DR3	21.12	3.05
DR4/DR4	21.98	3.09
DR4/X	7.03	1.95
DR3/X	4.53	1.51

Table 1: Odds ratios of HLA haplotypes conferring high risk for type 1 diabetes. 'X' refers to any HLA DQ allele that is not DR3 or DR4. Odds ratios taken from Winkler et al.

To generate a genetic risk score genotyping for the target SNPs is undertaken and the weighted odds ratios (ln(OR)) multiplied by the number of risk alleles (0, 1 or 2) at that loci. The total number is divided by the number of alleles to achieve a single score wherein each risk allele has a log-additive effect (103). This number can then be compared to large control cohorts to estimate the likelihood of a patient having a disorder – for example in T1D the score can be compared to the range of scores of gold standard WTCCC T1D patients and non-diabetic controls.

The T1D genetic risk score (T1D-GRS) has proven to be useful in distinguishing T1D from T2D (103) in young people, where discrimination is becoming increasing complex with rising obesity rates increasing the incidence of type 2 diabetes in those under 30. Patel et al used a similar approach to show that the T1D-GRS could distinguish non-autoimmune monogenic diabetes from T1D, which has important applications both in

clinical practise as well as in research settings to look for novel monogenic diabetes genes (104). In MODY the T1D-GRS is currently in clinical use in combination with biomarkers and clinical features to select patients for testing of the known genes (105). The correct classification of diabetes subtype at diagnosis allows for patients to be placed on the most effective treatment early on and may inform families and clinicians of recurrence risk.

Conclusion

Biomarkers are effectively used in diagnosing existing types of monogenic autoimmunity and can help select some patients for sequencing of known genes. The established biomarkers for T1D (islet autoantibodies and C-peptide) can differentiate T1D from non-autoimmune monogenic diabetes but are unlikely to be effective to identify monogenic autoimmune from T1D as both have autoimmune destruction of pancreas. Genetic risk scores have proven utility in the identification of T1D from young-onset T2D and in identifying T1D from non-autoimmune monogenic diabetes. Correct classification of diabetes allows for the most effective treatment to be utilised early on in disease course.

REFERENCES

1. Bousfiha A, Jeddane L, Al-Herz W, Ailal F, Casanova JL, Chatila T, et al. The 2015 IUIS Phenotypic Classification for Primary Immunodeficiencies. J Clin Immunol. 2015;35(8):727-38.

2. Hayakawa S, Okada S, Tsumura M, Sakata S, Ueno Y, Imai K, et al. A Patient with CTLA-4 Haploinsufficiency Presenting Gastric Cancer. J Clin Immunol. 2016;36(1):28-32.

3. Kuehn HS, Ouyang W, Lo B, Deenick EK, Niemela JE, Avery DT, et al. Immune dysregulation in human subjects with heterozygous germline mutations in CTLA4. Science. 2014;345(6204):1623-7.

4. Schubert D, Bode C, Kenefeck R, Hou TZ, Wing JB, Kennedy A, et al. Autosomal dominant immune dysregulation syndrome in humans with CTLA4 mutations. Nature medicine. 2014;20(12):1410-6.

5. Jago CB, Yates J, Olsen Saraiva CÂMara N, Lechler RI, Lombardi G. Differential expression of CTLA-4 among T cell subsets. Clinical and Experimental Immunology. 2004;136(3):463-71.

6. Lee KM, Chuang E, Griffin M, Khattri R, Hong DK, Zhang W, et al. Molecular basis of T cell inactivation by CTLA-4. Science. 1998;282(5397):2263-6.

7. Lindsten T, Lee KP, Harris ES, Petryniak B, Craighead N, Reynolds PJ, et al. Characterization of CTLA-4 structure and expression on human T cells. Journal of immunology (Baltimore, Md : 1950). 1993;151(7):3489-99.

8. Tai X, Van Laethem F, Pobezinsky L, Guinter T, Sharrow SO, Adams A, et al. Basis of CTLA-4 function in regulatory and conventional CD4(+) T cells. Blood. 2012;119(22):5155-63.

9. Qureshi OS, Zheng Y, Nakamura K, Attridge K, Manzotti C, Schmidt EM, et al. Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. Science. 2011;332(6029):600-3.

10. Lo B, Zhang K, Lu W, Zheng L, Zhang Q, Kanellopoulou C, et al. AUTOIMMUNE DISEASE. Patients with LRBA deficiency show CTLA4 loss and immune dysregulation responsive to abatacept therapy. Science. 2015;349(6246):436-40.

11. Lee S, Moon JS, Lee CR, Kim HE, Baek SM, Hwang S, et al. Abatacept alleviates severe autoimmune symptoms in a patient carrying a de novo variant in CTLA-4. J Allergy Clin Immunol. 2016;137(1):327-30.

12. Slatter MA, Engelhardt KR, Burroughs LM, Arkwright PD, Nademi Z, Skoda-Smith S, et al. Hematopoietic stem cell transplantation for CTLA4 deficiency. J Allergy Clin Immunol. 2016;138(2):615-9.e1.

13. Canale VC, Smith CH. Chronic lymphadenopathy simulating malignant lymphoma. J Pediatr. 1967;70(6):891-9.

14. Li P, Huang P, Yang Y, Hao M, Peng H, Li F. Updated Understanding of Autoimmune Lymphoproliferative Syndrome (ALPS). Clin Rev Allergy Immunol. 2016;50(1):55-63.

15. Oliveira JB. The expanding spectrum of the autoimmune lymphoproliferative syndromes. Curr Opin Pediatr. 2013;25(6):722-9.

16. Fisher GH, Rosenberg FJ, Straus SE, Dale JK, Middleton LA, Lin AY, et al. Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. Cell. 1995;81(6):935-46.

17. Dowdell KC, Niemela JE, Price S, Davis J, Hornung RL, Oliveira JB, et al. Somatic FAS mutations are common in patients with genetically undefined autoimmune lymphoproliferative syndrome. Blood. 2010;115(25):5164-9.

18. Agrebi N, Ben-Mustapha I, Matoussi N, Dhouib N, Ben-Ali M, Mekki N, et al. Rare splicing defects of FAS underly severe recessive autoimmune lymphoproliferative syndrome. Clin Immunol. 2017;183:17-23.

19. van der Burg M, de Groot R, Comans-Bitter WM, den Hollander JC, Hooijkaas H, Neijens HJ, et al. Autoimmune lymphoproliferative syndrome (ALPS) in a child from consanguineous parents: a dominant or recessive disease? Pediatr Res. 2000;47(3):336-43.

20. Bleesing JJH, Nagaraj CB, Zhang K. Autoimmune Lymphoproliferative Syndrome. In: Adam MP, Ardinger HH, Pagon RA, Wallace SE, Bean LJH, Mefford HC, et al., editors. GeneReviews((R)). Seattle (WA): University of Washington, Seattle

University of Washington, Seattle. GeneReviews is a registered trademark of the University of Washington, Seattle. All rights reserved.; 1993.

21. Waring P, Mullbacher A. Cell death induced by the Fas/Fas ligand pathway and its role in pathology. Immunol Cell Biol. 1999;77(4):312-7.

22. Bi LL, Pan G, Atkinson TP, Zheng L, Dale JK, Makris C, et al. Dominant inhibition of Fas ligand-mediated apoptosis due to a heterozygous mutation associated with autoimmune lymphoproliferative syndrome (ALPS) Type Ib. BMC Med Genet. 2007;8:41.

23. Magerus-Chatinet A, Stolzenberg MC, Lanzarotti N, Neven B, Daussy C, Picard C, et al. Autoimmune lymphoproliferative syndrome caused by a homozygous null FAS ligand (FASLG) mutation. J Allergy Clin Immunol. 2013;131(2):486-90.

24. Wu J, Wilson J, He J, Xiang L, Schur PH, Mountz JD. Fas ligand mutation in a patient with systemic lupus erythematosus and lymphoproliferative disease. J Clin Invest. 1996;98(5):1107-13.

25. Wang J, Zheng L, Lobito A, Chan FK, Dale J, Sneller M, et al. Inherited human Caspase 10 mutations underlie defective lymphocyte and dendritic cell apoptosis in autoimmune lymphoproliferative syndrome type II. Cell. 1999;98(1):47-58.

26. Zhu S, Hsu AP, Vacek MM, Zheng L, Schaffer AA, Dale JK, et al. Genetic alterations in caspase-10 may be causative or protective in autoimmune lymphoproliferative syndrome. Hum Genet. 2006;119(3):284-94.

27. Belot A, Kasher PR, Trotter EW, Foray AP, Debaud AL, Rice GI, et al. Protein kinase cdelta deficiency causes mendelian systemic lupus erythematosus with B cell-defective apoptosis and hyperproliferation. Arthritis Rheum. 2013;65(8):2161-71.

28. Salzer E, Santos-Valente E, Klaver S, Ban SA, Emminger W, Prengemann NK, et al. B-cell deficiency and severe autoimmunity caused by deficiency of protein kinase C delta. Blood. 2013;121(16):3112-6.

29. Kuehn HS, Niemela JE, Rangel-Santos A, Zhang M, Pittaluga S, Stoddard JL, et al. Loss-of-function of the protein kinase C delta (PKCdelta) causes a B-cell lymphoproliferative syndrome in humans. Blood. 2013;121(16):3117-25.

30. Andre J, Cimaz R, Ranchin B, Galambrun C, Bertrand Y, Bouvier R, et al. Overexpression of the antiapoptotic gene Bfl-1 in B cells from patients with familial systemic lupus erythematosus. Lupus. 2007;16(2):95-100.

31. Ono Y, Kurokawa T, Fujii T, Kawahara K, Igarashi K, Kikkawa U, et al. Two types of complementary DNAs of rat brain protein kinase C. Heterogeneity determined by alternative splicing. FEBS Lett. 1986;206(2):347-52.

32. Miyamoto A, Nakayama K, Imaki H, Hirose S, Jiang Y, Abe M, et al. Increased proliferation of B cells and auto-immunity in mice lacking protein kinase Cdelta. Nature. 2002;416(6883):865-9.

33. Mecklenbrauker I, Saijo K, Zheng NY, Leitges M, Tarakhovsky A. Protein kinase Cdelta controls self-antigen-induced B-cell tolerance. Nature. 2002;416(6883):860-5.

34. MacArthur DG, Manolio TA, Dimmock DP, Rehm HL, Shendure J, Abecasis GR, et al. Guidelines for investigating causality of sequence variants in human disease. Nature. 2014;508(7497):469-76.

35. Stepensky P, Rensing-Ehl A, Gather R, Revel-Vilk S, Fischer U, Nabhani S, et al. Early-onset Evans syndrome, immunodeficiency, and premature immunosenescence associated with tripeptidyl-peptidase II deficiency. Blood. 2015;125(5):753-61.

36. Huai J, Firat E, Nil A, Million D, Gaedicke S, Kanzler B, et al. Activation of cellular death programs associated with immunosenescence-like phenotype in TPPII knockout mice. Proc Natl Acad Sci U S A. 2008;105(13):5177-82.

37. Bolze A, Byun M, McDonald D, Morgan NV, Abhyankar A, Premkumar L, et al. Whole-exome-sequencing-based discovery of human FADD deficiency. Am J Hum Genet. 2010;87(6):873-81.

38. Kabra NH, Kang C, Hsing LC, Zhang J, Winoto A. T cell-specific FADD-deficient mice: FADD is required for early T cell development. Proc Natl Acad Sci U S A. 2001;98(11):6307-12.

39. Balachandran S, Thomas E, Barber GN. A FADD-dependent innate immune mechanism in mammalian cells. Nature. 2004;432(7015):401-5.

40. Balachandran S, Venkataraman T, Fisher PB, Barber GN. Fas-associated death domain-containing protein-mediated antiviral innate immune signaling involves the regulation of Irf7. Journal of immunology (Baltimore, Md : 1950). 2007;178(4):2429-39.

41. Chun HJ, Zheng L, Ahmad M, Wang J, Speirs CK, Siegel RM, et al. Pleiotropic defects in lymphocyte activation caused by caspase-8 mutations lead to human immunodeficiency. Nature. 2002;419(6905):395-9.

42. Iafusco D, Massa O, Pasquino B, Colombo C, Iughetti L, Bizzarri C, et al. Minimal incidence of neonatal/infancy onset diabetes in Italy is 1:90,000 live births. Acta Diabetol. 2012;49(5):405-8.

43. De Franco E, Flanagan SE, Houghton JA, Lango Allen H, Mackay DJ, Temple IK, et al. The effect of early, comprehensive genomic testing on clinical care in neonatal diabetes: an international cohort study. Lancet (London, England). 2015;386(9997):957-63.

44. Caudy AA, Reddy ST, Chatila T, Atkinson JP, Verbsky JW. CD25 deficiency causes an immune dysregulation, polyendocrinopathy, enteropathy, X-linked-like syndrome, and defective IL-10 expression from CD4 lymphocytes. J Allergy Clin Immunol. 2007;119(2):482-7.

45. Flanagan SE, Haapaniemi E, Russell MA, Caswell R, Allen HL, De Franco E, et al. Activating germline mutations in STAT3 cause early-onset multi-organ autoimmune disease. Nat Genet. 2014;46(8):812-4.

46. Oslowski CM, Urano F. A Switch From Life To Death in ER stressed β cells. Diabetes Obes Metab. 2010;12(0 2):58-65.

47. Temple IK, Mackay DJG, Docherty LE. Diabetes Mellitus, 6q24-Related Transient Neonatal. In: Adam MP, Ardinger HH, Pagon RA, Wallace SE, Bean LJH,

Mefford HC, et al., editors. GeneReviews((R)). Seattle (WA): University of Washington, Seattle

University of Washington, Seattle. GeneReviews is a registered trademark of the University of Washington, Seattle. All rights reserved.; 1993.

48. Pearson ER, Flechtner I, Njolstad PR, Malecki MT, Flanagan SE, Larkin B, et al. Switching from insulin to oral sulfonylureas in patients with diabetes due to Kir6.2 mutations. N Engl J Med. 2006;355(5):467-77.

49. Fendler W, Borowiec M, Baranowska-Jazwiecka A, Szadkowska A, Skala-Zamorowska E, Deja G, et al. Prevalence of monogenic diabetes amongst Polish children after a nationwide genetic screening campaign. Diabetologia. 2012;55(10):2631-5.

50. Irgens HU, Molnes J, Johansson BB, Ringdal M, Skrivarhaug T, Undlien DE, et al. Prevalence of monogenic diabetes in the population-based Norwegian Childhood Diabetes Registry. Diabetologia. 2013;56(7):1512-9.

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

52. Shepherd M, Shields B, Hammersley S, Hudson M, McDonald TJ, Colclough K, et al. Systematic Population Screening, Using Biomarkers and Genetic Testing, Identifies 2.5% of the U.K. Pediatric Diabetes Population With Monogenic Diabetes. Diabetes care. 2016;39(11):1879-88.

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

54. Amed S, Oram R. Maturity-Onset Diabetes of the Young (MODY): Making the Right Diagnosis to Optimize Treatment. Canadian journal of diabetes. 2016;40(5):449-54.

55. Hattersley AT, Patel KA. Precision diabetes: learning from monogenic diabetes. Diabetologia. 2017;60(5):769-77.

56. Katsarou A, Gudbjornsdottir S, Rawshani A, Dabelea D, Bonifacio E, Anderson BJ, et al. Type 1 diabetes mellitus. Nature reviews Disease primers. 2017;3:17016.

57. Burdett T (EBI) HPN, Hastings E (EBI), Hindorff LA (NHGRI), Junkins HA (NHGRI), Klemm AK (NHGRI), MacArthur J (EBI), Manolio TA (NHGRI), Morales J (EBI), Parkinson H (EBI) and Welter D (EBI). The NHGRI-EBI Catalog of published genome-wide association studies 2017 [Available from: www.ebi.ac.uk/gwas.

58. Noble JA. Immunogenetics of type 1 diabetes: A comprehensive review. Journal of autoimmunity. 2015;64:101-12.

59. Atkinson MA, Eisenbarth GS. Type 1 diabetes: new perspectives on disease pathogenesis and treatment. Lancet (London, England). 2001;358(9277):221-9.

60. Usher-Smith JA, Thompson M, Ercole A, Walter FM. Variation between countries in the frequency of diabetic ketoacidosis at first presentation of type 1 diabetes in children: a systematic review. Diabetologia. 2012;55(11):2878-94.

61. Incidence and trends of childhood Type 1 diabetes worldwide 1990-1999. Diabetic medicine : a journal of the British Diabetic Association. 2006;23(8):857-66.

62. Hussen HI, Moradi T, Persson M. The risk of type 1 diabetes among offspring of immigrant mothers in relation to the duration of residency in Sweden. Diabetes care. 2015;38(5):934-6.

63. Beyerlein A, Donnachie E, Jergens S, Ziegler AG. Infections in Early Life and Development of Type 1 Diabetes. Jama. 2016;315(17):1899-901.

64. Lonnrot M, Lynch KF, Elding Larsson H, Lernmark A, Rewers MJ, Torn C, et al. Respiratory infections are temporally associated with initiation of type 1 diabetes autoimmunity: the TEDDY study. Diabetologia. 2017;60(10):1931-40.

65. Richardson SJ, Willcox A, Bone AJ, Foulis AK, Morgan NG. The prevalence of enteroviral capsid protein vp1 immunostaining in pancreatic islets in human type 1 diabetes. Diabetologia. 2009;52(6):1143-51.

66. Nygren M, Carstensen J, Koch F, Ludvigsson J, Frostell A. Experience of a serious life event increases the risk for childhood type 1 diabetes: the ABIS population-based prospective cohort study. Diabetologia. 2015;58(6):1188-97.

67. Knip M, Virtanen SM, Akerblom HK. Infant feeding and the risk of type 1 diabetes. The American journal of clinical nutrition. 2010;91(5):1506s-13s.

68. Banting FG, Best CH, Collip JB, Campbell WR, Fletcher AA. Pancreatic Extracts in the Treatment of Diabetes Mellitus. Canadian Medical Association journal. 1922;12(3):141-6.

69. Relative effectiveness of insulin pump treatment over multiple daily injections and structured education during flexible intensive insulin treatment for type 1 diabetes: cluster randomised trial (REPOSE). BMJ (Clinical research ed). 2017;356:j1285.

70. Beck RW, Hirsch IB, Laffel L, Tamborlane WV, Bode BW, Buckingham B, et al. The effect of continuous glucose monitoring in well-controlled type 1 diabetes. Diabetes care. 2009;32(8):1378-83.

71. Mastrandrea L, Yu J, Behrens T, Buchlis J, Albini C, Fourtner S, et al. Etanercept treatment in children with new-onset type 1 diabetes: pilot randomized, placebo-controlled, double-blind study. Diabetes care. 2009;32(7):1244-9.

72. Shapiro AM, Pokrywczynska M, Ricordi C. Clinical pancreatic islet transplantation. Nature reviews Endocrinology. 2017;13(5):268-77.

73. Hering BJ, Clarke WR, Bridges ND, Eggerman TL, Alejandro R, Bellin MD, et al. Phase 3 Trial of Transplantation of Human Islets in Type 1 Diabetes Complicated by Severe Hypoglycemia. Diabetes care. 2016;39(7):1230-40.

74. Millman JR, Xie C, Van Dervort A, Gurtler M, Pagliuca FW, Melton DA. Generation of stem cell-derived beta-cells from patients with type 1 diabetes. Nature communications. 2016;7:11463.

75. Richardson SJ, Morgan NG, Foulis AK. Pancreatic pathology in type 1 diabetes mellitus. Endocrine pathology. 2014;25(1):80-92.

76. Krogvold L, Edwin B, Buanes T, Ludvigsson J, Korsgren O, Hyoty H, et al. Pancreatic biopsy by minimal tail resection in live adult patients at the onset of type 1 diabetes: experiences from the DiViD study. Diabetologia. 2014;57(4):841-3.

77. Leiter EH. The NOD mouse: a model for insulin-dependent diabetes mellitus. Current protocols in immunology. 2001;Chapter 15:Unit 15.9.

78. Leiter EH, von Herrath M. Animal models have little to teach us about type 1 diabetes: 2. In opposition to this proposal. Diabetologia. 2004;47(10):1657-60.

79. Mestas J, Hughes CC. Of mice and not men: differences between mouse and human immunology. Journal of immunology (Baltimore, Md : 1950). 2004;172(5):2731-8.

80. Dolensek J, Rupnik MS, Stozer A. Structural similarities and differences between the human and the mouse pancreas. Islets. 2015;7(1):e1024405.

81. Roep BO, Atkinson M. Animal models have little to teach us about type 1 diabetes: 1. In support of this proposal. Diabetologia. 2004;47(10):1650-6.

82. Strimbu K, Tavel JA. What are biomarkers? Curr Opin HIV AIDS. 2010;5(6):463-6.

83. Naylor S. Biomarkers: current perspectives and future prospects. Expert Rev Mol Diagn. 2003;3(5):525-9.

84. Manninen V, Tenkanen L, Koskinen P, Huttunen JK, Manttari M, Heinonen OP, et al. Joint effects of serum triglyceride and LDL cholesterol and HDL cholesterol concentrations on coronary heart disease risk in the Helsinki Heart Study. Implications for treatment. Circulation. 1992;85(1):37-45.

85. Feldt-Rasmussen U, Hoier-Madsen M, Bech K, Blichert-Toft M, Bliddal H, Date J, et al. Anti-thyroid peroxidase antibodies in thyroid disorders and non-thyroid autoimmune diseases. Autoimmunity. 1991;9(3):245-54.

86. Ascierto PA, Kirkwood JM, Grob JJ, Simeone E, Grimaldi AM, Maio M, et al. The role of BRAF V600 mutation in melanoma. J Transl Med. 2012;10:85.

87. Drucker E, Krapfenbauer K. Pitfalls and limitations in translation from biomarker discovery to clinical utility in predictive and personalised medicine. EPMA J. 2013;4(1):7.

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

89. Ludvigsson J, Carlsson A, Deli A, Forsander G, Ivarsson SA, Kockum I, et al. Decline of C-peptide during the first year after diagnosis of Type 1 diabetes in children and adolescents. Diabetes Res Clin Pract. 2013;100(2):203-9.

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

91. Achenbach P, Warncke K, Reiter J, Naserke HE, Williams AJ, Bingley PJ, et al. Stratification of type 1 diabetes risk on the basis of islet autoantibody characteristics. Diabetes. 2004;53(2):384-92.

92. Tsuda M, Torgerson TR, Selmi C, Gambineri E, Carneiro-Sampaio M, Mannurita SC, et al. The spectrum of autoantibodies in IPEX syndrome is broad and includes anti-mitochondrial autoantibodies. Journal of autoimmunity. 2010;35(3):265-8.

93. Bruserud Ø, Oftedal BE, Landegren N, Erichsen MM, Bratland E, Lima K, et al. A Longitudinal Follow-up of Autoimmune Polyendocrine Syndrome Type 1. The Journal of Clinical Endocrinology and Metabolism. 2016;101(8):2975-83.

94. Zennaro D, Scala E, Pomponi D, Caprini E, Arcelli D, Gambineri E, et al. Proteomics plus genomics approaches in primary immunodeficiency: the case of immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome. Clinical and Experimental Immunology. 2012;167(1):120-8.

95. Passerini L, Di Nunzio S, Gregori S, Gambineri E, Cecconi M, Seidel MG, et al. Functional type 1 regulatory T cells develop regardless of FOXP3 mutations in patients with IPEX syndrome. European Journal of Immunology. 2011;41(4):1120-31.

96. Gamez-Diaz L, August D, Stepensky P, Revel-Vilk S, Seidel MG, Noriko M, et al. The extended phenotype of LPS-responsive beige-like anchor protein (LRBA) deficiency. J Allergy Clin Immunol. 2016;137(1):223-30.

97. Lampasona V, Passerini L, Barzaghi F, Lombardoni C, Bazzigaluppi E, Brigatti C, et al. Autoantibodies to harmonin and villin are diagnostic markers in children with IPEX syndrome. PLoS One. 2013;8(11):e78664.

98. d'Hennezel E, Bin Dhuban K, Torgerson T, Piccirillo CA. The immunogenetics of immune dysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome. J Med Genet. 2012;49(5):291-302.

99. Meloni A, Furcas M, Cetani F, Marcocci C, Falorni A, Perniola R, et al. Autoantibodies against type I interferons as an additional diagnostic criterion for autoimmune polyendocrine syndrome type I. J Clin Endocrinol Metab. 2008;93(11):4389-97.

100. Zhang L, Barker JM, Babu S, Su M, Stenerson M, Cheng M, et al. A robust immunoassay for anti-interferon autoantibodies that is highly specific for patients with autoimmune polyglandular syndrome type 1. Clin Immunol. 2007;125(2):131-7.

101. Redondo MJ, Jeffrey J, Fain PR, Eisenbarth GS, Orban T. Concordance for islet autoimmunity among monozygotic twins. N Engl J Med. 2008;359(26):2849-50.

102. Winkler C, Krumsiek J, Buettner F, Angermuller C, Giannopoulou EZ, Theis FJ, et al. Feature ranking of type 1 diabetes susceptibility genes improves prediction of type 1 diabetes. Diabetologia. 2014;57(12):2521-9.

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

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

105. Colclough K. Type 1 Diabetes Genetic Risk Score 2017 [Available from: http://www.diabetesgenes.org/content/type-1-diabetes-genetic-risk-score.

Methods

This chapter summarises the methods used throughout this thesis. Each published data chapter also contains information on specific methodologies used for that chapter.

Immunoglobulin testing

To perform immunoglobulin testing (chapters 1, 2, 3 and 4) plasma was separated from whole blood in EDTA blood tubes. This was performed by the Exeter Molecular Genetics laboratory and the Royal Devon and Exeter Hospital biochemistry department. Briefly, samples were centrifuged at 1300g for 8 minutes and the resulting plasma layer was aliquoted into additional tubes and stored at -80°C. Plasma was converted to serum by the addition thrombin to remove fibrinogen and then calcium chloride to induce clotting [56].

Automated Immunoglobulin (Ig) measurement was performed on an ImmunoCAP 1000 instrument (Phadia, Uppsala, Sweden), which uses an enzyme-linked immunosorbent assay (ELISA). This involves using covalently bonded antibodies against the analyte of interest to specifically bind the analyte, in this case an immunoglobulin. Enzyme-labelled antibodies against the analyte are then added forming a complex. The substrate for the enzyme is then added and the product of its digestion measured. Thus, a quantitative measurement of the analyte can be achieved. General assays were used for the measurement of total IgE (Chapter 4) and specific assays were used for the measurement of anti-glutamic acid decarboxylase, anti-islet antigen-2 and anti-zinc transporter 8 antibodies (RSR, Cardiff, UK) (Chapters 1, 2 and 3).

DNA extraction and quantification

DNA extraction from whole blood leukocytes was used in chapters 1, 2 and 3. Most of this was performed by the Exeter Molecular Genetics laboratory and the Royal Devon and Exeter Hospital. The process was largely automated on the ChemagicSTAR instrument (Perkin Elmer, Waltham MA, USA). Briefly, this involved lysis of the leukocyte cells from EDTA-whole blood then binding of the nucleic acids to magnetic beads. Successive washes to remove cell components and other contaminants was performed on the beads before a final elution step into Tris-EDTA buffer.

Quantitation was initially performed by UV-Vis spectrophotometry on the NanoDrop 8000 (Thermo Scientific, Waltham MA, USA). This measures the absorbance of light at 260nm and 280nm to determine the concentration of nucleic acids within the sample. It can also give an estimation of purity by the ratio of absorbance at 260/280nm and 260/230nm – with a 260/280nm ratio of 1.8 indicative of highly pure DNA against RNA and a 260/230 ratio of between 2.0 and 2.2 is indicative of pure nucleic acid against protein.

As UV-Vis spectrophotometry is non-specific for DNA and applications such as droplet digital PCR (chapter 3) and Next Generation Sequencing (chapters 1, 2 and 3) require highly accurate quantitation of double stranded DNA (dsDNA), further quantification was performed with the Qubit Fluorometer (Thermo Scientific, Waltham MA, USA). This assay uses a specific intercalating dye that binds to dsDNA and fluoresces only when bound. The measured fluorescence was therefore compared to a standard curve created from samples with known concentration to obtain an accurate concentration of dsDNA.

Polymerase Chain Reaction (PCR)

Polymerase chain reaction, followed by Sanger sequencing was used throughout this thesis to confirm findings from Next Generation Sequencing, screen genes where a specific phenotype indicated testing or for the testing of family members to confirm carrier status. Polymerase chain reaction amplifies a region of the DNA sample by several orders of magnitude, meaning that after around 35 cycles, millions of copies of the target region are present. It relies on DNA polymerase which creates a complimentary copy of the template within the target region. Specific primers for the relevant genomic regions (coding regions -50 and +10 to capture branch sites and canonical splice sites) were designed *in silico* using primer3plus software, the NGRL SNPcheck tool and NCBI primer BLAST to ensure specificity [57]. An M13 tail was added to all primers to enable high-throughput Sanger sequencing to be undertaken (table 1)

M13 primer	Sequence (5' – 3')
Forward	TGTAAAACGACGGCCAGT
Reverse	CAGGAAACAGCTATGACC

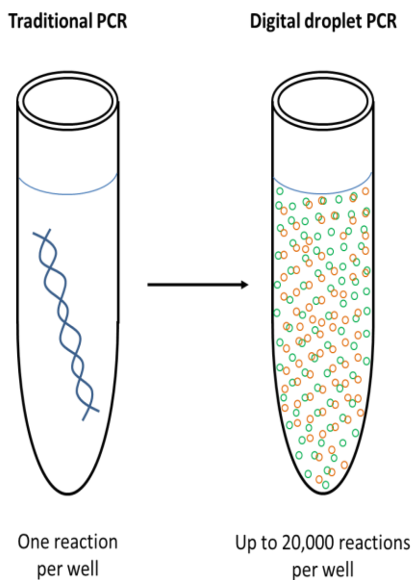
Table 1: M13 primer sequences. The M13 sequence was added to all PCR primers to incorporate the sequence into the resulting amplicons.

Droplet digital PCR (ddPCR)

Digital droplet PCR was used in chapters 1 and 3 to confirm putative mutations identified by targeted NGS. The BioRad ddPCR protocol is a technique based on partitioned polymerase chain reaction. The sample and reagents are partitioned into ~20,000 individual nanolitre droplets such that each droplet acts as an individual reaction (figure 1A). This means that the reaction can be measured as thousands of individual amplification events within a single genomic sample and the mosaicism of variants and copy number of deletions/insertions can be accurately and absolutely quantified (figure 1B). The reaction incorporates fluorescent dyes which can be detected to determine the presence/absence of the target defined by the PCR primers.

Briefly, the sample is quantified using the Qubit fluorometer and diluted to 4ng/µL before adding to a mastermix containing a TaqMan reporter dye, polymerase and assay-specific primers. The sample and droplet generation oil are loaded into a cassette and droplets are then generated using the BioRad Droplet Generator. The reaction is then run on a thermocycler and when complete is placed into the droplet reader. This pulls the droplets in single file past a detector to detect the presence or absence of the intercalated dye and thus the target sequence.

A



B

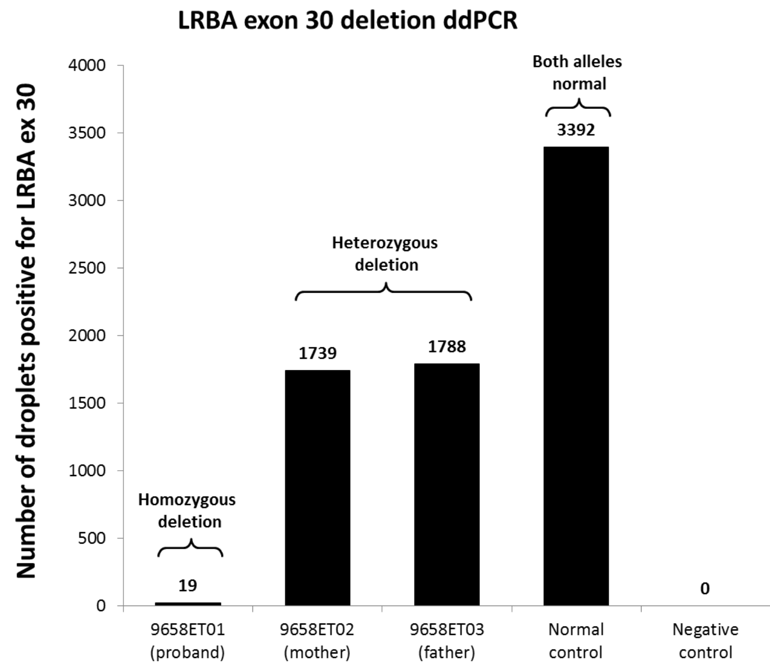


Figure 1 ddPCR. A: Droplet digital PCR can offer absolute quantification as each droplet can be counted as a separate reaction within a single well. **B**: Data from the confirmation of an *LRBA* exon 30 deletion identified in a proband by targeted NGS. The proband has 19 positive droplets, in keeping with the false positive rate due to non-specific amplification by the PCR primers. Both parents have ~50% positive droplets compared to the normal control.

Dideoxy chain terminator sequencing (Sanger sequencing)

Dideoxy chain terminator sequencing (Sanger sequencing), as pioneered by Frederick Sanger and colleagues in 1977, remains the gold standard for the detection of single nucleotide polymorphisms and small insertions and deletions within DNA and was used in chapters 1, 2 and 3. Usually used following PCR, it involves the use of chain-terminating fluorescently labelled di-deoxynucleotides to prevent elongation of the complementary strand by DNA polymerase. When used through multiple cycles, this creates a series of DNA fragments which differ in size by one nucleotide. Each fragment has a fluorescently labelled nucleotide at its 3' end and a capillary electrophoresis machine (3730 DNA analyser, Applied Biosystems, Waltham MA, USA) can be used to separate the fragments by length. The fragments are passed in front of an excitatory laser and the fluorescence emitted is read by a detector. Thus, an image (electropherogram) is formed of the sequence which can be compared to the reference sequence.

Next Generation Sequencing

Massively parallel high throughput sequencing (Next Generation Sequencing, NGS) has revolutionised the way in which human molecular genetic research is performed. Using this methodology enables vast amounts of sequencing data to be generated, with a single NextSeq500 run generating approximately 120 billion bases of data in ~24 hours. This compares to approximately 700 thousand bases of data generated in a similar timeframe by Sanger sequencing.

This technique has been utilised in this thesis to test multiple genes, including in combination with in-solution hybridisation to test all coding genes (exome sequencing), and for the custom targeted capture of genetic regions of interest (chapters 1, 2 and

3). We utilised the Illumina sequencing by synthesis method on an Illumina HiSeq 2500 (Exeter Sequencing Service, University of Exeter) or NextSeq 500 (in house) for exome sequencing and targeted NGS.

Custom Targeted Next Generation Sequencing

In order to rapidly assess the multiple genes causing monogenic autoimmune disease, as well as rule out the known causes of monogenic diabetes, in-solution hybridisation to biotinylated RNA baits complementary to the target region was used, relying on a customised version of the Agilent SureSelect protocol (figure 2; Chapters 1, 2, 3 and 4; [58]). In brief, this involved fragmenting DNA samples to ~200bp, repairing the ends of the fragments to blunt ends, the addition of an adenosine base to the end of each fragment, ligation to adaptors containing universal primer sequences, illumina sequencing compatible hybridisation regions and unique molecular barcodes of 6bp, and a PCR step to enrich the sample for adapted molecules. Samples were then multiplexed (12-plex) and hybridised overnight with the biotinylated RNA baits to capture adapted fragments within the region of interest. Capture on streptavidin coated beads followed by washing and amplification of the captured fragments was performed before QC analysis and pooling for sequencing. A list of the genes in our custom targeted panel is provided in appendix 1.

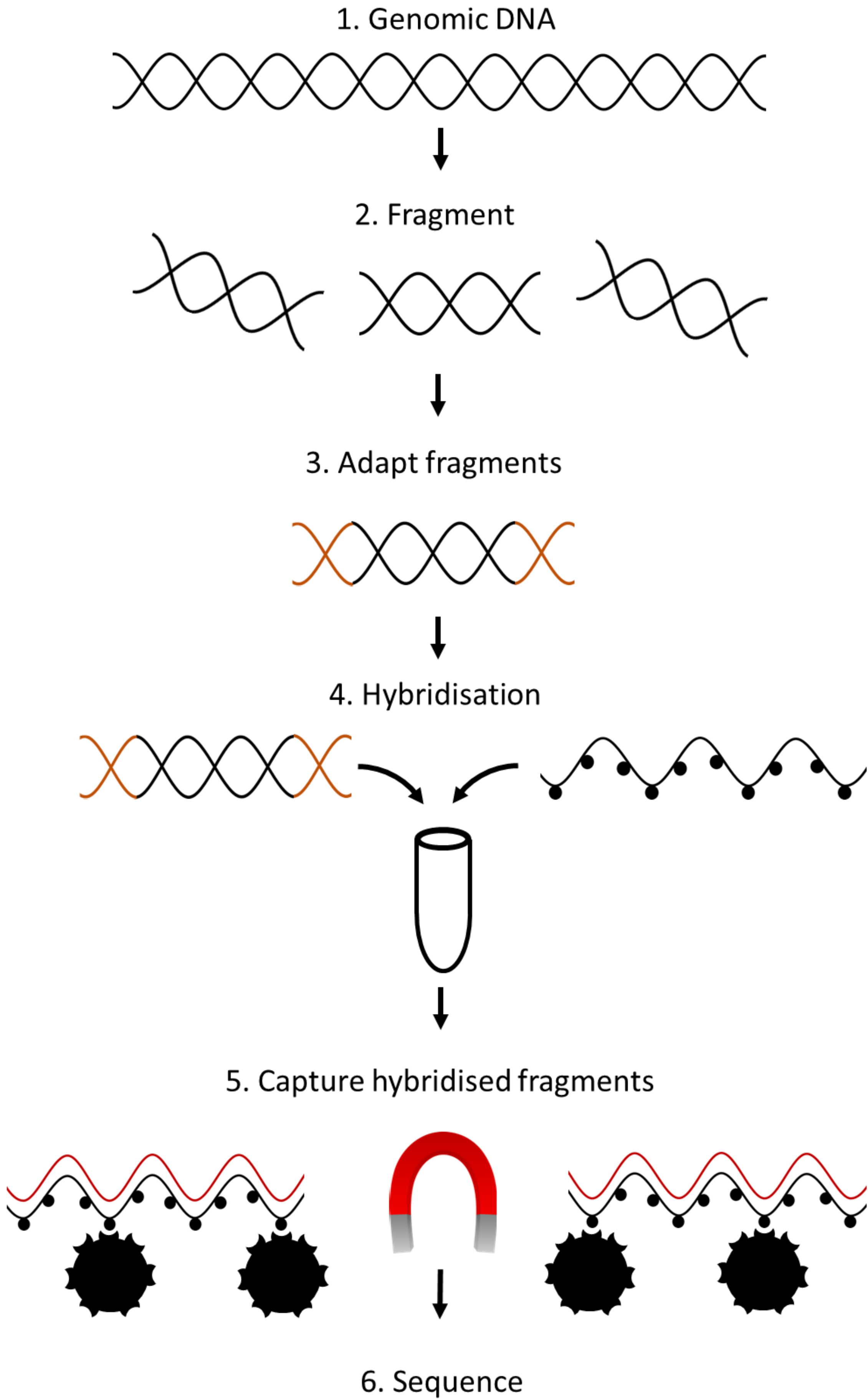

1. Genomic DNA
2. Fragment
3. Adapt fragments
4. Hybridisation
5. Capture hybridised fragments
6. Sequence

Figure 2: Stylised diagram of SureSelect enrichment protocol for exome sequencing and targeted NGS. 1. Genomic DNA is fragmented to ~200bp. 2. Adapter sequences are added that include a patient specific indexing sequence and Illumina sequencer hybridisation regions. 4. Adapted fragments are hybridised overnight to biotinylated RNA baits specific for the target sequence. 5. Hybridised DNA is captured on streptavidin coated paramagnetic beads which are magnetically separated and purified. 6. After amplification and quantification, the captured library is sequenced.

Exome Capture

Exome sequencing (chapter 3) is a targeted sequencing method that captures the majority of the coding regions of the genome. While massively parallel sequencing is cheaper per-base than dideoxy chain terminator sequencing, the cost of whole genome sequencing remains prohibitively expensive and the storage, processing and analysis of data is cumbersome. Moreover, the ~1% of the human genome that encodes proteins has been extensively characterised meaning interpretation of sequence variation in this region is easier. Exome sequencing therefore offers a cost-effective strategy to identify Mendelian disease genes. Exome sequencing was used based on the Agilent SureSelect protocol (see targeted sequencing above and figure 2), using the exome capture library all-exon v5 or v6 (total region targeted: 50 or 60Mb respectively, details available at http://www.genomics.agilent.com/en/SureSelect-DNA-Target-Enrichment-Baits-NEW/SureSelect-Human-All-Exon-V6/?cid=AG-PT-124&tabId=AG-PR-1308).

Massively parallel high throughput sequencing (Illumina)

Massively parallel high throughput sequencing for both the custom targeted capture panels and exome sequencing was undertaken on either the HiSeq 2500 instrument or NextSeq500 instrument (Illumina, San Diego CA, USA). Adapted fragments (the sequencing library) are washed across a flow cell which contains covalently bonded oligonucleotides which are complementary to the ends of the adapters to hybridise the library to the flow cell. Clonal amplification is used to create thousands of identical copies of each of the fragments in a discrete space, termed clusters. Fluorescently labelled nucleotides which have reversible chain terminating regions are then added to the flow cell and an image taken of the growing DNA molecules between each

addition. This allows for the base that is added to be measured and a read of the sequence to be generated.

Bioinformatics analysis

To analyse the large quantities of data generated by next generation sequencing specialised computer hardware and software is required. An overview of a generic pipeline for the analysis of NGS data is given in figure 3 with a brief description of each step, and specific information on the pipelines used in this thesis is included in sections below.

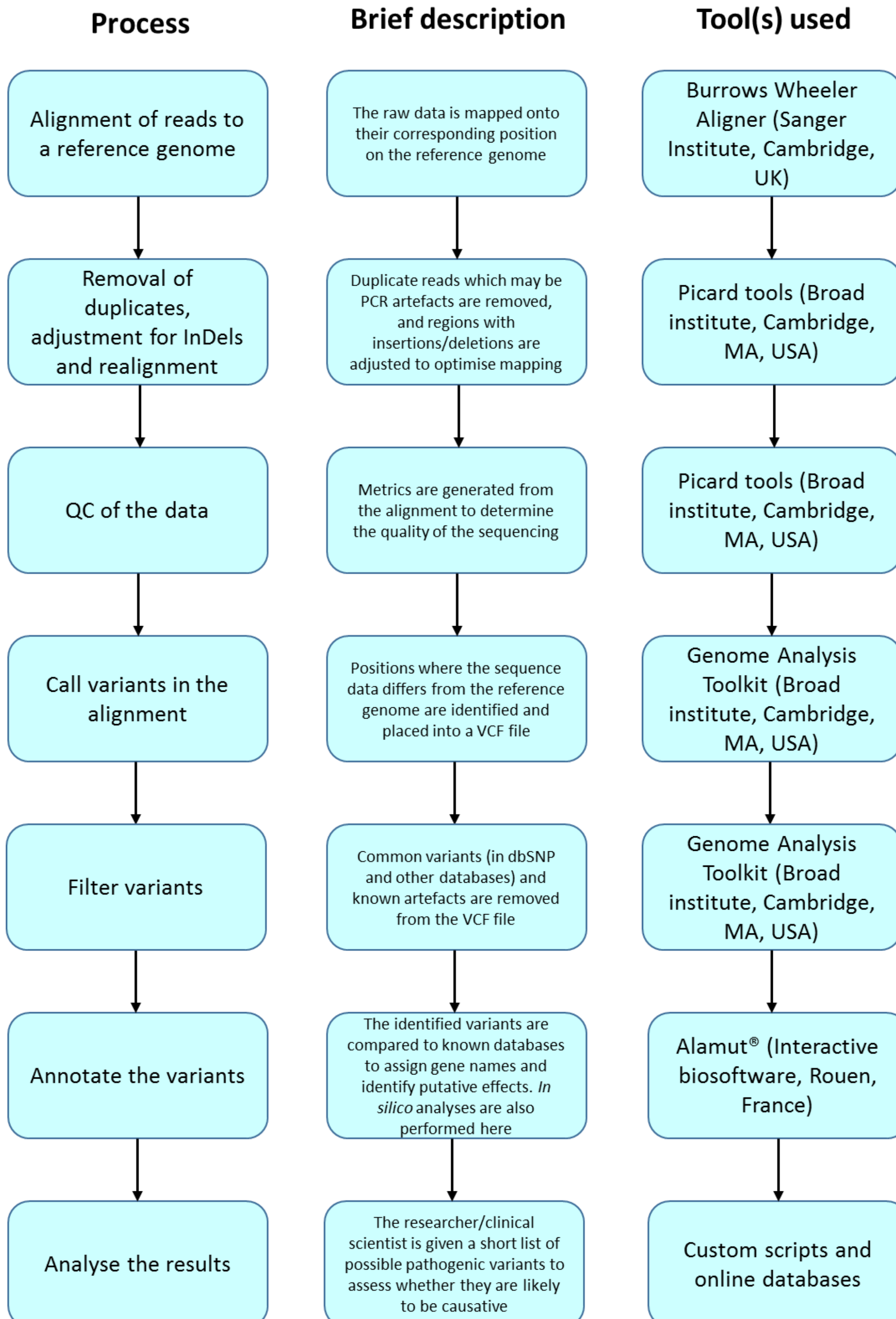

Process
Brief description
Tool(s) used
Alignment of reads to a reference genome
The raw data is mapped onto their corresponding position on the reference genome
Burrows Wheeler Aligner (Sanger Institute, Cambridge, UK)
Removal of duplicates, adjustment for InDels and realignment
Duplicate reads which may be PCR artefacts are removed, and regions with insertions/deletions are adjusted to optimise mapping
Picard tools (Broad institute, Cambridge, MA, USA)
QC of the data
Metrics are generated from the alignment to determine the quality of the sequencing
Picard tools (Broad institute, Cambridge, MA, USA)
Call variants in the alignment
Positions where the sequence data differs from the reference genome are identified and placed into a VCF file
Genome Analysis Toolkit (Broad institute, Cambridge, MA, USA)
Filter variants
Common variants (in dbSNP and other databases) and known artefacts are removed from the VCF file
Genome Analysis Toolkit (Broad institute, Cambridge, MA, USA)
Annotate the variants
The identified variants are compared to known databases to assign gene names and identify putative effects. In silico analyses are also performed here
Alamut® (Interactive biosoftware, Rouen, France)
Analyse the results
The researcher/clinical scientist is given a short list of possible pathogenic variants to assess whether they are likely to be causative
Custom scripts and online databases

Figure 3: overview of bioinformatics pipeline. A brief description of the individual steps is given, as well as the software/tool used. The underlying code of the specific pipeline used to analyse data can be found at [https://github.com/matt0johnson/tNGS_v5]

Targeted NGS pipeline

This pipeline was initially developed by Hana Lango-Allen (University of Exeter Medical school, Exeter, UK), with ongoing maintenance and additions to the code by Andrew Parrish and Garan Jones, Thomas Laver and Matthew Johnson (University of Exeter Medical school, Exeter, UK and Royal Devon and Exeter NHS Trust, Exeter, UK). It utilises the core steps outlined in figure 3 however includes the following additional analyses;

i) Copy number variation analysis using ExomeDepth software [59]

ii) Parsing of the data into a customised and accessible format which includes relevant detail for analysis and key metrics for the sample

iii) Calling of variants only in regions of interest to minimise incidental findings

Exome pipeline

This pipeline was primarily developed by Matthew Wakeling (University of Exeter Medical School, Exeter, UK). It is similar to the tNGS pipeline but includes the following information;

i) Homozygosity mapping by looking for blocks of homozygous single nucleotide variants to identify regions putatively identical by descent

ii) Analysis of relatedness of parent-proband trios to ensure correct identification of *de novo* variants

iii) Analysis of off-target reads (those not in coding regions) to look for large structural variants and karyotype changes

iv) Contamination analysis by looking at the balance of heterozygous calls (true heterozygous calls should have read depths of approximately 50%).

Type 1 Diabetes Genetic Risk Score (T1D-GRS)

To generate the T1D-GRS (chapter 1) samples were first genotyped for the top 10 risk SNPs/alleles (table 2) either by Sanger sequencing, targeted NGS or externally by LGC genomics' proprietary KASP assay (Middlesex, UK). The resulting genotypes were then scored based on their weighted risk (ln(odds ratio)) and allele count (0, 1 or 2). The scores were summed and divided by the total number of alleles to achieve a log additive risk score. This was compared to controls (WTCCC type 1 diabetes or non-diabetic control cohorts) to provide an indication of the likelihood of patients having type 1 diabetes or a monogenic cause.

SNP(s)	Gene/allele	Odds Ratio	Weight
rs2187668, rs7454108	DR3/DR4-DQ8	48.18	3.87
	DR3/DR3	21.12	3.05
	DR4- DQ8/DR4-DQ8	21.98	3.09
	DR4-DQ8/X	7.03	1.95
	DR3/X	4.53	1.51
rs1264813	HLA_A_24	1.54	0.43
rs2395029	HLA_B_5701	2.5	0.92
rs3129889	HLA_DRB1_15	14.88	2.7
rs2476601	PTPN22	1.96	0.67
rs689	INS	1.75	0.56
rs12722495	IL2RA	1.58	0.46
rs2292239	ERBB3	1.35	0.3
rs10509540	C10orf59	1.33	0.29

Table 2: top 10 risk SNPs for type 1 diabetes and their weighted risk.

Statistical analysis

Statistical analyses were undertaken in Stata 14 (Statacorp, TX, USA) and the tests used are detailed in each chapter. These included parametric and non-parametric tests as appropriate.

Ethical approval

Specific ethical approval was not required for this thesis as the studies herein were undertaken using samples from the Exeter beta cell research bank with ethical approval from the North Wales Ethical Committee, who specialise in genetic studies into rare diseases.

Patient recruitment and follow up

Referrals were taken for all patients with diabetes that was diagnosed in the first six months of life, or where diabetes was diagnosed in a patient before the age of 5 years and an additional autoimmune disease was present, also diagnosed before 5 years.
To obtain accurate clinical information from the patients a bespoke request form was designed and used to collect information from clinicians about incident and prevalent cases of multiple early-onset autoimmune disease (available at http://www.diabetesgenes.org/content/early-onset-diabetes-and-autoimmunity and appendix 2). This allowed for standardised information to be collected, while also allowing for free text to be added. The samples are securely stored in Exeter Molecular Genetics laboratory at the Royal Devon and Exeter NHS foundation trust.

Clinical information was collated in a password protected database stored on the on the Royal Devon and Exeter NHS foundation trust's secure servers. Follow up and correspondence was by email enabling rapid communication of results and new

clinical manifestations. For international referrals consent was taken and held locally by the referring clinician, and for UK referrals consent was obtained as part of the Exeter beta cell research bank (http://www.diabetesgenes.org/content/genetic-beta-cell-research-bank).

References

1. Nilson E, Ekholm B, Rees Smith B, Torn C, Hillman M. Calcium addition to EDTA plasma eliminates falsely positive results in the RSR GADAb ELISA. Clinica chimica acta; international journal of clinical chemistry. 2008;388(1-2):130-4.

2. Yu B, Zhang C. In silico PCR analysis. Methods in molecular biology (Clifton, NJ). 2011;760:91-107.

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

4. Plagnol V, Curtis J, Epstein M, Mok KY, Stebbings E, Grigoriadou S, et al. A robust model for read count data in exome sequencing experiments and implications for copy number variant calling. Bioinformatics (Oxford, England). 2012;28(21):2747-54.

Chapter 1

A Type 1 diabetes genetic risk score can discriminate monogenic autoimmunity with diabetes from early onset clustering of polygenic autoimmunity with diabetes

Matthew B Johnson, Kashyap E Patel, Elisa De Franco, Jayne A L Houghton, Sian Ellard[1], Sarah E Flanagan, & Andrew T Hattersley

Diabetologia 2017 *under review*

Acknowledgments

A.T.H, S.E, S.E.F and K.A.P. assisted with the design of the study. K.A.P., E.D.F and J.A.H. assisted with the interpretation of clinical information and contributed to discussion. A.T.H. gave feedback on drafts of the manuscript which was reviewed and edited by all authors.

My contributions to the chapter

I designed the study, selected the cohorts for testing from our database of patients with early-onset diabetes, performed the genetic testing for the cases referred between 2014-2016 and analysed the genetic data for patients tested by targeted NGS. I collated the clinical information from the patients' clinical notes, created the figures and tables and performed the statistical analysis. I performed the genotyping on ~50% of the cohort and generated the genetic risk score from genotyping data for all of the cohort. I harmonised the genotyping data from multiple sources into a comparable genetic risk score, wrote the manuscript, revised it according to comments from the co-authors and submitted the manuscript.

ABSTRACT

Aims/hypothesis

Identifying patients suitable for monogenic autoimmunity testing and gene discovery studies is challenging: early-onset type 1 diabetes mellitus (T1D) can cluster with additional autoimmune diseases due to shared polygenic risk and islet and other organ specific autoantibodies are present in patients with both monogenic and polygenic aetiologies. We aimed to assess if a type 1 diabetes genetic risk score (T1D-GRS) could identify monogenic autoimmune diabetes and be useful to prioritise patients for gene discovery studies.

Methods

We studied 79 patients with diabetes and at least 1 additional autoimmune disease diagnosed before 5 years. We screened all patients for variants in 7 genes known to cause monogenic autoimmunity that can include diabetes (*AIRE*, *IL2RA*, *FOXP3*, *LRBA, STAT1*, *STAT3*, *STAT5b*). We genotyped all patients for the top 10 risk alleles for T1D, including HLA and non-HLA loci, to generate a T1D-GRS.

Results

47% (37/79) of individuals had mutations in the monogenic autoimmunity genes. The T1D-GRS was lower in these patients compared to individuals without mutations in these genes (median 9th vs. 49th centile T1D controls ($p<0.0001$). Age of diabetes diagnosis and T1D-GRS combined to be highly discriminatory of monogenic autoimmunity (ROC-AUC: 0.88). Most patients without a mutation in a known gene had a high T1D-GRS, suggesting they have polygenic clustering of T1D and additional autoimmunity and should not be included in gene discovery studies.

Conclusions

We have shown that the T1D-GRS can identify patients likely to have monogenic autoimmunity helping both diagnostic testing and novel monogenic autoimmunity gene discovery. Patients with monogenic autoimmunity have a different clinical course to those with polygenic type 1 diabetes (T1D) and can respond well to therapies targeting the underlying genetic defect.

KEYWORDS

Monogenic autoimmune diabetes, Genetic Risk Score, Type 1 diabetes, Gene discovery

ABBREVIATIONS

- AITD – autoimmune thyroid disease
- CD – coeliac disease
- GRS – genetic risk score
- IPEX – immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
- NGS – next generation sequencing
- T1D – type 1 diabetes mellitus
- T2D – type 1 diabetes mellitus

Monogenic autoimmune disease often presents with very early-onset diabetes. For example, hemizygous mutations in *FOXP3* cause IPEX (Immunodysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome, which presents in the neonatal period with diabetes, protein-losing enteropathy and severe eczema [1] and patients with infantile-onset multisystem autoimmune disease due to dominant gain-of-function *STAT3* mutations or common variable immunodeficiency 8 with autoimmunity due to recessively inherited *LRBA* mutations may present with neonatal diabetes [2, 3].

While some patients harbour a causative mutation in a single gene, the clustering of very early-onset diabetes with autoimmune disease is often due to a strong polygenic risk resulting from shared predisposing genetic loci. It is well established that the HLA-DR3 haplotype is associated with the development of type 1 diabetes mellitus (T1D) [4] and coeliac disease (CD) through its strong linkage with the HLA-DQ2 haplotype [5]. Outside the HLA region the *IL2RA* polymorphism rs706778 is associated with increased risk of T1D, autoimmune thyroid disease (AITD) and CD, as well as other paediatric-onset autoimmune disorders [6].

The phenotypic overlap between the two groups means identifying patients for testing is difficult using clinical features or biomarkers. While islet auto-antibodies are highly discriminatory of T1D against type 2 diabetes (T2D) and maturity onset diabetes of the young (MODY) [7, 8], they are often present in patients with monogenic autoimmunity. For example, multiple islet autoantibodies are present in more than half of individuals with IPEX syndrome [9]. Moreover, as it is thought that these patients have autoimmune destruction of the pancreatic β cells [10], serum C-peptide levels and treatment type or dose is also likely to be similar in the two groups.

The type 1 genetic risk score (T1D-GRS) is calculated by genotyping the top risk alleles and summing their effective weight to assign a numerical score to the patient that can be compared to control samples [11]. It was recently shown to be highly discriminatory of non-autoimmune monogenic diabetes and T2D from T1D [11, 12]. We sought to determine if the T1D-GRS could distinguish between monogenic autoimmunity and polygenic clustering of autoimmune disease. Our results show that it performs better than clinical features or biomarkers in this patient group.

RESEARCH DESIGN AND METHODS

Study cohorts

Patients with early-onset autoimmunity

We studied 79 patients diagnosed with diabetes and ≥1 additional autoimmune disorder before the age of 5 years referred to Exeter Molecular Genetics laboratory between 2005-2017(table 1). All patients had previously been screened for all known monogenic diabetes genes [13]. Clinical information was supplied by the referring clinician from the patient's medical notes.

T1D controls

As previously described [12], we used T1D controls from the Wellcome Trust Case Control Consortium (WTCCC) [14]. These 1963 patients from the WTCCC T1D cohort have a clinical diagnosis of T1D, were diagnosed before 17 years and insulin treated from diagnosis.

Methods

Genetic Testing

We used targeted next generation sequencing (NGS) as previously described [13] to test the 7 genes known to cause monogenic diabetes with autoimmunity (*AIRE*, *IL2RA*, *FOXP3*, *LRBA, STAT1*, *STAT3*, *STAT5b*) in 79 individuals. All putative mutations were confirmed by Sanger sequencing or digital droplet PCR (primers available on request).

T1 Genetic Risk Score

In order to generate a T1D-GRS we genotyped the top 10 SNPs with the largest effect size as previously described, including both HLA and non-HLA regions [11, 12]

(supplementary table S1) by targeted NGS, Sanger sequencing (primer sequences available on request) or the KASP assay (LGC Limited, Middlesex, UK).

Statistical analysis

Logistic regression and receiver operating characteristic (ROC) curve analysis was used to assess the discriminatory power of biomarkers, clinical features and the T1D-GRS. Parametric (*t* test) and non-parametric (Mann-Whitney *U*) tests were used for continuous variables and the Fishers Exact test was used to compare categorical variables. Statistical analyses were performed in Stata 14 (StataCorp LP, College Station, TX).

Antibody testing

Antibody testing for anti-Glutamic Acid Decarboxylase (GAD), anti-Zinc Transporter 8 (ZnT8) and anti-Islet Antigen 2 (IA-2) was performed as previously described when serum was available (n=43) [8].

Ethical approval

The study was approved by the Genetic Beta Cell Research Bank, Exeter, U.K. with ethical approval from the North Wales Research Ethics Committee, U.K.

RESULTS

Molecular genetics

A mutation in a known monogenic autoimmunity gene was identified in 47% (37/79) of the individuals with diabetes and ≥1 autoimmune disorder diagnosed before 5 years; 25 males had a hemizygous mutation in *FOXP3*, 8 patients had recessively inherited mutations in *LRBA*, two had recessively inherited *IL2RA* mutations and two patients had heterozygous gain-of-function *STAT3* mutations. 12 of these patients have been reported previously [2, 3, 15]. The remaining 42 patients have early-onset multiple autoimmunity but do not have a mutation in a known gene. The group of individuals with “unknown aetiology” will either have a polygenic predisposition to diabetes and other autoimmune disease or a monogenic cause of autoimmunity, which includes diabetes, which has not been described to date.

The T1D-GRS is lower in monogenic autoimmunity than in patients with multiple autoimmune disease of unknown aetiology

Patients with confirmed monogenic autoimmunity had a markedly lower median T1D-GRS than those with early-onset autoimmunity of unknown aetiology (9th v 49th centile of T1D p = <0.0001); figure 1. Patients with unknown aetiology had a similar median T1D-GRS as the T1D controls (49th v 50th T1D centile p = 0.63).

The likelihood of identifying monogenic autoimmunity increases with decreasing T1D-GRS

When the entire cohort of 79 patients was split into quartiles that were defined by the T1D controls the likelihood of identifying monogenic autoimmunity decreased as the T1D-GRS increased. 69% (29/42) with a score below the 25th centile had a mutation in a known gene whilst 0% (0/11) with a T1D-GRS above the 75th centile had a

mutation in a known gene (Figure 2B). 79% (11/14) of those below the 5th centile had a mutation in a known gene and 0% (0/8) above the 95th centile had a mutation in a known gene (data not shown).

Most of those with unknown aetiology are likely to have polygenic clustering of type 1 diabetes and additional autoimmunity

The 42 patients who do not have a known cause of monogenic autoimmunity have a similar distribution between the four T1D-GRS quartiles as seen in T1D controls (p=0.38, figure 2A). This would fit with the majority of the patients, where a known cause was not found, having polygenic Type 1 diabetes. The 37 patients with confirmed monogenic autoimmunity were most likely to have a low T1D-GRS: 78% (29/37) of those with monogenic autoimmunity were in the first quartile of T1D-GRS while none (0/37) were in the fourth quartile (figure 2B, p<0.0001).

Those with monogenic autoimmunity developed diabetes earlier and had broadly different clinical features to those with unknown aetiology

Clinical features of those with and without a known cause of monogenic diabetes are shown in table 1. The patients with confirmed monogenic autoimmunity were typically diagnosed earlier than those with unknown aetiology (5 weeks [IQR: 1-18] vs. 36 weeks [IQR: 26-44], p<0.0001). A similar proportion of patients had a positive result for at least one of anti-GAD, IA-2 or ZnT8 autoantibodies: 44% (8/18) with mutation and 44% (11/25) unknown aetiology p = 1.00). When restricted to patients positive for ≥1 islet autoantibody (n=19), the GRS was lower in those with monogenic autoimmunity (0.558 [IQR: 0.528-0.613] vs. 0.716 [IQR: 0.670-0.819], p = 0.0005). Insulin dose and the median number of autoimmune features were similar.

Organ specific disorders showed different frequencies in the two groups (Table 1, overall p = 0.0002). Patients with monogenic autoimmunity were more likely to have autoimmune enteropathy (*p*=0.01 OR 3.8 [95% CI 1.3-10.8]) or glomerulonephritis (p=0.008 OR17.5 [95% CI 0.95-323.0]) and less likely to have thyroid disease (AITD) and/or coeliac disease (CD)) compared to patients with autoimmunity of unknown aetiology (*p*=0.001, OR 5.3 [95% CI 1.8-16.6]) The clustering of T1D, coeliac and thyroid disease in those without a known cause of monogenic autoimmunity is likely to reflect the shared predisposition resulting from HLA-DR3 for Type 1 diabetes, thyroid disease and coeliac disease. Of the patients with diabetes and AITD or CD, 18/25 (72%) of those with unknown aetiology and 3/8 of those with a monogenic aetiology carry at least one copy of DR3 (supplementary table S2).

A combination of clinical features and T1D-GRS is highly discriminative of monogenic autoimmunity

The T1D-GRS was highly discriminatory for identifying those with monogenic autoimmunity against those with unknown aetiology (figure 3). Receiver operating characteristic (ROC) curve analysis gave a ROC area under the curve (ROC-AUC) for the T1D-GRS of 0.80 (95% CI: 0.70-0.90). Age of diagnosis had similar ROC-AUC (0.79 [95% CI: 0.69-0.90], p = 0.91) and when these 2 features were combined the discrimination improved against the T1D-GRS alone (ROC-AUC 0.88 [95% CI: 0.80-0.95], $p = 0.04$).

DISCUSSION

We have shown that a T1D-GRS can be used to discriminate patients most likely to have a mutation in a monogenic autoimmune gene and could be used to prioritise patients for gene discovery studies and, in combination with clinical features, genetic testing. Patients with confirmed monogenic autoimmune disease have a markedly lower T1D-GRS than those with isolated Type 1 diabetes or Type 1 diabetes associated with other autoimmune disease, even when both conditions are diagnosed very young.

The T1D associated antibodies have no discriminatory value, being present both in patients with and without monogenic autoimmunity. Whilst pancreatic autoantibodies have been previously shown to be specific (>57%) and highly sensitive (>99%) for discriminating T1D from non-autoimmune monogenic diabetes [8], we did not observe this in our cohort as monogenic autoimmunity often leads to autoantibody production. When islet autoantibodies were present, the T1D-GRS was lower in those with confirmed monogenic autoimmunity than in patients with an unknown aetiology (0.558 v 0.716). There is evidence that autoantibodies to harmonin and villin are diagnostic markers for patients with IPEX syndrome [16], however we were unable to test this in our patients with hemizygous *FOXP3* mutations. C-peptide testing is useful for identifying type 2 diabetes and MODY from T1D [17], however as monogenic autoimmunity is likely to result in destruction of the pancreatic beta cells (as evidenced by post-mortem histological studies of individuals with IPEX syndrome [15, 18, 19]) it is unlikely to be useful in this patient group and we were unable to assay serum C-peptide in our patients. The T1D-GRS (ROC-AUC: 0.80) gave similar discrimination of monogenic autoimmunity from unknown aetiology than clinical features (ROC-AUC

Age of diagnosis: 0.79) and a combination of these two features gave the best discrimination (ROC-AUC 0.88).

The overlap in clinical features may preclude their use to identify patients with monogenic autoimmunity. Age at diabetes onset was a good discriminator between the two patient groups, however the range of age of diabetes diagnosis overlapped (monogenic autoimmunity: 0-83 weeks, unknown aetiology: 1-258 weeks). While autoimmune enteropathy and CD showed different prevalence in those with and without a mutation (Table 1) both groups included patients with CD and autoimmune enteropathy. Furthermore, at the onset of symptoms these disorders can be challenging to distinguish clinically, particularly in very young patients.

Those with confirmed monogenic autoimmunity were less likely to have AITD or CD in addition to T1D than those with an unknown aetiology (22% vs 60%, OR 5.33). This is driven by the strong predisposing HLA allele DR3 (through linkage with DQ2) in keeping with previous studies on shared HLA risk for these disorders [20]. The same effect does not appear to modulate disease in monogenic autoimmunity as none of the 5 individuals carrying the highest risk alleles for concurrent T1D and CD - DR3/DR3 and DR3/DR4 [20] - have CD, and only 3/14 with DR3/X has CD or AITD. Further study of a larger group of patients is needed to confirm this effect as it may be that they go on to develop CD or AITD later in childhood. We have selected patients with an extreme phenotype (diabetes and $\geq$1 autoimmune disease diagnosed before 5 years) hence we have found the extreme genotypes, both for monogenic and polygenic disease.

This study provides evidence that the polygenic risk of developing autoimmune diabetes does not affect the development of diabetes in patients with monogenic

autoimmunity. Previous reports of individuals with monogenic autoimmunity have shown that many patients do not develop diabetes, for example 70% of those reported with gain-of-function *STAT3* mutations are not diabetic [2, 21, 22]. The known risk alleles are not modifying the phenotype in these patients as the polygenic risk of developing autoimmune diabetes in our cohort of *with* diabetes is similar to healthy controls (p=0.162, data not shown). Further study of non-diabetic patients with monogenic autoimmunity is warranted.

We propose that the T1D-GRS could be used to prioritise patients for gene discovery studies. Our results suggest that a cut-off based on the 25th centile of T1D controls would be suitable to guide selection of patients for initial discovery as the majority in this group have a monogenic cause. Furthermore, there was a small enrichment of individuals in the first quartile of the unknown patients (figure 2A) suggesting some patients in this group may have monogenic autoimmunity. These novel causes may be mutations in genes not previously associated with disease or deep-intronic/regulatory mutations in known genes. Identifying these novel aetiologies will further the understanding of the adaptive immune system and could provide new therapeutic targets as knowledge of the underlying pathway defect can allow personalised therapies. This is already happening for patients with recessive *LRBA* mutations who can be treated with abatacept which replaces the lost receptor molecule [23] and patients with IPEX syndrome who are amenable to hematopoietic stem cell transplantation which, if performed early, can prevent the onset of organ-specific autoimmunity. Furthermore, identifying novel aetiologies will assist with research by preventing patients with monogenic disease from taking part in clinical trials aimed at those with a polygenic aetiology.

The numbers of patients with monogenic autoimmune disease available to study in our cohort is low (n=37) however, to our knowledge, this is the largest series of patients with monogenic autoimmune diabetes described in the literature to date. Interestingly we did not identify any patients with autoimmune polyendocrine syndrome type I (APS1) due to biallelic *AIRE* mutations. The onset of autoimmune diabetes in APS1 is typically later (30-50 years) [24, 25] and the specific clinically defining features, namely chronic mucocutaneous candidiasis and hypoparathyroidism, mean their identification may present less of a challenge.

Seven of the 10 genotyped SNPs in this T1D-GRS cover loci that are associated (positively or negatively) with >1 autoimmune disease (supplementary table S1) however some variants that predispose to multiple clinically distinct autoimmune disorders were not included in our panel. A recent meta-analysis of associations with childhood onset autoimmune disease, including T1D, identified 22 loci which associated with two or more of the disorders in our patient group [6]. A GRS tailored for regions with pleiotropic effects could offer higher discrimination of polygenic clustering of autoimmune disease and monogenic autoimmunity.

In conclusion we have demonstrated that the T1D-GRS is useful to discriminate clustering of early-onset type 1 diabetes with autoimmunity from monogenic autoimmune disease and could be used to prioritise patients for gene discovery studies and follow up genetic testing. Identifying these patients can allow for targeted treatment, inform families and clinicians of the likely clinical course and increase understanding of the human immune system.

Acknowledgements

The authors thank the referring clinicians, patients and their families. This work was supported by a Wellcome Trust Senior Investigator Award to SE and ATH (grant number: 098395/Z/12/Z). ATH is an NIHR senior investigator. SEF has a Sir Henry Dale Fellowship jointly funded by the Wellcome Trust and the Royal Society (grant Number: 105636/Z/14/Z). Additional support came from the Helmsley Foundation's Breakthrough Initiative, University of Exeter and the NIHR Exeter Clinical Research Facility. A.T.H is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Duality of Interest

No potential conflicts of interest relevant to this article are reported.

REFERENCES

[1] d'Hennezel E, Bin Dhuban K, Torgerson T, Piccirillo CA (2012) The immunogenetics of immune dysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome. J Med Genet 49: 291-302

[2] Flanagan SE, Haapaniemi E, Russell MA, et al. (2014) Activating germline mutations in STAT3 cause early-onset multi-organ autoimmune disease. Nat Genet 46: 812-814

[3] Johnson MB, De Franco E, Lango-Allen H, et al. (2017) Recessively Inherited Lrba Mutations Cause Autoimmunity Presenting as Neonatal Diabetes. Diabetes 66 2316-2322

[4] Devendra D, Eisenbarth GS (2003) 17. Immunologic endocrine disorders. J Allergy Clin Immunol 111: S624-636

[5] Tosi R, Vismara D, Tanigaki N, et al. (1983) Evidence that celiac disease is primarily associated with a DC locus allelic specificity. Clin Immunol Immunopathol 28: 395-404

[6] Li YR, Li J, Zhao SD, et al. (2015) Meta-analysis of shared genetic architecture across ten pediatric autoimmune diseases. Nat Med 21: 1018-1027

[7] Batstra MR, Aanstoot HJ, Herbrink P (2001) Prediction and diagnosis of type 1 diabetes using beta-cell autoantibodies. Clin Lab 47: 497-507

[8] McDonald TJ, Colclough K, Brown R, et al. (2011) Islet autoantibodies can discriminate maturity-onset diabetes of the young (MODY) from Type 1 diabetes. Diabet Med 28: 1028-1033

[9] Tsuda M, Torgerson TR, Selmi C, et al. (2010) The spectrum of autoantibodies in IPEX syndrome is broad and includes anti-mitochondrial autoantibodies. J Autoimmun 35: 265-268

[10] Xavier-da-Silva MM, Moreira-Filho CA, Suzuki E, Patricio F, Coutinho A, Carneiro-Sampaio M (2015) Fetal-onset IPEX: report of two families and review of literature. Clin Immunol 156: 131-140

[11] Oram RA, Patel K, Hill A, et al. (2016) A Type 1 Diabetes Genetic Risk Score Can Aid Discrimination Between Type 1 and Type 2 Diabetes in Young Adults. Diabetes Care 39: 337-344

[12] Patel KA, Oram RA, Flanagan SE, et al. (2016) Type 1 Diabetes Genetic Risk Score: A Novel Tool to Discriminate Monogenic and Type 1 Diabetes. Diabetes 65: 2094-2099

[13] Ellard S, Lango Allen H, De Franco E, et al. (2013) Improved genetic testing for monogenic diabetes using targeted next-generation sequencing. Diabetologia 56: 1958-1963

[14] WTCCC (2007) Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. Nature 447: 661-678

[15] Rubio-Cabezas O, Minton JA, Caswell R, et al. (2009) Clinical heterogeneity in patients with FOXP3 mutations presenting with permanent neonatal diabetes. Diabetes Care 32: 111-116

[16] Lampasona V, Passerini L, Barzaghi F, et al. (2013) Autoantibodies to harmonin and villin are diagnostic markers in children with IPEX syndrome. PLoS One 8: e78664

[17] Ludvigsson J, Carlsson A, Forsander G, et al. (2012) C-peptide in the classification of diabetes in children and adolescents. Pediatr Diabetes 13: 45-50

[18] Smigoc Schweiger D, Mendez A, Kunilo Jamnik S, et al. (2016) High-risk genotypes HLA-DR3-DQ2/DR3-DQ2 and DR3-DQ2/DR4-DQ8 in co-occurrence of type 1 diabetes and celiac disease. Autoimmunity 49: 240-247

[19] Haapaniemi EM, Kaustio M, Rajala HL, et al. (2015) Autoimmunity, hypogammaglobulinemia, lymphoproliferation, and mycobacterial disease in patients with activating mutations in STAT3. Blood 125: 639-648

[20] Milner JD, Vogel TP, Forbes L, et al. (2015) Early-onset lymphoproliferation and autoimmunity caused by germline STAT3 gain-of-function mutations. Blood 125: 591-599

[21] Lo B, Zhang K, Lu W, et al. (2015) AUTOIMMUNE DISEASE. Patients with LRBA deficiency show CTLA4 loss and immune dysregulation responsive to abatacept therapy. Science 349: 436-440

[22] Husebye ES, Perheentupa J, Rautemaa R, Kampe O (2009) Clinical manifestations and management of patients with autoimmune polyendocrine syndrome type I. J Intern Med 265: 514-529

[23] Perheentupa J (2006) Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy. J Clin Endocrinol Metab 91: 2843-2850

End of text of article

Table 1: Summary of the main clinical and demographic features of the cohort. *Either the result of consanguineous union or from regions with a high rate of consanguinity as previously described. †IQR = Inter-quartile range. ‡IPEX syndrome, caused by hemizygous mutations in *FOXP3*, is an X-linked recessive disorder and therefore only presents in males, hence the bias toward males in those with confirmed monogenic autoimmunity.

Clinical/demographic feature	Monogenic autoimmunity (n=37)	Unknown aetiology (n=42)	p value
Consanguineous*	19/37 (51%)	11/42 (26%)	0.04
Male: Female ratio	31:6	25:17	0.03‡
Diabetes characteristics			
Median age of diabetes diagnosis, weeks (range)	5 (0 - 83)	36 (1 - 252)	<0.001
Median insulin dose (U/Kg/Day)	1.0 (0.6-1.2)	0.8 (0.5-1.1)	0.33
Islet autoantibody status (n = 43)			
Positive for ≥1 antibody	8/18 (44%)	11/25 (44%)	1.00
GAD positive	5/18 (28%)	8/25 (32%)	1.00
IA2 positive	2/18 (11%)	2/25 (8%)	1.00
ICA positive	2/18 (11%)	3/25 (12%)	1.00
ZnT8 positive	1/18 (5%)	0/25 (0%)	0.42
Additional autoimmune diseases			
Median number of additional disorders (IQR†)	2.0 (1.0 – 2.0)	1.5 (1.0 – 2.0)	0.51
Autoimmune enteropathy	16/37 (43%)	7/42 (17%)	0.01
Coeliac disease	2/37 (5%)	12/42 (29%)	0.008
Autoimmune thyroid disease (Hypo-/hyperthyroidism)	6/37 (16%)	17/42 (40%)	0.025
Autoimmune haematological disease (Thrombocytopenia, lymphoproliferative disease or hepatosplenomegaly)	5/37 (14%)	6/42 (14%)	1.00
Atopic dermatitis	6/37 (16%)	5/42 (12%)	0.75
Alopecia	0/37 (0%)	3/42 (7%)	0.24
Glomerulonephritis	6/37 (16%)	0/42 (0%)	0.008

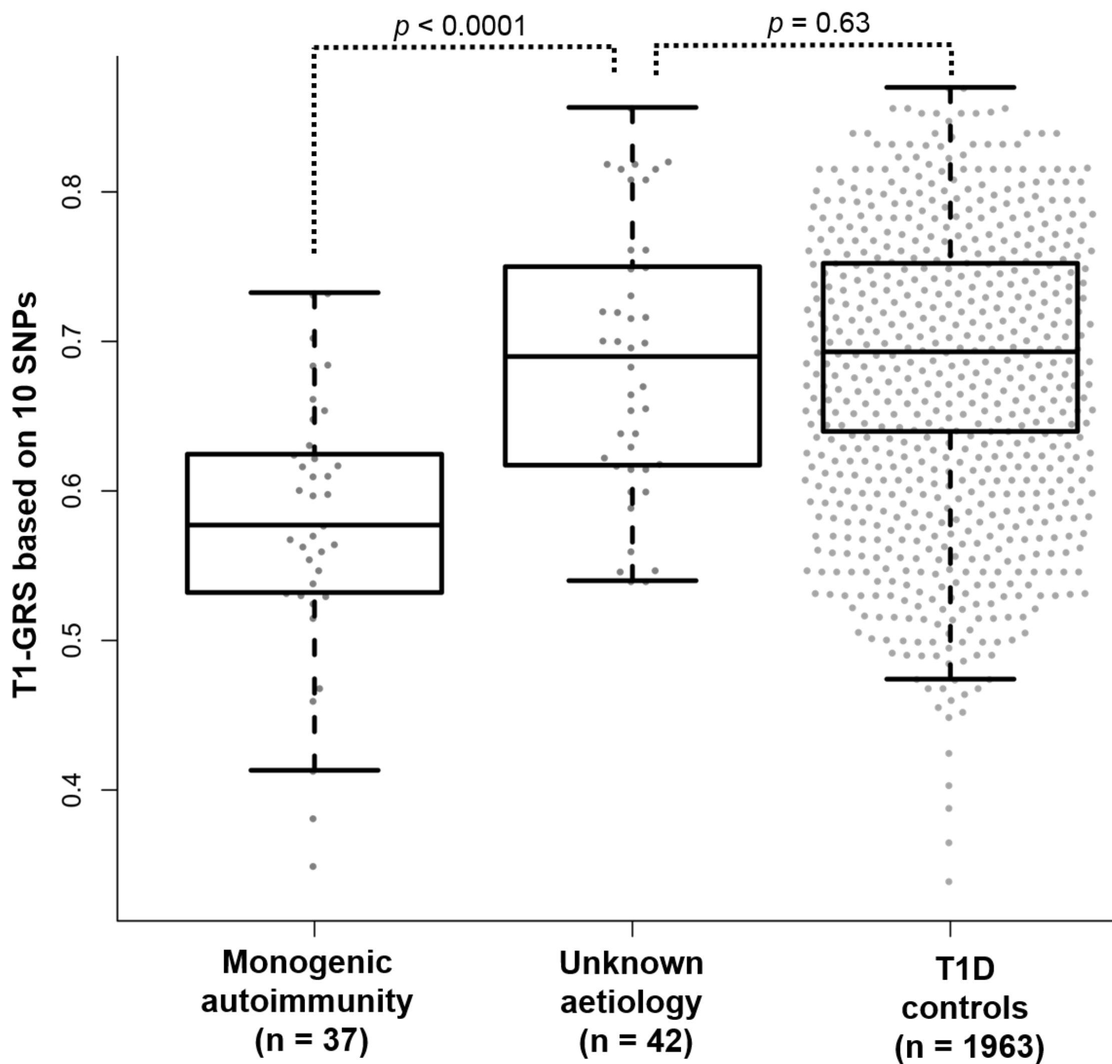


Figure 1: Boxplot of the T1D-GRS in confirmed monogenic autoimmunity, patients with unknown aetiology and T1D controls. The central line within the box represents the median and the upper and lower limits of the box represent the interquartile range. The whiskers are the most extreme values within 1.5x the interquartile range from the 1st and 2nd quartiles. Those with confirmed monogenic autoimmunity have a lower median score than T1D controls, while those with unknown aetiology have a similar score to the T1D controls.

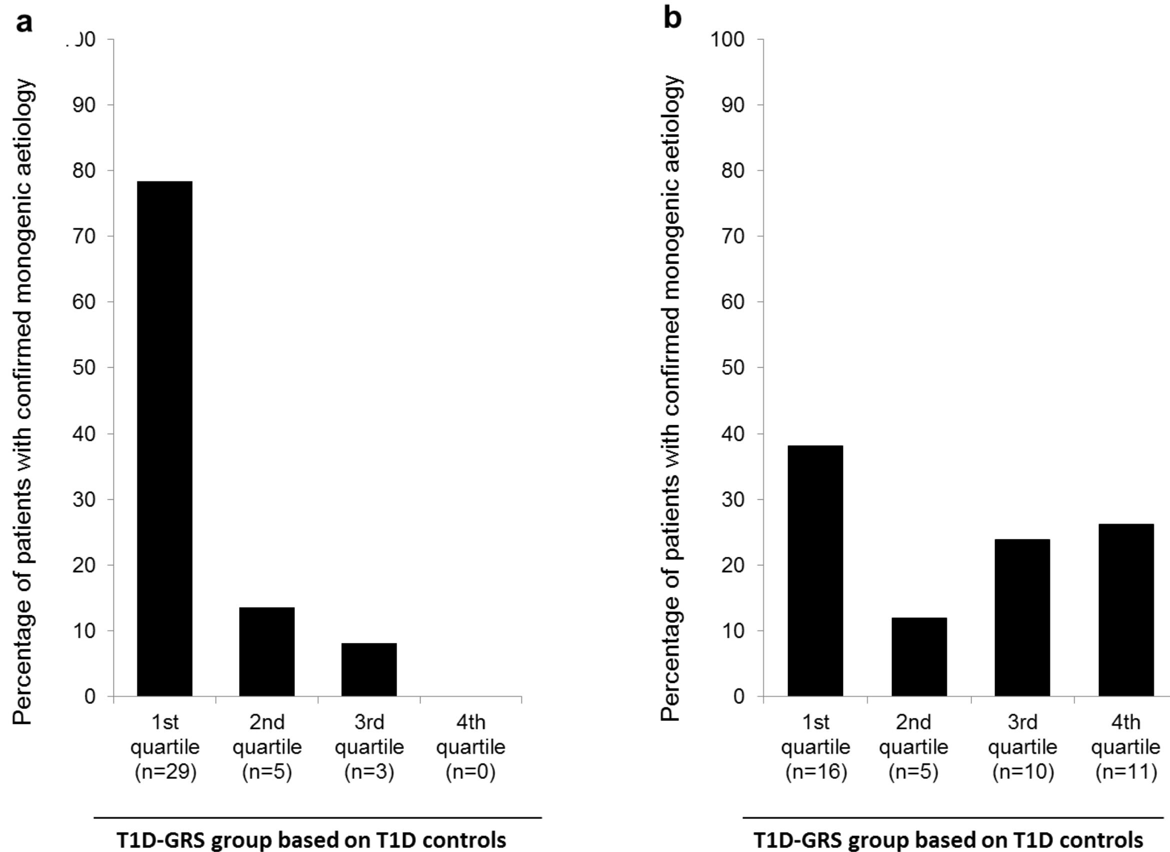


Figure 2: The T1D-GRS in patients with monogenic autoimmunity and patients with unknown aetiology. A) The proportion of patients with early-onset multiple autoimmunity of unknown aetiology (n= 42) in each quartile based on T1D controls. There is an over-representation of individuals with a low T1D-GRS, suggesting there are novel monogenic causes remaining to be found in our cohort. **B)** The proportion of patients with confirmed monogenic autoimmunity (n=37) in each quartile based on T1D controls. The proportion of patients with a confirmed monogenic cause was higher in patients with a low T1D-GRS.

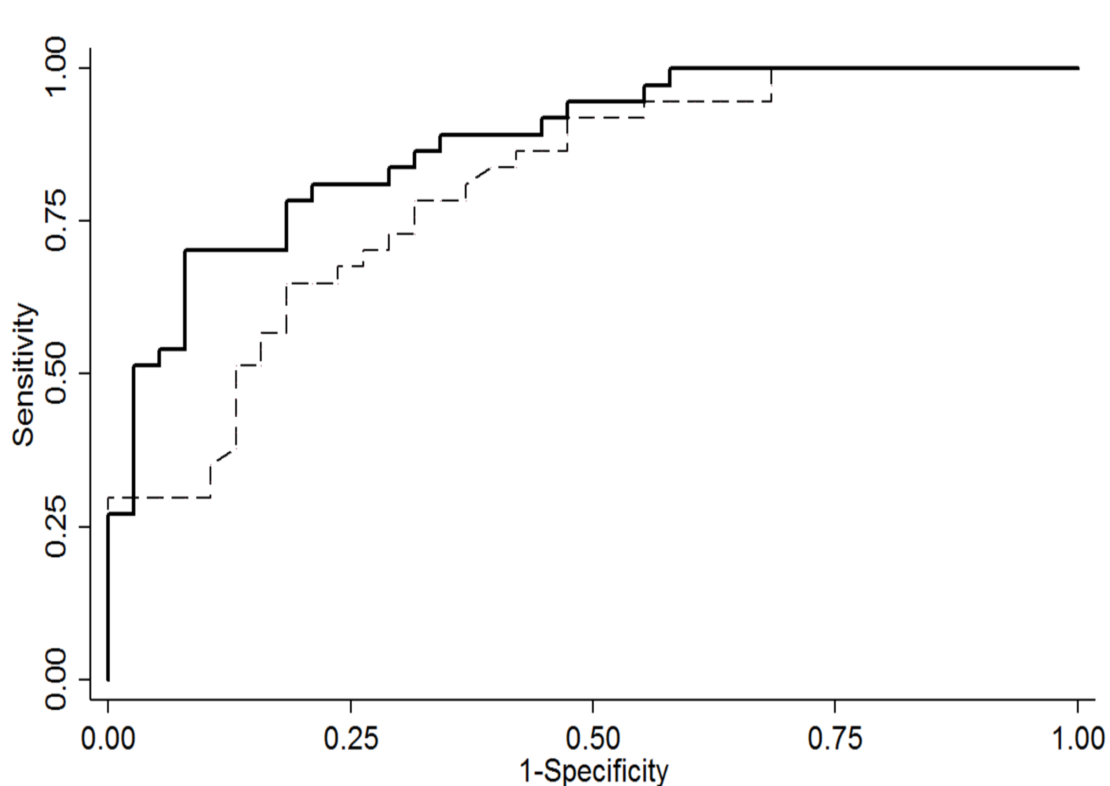


Figure 3: Receiver Operating Characteristic (ROC) curve T1D-GRS and T1D-GRS combined with age of diabetes diagnosis in the discrimination of patients with monogenic autoimmunity from those with unknown aetiology (n=79). The dashed line shows T1D-GRS (AUC: 0.80 [95% CI: 0.70-0.90]) and the black line shows T1D-GRS combined with age of diabetes diagnosis (AUC: 0.88 [95% CI: 0.80-0.95]). For age of diabetes diagnosis alone (AUC: 0.79 [95% CI: 0.69-0.90]) and the presence of autoantibodies (AUC 0.49, [95% CI: 0.34-0.65]) data not shown.

Supplementary data:

A Type 1 genetic risk score can discriminate monogenic autoimmunity with diabetes from early onset clustering of polygenic autoimmunity with diabetes

Matthew B Johnson, Kashyap E Patel, Elisa De Franco, Jayne A L Houghton, Sian Ellard, Sarah E Flanagan, & Andrew T Hattersley

Supplementary table S1: SNPs used for the T1-GRS calculation. Disease associations taken from www.gwascatalog.com.

SNP(s)	Gene	Odds Ratio	Weight	Autoimmune disease associations
rs2187668, rs7454108	DR3/DR4-DQ8	48.18	3.87	Type 1 diabetes, Coeliac disease, Autoimmune thyroid disease, Autoimmune hepatitis
	DR3/DR3	21.12	3.05	
	DR4- DQ8/DR4-DQ8	21.98	3.09	
	DR4-DQ8/X	7.03	1.95	
	DR3/X	4.53	1.51	
rs1264813	HLA_A_24	1.54	0.43	Type 1 diabetes, Myasthenia gravis
rs2395029	HLA_B_5701	2.5	0.92	Type 1 diabetes, Psoriasis
rs3129889	HLA_DRB1_15	14.88	2.7	Type 1 diabetes (protective), Multiple sclerosis
rs2476601	PTPN22	1.96	0.67	Type 1 diabetes, Autoimmune thyroid disease Crohn’s disease, Myasthenia gravis, Systemic lupus erythematosus, Rheumatoid arthritis
rs689	INS	1.75	0.56	Type 1 diabetes
rs12722495	IL2RA	1.58	0.46	Type 1 diabetes, coeliac disease, Systemic sclerosis
rs2292239	ERBB3	1.35	0.3	Type 1 diabetes
rs10509540	C10orf59	1.33	0.29	Type 1 diabetes

Supplementary table S2: HLA DR3 status of patients with coeliac disease and autoimmune thyroid disease in those with confirmed monogenic autoimmunity and unknown aetiology. X = any HLA allele other than DR3.

	Monogenic autoimmunity (n=37)			Unknown aetiology (n=42)		
	Coeliac disease (n=2)	Autoimmune thyroid disease (n=6)	Either/ both (n=8)	Coeliac disease (n=12)	Autoimmune thyroid disease (n=17)	Either/ both (n=25)
DR3/DR3	0	0	0	4	2	6
DR3/X	1	2	3	7	9	16
X/X	1	4	5	1	6	7

Chapter 2

Genotype and clinical phenotype do not predict prognosis in IPEX syndrome

M. B. Johnson, J. A. L. Houghton, E. De Franco, A. T. Hattersley, S. E. Flanagan & S. Ellard

For publication in the Journal of Clinical Immunology

Contributions

J.A.L.H assisted with the collection of clinical information from NHS diagnostic referrals and assisted with the analysis of the families with hypomorphic variants. E.D.F assisted with the statistical analysis of the patient data and presentation of the tables. A.T.H, S.E and S.E.F recruited patients into the study, assisted with the design of the study and oversaw the overall project into monogenic autoimmune diabetes. All authors assisted with the interpretation of the clinical information, contributed to discussion and gave feedback on drafts of the manuscript and figures.

My contributions to the chapter

I designed the study with A.T.H, S.E and S.E.F. I reviewed the patients' medical case notes held in Exeter, requested additional clinical information for all patients via email and collated and transcribed the data into tables. I also performed the targeted next generation sequencing genetic analysis in cases referred 2014-2016, classified the *FOXP3* variants identified according to the ACMG guidelines, performed the statistical analysis to look for genotype/phenotype relationships and predictors of prognosis, wrote the manuscript and revised it according to feedback from co-authors and created all figures and tables.

ABSTRACT

Immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, caused by hemizygous variants in *FOXP3*, is challenging to manage for clinicians and represents a devastating diagnosis for families. Classically IPEX was thought to be severe and fatal in infancy for most cases, however recent studies have identified patients with a milder clinical course. The only curative treatment for IPEX is haematopoietic stem cell transplantation (HSCT), however the risks associated with this procedure, especially in infants with severe autoimmunity, mean the decision to transplant is fraught with difficulty. We collected clinical and genetic information on a cohort of patients with IPEX syndrome (n=48) referred to our centre since 2005. We sought to identify genotype/phenotype relationships or clinical characteristics that could predict prognosis or identify patients who had a milder clinical course. We did not find evidence of a genotype/phenotype relationship, as patients with missense or null variants did not consistently differ in their presentation, disease severity or prognosis, and patients with the same variant had disparate clinical features. Furthermore, presenting feature (diabetes or enteropathy) did not predict clinical outcome. We also report the longest surviving male with a FOXP3 mutation, who is in his seventh decade of life, despite his grandson having the classical features of IPEX in infancy. The decision to undertake HSCT should therefore be based on individual clinical need and not on the variant identified or family history.

INTRODUCTION

Immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome is a severe and complex condition of autoimmunity with onset usually in the first months of life. IPEX was first reported in 1982 in a large non-consanguineous pedigree with multiple affected males [1]. Enteropathy is the most common clinical manifestation (>90% of patients) and is the presenting feature in >50% of individuals [2, 3]. Diabetes, often diagnosed in the neonatal period, is present in ~70% of patients and severe atopic dermatitis is also seen in ~70%. The estimated prevalence of IPEX syndrome is <1/1 million live births [4], though this is likely to be an underestimate; many affected individuals will not receive a genetic diagnosis due to the high rate of mortality and disparity in the availability of genetic testing around the world.

In 2000 the causative gene for IPEX was identified as forkhead-box P3 (*FOXP3*) [5]. Since its implication in IPEX syndrome >75 disease-causing variants have been reported [6]. The loss of regulatory T cells and/or their inability to supress inappropriate immune reactions underlies the disease; FOXP3 is the master transcription factor of the regulatory T cell (Treg) lineage, important not only for their development but also for their continued suppressive function [7]. Tregs are essential for maintaining immune tolerance, removing or deactivating T cells reactive to autoantigens and suppressing inappropriate inflammation.

Classically, a clinical diagnosis of IPEX was made based on pathognomonic features (enteropathy, diabetes and atopic dermatitis) and subsequently confirmed by genetic testing. Due to reductions in cost and improvements in speed and accuracy of genetic testing a genetic diagnosis of IPEX can pre-empt a clinical diagnosis [8]. Recent reports have shown that the clinical manifestation of IPEX syndrome is highly variable,

ranging from prenatal death caused by foetal hydrops [9, 10] to diabetes with enteropathy that resolved spontaneously in the 3rd decade of life [11]. Furthermore, the use of gene panel tests for diseases with clinical overlap is identifying patients with disease causing variants that do not have the classically associated phenotype [12]. Rather than representing atypical cases these are likely to represent the true spectrum of IPEX which was previously under-recognised.

Making a genetic diagnosis of IPEX syndrome can improve treatment for patients. The only curative treatment for IPEX syndrome is haematopoietic stem cell transplantation (HSCT) and there is evidence that those receiving transplantation early (<5.5 years) have significantly greater chance of survival than those who undergo transplantation later in life [13]. If undertaken before the onset of specific autoimmune manifestations HSCT can prevent irreversible damage to target tissues such as the pancreatic islets. Despite improvements in patient outcomes after HSCT, the procedure still has substantial mortality risk; approximately 1 in 6 (16%) patients undergoing HSCT do not survive to 200 days' post-transplant [14].

Taken together, the increased speed to diagnosis and recognition of atypical 'milder' cases pose a problem for clinicians involved in the management of patients with IPEX. Genetic counselling for families is challenging as whilst it can inform on recurrence risk, it cannot accurately predict clinical phenotype of individual patients. A genetic diagnosis of IPEX syndrome may lead to increased psychological stress for families, as the disease was thought to be fatal in infancy and information available generally pertains to severe cases that have been recognised by the classical phenotype. Furthermore, for patients with a milder clinical course and who may survive to adulthood the benefits of HSCT may not outweigh the risk of the procedure. A genetic diagnosis may now occur before a clinical diagnosis of IPEX such as when patients

have presented with isolated diabetes. The decision to undertake HSCT may therefore be taken before the full clinical course of the patient has been understood.

We report the largest series of probands with IPEX syndrome to date (n=48). We aimed to determine if the type of mutation (i.e. missense vs protein truncating mutations) or clinical characteristics that could be used to predict prognosis in patients with IPEX syndrome and therefore inform medical management.

METHODS

Study population

48 patients with a clinical diagnosis at referral of either isolated neonatal diabetes (NDM; n=20), IPEX syndrome (n=21), early-onset autoimmune disease (n=5) or a history of multiple affected male foetuses (n=2) were referred to the Exeter Molecular Genetics Laboratory between 2005 and 2017. Clinical information was initially taken from the patients genetic testing request form. Further information was requested from the referring clinician when results were reported and detailed follow up was requested for this study.

Molecular genetics

Where a clinical diagnosis of IPEX syndrome was made prior to referral, rapid screening of the *FOXP3* gene was performed by Sanger sequencing (n=21). When patients had isolated neonatal diabetes or the onset of multiple autoimmunity was outside infancy (>12 months) comprehensive panel testing of genes causing neonatal diabetes and monogenic autoimmune diabetes was undertaken by targeted next generation sequencing (NGS) as previously described (methods section 6.1 and [15]). All putative disease-causing variants found by targeted NGS were confirmed by Sanger sequencing. Where samples were available family member testing was undertaken to assess co-segregation.

Novel/rare variant interpretation

All novel variants identified were classified using the American College of Medical Genetics guidelines [16]. Variants which were classified as pathogenic or likely pathogenic were included in this study.

Immunoglobulin testing

Where serum samples (n = 16) were available immunoglobulin E (IgE) testing was performed in house as previously described [17].

Statistical analysis

The appropriate non-parametric (Gehan-Breslow-Wilcoxon and Wilcoxon rank) tests were used for continuous variables and the Fishers exact test was used to compare categorical variables. Statistical analyses were performed in Stata 14 (StataCorp LP, College Station, TX).

Ethical approval

The study was approved by the Genetic Beta Cell Research Bank, Exeter, U.K. with ethical approval from the North Wales Research Ethics Committee, U.K.

RESULTS

Disease causing variants in *FOXP3*

We report 34 different disease causing variants in the *FOXP3* gene identified in 48 probands and 2 family members. Seventeen novel variants were identified in 18 individuals and were all classified as pathogenic or likely pathogenic (table 1). In the 30 remaining patients, we identified 17 previously reported disease causing variants (see table 1 for HGMD/ClinVar identifiers).

Clinical characteristics of patients

In 2 families, genetic testing was undertaken on a foetal DNA sample due to a history of multiple spontaneous miscarriages of males (patient 1) and foetal hydrops (patient 2). Most of the remaining individuals presented with either diabetes (n=32, 70%) or enteropathy (n=11, 24%). Three patients (7%) presented with autoimmune hypothyroidism, recurrent severe sepsis and respiratory insufficiency, respectively. The age of presentation was similar for those presenting with diabetes or enteropathy (4 weeks [IQR 2-12] vs 4 weeks [IQR 0.3-16] respectively, $p = 0.86$). The median age of latest follow up was 1.33 years (IQR: 0.5-5.33).

The most common clinical feature was diabetes in 38/46 individuals (83%; table 1). Enteropathy was seen in 28 individuals (61%) and atopic dermatitis was seen in 20/46 (43%). Common additional features were anaemia (haemolytic [n=2] and of unknown type [n=8]); 10/46, 22%), hypothyroidism (9/46, 20%), recurrent infections (9 individuals) and nephrotic disease (9/46, 20%). Rarer features included, pulmonary disease (6 individuals) and alopecia (2 individuals). In the 21 patients where measurement of IgE was undertaken (16 tested internally) 18 (86%) had values above

the age specific reference range in keeping with previous reports of patients with IPEX syndrome having raised IgE.

Demographic information				Variant details			Clinical features														
Patient	Country of origin	Birthweight/g (gestation/weeks)	Age at latest follow up	Protein change	Nucleotide Change	HGMD ID; ClinVar ID	Presenting feature	Age at presentation	Diabetes	Autoimmune enteropathy	Atopic dermatitis	Hypothyroidism	Anaemia	Nephrotic disease	Recurrent infections	Pulmonary disease	IgE level [reference range]/ IU/L	Other clinical features	Treatment details	Current clinical condition
1	UK	NA	NA	p.Asp213fs	c.636_646del	Novel	NA	NA									ND			NA
2	UK	NA	NA	p.Arg397trp	c.1189C>T	CM010059; RCV000012160.2	NA	NA									ND			NA
3	Argentina	3180 (38)	1y 4m	p.Arg337Gln	c.1010G>A	CM0911379	Diabetes	5w	✓	✓				✓			2266 [2-97]		Insulin, meprednisone, tacrolimus, azathioprine	Died aged 1y 4m
4	Bulgaria	3420 (39)	2y 6m	p.Arg337Gln	c.1010G>A	CM0911379	Recurrent infections	<1y	✓	✓	✓				✓		*Raised	Hydronephrosis, Joint hyperflexibility, Macroglossia, Eosinophilia.		Died aged 2ys 6m
5	UK	ND (ND)	5y	p.Arg337Gln	c.1010G>A	CM0911379	Enteropathy	<4y		✓	✓				✓		34000 [2-97]	Hepato-splenomegaly	HSCT undertaken, age unknown	Died aged 5ys
6	Finland	4300 (40)	22y	p.Arg337Gln	c.1010G>A	CM0911379	Diabetes	2w	✓	✓						✓	ND	Growth hormone deficiency diagnosed at 5y, Epilepsy	Curative BMT undertaken at 18y. Now healthy, on full replacement insulin dose	Alive
7	Venezuela	3000 (38)	8m	p.Arg337Gln	c.1010G>A	CM0911379	Diabetes	4w	✓	✓	✓					✓	259 [2-34]	Died from disseminated intravascular coagulation at 8 months of age	Methotrexate and insulin therapy for diabetes	Died aged 8m
8	Germany	1590 (38)	1y 6m	p.Pro339Ala	c.1015C>G	CM086632; RCV000387030.1	Diabetes	1w	✓	✓		✓					ND	Developmental delay		Died aged 1y 6m
9	UK	ND (ND)	12 w	p.Ile346Thr	c.1037T>C	CM1110994	Enteropathy	1d		✓	✓						ND			Alive
10	UK	3050 (ND)	1y 4m	p.Arg347His	c.1040G>A	CM086633	Diabetes	8w	✓								ND	Polydactyly, delayed dentition		Alive
11	Ukraine	3880 (40)	6m	p.Arg347His	c.1040G>A	CM086633	Diabetes	4w	✓				✓				ND	Respiratory insufficiency and diaphragmatic hernia at birth		Died aged 6m
12	Turkey	3300 (39)	1y 6m	p.Arg347His	c.1040G>A	CM086633	Diabetes	4w	✓								45 [2-34]			Alive
13	Australia	3928 (39)	5y 4m	p.Arg347His	c.1040G>A	CM086633	Diabetes	8w	✓								ND			Alive
14	Morocco	3200 (39)	8m	p.?	c.1044+4A>G	CS003179	Diabetes	5d	✓	✓	✓						ND			Died aged 8m

15	UK	ND (ND)	7m	p.Ala349Thr	c.1054G>A	Novel	Enteropathy	6m		✓							*Raised	Alopecia, pancreatic exocrine dysfunction, metaphyseal chondrodysplasia		Died aged 11w
16	India	1700 (36)	30y	p.Ile363Leu	c.1087A>C	Novel	Diabetes	6w	✓								ND	b12 deficiency (without evidence of celiac disease)		Alive
17	UK	ND (ND)	9m	p.Met370Val	c.1108A>G	RCV000414229.1	Respiratory insufficiency	5m			✓				✓	✓	2396 [2-34]			Alive
18	Vietnam	2400 (41)	4 w	p.Pro378Leu	c.1133C>T	Novel	Diabetes	2w	✓			✓	✓				ND			Alive
19	India	ND (ND)	2m	p.Ala384Thr	c.1150G>A	CM010058; RCV000012163.13	Diabetes	1w	✓								ND			Alive
20	India	3600 (39)	4m	p.Ala384Thr	c.1150G>A	CM010058; RCV000012163.13	Hypothyroidism	1d	✓		✓	✓					ND			Died aged 4m
21	UK	ND (ND)	25y	p.Ala384Thr	c.1150G>A	CM010058; RCV000012163.13	Diabetes	2w	✓	✓	✓	✓			✓	✓	*Raised	Malnutrition		Alive
22	South Africa	2500 (32)	4y 2m	p.Arg386His	c.1157G>A	CM109690	Enteropathy	1w		✓	✓		✓		✓		*Raised	Hepatosplenomegaly, Thrombocytopenia, presented as neonate with hyperinsulinaemia which spontaneously resolved after 3 weeks	Prednisolone (effective), then rituximab, tacrolimus and oral steroids. Curative HSCT undertaken at 10mo.	Alive
23	Pakistan	2600 (37)	3 w	p.Asn388Ser	c.1163A>G	Novel	Diabetes	1w	✓	✓							ND	Exocrine pancreatic deficiency		Died aged 6m
24	Canada	ND (ND)	7m	p.Arg397Gln	c.1190G>A	CM109691	Enteropathy	16w		✓							ND			Alive
25	Netherlands	ND (ND)	18y	p.Glu399Lys	c.1195G>A	Novel	Diabetes	11m	✓	✓	✓						0.1 [2-214]	IgA deficiency, muscle weakness		Alive
26	Czech Republic	2400 (36)	25y	p.Val408Met	c.1222G>A	CM0911380	Diabetes	1d	✓					✓			ND			Alive
27	El Salvador	ND (ND)	23 w	p.Val408Met	c.1222G>A	CM0911380	Diabetes	7w	✓								ND			Alive
28	Germany	3720 (40)	9y	p.Val408Met	c.1222G>A	CM0911380	Diabetes	3w	✓	✓		✓					ND	Developmental delay		Alive
29	Turkey	4300 (38)	14 w	p.Glu412_Arg420del	c.1234_1260del	Novel	Diabetes	9w	✓	✓	✓		✓		✓		ND	Thrombocytopenia		Alive
30	Sri Lanka	ND (40)	1y 3m	p.Glu412Asp	c.1236G>C	Novel	Diabetes	18w	✓					✓			ND		Prednisolone for nephrotic syndrome	Died aged 2ys
31	Guatemala	2720 (38)	23 w	p.Arg414fs	c.1240del	Novel	Enteropathy	9w	✓	✓	✓	✓			✓		*Raised			Died aged 5m

32	Israel	2000 (38)	2m	p.Cys424Tyr	c.1271G>A	CM078706	Diabetes	1w	✓				✓	✓		✓	ND	Persistent metabolic acidosis		Died aged ?
33	UK	ND (ND)	6 w	p.Asn426fs	c.1276_1286del	Novel	Enteropathy	2d		✓	✓						4899 [2-34]	Osteopenia, left ventricle hypertrophy		Died aged <3ys
34	USA	2995 (38)	14y	p.?	c.210+2T>C	Novel	Enteropathy	4y	✓	✓			✓				ND	hypogonadism secondary to malnourishment		Died aged 14ys
35	UK	ND (ND)	9m	p.Leu76fs	c.227delT	CD013979	Diabetes	1d	✓	✓		✓			✓		ND	Apnoea and bradycardia, osteopenia, seizures, cerebral haemorrhage		Died aged 9m
36	UK	ND (ND)	2y	p.?	c.-23G>A	Novel	Enteropathy	3w	✓	✓	✓						>1000 [2-34]	Rhinitis, failure to thrive	Partially curative HSCT undertaken at 2 years	Alive
37	Australia	4000 (40)	20y	p.Phe102fs	c.305delT	Novel	Diabetes	6m	✓								ND	Mild gastritis at 17y. Raised LFTs, diagnosed with primary sclerosing cholangitis at 19y. Had recurrent boils, now resolved.	Ursofalk for liver disease, mesalazine for gastritis	Alive
38	USA	3180 (40)	8m	p.Thr108Met	c.323C>T	CM066087	Diabetes	25w	✓								2950 [2-34]			Alive
39	USA	2390 (39)	8m	p.Ser181Thr	c.542G>C	Novel	Diabetes	1d	✓		✓						6.3 [2-34]	Milk protein allergy		Alive
40	Indonesia	3600 (39)	2y 4m	p.Lys250del	c.748_750del	CD096416	Diabetes	5m	✓	✓	✓	✓	✓				7173 [<144]		Immunosuppressive (prednisolone then cyclosporine)	Alive
41	Indonesia	2400 (36)	2y 2m	p.Lys250del	c.748_750del	CD096416	Diabetes	2w	✓	✓			✓				>10,000 (<45)	Hepatosplenomegaly, Developmental delay		Alive
42	Iran	2150 (38)	4y 5m	p.Glu251del	c.751_753del	CM003191; RCV000012166.11	Diabetes	2w	✓	✓				✓		✓	2089 [2-199]			Alive
43	Sri Lanka	2900 (40)	9y 10m	p.Glu251del	c.751_753del	CM003191; RCV000012166.11	Diabetes	9m	✓		✓		✓	✓			ND	Chronic cutaneous candidiasis, Alopecia		Alive
44	USA	4307 (37)	4y	p.Met256Val	c.766A>G	Novel	Diabetes	7w	✓		✓			✓			ND	Inflammatory arthritis, eosinophilia		Alive
45	Netherlands	3850 (41)	9y 2m	p.Met256Val	c.766A>G	Novel	Diabetes	10m	✓		✓			✓			0.1 [2-696]			Alive
46	Iran	3150 (40)	14 w	p.?	c.816+7G>C	CS107134	Enteropathy	1d	✓	✓				✓			ND	Hypoxic brain injury at birth		Died aged 3m
47	USA	2865 (38)	6y 1m	p.Glu323Lys	c.967G>A	Novel	Enteropathy	5w	✓	✓	✓				✓		>3000 [2-34]		Curative HSCT undertaken at 6mo	Alive
48	Vietnam	3500 (39)	10m	p.Phe331Val	c.991T>G	Novel	Diabetes	12w	✓	✓		✓					730 [<130]			Died aged 10m

Table 1: Clinical and genetic characteristics of 48 probands with *FOXP3* variants. ND – no data available. NA – not applicable as testing performed on foetal DNA. Variants are described based on HGVS guidelines and refer to *FOXP3* transcript NM_014009.3.

Identifying *FOXP3* disease-causing variants before clinical IPEX

Twenty patients were referred with diabetes in the absence of any notable additional clinical manifestations at referral. Of these, 8 (40%) went on to develop the classical features of IPEX syndrome after a genetic diagnosis was made and within the first 12 months of life. The time from referral to diagnosis was faster in those with classical IPEX at referral (n=21) compared to those with isolated diabetes (n=20) (5.3 weeks v 21.6 weeks, p=0.001). The median age at referral was similar in the two groups (34.7 weeks v 58.3 weeks, p=0.51).

No evidence for a genotype/phenotype relationship

Variant type (protein truncating [n=9], in-frame deletions [n=5] or missense [n=34]) did not predict clinical presentation or development of the classic features of IPEX syndrome (figure 1). The proportion of different variant types was the same in those with isolated diabetes at testing and those with other features indicative of IPEX syndrome (p=0.239). The spectrum of variant types was also the same in those who presented with diabetes compared to those presenting with enteropathy (p=0.361). Mortality rates for those with missense variants (12/34 deceased) and those with protein truncating variants (6/9 deceased) were similar (p=0.24), including when considering only those with limited clinical information (last update <2 years, p=0.08).

Assessment of a larger group of patients with IPEX syndrome is warranted to increase statistical power, as the number of patients with protein truncating and in-frame deletion mutations is low. Furthermore, studies to assess the function of the missense mutations by DNA binding assays could be performed to identify missense variants that are hypomorphic and retain some function against those

that abrogate FOXP3 function completely, as these may represent a separate group which are skewing these results.

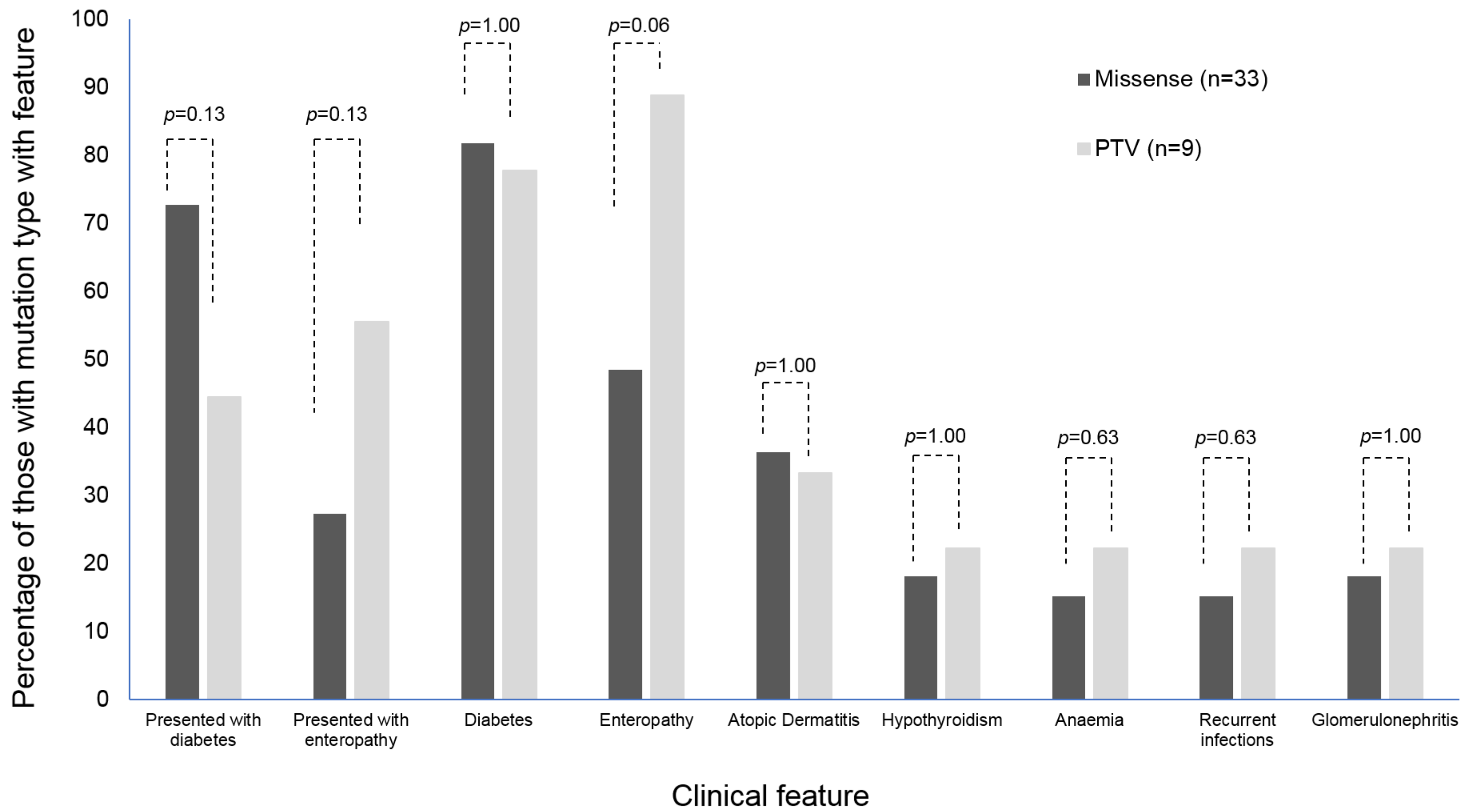

Percentage of those with mutation type with feature
100
90
80
70
60
50
40
30
20
10
0
p=0.13
p=0.13
p=1.00
p=0.06
p=1.00
p=1.00
p=0.63
p=0.63
p=1.00
Missense (n=33)
PTV (n=9)
Presented with diabetes
Presented with enteropathy
Diabetes
Enteropathy
Atopic Dermatitis
Hypothyroidism
Anaemia
Recurrent infections
Glomerulonephritis
Clinical feature

Figure 1: Proportion of patients with phenotypic characteristics by their variant type. Black bars show patients with missense variants (n=33). Grey bars show patients with protein truncating variants. None of the phenotypes/clinical characteristics show significant difference between the two groups.

Furthermore, and in keeping with previous published cases of IPEX syndrome, identical disease causing variants were identified in patients with disparate clinical features. For example, the phenotype associated with the p.Val408Met variant ranged from diabetes and nephrotic syndrome at 25 years (patient 26) to classical IPEX syndrome (including neonatal diabetes, autoimmune enteropathy and hypothyroidism) diagnosed in infancy (patient 28).

Variation in presentation within the same family

Even in members of the same family with the same variant, there was wide variation in the clinical presentation and severity of disease between affected males (figure 2). The proband (patient 13, 3:iii) presented with neonatal diabetes, eczema (which subsequently resolved) and chronic diarrhoea resulting in a clinical diagnosis of IPEX syndrome. Genetic testing identified the previously reported missense variant p.Arg347His. Family member testing identified that his mother and aunt were carriers of the variant; his male cousin had also inherited the variant. This cousin was affected with isolated eczema in infancy which resolved spontaneously. He is currently clinically well and free from any conditions associated with IPEX at the age of 10 years. Intriguingly, testing of the maternal grandparents identified the variant in the maternal grandfather who had been affected with eczema which resolved in infancy and was diagnosed with ulcerative colitis aged 13 years. His colitis is currently described as manageable with treatment, and his symptoms reportedly do not affect his day to day life. He is currently 61 years old which, to our knowledge, makes him the oldest surviving male carrier of a *FOXP3* variant.

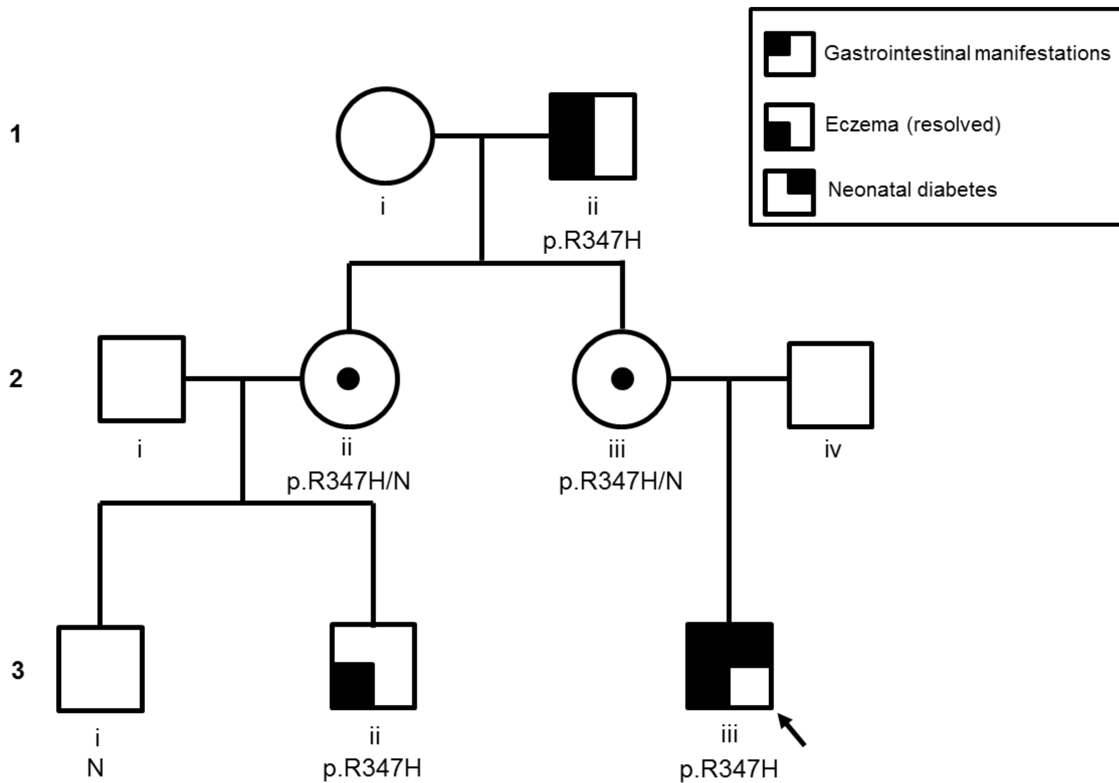


Figure 2: Clinical manifestations in a family with a *FOXP3* variant. The proband (3:iii, patient 13) has classical IPEX syndrome diagnosed in infancy. His cousin (3:ii), who is also a variant carrier, had eczema in infancy that resolved spontaneously. Strikingly, the 61-year-old maternal grandfather has the variant and had eczema in infancy which resolved and had ulcerative colitis diagnosed at age 13.

Milder clinical course despite predicted loss of FOXP3

One patient with a protein truncating variant (PTV) (c.305delT, p.Phe201fs), predicted to result in the complete loss of FOXP3, had a milder clinical course having presented with isolated diabetes at 6 months (figure 3). Routine antibody screening identified persistently positive tissue transglutaminase antibody and, due to a history of lethargy and abdominal pain, the patient underwent a ileocolonoscopy and upper gastrointestinal (GI) endoscopy at age at 17 years. This identified patchy inflammation and a diagnosis of gastritis was made. Villous atrophy, found in most cases of IPEX-associated autoimmune enteropathy, was not present. The patient also had abnormal liver function test results from age 17 and a diagnosis of primary sclerosing cholangitis was made at age 19, which is responding well to treatment with ursodeoxycholic acid. The patient is currently clinically stable at 23 years and has had improvement in his liver function and GI symptoms.

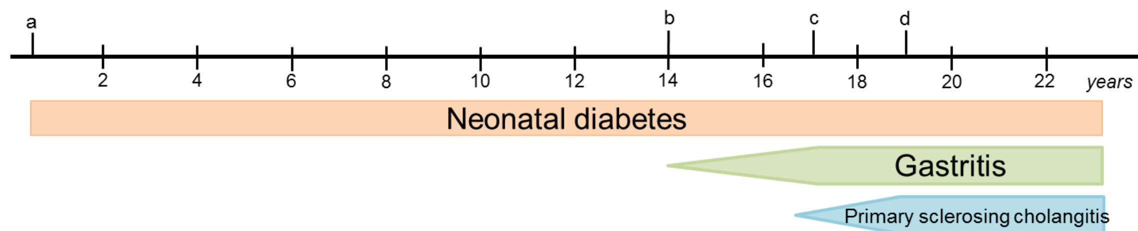


Figure 3: clinical course of patient 37 (c.305del). a – diagnosis of neonatal diabetes. b – onset of GI symptoms and positive tissue transglutaminase antibody result. c – diagnosis of gastritis and abnormal liver function detected. d – diagnosis of primary sclerosing cholangitis.

Presenting feature does not predict clinical course

Having diabetes as the presenting feature did not predict a different clinical course compared to those who presented with enteropathy (table 2). A similar proportion of those presenting with diabetes (n=32) and those presenting with enteropathy (n=11) developed classical IPEX within the first 12 months of life (19/32, 59% v 6/11, 55%; p=1.00). The distribution of other clinical features (atopic dermatitis, hypothyroidism, anaemia, glomerulonephritis, recurrent infections and pulmonary disease) was also similar in the two groups (p = 0.55). Eighteen patients are deceased in our cohort, highlighting the clinical complexity and severity of IPEX syndrome. Mortality rates were similar for those presenting with diabetes compared to those presenting with enteropathy (10/32 v 4/11 deceased by age 5, p=0.61).

Clinical/demographic feature	All patients (n=46)	Diabetes presenting feature (n=32)	Enteropathy presenting feature (n=11)
Demographic features			
Median age at onset of 1st disease/weeks [IQR]	4 [0.1-14]	4 [2-12]	4 [0.3-16]
Median age at referral or follow up/years [IQR]	1.3 [0.5-5.3]	1.5 [0.7-9.2]	0.6 [0.3-5]
Median time ref to dx/weeks [IQR]	11.7 [2.3-25.1]	17.1 [6-36.1]	1.9 [1-12.9]
Median birthweight/g (gestation/weeks) n=31	3165 (39)	3180 (39)	2865 (38)
Clinical features			
Classical IPEX*	31 (67%)	20 (63%)	9 (82%)
Diabetes	39 (85%)	32 (100%)	5 (45%)
Autoimmune enteropathy	28 (61%)	16 (50%)	11 (100%)
Atopic dermatitis	20 (43%)	10 (31%)	7 (64%)
Hypothyroidism	9 (20%)	7 (22%)	1 (9%)
Anaemia	10 (22%)	8 (25%)	2 (18%)
Nephrotic disease	9 (20%)	8 (25%)	1 (9%)
Recurrent infections	9 (20%)	3 (9%)	4 (36%)
Pulmonary disease	6 (13%)	5 (16%)	0 (0%)
Hyper IgE	18/19 (95%)	9/10 (90%)	9/9 (100%)

Table 2: clinical features of patients with disease-causing variants in *FOXP3*.

*classical IPEX is defined as 2 or more of enteropathy, diabetes and atopic dermatitis.

DISCUSSION

We report 34 distinct disease-causing variants in *FOXP3* in 48 families, the largest series of IPEX patients to date. We have shown that clinical presentation does not predict clinical course or prognosis, and did not find evidence of phenotype-genotype relationships in IPEX syndrome as the type of variant identified (i.e. missense or protein truncating) did not predict clinical phenotype.

We observed highly variable clinical manifestations in our cohort, however most patients had diabetes and/or enteropathy in keeping with previous reports of IPEX syndrome. A higher proportion of our cohort had diabetes compared to previous reports [1, 2]. This is likely to be due to referral bias as we actively recruit patients with either neonatal diabetes and/or early-onset autoimmune diabetes for research-funded genetic testing (www.diabetesgenes.com). Enteropathy was reported in 61% of our cohort (28/46), lower than previous reports of IPEX where >90% have enteropathy [1, 2]. This is likely to reflect the increasing recognition of the variability in IPEX syndrome.

We did not observe any correlation between the genotype of patients and their phenotype; those with missense variants did not have different clinical features or prognosis to those with protein truncating variants. The mortality rate for missense variants and PTVs was similar (p=0.24). This is in keeping with previously reported patients where data on mutation type and survival was available (n=65; missense 21/44 deceased vs PTV 8/21 deceased, p=0.56) (3). Previous studies have identified a normal number of Tregs in some patients with missense or small in-frame deletions in *FOXP3* [3, 4]. These were proposed to result in residual FOXP3 function, although further studies on different patients with the same variants did not detect FOXP3$^+$ Tregs [5], and the observed phenotype was not consistently milder in these patients.

The resulting disease manifestations in IPEX must therefore depend on more than the function of FOXP3 and the Treg compartment.

We have identified the oldest surviving patient with IPEX syndrome reported to date (figure 1) (current age 61). He had eczema in infancy which resolved and episodes of anaemia, and has a diagnosis of ulcerative colitis which is reportedly managed well with treatment. His grandson, patient 13, presented with neonatal diabetes at 8 weeks leading to genetic testing and went on to develop enteropathy in infancy. The variant this family carries is the p.Arg347His variant that has been previously described as causing a milder form of IPEX syndrome, though some patients have classic features in infancy [3, 4, 6-10].

The variability in phenotype is not restricted to patients with missense variants. We identified a frameshift variant in patient 36 which is predicted to result in complete loss of FOXP3 via nonsense mediated decay. Despite this, the patients' only feature until 19 years of age was diabetes (diagnosed at 6 months) and he is clinically stable at the age of 23 years. A previously reported patient with a PTV spanning the same base (c.304_305del, p.Phe102fs) had severe IPEX syndrome (enteropathy, atopic dermatitis, autoimmune haemolytic anaemia and neonatal diabetes) diagnosed before the age of 6 months which was successfully treated by HSCT [11].

IPEX syndrome was classically defined as a triad of early-onset diabetes, autoimmune enteropathy and severe atopic dermatitis with additional immunodysregulatory features. Our study, along with other recent reports, has extended the phenotype which now ranges from fatal autoimmunity with prenatal onset to isolated diabetes in adulthood [8, 12, 13]. These cases represent a spectrum of disease associated with

variants in *FOXP3* and it is likely that IPEX syndrome is under-recognised; patients without severe disease in infancy are unlikely to undergo genetic testing.

The wide variability of clinical manifestations, even in families with the same variant, and lack of features predictive of prognosis means a genetic diagnosis can be distressing for families and medical management is challenging for clinicians. The decision to undertake HSCT in patients with IPEX carries substantial risk and prognosis cannot accurately be predicted by genetic or clinical information. Treatment must therefore remain based on the clinical condition of the patient. An early genetic diagnosis can facilitate careful monitoring of the patient's clinical condition and allow the search for a matched donor to begin before possible progression of disease, even if the prognosis of the patient remains unknown. A genetic diagnosis for families allows genetic counselling to inform them of recurrence risk and will facilitate pre-natal testing, however prediction of prognosis for individuals affected is not possible.

Whilst we report the largest single cohort of patients with IPEX syndrome, the numbers are still relatively low and longitudinal data on clinical course is not available for many patients (average age at follow up; 1.33 years [IQR: 0.5-5.33]). A longitudinal study of the patients with IPEX syndrome that survive into adulthood may offer better insights into the clinical course and identify characteristics of patients with milder disease. This has been beneficial for patients with autoimmune polyendocrine syndrome type 1 (APS1) caused by biallelic variants in *AIRE* [14-17]. Studies of patients with APS1 across their lifetime have enabled optimal treatment and follow-up strategies to be proposed and identified a putative association with disease course and human leukocyte antigen (HLA) alleles.

In conclusion, whilst faster and more comprehensive genetic testing can allow for rapid treatment intervention, as more atypical cases are identified the medical management and decision to undertake HSCT for patients with IPEX has become increasingly difficult. We did not find evidence for a genotype/phenotype relationship in our cohort, in keeping with previous reports, and clinical presentation did not predict prognosis. The decision to intervene with HSCT cannot be made based on *FOXP3* genotype or clinical presentation alone and must be undertaken based on individual clinical need. Further studies of the factors which dictate clinical phenotype in IPEX syndrome are warranted.

References

1. Powell BR, Buist NR, Stenzel P. An X-linked syndrome of diarrhea, polyendocrinopathy, and fatal infection in infancy. The Journal of pediatrics. 1982;100(5):731-7.

2. Johnson MB, Hattersley AT, Flanagan SE. Monogenic autoimmune diseases of the endocrine system. The lancet Diabetes & endocrinology. 2016;4(10):862-72.

3. d'Hennezel E, Bin Dhuban K, Torgerson T, Piccirillo CA. The immunogenetics of immune dysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome. Journal of medical genetics. 2012;49(5):291-302.

4. Hannibal MC, Torgerson, T. GeneReviews Internet: University of Washington; 2011 [updated 27/01/2011. Available from: https://www.ncbi.nlm.nih.gov/books/NBK1118.

5. Chatila TA, Blaeser F, Ho N, Lederman HM, Voulgaropoulos C, Helms C, et al. JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic disregulation syndrome. The Journal of clinical investigation. 2000;106(12):R75-81.

6. Stenson PD, Ball EV, Mort M, Phillips AD, Shiel JA, Thomas NS, et al. Human Gene Mutation Database (HGMD): 2003 update. Human mutation. 2003;21(6):577-81.

7. Li X, Zheng Y. Regulatory T cell identity: formation and maintenance. Trends in immunology. 2015;36(6):344-53.

8. Rubio-Cabezas O, Minton JA, Caswell R, Shield JP, Deiss D, Sumnik Z, et al. Clinical heterogeneity in patients with FOXP3 mutations presenting with permanent neonatal diabetes. Diabetes care. 2009;32(1):111-6.

9. Shehab O, Tester DJ, Ackerman NC, Susan Cowchock F, Ackerman MJ. Whole Genome Sequencing Identifies Etiology of Recurrent Male Intrauterine Fetal Death. Prenatal diagnosis. 2017.

10. Xavier-da-Silva MM, Moreira-Filho CA, Suzuki E, Patricio F, Coutinho A, Carneiro-Sampaio M. Fetal-onset IPEX: report of two families and review of literature. Clinical immunology (Orlando, Fla). 2015;156(2):131-40.

11. Seidel MG, Boztug K, Haas OA. Immune Dysregulation Syndromes (IPEX, CD27 Deficiency, and Others): Always Doomed from the Start? Journal of clinical immunology. 2016;36(1):6-7.

12. De Franco E, Flanagan SE, Houghton JA, Lango Allen H, Mackay DJ, Temple IK, et al. The effect of early, comprehensive genomic testing on clinical care in neonatal diabetes: an international cohort study. Lancet (London, England). 2015;386(9997):957-63.

13. Kucuk ZY, Bleesing JJ, Marsh R, Zhang K, Davies S, Filipovich AH. A challenging undertaking: Stem cell transplantation for immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome. The Journal of allergy and clinical immunology. 2016;137(3):953-5.e4.

14. Gooley TA, Chien JW, Pergam SA, Hingorani S, Sorror ML, Boeckh M, et al. Reduced mortality after allogeneic hematopoietic-cell transplantation. The New England journal of medicine. 2010;363(22):2091-101.

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

16. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus

recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genetics in medicine : official journal of the American College of Medical Genetics. 2015;17(5):405-24.

17. Johnson MB, Flanagan SE, Martins TB, Hill HR, Hattersley AT, McDonald TJ. Low IgE Is a Useful Tool to Identify STAT3 Gain-of-Function Mutations. Clinical chemistry. 2016;62(11):1536-8.

18. McMurchy AN, Gillies J, Allan SE, Passerini L, Gambineri E, Roncarolo MG, et al. Point mutants of forkhead box P3 that cause immune dysregulation, polyendocrinopathy, enteropathy, X-linked have diverse abilities to reprogram T cells into regulatory T cells. The Journal of allergy and clinical immunology. 2010;126(6):1242-51.

19. Passerini L, Di Nunzio S, Gregori S, Gambineri E, Cecconi M, Seidel MG, et al. Functional type 1 regulatory T cells develop regardless of FOXP3 mutations in patients with IPEX syndrome. European journal of immunology. 2011;41(4):1120-31.

20. Otsubo K, Kanegane H, Kamachi Y, Kobayashi I, Tsuge I, Imaizumi M, et al. Identification of FOXP3-negative regulatory T-like (CD4(+)CD25(+)CD127(low)) cells in patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome. Clinical immunology (Orlando, Fla). 2011;141(1):111-20.

21. Gambineri E, Perroni L, Passerini L, Bianchi L, Doglioni C, Meschi F, et al. Clinical and molecular profile of a new series of patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome: inconsistent correlation between forkhead box protein 3 expression and disease severity. The Journal of allergy and clinical immunology. 2008;122(6):1105-12.e1.

22. Scaillon M, Van Biervliet S, Bontems P, Dorchy H, Hanssens L, Ferster A, et al. Severe gastritis in an insulin-dependent child with an IPEX syndrome. Journal of pediatric gastroenterology and nutrition. 2009;49(3):368-70.

23. Wildin RS, Smyk-Pearson S, Filipovich AH. Clinical and molecular features of the immunodysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome. Journal of medical genetics. 2002;39(8):537-45.

24. Zama D, Cocchi I, Masetti R, Specchia F, Alvisi P, Gambineri E, et al. Late-onset of immunodysregulation, polyendocrinopathy, enteropathy, x-linked syndrome (IPEX) with intractable diarrhea. Italian journal of pediatrics. 2014;40:68.

25. Moudgil A, Perriello P, Loechelt B, Przygodzki R, Fitzerald W, Kamani N. Immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome: an unusual cause of proteinuria in infancy. Pediatric nephrology (Berlin, Germany). 2007;22(10):1799-802.

26. Bruserud O, Oftedal BE, Landegren N, Erichsen MM, Bratland E, Lima K, et al. A Longitudinal Follow-up of Autoimmune Polyendocrine Syndrome Type 1. The Journal of clinical endocrinology and metabolism. 2016;101(8):2975-83.

27. Meloni A, Willcox N, Meager A, Atzeni M, Wolff AS, Husebye ES, et al. Autoimmune polyendocrine syndrome type 1: an extensive longitudinal study in Sardinian patients. The Journal of clinical endocrinology and metabolism. 2012;97(4):1114-24.

28. Perheentupa J. Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy. The Journal of clinical endocrinology and metabolism. 2006;91(8):2843-50.

29. Zaidi G, Bhatia V, Sahoo SK, Sarangi AN, Bharti N, Zhang L, et al. Autoimmune polyendocrine syndrome type 1 in an Indian cohort: a longitudinal study. Endocrine connections. 2017;6(5):289-96.

Chapter 3

Recessively inherited *LRBA* mutations cause autoimmunity presenting as neonatal diabetes

Matthew B Johnson BSc, Elisa De Franco PhD, Hana Lango Allen PhD, Aisha Al Senani MD, Nancy Elbarbary MD, Zeynep Siklar MD, Merih Berberoglu MD, Zineb Imane MD, Alireza Haghighi DPhil, Zahra Razavi DM, Irfan Ullah MD, Saif Alyaarubi MD, Daphne Gardner MD, Sian Ellard PhD, Andrew T Hattersley DM & Sarah E Flanagan PhD

Diabetes 2017 66(8):2316-2322

Acknowledgments

S.E, A.T.H and S.E.F assisted with the design of the study. E.D-F lead on the genetic analysis and the interpretation of the exome sequencing the data. H.L-A performed bioinformatics analysis for the index case, A.A-S, N.E, Z.S, M.B, Z.I, A.H, I.U, S.A and D.G recruited patients, provided clinical information and contributed to discussion, E.D-F, A.T.H and S.E.F. gave feedback on drafts of the manuscript which was reviewed/edited by all authors.

My contributions to the chapter

I contributed to the design of the study, performed the exome library preparation for the index case, analysed the exome data with EDF, selected the replication cohort, performed wet lab work, designed the primers, designed the ddPCR assay and performed the wet-lab work and analysis of data. I collected and collated the patients' clinical information provided by their clinician, contacted the clinicians and generated formal reports for the patients, performed statistical analysis, performed a literature review of published cases and collated the data, wrote the manuscript and created the figures, and revised according to feedback from co-authors, submitted the manuscript and responded to reviewers' comments and checked the proof for typographical mistakes.

ABSTRACT

Young-onset autoimmune diabetes associated with additional autoimmunity usually reflects a polygenic predisposition but rare cases result from monogenic autoimmunity. Diagnosing monogenic autoimmunity is crucial for patients' prognosis and clinical management. We sought to identify novel genetic causes of autoimmunity presenting with neonatal diabetes (NDM; diagnosis <6 months).

We performed exome sequencing in a patient with NDM and autoimmune lymphoproliferative syndrome and his unrelated, unaffected parents and identified compound heterozygous null mutations in *LRBA*. Biallelic *LRBA* mutations cause Common Variable Immunodeficiency-8, however NDM has not been confirmed in this disorder. We sequenced *LRBA* in 169 additional patients with diabetes diagnosed <1 year without mutations in the 24 known NDM genes. We identified recessive null mutations in 8 additional probands, of which 3 had NDM (<6 months). Diabetes was the presenting feature in 6 of 9 probands. Six of 17 (35%) patients both born to consanguineous parents and with additional early-onset autoimmunity had recessive *LRBA* mutations.

LRBA testing should be considered in patients with diabetes diagnosed <12 months, particularly if they have additional autoimmunity or are born to consanguineous parents. A genetic diagnosis is important as it can enable personalized therapy with abatacept, a CTLA4 mimetic, and inform genetic counselling.

Clustering of diabetes with early-onset autoimmunity in very early childhood is usually due to a combination of extreme polygenic risk and environmental exposure. Rarely, a mutation in a single gene is the aetiological cause and the identification of the underlying monogenic defect can give important insights into mechanisms of beta-cell autoimmunity and pathways of immune tolerance[1, 18-30]. Due to significant clinical overlap, discriminating patients with causative mutations in a single gene from those with a polygenic aetiology remains a challenge.

A prompt diagnosis of monogenic autoimmunity is crucial as it informs clinical management and targeted therapies may be possible. *FOXP3* mutations in males cause Immunodysregulation, Polyendocrinopathy, Enteropathy, X-Linked (IPEX) syndrome[1] which can be treated with a haematopoietic stem cell transplant (HSCT). If performed early HSCT can cure the life-threatening enteropathy as well as prevent the onset of autoimmune-mediated diabetes[31]. In an individual with polyarthritis, scleroderma and autoimmune haemolytic anaemia resulting from an activating *STAT3* mutation, treatment with tocilizumab, a monoclonal antibody against IL-6, resulted in marked improvement in their symptoms[32]. Patients with Common Variable Immunodeficiency-8 (CVID-8), caused by recessively inherited mutations in Lipopolysaccharide-responsive Beige-like Anchor protein (*LRBA*), can be successfully treated with Abatacept, a mimetic for CTLA-4. CTLA-4 is a potent suppressive receptor that acts as an immune checkpoint and is post-translationally regulated by LRBA. [18].

Monogenic autoimmune disease often presents extremely early; for example, mutations in the *STAT3*, *FOXP3* or *IL2RA* genes commonly present with neonatal diabetes[1, 30, 33]. Mutations in *LRBA* typically presents with severe autoimmune disease early in childhood and diabetes is a feature in 22% of patients, however neonatal diabetes has not been confirmed [19].

We identified biallelic mutations in *LRBA* in an individual with neonatal diabetes diagnosed at 7 weeks and additional early-onset autoimmunity of unknown cause. We go on to show that this is a relatively common aetiology of neonatal or infancy-onset diabetes when patients have additional early-onset autoimmune disease and are born to consanguineous parents.

RESEARCH DESIGN AND METHODS

Gene discovery using exome sequencing: The initial case presented in diabetic keto-acidosis (blood glucose concentration: 53 mmol/L) at the age of seven weeks and developed thrombocytopenia and autoimmune lymphoproliferative disease aged three years. To define the genetic aetiology, having excluded all 24 known causes of neonatal diabetes, we used exome sequencing and trio analysis of the proband and his unaffected, unrelated parents to search for *de novo* heterozygous mutations and/or compound heterozygous mutations. Exome sequencing was performed using Agilent's SureSelect Human All Exon kit (v5) with paired end 100bp read length sequencing undertaken on an Illumina HiSeq 2500. For single nucleotide variant identification, the resulting reads were aligned to the hg19 reference genome according to GATK[34, 35] best practice guidelines. An in house script was used to remove synonymous variants, those outside the coding region or conserved splice site, and variants present in dbSNP131 or the ExAC database with a MaF greater than 0.1%, as previously reported[30]. We used the R software package ExomeDepth[36] to detect copy number variation. Coverage and read depth data for the trio is provided in supplementary table S1.

Follow up testing in selected neonatal/infancy onset diabetes: In our cohort of 1561 patients diagnosed with diabetes before the age of 12 months, 169 did not have a mutation in a known gene and were screened for mutations in *LRBA* (figure 2). Of these, 54 patients were consanguineous and within this group 17 individuals had autoimmune disease. Autoimmune disease was also present in 25 of 116 non-consanguineous patients. Consanguineous unions were defined as previously described [37], either known related parents (n=25) or patients who were from regions with high levels of consanguineous unions (n=29)[37]. The additional autoimmune

disease was diagnosed before 5 years and included hypothyroidism (15/42), Coeliac disease/autoimmune enteropathy (16/42) and inflammatory arthritis (3/42) (further details are provided in supplementary table S2).

The 24 known causes of neonatal diabetes had been previously excluded by next generation-sequencing [30, 38], and methylation specific MLPA (MRC Holland) in all 169 patients. Targeted next generation sequencing of the 58 exons and flanking intronic regions of *LRBA* (NM_006726.4) was performed as previously described[39] in the 169 patients with diabetes diagnosed before 1 year. Putative mutations were confirmed by Sanger sequencing or by droplet digital PCR (details available on request). When available samples from affected siblings and unaffected parents underwent mutation testing. Clinical information was collected from the patient's medical records by the referring clinician. All subjects and/or their parents gave informed consent for genetic testing. The study was approved by the Genetic Beta Cell Research Bank, Exeter, U.K. with ethical approval from the North Wales Research Ethics Committee, U.K.

RESULTS

Molecular Genetics

We initially searched for *de novo* mutations in the proband and unrelated, unaffected parent trio. This identified four coding variants in the proband, all of which were present in the Exome Aggregation Consortium (ExAC) database[40] of >60,000 patients not diagnosed with any severe paediatric disease (see supplementary table S3). We considered these unlikely to be causative and switched our analysis to look for recessive causes.

We identified compound heterozygous mutations in *LRBA* and *PKHD1L1*. The two novel null mutations in *LRBA* (p.D1053fs*2; c.3156del and p.S2659*; c.7976C>G) were considered likely to be pathogenic as bi-allelic mutations in this gene are known to cause Common Variable Immunodeficiency-8 (CVID-8)[24]. Variants in *PKHD1L1* have not been associated with Mendelian disease and there are 549 individuals in the ExAC database (controls without severe paediatric disease) with homozygous loss of function mutations [40], suggesting that loss of *PKHD1L1* does not cause childhood-onset disease.

Whilst diabetes has been reported as a feature in 11/57 patients[18-29] with *LRBA* mutations, only two patients were diagnosed before the age of one year; one at 4 months and one at 7 months. The median age of diabetes diagnosis in the other patients was two years (range 1-9 years). *LRBA* encodes the Lipopolysaccharide-responsive beige-like anchor protein - an essential post-translational regulator of the CTLA-4 receptor involved in the suppression of regulatory T cells[18].

Sequence analysis of *LRBA* in 169 patients diagnosed with diabetes before 12 months identified homozygous null mutations in eight additional probands and one affected

sibling (see table 1, figure 1, supplementary figure S2). All mutations introduce premature termination codons (3 nonsense, 4 frameshifts, 2 mutations affecting splicing and one whole exon deletion) and are predicted to result in complete loss of the LRBA protein. Carrier status was confirmed in parents when samples were available (see figure 1).

Table 2 shows the distribution of individuals with *LRBA* mutations by age of diabetes diagnosis and parental consanguinity. Interestingly the highest proportion of those with a mutation were diagnosed with diabetes between 6 and 12 months. We identified *LRBA* mutations in 9 of 1561 patients diagnosed with diabetes before 12 months, giving a minimum prevalence of 0.6% in our cohort (Table 2).

Seven of 54(13%) consanguineous patients had *LRBA* mutations whilst a mutation was identified in only 2 of 25 (8%) non-consanguineous patients with additional autoimmunity. Strikingly, when the criteria of consanguinity and additional autoimmunity were combined, 6 of 17 (35%) patients harboured mutations in *LRBA* (figure 2). Using these two criteria therefore greatly increased the likelihood of identifying an *LRBA* mutation.

Clinical characteristics

The proband presented in severe diabetic ketoacidosis at the age of seven weeks (blood glucose 53 mmol/L). He was treated with a full replacement dose of insulin, was negative for anti-GAD antibodies and had a HbA1c prior to his death of 7.0% (53 mmol/mol). Thrombocytopenia and autoimmune lymphoproliferative disease were reported at the age of three years with additional features including right hemiparesis and neuromotor retardation also noted at this time. The patient died shortly before his fourth birthday due to an intracranial haemorrhage caused by thrombocytopenia.

Detailed follow up after genetic analysis revealed the proband's three elder siblings had also died in childhood at another hospital; two older sisters died at ages 12 years and 3.5 years due to complications relating to immunodeficiency and his older brother died at the age of 6.5 years due to immunodeficiency and severe enteropathy. Diabetes was not reported in these individuals and DNA was not available for testing. This family history suggests the siblings were also compound heterozygous for the *LRBA* mutations, fitting with the inheritance pattern of *LRBA.* The parents were unaffected in keeping with previous reports that haploinsufficiency of *LRBA* does not cause CVID-8 [22, 23]

All 10 patients with bi-allelic *LRBA* mutations were diagnosed with diabetes in the first 15 months of life (median: 7.5 months, range: 6 weeks – 15 months) and four of these patients met the criteria for neonatal diabetes, having been diagnosed with diabetes before the age of 6 months. All had insulin doses suggesting full replacement was required (table 1). Positivity for anti-GAD antibodies (90 U/mL; normal range <25 U/mL) was detected in just 1 of the 6 patients in whom pancreatic antibody screening for anti-GAD/IA2/ZnT8 antibodies was possible (table 1). This low prevalence of autoantibodies is in keeping with these patients having autoimmunity with a distinct mechanism to that seen in type 1 diabetes.

In 6 of the 9 probands diabetes was the presenting feature. Autoimmune disorders were present in 8 of the 9 probands (table 1) and included haematological manifestations (5/8), autoimmune enteropathy (3/8) and hypothyroidism (1/8). The remaining proband is the result of a consanguineous union and has presented with diabetes at 9 months, which is still the only clinical feature at 2 years. In three patients, recurrent respiratory infections were reported. The prognosis was poor as three of the patients are deceased; the original proband died from a cerebral haemorrhage likely

caused by thrombocytopenia, a second patient developed complications associated with nephroblastoma and a third child died of sepsis (table1).

Conclusions

We have identified 10 different loss of function *LRBA* mutations in nine probands and one sibling (figure 1 and table 1) with 4 cases having neonatal diabetes. This study increases the total number of genetic causes of neonatal diabetes to 25, and the genetic causes of severe early-onset autoimmunity that includes neonatal diabetes to 4; the others being *FOXP3* [1], *IL2RA* [33], and *STAT3* [30].

In our cohort of patients with diabetes diagnosed before 12 months, 0.6% have recessively inherited *LRBA* mutations. In contrast to other monogenic autoimmune diabetes subtypes the highest pick-up rate for *LRBA* mutations was in those individuals diagnosed with diabetes between 6 and 12 months. The combined criteria of autoimmune disease and consanguinity identified a high proportion of patients with *LRBA* mutations. Six of the 9 probands with *LRBA* mutations were suspected or proven to be consanguineous and have additional autoimmune disease. Using these criteria in our patients with infancy-onset diabetes we identified a causative mutation in 35% (6/17) of patients; (see figure 2). We therefore recommend testing for *LRBA* mutations is considered in all patients diagnosed with diabetes before the age of 12 months, particularly those who have diabetes and additional autoimmunity and are the result of consanguineous union.

Identifying *LRBA* mutations early is crucial as it may allow for the introduction of optimal treatment strategies before the disease progresses. Genetic testing of all causes of neonatal diabetes is now predominantly by targeted panels (e.g. [39, 41, 42]) occurring immediately after the diagnosis of diabetes with in the first 6 months of life[38]. Targeted sequencing should include *LRBA* as in all 4 probands with neonatal

diabetes this was the first feature of their multisystem autoimmune disorder and for one proband is currently the only feature at the age of two years.

Recessive mutations in *LRBA* are a known cause of Common Variable Immunodeficiency 8 (CVID-8, MIM #614700)[24] which often includes early-onset autoimmunity, immune dysregulation, recurrent infections and hypogammaglobinaemia with variable penetrance [18-29, 43]. Neonatal diabetes had not been observed as a feature of this disorder. The extra-pancreatic features observed in our cohort are at a similar prevalence to those reported in patients with biallelic *LRBA* mutations (supplementary Figure S1), consistent with a diagnosis of CVID-8.

Five of the 6 patients in whom testing was possible were negative for pancreatic antibodies. This suggests that the mechanism of autoimmunity may be distinct to that observed in early-onset type 1 diabetes. It may be that the autoantigens which are the target of the immune response are as yet uncharacterised, that they are not islet specific, or that the autoimmunity is cell-based rather than antibody driven. Further work to elucidate the true mechanism underlying the development of diabetes in CVID-8 is warranted and may give new insights into the pathophysiology of type 1 diabetes.

All patients we describe have functionally similar bialleleic null mutations but despite this there is considerable variation in their phenotype. For example, patient 3 presented with neonatal diabetes (diagnosed at 4 months) and has immunodeficiency, autoimmune lymphoproliferative disease, hepatosplenomegaly, lymphocytic interstitial pneumonia and recurrent chest infections diagnosed before the age of 8 years, whereas patient 7 was diagnosed with diabetes at the age of 10 months and has coeliac disease, pernicious anaemia and subclinical hypothyroidism at the age of

26 years (see table 1). The variable phenotype of patients with homozygous missense mutations seen in previous studies was not statistically different from those with protein truncating mutations; the age of onset of the first symptom is similar in both groups (median age of onset; missense mutations: 1.75 years versus nonsense mutations: 2 years, p = 0.79) [18, 22, 24, 25, 28]. It therefore seems likely that additional genetic and/or environmental factors influence the severity of the disease and the specific organs affected in these patients.

The autoimmunity observed in patients with *LRBA* mutations is considered to result from the loss of an essential immune regulatory pathway and a reduction in the suppressive action of regulatory T cells, therefore a disruption of immune tolerance[18]. It was recently shown that LRBA prevents the lysosomal degradation of the CTLA-4 receptor, facilitating its trafficking to the surface of T cells during T cell receptor (TCR) stimulation[18]. CTLA-4 is a potent suppressor, blocking co-stimulation of the TCR and therefore negatively regulating immune responses [44]. A loss of LRBA therefore results in increased CTLA-4 degradation diminishing this inhibitory pathway on T-cell activation and resulting in unchecked activation of immunologic responses.

Identifying the underlying genetic aetiology is clinically important for these patients as understanding the disease mechanism may allow the use of personalized therapy. Abatacept, a CTLA-4 mimetic that replaces the action of the lost suppressive receptor, has been used to treat 12 patients with *LRBA* mutations so far and all showed improvement in their autoimmune features[18, 22]. Therapy with abatacept had not been attempted in our patients at the time of reporting. HSCT is also an option for these patients, with successful outcome reported in 3 of 4 patients with *LRBA* mutations in whom it has been attempted[22, 27, 29].

In conclusion, we have identified *LRBA* mutations in 9 probands with early-onset diabetes (< 1 year) of whom 8 had additional autoimmune features. In 4 of these patient's diabetes was diagnosed before 6 months confirming the role of this gene in the aetiology of neonatal diabetes. As diabetes was the presenting feature in 6/9 individuals we recommend that testing for *LRBA* mutations is considered in all patients with newly diagnosed neonatal diabetes, and in those with infancy onset (< 12 months) diabetes, especially when a recessive inheritance is suspected or additional autoimmune features are present. A genetic diagnosis is critical not only for counselling on recurrence risk but it can also allow for immunomodulatory agents such as abatacept to be considered as part of the treatment regimen.

Acknowledgements

The authors thank Benjamin Bunce of The Royal Devon and Exeter NHS Foundation Trust and Richard Caswell, Matthew Wakeling and Thomas Laver of the University of Exeter Medical School for their assistance. This work was supported by a Wellcome Trust Senior Investigator Award to SE and ATH (grant number: 098395/Z/12/Z). ATH is an NIHR senior investigator. EDF is a Naomi Berrie Fellow in Diabetes Research. SEF has a Sir Henry Dale Fellowship jointly funded by the Wellcome Trust and the Royal Society (grant Number: 105636/Z/14/Z). Additional support came from the University of Exeter and the NIHR Exeter Clinical Research Facility.

Guarantor statement

A.T.H is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Conflict of interest statement

No potential conflicts of interest relevant to this article were reported.

References

1. Lo B, Zhang K, Lu W, Zheng L, Zhang Q, Kanellopoulou C, et al. AUTOIMMUNE DISEASE. Patients with LRBA deficiency show CTLA4 loss and immune dysregulation responsive to abatacept therapy. Science. 2015 Jul 24;349(6246):436-40.

2. Charbonnier LM, Janssen E, Chou J, Ohsumi TK, Keles S, Hsu JT, et al. Regulatory T-cell deficiency and immune dysregulation, polyendocrinopathy, enteropathy, X-linked-like disorder caused by loss-of-function mutations in LRBA. J Allergy Clin Immunol. 2015 Jan;135(1):217-27.

3. Alangari A, Alsultan A, Adly N, Massaad MJ, Kiani IS, Aljebreen A, et al. LPS-responsive beige-like anchor (LRBA) gene mutation in a family with inflammatory bowel disease and combined immunodeficiency. J Allergy Clin Immunol. 2012 Aug;130(2):481-8 e2.

4. Burns SO, Zenner HL, Plagnol V, Curtis J, Mok K, Eisenhut M, et al. LRBA gene deletion in a patient presenting with autoimmunity without hypogammaglobulinemia. J Allergy Clin Immunol. 2012 Dec;130(6):1428-32.

5. Gamez-Diaz L, August D, Stepensky P, Revel-Vilk S, Seidel MG, Noriko M, et al. The extended phenotype of LPS-responsive beige-like anchor protein (LRBA) deficiency. J Allergy Clin Immunol. 2016 Jan;137(1):223-30.

6. Levy E, Stolzenberg MC, Bruneau J, Breton S, Neven B, Sauvion S, et al. LRBA deficiency with autoimmunity and early onset chronic erosive polyarthritis. Clin Immunol. 2016 Jul;168:88-93.

7. Lopez-Herrera G, Tampella G, Pan-Hammarstrom Q, Herholz P, Trujillo-Vargas CM, Phadwal K, et al. Deleterious mutations in LRBA are associated with a

syndrome of immune deficiency and autoimmunity. Am J Hum Genet. 2012 Jun 8;90(6):986-1001.

8. Revel-Vilk S, Fischer U, Keller B, Nabhani S, Gamez-Diaz L, Rensing-Ehl A, et al. Autoimmune lymphoproliferative syndrome-like disease in patients with LRBA mutation. Clin Immunol. 2015 Jul;159(1):84-92.

9. Schreiner F, Plamper M, Dueker G, Schoenberger S, Gamez-Diaz L, Grimbacher B, et al. Infancy-Onset T1DM, Short Stature, and Severe Immunodysregulation in Two Siblings With a Homozygous LRBA Mutation. J Clin Endocrinol Metab. 2016 Mar;101(3):898-904.

10. Seidel MG, Hirschmugl T, Gamez-Diaz L, Schwinger W, Serwas N, Deutschmann A, et al. Long-term remission after allogeneic hematopoietic stem cell transplantation in LPS-responsive beige-like anchor (LRBA) deficiency. J Allergy Clin Immunol. 2015 May;135(5):1384-90 e1-8.

11. Serwas NK, Kansu A, Santos-Valente E, Kuloglu Z, Demir A, Yaman A, et al. Atypical manifestation of LRBA deficiency with predominant IBD-like phenotype. Inflamm Bowel Dis. 2015 Jan;21(1):40-7.

12. Tesi B, Priftakis P, Lindgren F, Chiang SC, Kartalis N, Lofstedt A, et al. Successful Hematopoietic Stem Cell Transplantation in a Patient with LPS-Responsive Beige-Like Anchor (LRBA) Gene Mutation. J Clin Immunol. 2016 Jul;36(5):480-9.

13. Flanagan SE, Haapaniemi E, Russell MA, Caswell R, Lango Allen H, De Franco E, et al. Activating germline mutations in STAT3 cause early-onset multi-organ autoimmune disease. Nat Genet. 2014 Aug;46(8):812-4.

14. d'Hennezel E, Bin Dhuban K, Torgerson T, Piccirillo CA. The immunogenetics of immune dysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome. J Med Genet. 2012 May;49(5):291-302.

15. Nademi Z, Slatter M, Gambineri E, Mannurita SC, Barge D, Hodges S, et al. Single centre experience of haematopoietic SCT for patients with immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome. Bone Marrow Transplant. 2014 Feb;49(2):310-2.

16. Milner JD, Vogel TP, Forbes L, Ma CA, Stray-Pedersen A, Niemela JE, et al. Early-onset lymphoproliferation and autoimmunity caused by germline STAT3 gain-of-function mutations. Blood. 2015 Jan 22;125(4):591-9.

17. Sharfe N, Dadi HK, Shahar M, Roifman CM. Human immune disorder arising from mutation of the alpha chain of the interleukin-2 receptor. Proc Natl Acad Sci U S A. 1997 Apr 1;94(7):3168-71.

18. Van der Auwera GA, Carneiro MO, Hartl C, Poplin R, Del Angel G, Levy-Moonshine A, et al. From FastQ data to high confidence variant calls: the Genome Analysis Toolkit best practices pipeline. Curr Protoc Bioinformatics. 2013;43:11 0 1-33.

19. DePristo MA, Banks E, Poplin R, Garimella KV, Maguire JR, Hartl C, et al. A framework for variation discovery and genotyping using next-generation DNA sequencing data. Nat Genet. 2011;43.

20. Plagnol V, Curtis J, Epstein M, Mok KY, Stebbings E, Grigoriadou S, et al. A robust model for read count data in exome sequencing experiments and implications for copy number variant calling. Bioinformatics. 2012 Nov 1;28(21):2747-54.

21. Bittles AH. A community genetics perspective on consanguineous marriage. Community Genet. 2008;11(6):324-30.

22. De Franco E, Flanagan SE, Houghton JA, Lango Allen H, Mackay DJ, Temple IK, et al. The effect of early, comprehensive genomic testing on clinical care in neonatal diabetes: an international cohort study. Lancet. 2015 Sep 5;386(9997):957-63.

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

24. Lek M, Karczewski KJ, Minikel EV, Samocha KE, Banks E, Fennell T, et al. Analysis of protein-coding genetic variation in 60,706 humans. Nature. 2016 Aug 17;536(7616):285-91.

25. Bonnefond A, Philippe J, Durand E, Muller J, Saeed S, Arslan M, et al. Highly sensitive diagnosis of 43 monogenic forms of diabetes or obesity through one-step PCR-based enrichment in combination with next-generation sequencing. Diabetes Care. 2014 Feb;37(2):460-7.

26. Gao R, Liu Y, Gjesing AP, Hollensted M, Wan X, He S, et al. Evaluation of a target region capture sequencing platform using monogenic diabetes as a study-model. BMC Genet. 2014 Jan 29;15:13.

27. Alkhairy OK, Abolhassani H, Rezaei N, Fang M, Andersen KK, Chavoshzadeh Z, et al. Spectrum of Phenotypes Associated with Mutations in LRBA. J Clin Immunol. 2016 Jan;36(1):33-45.

28. Lee KM, Chuang E, Griffin M, Khattri R, Hong DK, Zhang W, et al. Molecular basis of T cell inactivation by CTLA-4. Science. 1998 Dec 18;282(5397):2263-6.

Table 1

Patient	1	2.1	2.2	3	4	5	6	7	8	9
Genotype*	p.D1053fs/p.S2659* (c.3156del/c.7976C>A)	p.R2348* (c.7042C>T)	p.R2348* (c.7042C>T)	p.R1271* (c.3811C>T)	p.? (c.5581-1G>A)	p.M589fs (c.1764dup)	p.P816fs (c.2447del)	p.? (c.(4729+1_4730-1)_(5171+1_5172-1)del)	p.? (c.5172-2A>G)	p.I1330fs (c.3988dup)
Birth weight [g] (gestation [weeks])	2600 (35)	3200 (39)	3200 (unknown)	2700 (40)	3200 (38)	2750 (39)	3200 (40)	2965 (40)	2970 (40)	3000 (40)
Sex	Male	Male	Male	Male	Female	Female	Male	Male	Female	Male
Current Age (years)	Deceased	1	Deceased	8	Deceased	2	6	26	1	4
Known consanguinity	No	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes
Ethnicity	Turkish	Moroccan	Moroccan	Omani	Omani	Iranian	Egyptian	Chinese	Turkish	Pakistani
Diabetic features										
Age at onset	7 weeks	6 weeks	15 months	4 months	5 months	9 months	9 months	10 months	8 months	7 months
Treatment (dose)	Insulin (1U/kg/day)	Insulin (1U/kg/day)	Insulin (1.2U/kg/day)	Insulin (1U/kg/day)	Insulin (0.6U/kg/day)	Insulin (0.7U/kg/day)	Insulin (2U/kg/day)	Insulin (0.6U/kg/day)	Insulin (0.9U/kg/day)	Insulin (1.7U/Kg/day)
HbA1c† (mmol/mol)	7.0% (53)	7.1% (54)	6.6% (49)	(7.1% (54)	8.3% (67)	ND	ND	8.7% (72)	7.2% (55)	ND
Antibody Status	GAD Negative	GAD/IA2/ZnT8 Negative	ND	GAD/IA2 Negative	GAD positive	ND	ND	GAD negative	GAD/IA2 Negative	ND
Immunodysregulatory features										
Haemato-logical disorders	Thrombocytopenia; Autoimmune lymphoproliferative disease	–	Thrombocytopenia; Autoimmune lymphoproliferative disease	Agammaglobulinaemia; Autoimmune lymphoproliferative disease	–	–	Thrombocytopenia; Autoimmune haemolytic anaemia	Pernicious anaemia	–	–
Gastro-intestinal disorders	–	Autoimmune enteropathy	Autoimmune enteropathy; Hepatosplenomegaly	Hepatosplenomegaly	Autoimmune enteropathy	–	Episodes of diarrhoea	Coeliac disease	–	Chronic diarrhoea
Endocrine disorders	–	–	–	–	–	–	Autoimmune hypothyroidism	TPO Ab positive (sub clinical hypothyroidism)	–	–
Recurrent infections	–	–	Died of septic shock following unknown infection	Recurrent chest infections (Aspergillus spp.)	–	–	Pneumonia	–	Pneumonia; Otitis media	URTI‡, septicaemia
Other features										
	Cleft lip; Developmental delay; Hemiparesis. Died from intracranial bleed	–	–	Lymphocytic interstitial pneumonia	Died from Nephroblastoma	–	History of convulsions; Multiple cerebral infarctions	Parenchymal calcification of kidneys	–	–

Table 1 – Clinical features of patients with *LRBA* mutations. *All mutations are homozygous unless otherwise indicated and are described according to HGVS guidelines based on the longest isoform, NM_006726.4. Disorders reported are based on the clinical diagnosis made by the patients' physician and were not always confirmed by diagnostic investigations such as biopsies. †Most recent HbA1c recorded. ‡Upper respiratory tract infections. ND – no data. TPO Ab – thyroid peroxidase antibody.

	<6 months		6-12 months		<12 months	
	Consang*	**Non-consang†**	**Consang**	**Non-consang**	**Consang**	**Non-consang**
Total number of patients	338	892	63	268	401	1160
Number with other known genetic cause	299	761	17	56	316	817
Number *LRBA* tested in	31	74	23	41	54	116
Number of *LRBA* cases identified	3	1	4	1	7	2
Minimum prevalence (%)	**0.9%**	**0.1%**	**6.3%**	**0.4%**	**1.7%**	**0.2%**

Table 2: Minimum prevalence of *LRBA* mutations in our cohort of patients diagnosed with diabetes before 12 months. *Born to consanguineous parents. †Born to unrelated parents.

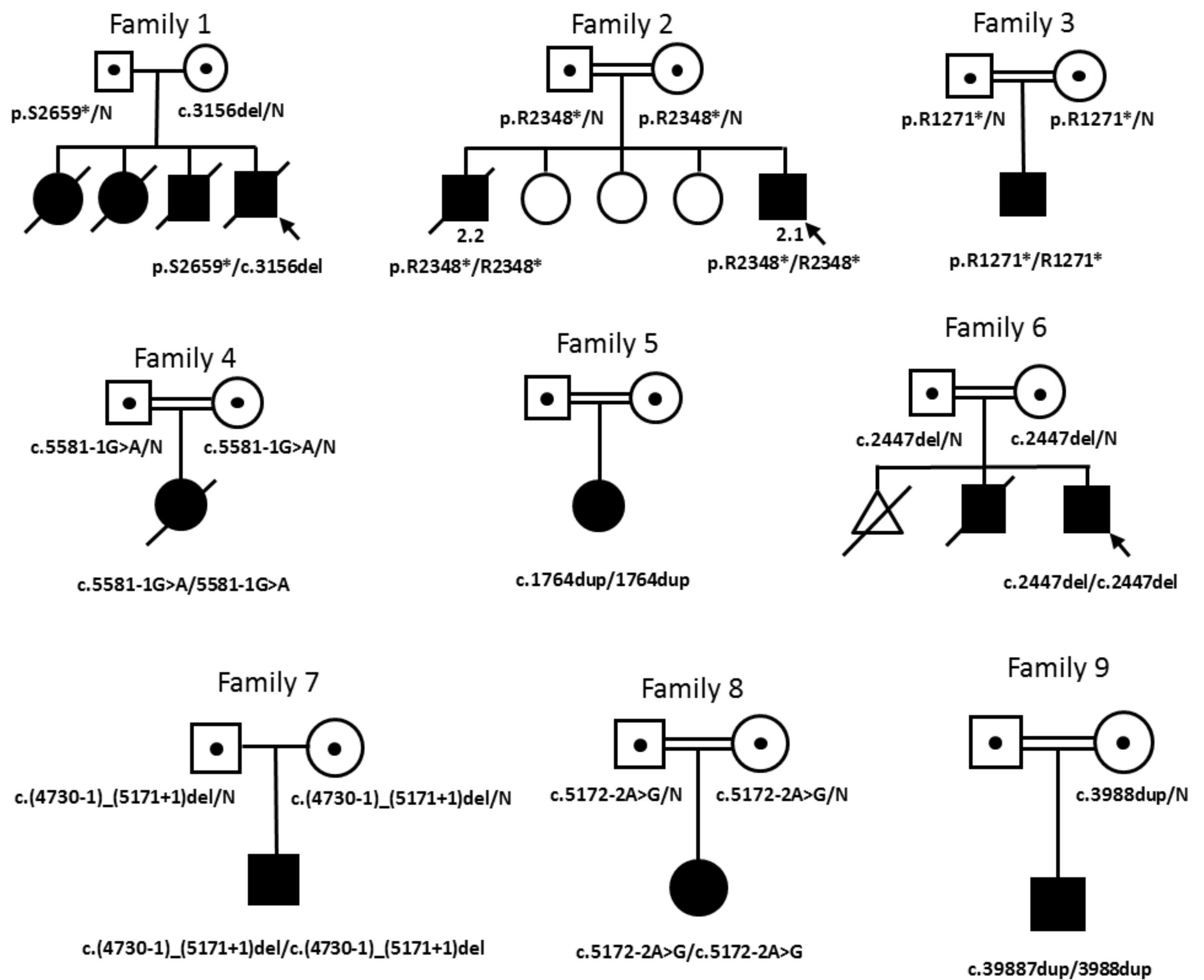


Figure 1 – Family pedigrees of patients with *LRBA* mutations. Filled symbols represent affected individuals and dots within symbols represent heterozygous unaffected carriers. Double lines signify parents are related. Genotypes are provided below affected individuals and carriers. When no genotype is given, samples were unavailable for testing.

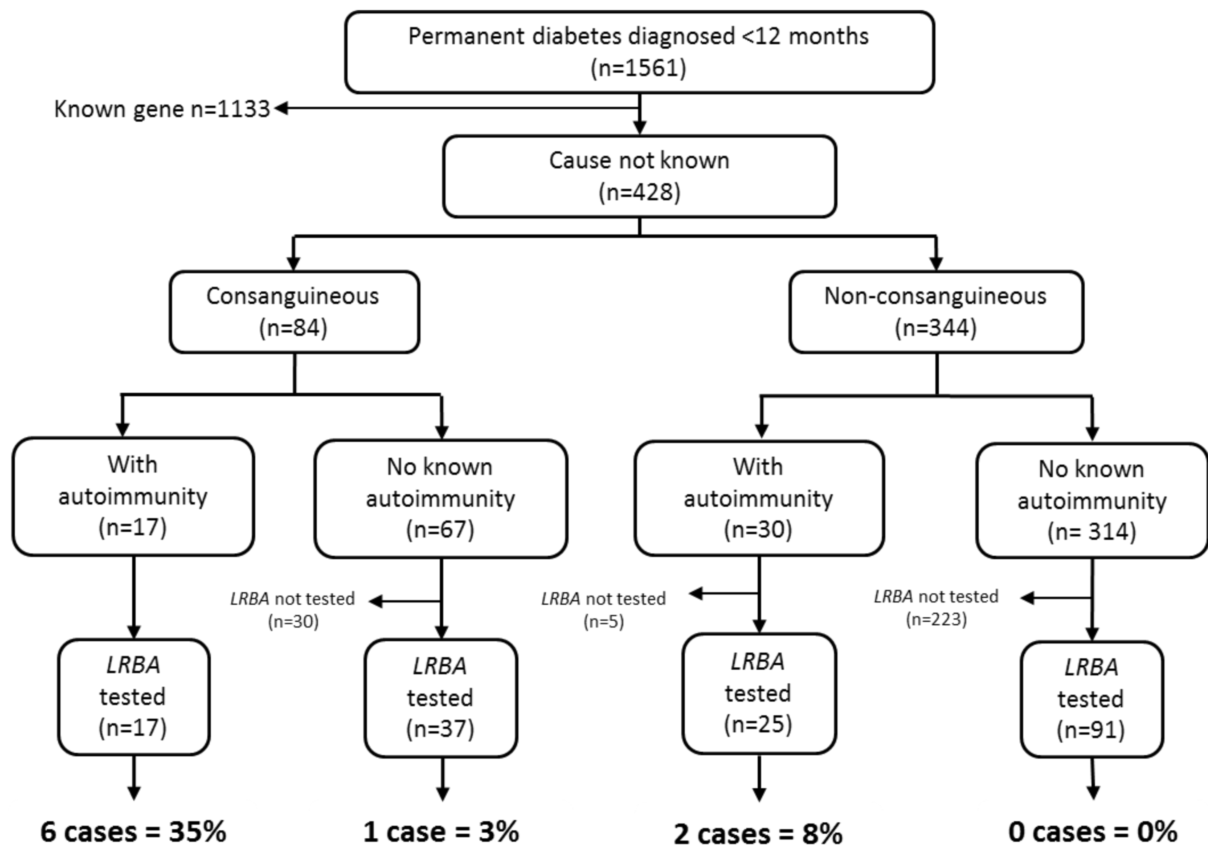


Figure 2 – Flow diagram showing the testing strategy for *LRBA* screening in individuals diagnosed with diabetes diagnosed before 12 months of age. The pick-up rates of *LRBA* mutations, when individuals are sub-grouped according to consanguinity and additional autoimmune disease, are provided.

Supplementary data

Recessively inherited *LRBA* mutations cause autoimmunity presenting as neonatal diabetes

Matthew B Johnson BSc, Elisa De Franco PhD, Hana Lango Allen PhD, Aisha Al Senani MD, Nancy Elbarbary MD, Zeynep Siklar MD, Merih Berberoglu MD, Zineb Imane MD, Alireza Haghighi DPhil, Zahra Razavi DM, Irfan Ullah MD, Saif Alyaarubi MD, Daphne Gardner MD, Sian Ellard PhD, Andrew T Hattersley DM & Sarah E Flanagan PhD

Diabetes 2017 66(8):2316-2322

Supplementary table S1: Exome sequencing metrics for family 1.

Sample	Proband	Mother	Father
Genes targeted	21,522		
Size of targeted region (Kb)	50,621		
% of target at 10x coverage	93.6	96.6	95.6
% of target at 20x coverage	79.2	89.8	86.0
% of target at 0x coverage	1.0	1.0	0.9
Mean coverage across target	46x	65x	56x

Supplementary table S2: Autoimmune features of the cohort tested for *LRBA* including the index patient (1) and sibling of a patient with a mutation (2.2). Individuals without autoimmune disease are not included (i.e. patient 5).

Patient	**Autoimmune Haematological Disorders**	**Autoimmune enteropathy**	**Autoimmune thyroid disease**	**Hepatosplenomegaly**	**Immunodeficiency**	**Myasthenia gravis**	**Arthritis**	**Psoriasis**	**Atopic dermatitis**	**Alopecia**
1	✓									
2.1		✓								
2.2	✓	✓			✓					
3	✓			✓	✓					
4		✓								
6	✓	✓	✓							
7	✓	✓								
8					✓					
9		✓			✓					
10			✓							
11	✓									
12		✓								
13			✓							
14		✓								
15						✓				

16	✓			✓						
17							✓			
18		✓	✓							
19		✓								
20		✓								
21		✓								
22	✓		✓							
23		✓								
24			✓							
25		✓							✓	✓
26			✓	✓						
27			✓							
28		✓								
29		✓								
30	✓	✓	✓						✓	
31			✓							
32		✓								
33			✓							
34		✓	✓							
35							✓			
36		✓		✓					✓	
37		✓			✓					
38	✓	✓								✓
39		✓								

40		✓	✓							
41	✓	✓	✓							
42	✓	✓	✓							

Supplementary table S3: Heterozygous *de novo* variants identified from exome sequencing data in patient 1

Gene	Variant	Consequence	ExAC frequency
OR4A16	NM_001005274.1:c.730G>A p.Val244Met	Missense	0.002%
TBP	NM_003194.4:c.273_281del p.Gln93_Gln95del	In-frame deletion	0.011%
STK19	NM_032454.1:c.59dup p.Asn20Lysfs*16	Frameshift	0.04%
HNRNPCL1	NM_001013631.2:c.830C>T p.Ala277Val	Missense	0.05%

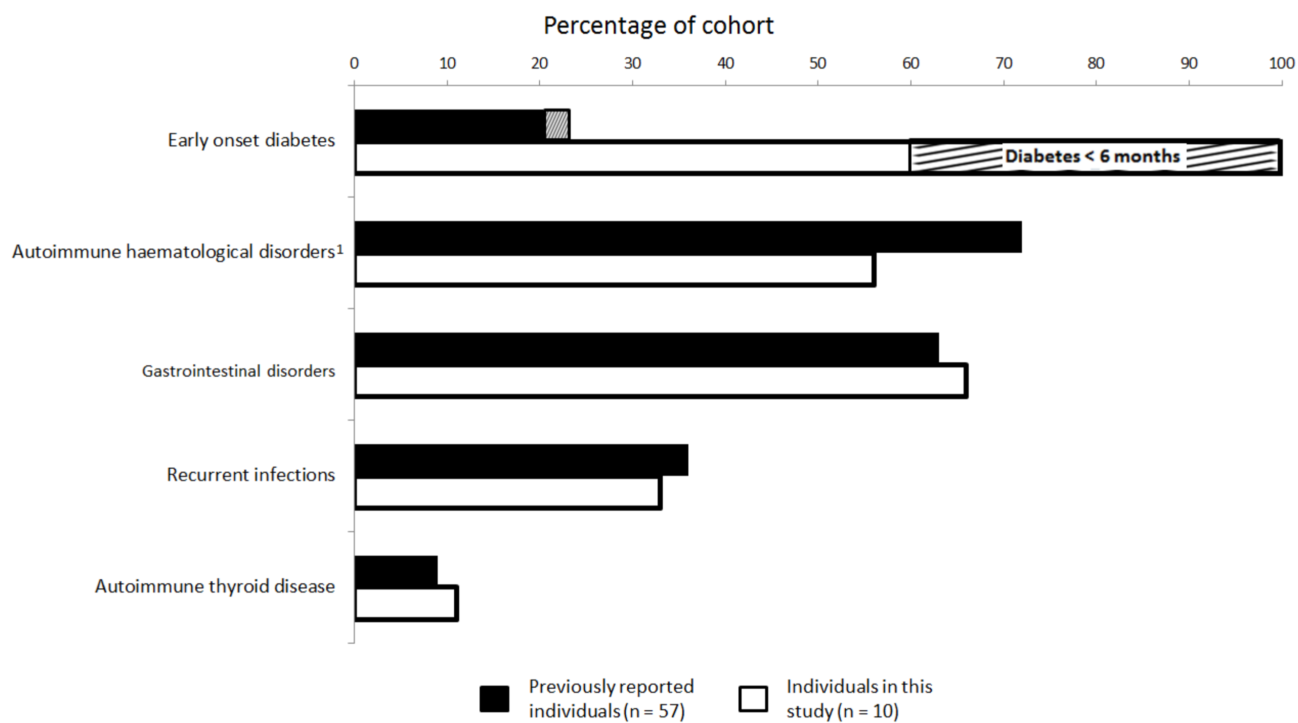


Figure S1: Features of CVID-8 in this cohort and previously identified patients. Hatched boxes represent individuals with neonatal diabetes (diagnosed <6 months).

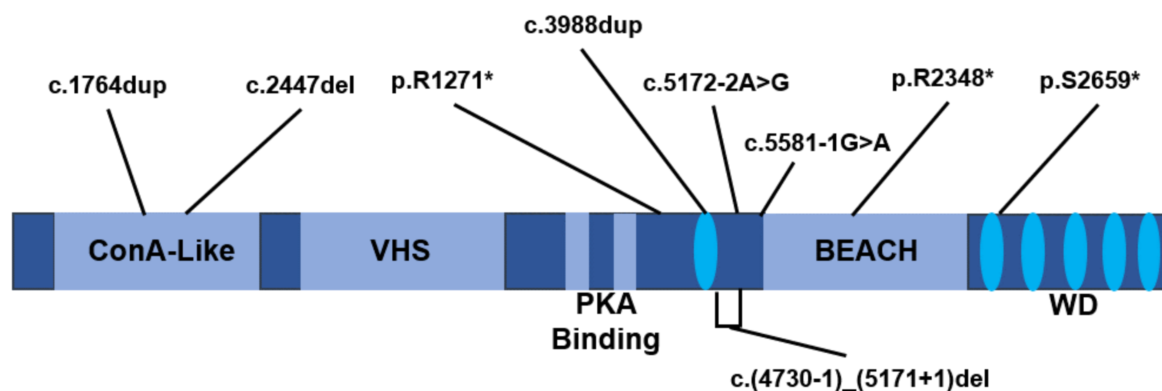


Figure S2 – Ideogram of LRBA protein showing functional domains and approximate location of mutations identified in our cohort. ConA-like: Concanavalin A (ConA)-like lectin binding domain; VHS: VPS (vacuolar protein sorting)-27, Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) domain and STAM (signal transducing adaptor molecule); PKA: Protein Kinase A; WD: structural motif of approximately 40 amino acids, often terminating in tryptophan-aspartic acid (W-D) dipeptides; BEACH: beige and CHS domain.

Chapter 4 Part A

Immunoglobulin E in health and disease

M. B. Johnson

Immunoglobulin E

Immunoglobulin E (IgE) is the lowest abundance immunoglobulin in healthy individuals, accounting for less than 1 x 10^{-4}% of total serum immunoglobulin [1]. Its physiological role is primarily in the defence against multicellular parasites, as demonstrated by the stimulation of specific IgE production by helminth infection and raised levels of serum IgE observed in people living in the tropics, where helminth's are endemic [2]. Excess production of non-specific IgE is also associated with atopic diseases and hyper-IgE syndrome (HIES; also known as Job's syndrome), caused predominantly by dominant negative mutations in *STAT3* [3].

IgE is produced after class switching of B-cells into short lived plasma cells. This mostly occurs in germinal centres but also in extra-follicular space in secondary lymph nodes [4]. These plasma cells then produce IgE in the lymphatic system and periphery. IgE primes the IgE-mediated response by binding to Fc receptors found on the surface of mast cells and basophils. Fc receptors are also found on eosinophils, monocytes, macrophages and platelets in humans [5].

IgE in medicine

Serum IgE is most commonly elevated in the context of allergic reactions[6]. Its use is limited in determining the specific allergen, other than in allergic bronchopulmonary Aspergillosis (ABPA) which is part of the diagnostic criteria [7]. Measurement of IgE may also be used to identify patients with severe atopic disease suitable for anti-IgE therapy with omalizumab and for monitoring efficacy and determining dosage [8] . Raised IgE is also used to identify primary immunodeficiencies such as HIES, Omenn syndrome and IPEX syndrome.

Hyper IgE syndrome

HIES is a multisystem disorder that commonly includes increased levels of serum IgE (>2000 IU/L; >95%), severe eczema (100%), eosinophilia (93%) characteristic facial dysmorphism (83%), susceptibility to Staphylococcal infections (>90%) and abnormal dentition (72%) [9]. Heterozygous dominant-negative mutations in *STAT3* are the primary cause. The vast majority of mutations identified in *STAT3* are missense, in keeping with a dominant negative mechanism of disease. Recessively inherited mutations in *DOCK8* also causing an immunodeficiency syndrome with raised IgE in a smaller number of cases [10].

Germline gain of function (GOF) mutations in *STAT3* were recently identified as the cause of early-onset multisystem autoimmune disease (OMIM: 615952) [11, 12]. This is characterised by the early-onset of multiple autoimmune diseases and contrasts to the phenotype associated with HIES, though severe eczema and dental anomalies may be present in both conditions.

IgE in IPEX

Previous studies have shown that the majority of patients with IPEX syndrome, caused by hemizygous mutations in *FOXP3,* have raised concentrations of serum IgE [13]. In our cohort of patients with IPEX syndrome, where serum was available and measurement was possible (n=16), 13/16 (81%) had serum IgE concentrations above the age specific reference range in keeping with previous reports (chapter 2 and table 1).

The main differential diagnosis in non-consanguineous males with very early-onset autoimmunity is hemizygous *FOXP3* mutations or GOF *STAT3* mutations. Our patients with *FOXP3* mutations had significantly higher serum IgE than those with *STAT3* mutations (median 2177.5 KU/L [IQR 152-3200] v 1 KU/L [IQR 0.7-2], p=0.002, figure 1). Measurement of IgE could therefore be extremely useful in

this patient group to guide genetic testing. Moreover, in those without a mutation in the coding region of *FOXP3*, it could facilitate the identification of regulatory mutations or novel genetic aetiologies by suggesting which pathway is most likely to be affected.

Patient	Gene	Age serum collected	IgE concentration (KU/L) [reference range] *	Interpretation
1	*FOXP3*	0	2950 [2-34]	Raised IgE
2	*FOXP3*	0	259.4 [2-34]	Raised IgE
3	*FOXP3*	0	44.5 [2-34]	Raised IgE
4	*FOXP3*	0	557.8 [2-34]	Raised IgE
5	*FOXP3*	0	3000 [2-34]	Raised IgE
6	*FOXP3*	1	2266 [2-97]	Raised IgE
7	*FOXP3*	6	2089 [2-199]	Raised IgE
8	*FOXP3*	25	0.1 [2-214]	Low IgE
9	*FOXP3*	0	5.6 [2-34]	Normal
10	*FOXP3*	0	6.3 [2-34	Normal
11	*FOXP3*	0	4899 [2-34]	Raised IgE
12	*FOXP3*	4	7173 [2-199]	Raised IgE
13	*FOXP3*	0	2396 [2-34]	Raised IgE
14	*FOXP3*	0	10000 [2-34]	Raised IgE
15	*FOXP3*	1	3400 [2-97]	Raised IgE
16	*FOXP3*	0	1000 [2-34]	Raised IgE
17	*STAT3*	0	0.5 [2-34]	Low IgE
18	*STAT3*	0	1 [2-34]	Low IgE
19	*STAT3*	0	1 [2-34]	Low IgE
20	*STAT3*	4	2 [2-199]	Low IgE
21	*STAT3*	7	2 [2-307]	Low IgE
22	*STAT3*	4	0.7 [2-199]	Low IgE

Table 1: IgE concentration for patients with hemizygous *FOXP3* mutations and GOF *STAT3* mutations. *age specific reference ranges taken from Martins et al.

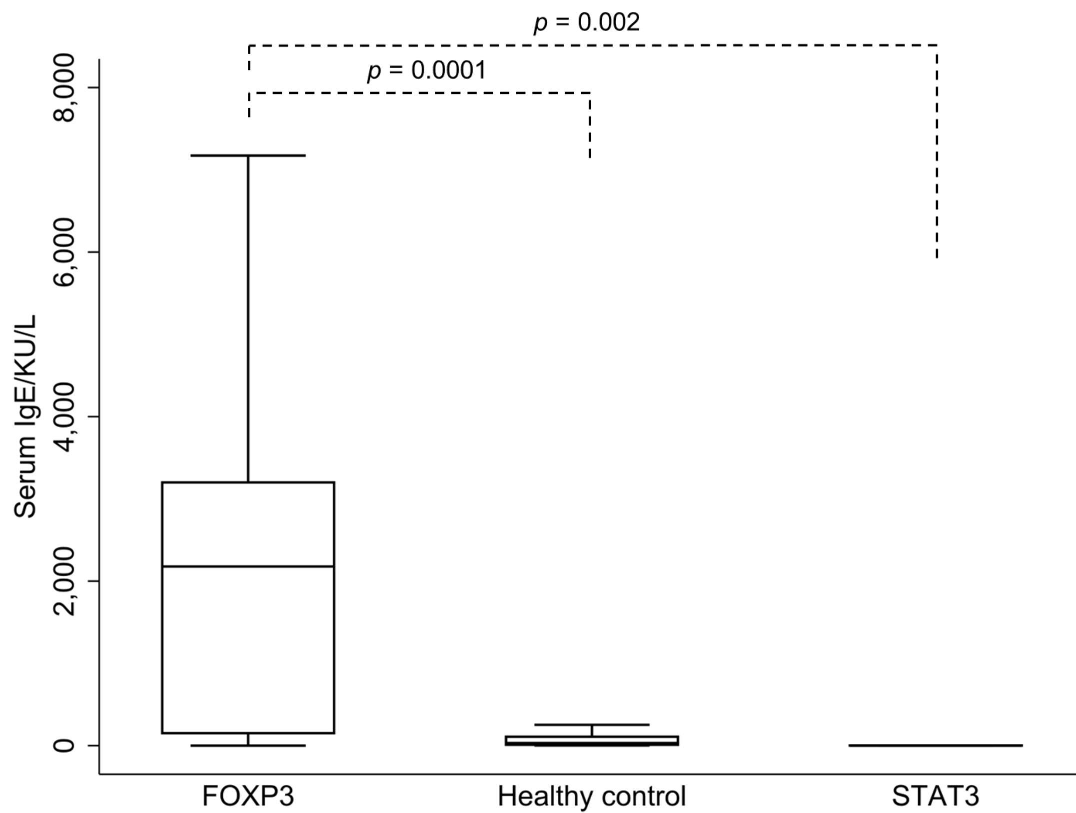


Figure 1: Boxplot showing serum IgE levels in patients with IPEX syndrome (n=16), healthy controls (n=1510) and patients with *STAT3* GOF mutations (n=6). Serum IgE was significantly higher in patients with IPEX (median 2177.5 KU/L) than healthy controls, and significantly lower in patients with STAT3 GOF mutations (median 1 KU/L).

STAT3 and IgE

The pathogenic mechanism(s) that underlies the increased production of IgE in patients with HIES has not been fully resolved, in part due to knockout of *STAT3* in mice being lethal during embryonic development and the overlapping and pleiotropic nature of STAT signalling [14, 15]. Furthermore, whilst in the murine model IL-21 down-regulates IgE production via STAT3 signalling [16], IL-21 increases production of IgE by human plasma cells in a STAT3 dependent manner [17].

The pathogenesis of HIES is in part due to defective IL-10 signalling leading to a reduction in immune tolerance [18]. IL-10 also has a role in the suppression of IgE production [19]. The loss of *STAT3* signalling may therefore increase IgE production by reducing IL-10 signalling. In patients with *STAT3* GOF mutations, increased IL-10 signalling may underlie decreased IgE production, however empirical data to support this is not available. Further study of this area is warranted to elucidate the role of STAT3 in the production of IgE. Cells from patients with *STAT3* GOF, in combination with those from patients with HIES, could offer an ideal experimental model for this.

Regulatory T cells (Tregs) have a role in the regulation of IgE production. Tregs suppress antibody production by B-cells via IL-10 signalling and TGF-B (transforming growth factor beta) [20]. Reduced numbers or function of regulatory T cells may therefore underlie the increase in IgE seen in patients with IPEX syndrome. In previous reports of patients with *STAT3* GOF mutations [21, 22], 6 patients were reported to have reduced numbers of Tregs however their serum IgE concentration was not available. Further study is warranted to elucidate a possible connection between STAT3 GOF mutations, the Treg compartment and IgE production.

Further study of IgE in *STAT3* GOF

Since the publication of our manuscript 'Low IgE is a useful tool to identify STAT3 gain of function mutations' (Chapter 4B) a response has been published by an international team of investigators following a larger cohort of patients with *STAT3* GOF [23]. The authors assessed the serum IgE concentration in an additional 23 patients with either previously published mutations or in whom GOF was confirmed by luciferase reporter assay. They found that 9 of the patients had values above 2KU/L (mean 28.3KU/L, median <2KU/L) and conclude that low serum IgE is therefore less useful for identifying patients with *STAT3* GOF mutations than our data suggested, with a sensitivity of 0.61 [95%CI 0.39-0.80].

When the patients we report are included (n=6, table 1), the sensitivity of IgE <2KU/L is 0.69 [95%CI 0.49-0.84]. This is still too low to be clinically useful for guiding genetic testing. As the authors note, as additional patients with *STAT3* GOF mutations have been reported the phenotypic spectrum observed has increased. It may therefore be that patients with low IgE represent a distinct subgroup, or have mutations that affect *STAT3* signalling in a specific way that denotes a change to IgE level, possibly via IL-10 signalling. Further study is needed to elucidate the mechanism that underlies aberrant IgE production in some individuals with GOF *STAT3* mutations.

References

1. Waldmann TA, Iio A, Ogawa M, McIntyre OR, Strober W. The metabolism of IgE. Studies in normal individuals and in a patient with IgE myeloma. Journal of immunology (Baltimore, Md : 1950). 1976;117(4):1139-44.

2. Yazdanbakhsh M, van den Biggelaar A, Maizels RM. Th2 responses without atopy: immunoregulation in chronic helminth infections and reduced allergic disease. Trends in immunology. 2001;22(7):372-7.

3. Minegishi Y, Saito M, Tsuchiya S, Tsuge I, Takada H, Hara T, et al. Dominant-negative mutations in the DNA-binding domain of STAT3 cause hyper-IgE syndrome. Nature. 2007;448(7157):1058-62.

4. Wu LC, Zarrin AA. The production and regulation of IgE by the immune system. Nature reviews Immunology. 2014;14(4):247-59.

5. Akula S, Mohammadamin S, Hellman L. Fc receptors for immunoglobulins and their appearance during vertebrate evolution. PloS one. 2014;9(5):e96903.

6. Amarasekera M. Immunoglobulin E in health and disease. Asia Pacific allergy. 2011;1(1):12-5.

7. Greenberger PA, Bush RK, Demain JG, Luong A, Slavin RG, Knutsen AP. Allergic bronchopulmonary aspergillosis. The journal of allergy and clinical immunology In practice. 2014;2(6):703-8.

8. Diaz RA, Charles Z, George E, Adler AI. NICE guidance on omalizumab for severe asthma. The Lancet Respiratory medicine. 2013;1(3):189-90.

9. Freeman AF, Holland SM. The Hyper IgE Syndromes. Immunology and allergy clinics of North America. 2008;28(2):277-viii.

10. Zhang Q, Davis JC, Lamborn IT, Freeman AF, Jing H, Favreau AJ, et al. Combined immunodeficiency associated with DOCK8 mutations. The New England journal of medicine. 2009;361(21):2046-55.

11. Flanagan SE, Haapaniemi E, Russell MA, Caswell R, Allen HL, De Franco E, et al. Activating germline mutations in STAT3 cause early-onset multi-organ autoimmune disease. Nature genetics. 2014;46(8):812-4.

12. Amberger JS, Bocchini CA, Schiettecatte F, Scott AF, Hamosh A. OMIM.org: Online Mendelian Inheritance in Man (OMIM(R)), an online catalog of human genes and genetic disorders. Nucleic acids research. 2015;43(Database issue):D789-98.

13. Hennezel E, Bin Dhuban K, Torgerson T, Piccirillo C. The immunogenetics of immune dysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome. Journal of Medical Genetics. 2012;49(5):291.

14. Akira S. Functional roles of STAT family proteins: lessons from knockout mice. Stem cells (Dayton, Ohio). 1999;17(3):138-46.

15. O'Shea JJ, Schwartz DM, Villarino AV, Gadina M, McInnes IB, Laurence A. The JAK-STAT pathway: impact on human disease and therapeutic intervention. Annual review of medicine. 2015;66:311-28.

16. Ozaki K, Spolski R, Feng CG, Qi CF, Cheng J, Sher A, et al. A critical role for IL-21 in regulating immunoglobulin production. Science (New York, NY). 2002;298(5598):1630-4.

17. Avery DT, Ma CS, Bryant VL, Santner-Nanan B, Nanan R, Wong M, et al. STAT3 is required for IL-21-induced secretion of IgE from human naive B cells. Blood. 2008;112(5):1784-93.

18. Saito M, Nagasawa M, Takada H, Hara T, Tsuchiya S, Agematsu K, et al. Defective IL-10 signaling in hyper-IgE syndrome results in impaired

generation of tolerogenic dendritic cells and induced regulatory T cells. The Journal of experimental medicine. 2011;208(2):235-49.

19. Punnonen J, de Waal Malefyt R, van Vlasselaer P, Gauchat JF, de Vries JE. IL-10 and viral IL-10 prevent IL-4-induced IgE synthesis by inhibiting the accessory cell function of monocytes. Journal of immunology (Baltimore, Md : 1950). 1993;151(3):1280-9.

20. Palomares O, Martin-Fontecha M, Lauener R, Traidl-Hoffmann C, Cavkaytar O, Akdis M, et al. Regulatory T cells and immune regulation of allergic diseases: roles of IL-10 and TGF-beta. Genes and immunity. 2014;15(8):511-20.

21. Haapaniemi EM, Kaustio M, Rajala HL, van Adrichem AJ, Kainulainen L, Glumoff V, et al. Autoimmunity, hypogammaglobulinemia, lymphoproliferation, and mycobacterial disease in patients with activating mutations in STAT3. Blood. 2015;125(4):639-48.

22. Milner JD, Vogel TP, Forbes L, Ma CA, Stray-Pedersen A, Niemela JE, et al. Early-onset lymphoproliferation and autoimmunity caused by germline STAT3 gain-of-function mutations. Blood. 2015;125(4):591-9.

23. Tangye SG, Forbes LR, Leiding J, Preece K, Kumar AR, Gambineri E, et al. Low IgE Is Insufficiently Sensitive to Guide Genetic Testing of STAT3 Gain-of-Function Mutations. Clinical chemistry. 2017;63(9):1539-40.

Chapter 4 Part B

Low IgE is a useful tool to identify *STAT3* gain-of-function mutations

M. B. Johnson, S. E. Flanagan, T. B. Martins, H. R. Hill, A. T. Hattersley & T. J. McDonald

Clinical chemistry 2016 62(11):1536-1538

Acknowledgments

S.E.F, T.J.M and A.T.H. assisted with the design of the study. T.B.M and H.R.H provided IgE data for the controls and developed age specific normal reference ranges. All authors contributed to discussion, reviewed drafts of the manuscript and provided feedback

My contributions to the chapter

I selected the patients for testing and retrieved and prepared serum samples for 2 cases. I analysed the data and generated the figures and performed statistical analysis, wrote the manuscript, responded to reviewers' comments and checked the proof for typographical errors. For the non-published section I wrote the chapter, created the figures and tables, analysed the data and performed statistical analyses.

To the Editor,

Germline gain-of-function mutations (GOF) in *STAT3* were recently identified as the cause of early-onset autoimmune disease (MIM: 615952). This rare monogenic autoimmune disorder commonly encompasses autoimmune haematological disorders (identified in 70% of patients), recurrent infections (60%) and autoimmune enteropathies (50%)[21, 22, 24]. The phenotype observed contrasts to that of patients with loss-of-function (LOF) *STAT3* mutations causing hyper-IgE (Job) syndrome, typically characterised by elevated immunoglobulin E (IgE), recurrent infections and eczema[25]. Identifying patients with *STAT3* GOF mutations informs families and clinicians of prognosis and facilitates personalised treatment.

There is substantial clinical variability between patients with GOF *STAT3* mutations and overlap in phenotype with other monogenic autoimmune disorders such as IPEX syndrome (immunodysregulation, polyendocrinopathy, enteropathy, X-linked; MIM: 304790) resulting from hemizygous loss-of-function *FOXP3* mutations, and early-onset polygenic autoimmune disease. Distinguishing the specific genetic aetiology using clinical characteristics alone is not possible and genetic testing, often performed sequentially until a pathogenic mutation is found, is essential for accurate diagnosis.

Genetic testing for *STAT3* is expensive and available only in a limited number of specialist centres. An inexpensive and widely available test to identify patients most likely to harbour GOF *STAT3* mutations is desirable as it will prevent inappropriate genetic testing. We assessed whether IgE would be a useful tool to aid in the identification of patients suitable for *STAT3* sequencing.

Total serum IgE concentration was measured by using the Immuno-CAP 1000 instrument (Phadia) for both healthy reference samples (n = 1510) and *STAT3*

patient samples (n = 6). Calibration was performed with the 2nd International Reference Preparation 75/502 of Human Serum IgE from the World Health Organization[26]. Statistical analysis was performed in Stata®; specificity, sensitivity and binomial confidence intervals were calculated. The age of the patients with *STAT3* GOF mutations ranged from 1-9 years, and the median time from the first clinical symptom to IgE testing was four years.

Immunological assessment of individuals with *STAT3* GOF mutations identified total serum IgE below the lower reference limit of age-matched controls in all six cases, (<2 KU/L, range 0.7-2 KU/L; See **Figure 1**) [24]. Using 2 KU/L as a cut off for the assay for identification against all age groups gave a sensitivity and specificity of 100% (95% CI: 54.1 – 100) and 97.2% (95% CI: 96.2-97.9), respectively.

We have demonstrated that serum IgE, an inexpensive and widely available diagnostic test, is likely to be very useful to facilitate identifying patients suitable for *STAT3* sequencing, and a 2 KU/L cut-off gives high sensitivity and specificity. The role we propose for IgE measurement is in patients with multi-system early-childhood autoimmune disease in whom *STAT3* GOF mutations are being considered. This is likely to be particularly helpful in distinguishing from multiple autoimmunity resulting from hemizygous *FOXP3* mutations as more than 90% of patients with IPEX syndrome have increased serum IgE. In this scenario raised IgE would be helpful to rule out *STAT3* GOF mutations, reducing total genetic testing expenditure. In addition, despite recent advances in sequencing technologies, identifying pathogenic mutations from benign variants remains a challenge. Measuring IgE could be a tool to aid molecular geneticists in classifying *STAT3* variants found by DNA sequencing; serum IgE concentration below 2 KU/L supports pathogenic GOF.

We have assessed IgE in a small number of patients with GOF *STAT3* mutations (n=6) and assessment in further patients is required to increase confidence in the diagnostic utility, but it is notable that all six patients had IgE concentrations below the 1st centile of the reference population. Two previous case series of patients with *STAT3* GOF mutations measured IgE in a total of nine additional individuals (four of those previously reported are included in this study[21, 24]). One described two additional individuals with serum IgE below 2 KU/L[21], supporting our findings. The other reported more variability in IgE than we have observed (0.1-58.5 mg/dL; n=7)[22], but we were unable to ascertain reference interval, testing platform or comparable units to our assay for these individuals and therefore, unable to make a direct comparison to our findings[22]. A potential limitation of the diagnostic accuracy of IgE to identify patients with *STAT3* GOF mutations is that an allergic response or parasitic infection may increase serum IgE. Given that allergies are relatively common (for example allergic rhinitis is present in >20% of the European population) this is an important consideration as this may decrease the performance of the test in these patients. In conclusion, total serum IgE concentration is an inexpensive and robust tool for determining which patients with multiple early-onset autoimmunity are likely to have a *STAT3* GOF mutation. In combination with the identification of clinical features of early-onset autoimmune disease IgE concentrations could be assayed locally to facilitate appropriate, cost-efficient and rapid genetic testing. Further work is warranted to determine IgE in other subtypes of monogenic autoimmunity.

M. B. Johnson[1], B.Sc.

S. E. Flanagan[1], Ph.D.

T. B. Martins[3], M.S

H. R. Hill[3], M.D

A. T. Hattersley[1] D.M

T. J. McDonald[1, 2], Ph.D.

From [1]The Institute of Biomedical and Clinical Research, University of Exeter Medical School, UK, [2]Molecular Genetics and Blood Sciences, Royal Devon and Exeter NHS foundation trust, Exeter, UK and [3]Department of Pathology, The ARUP Institute for Clinical and Experimental Pathology, University of Utah, USA

Acknowledgements

This work was supported by the Wellcome Trust. SF has a Sir Henry Dale Fellowship jointly funded by the Wellcome Trust and the Royal Society (Grant Number 105636/Z/14/Z). ATH is a Wellcome Trust Senior Investigator. TM is an NIHR Chief Scientific Officer fellow.

The authors would like to thank Dr T Laver, Dr E De Franco for their useful comments and suggestions and Dr B Shields for assistance with statistical analyses.

Disclosure of conflicts of interest: the authors declare no conflict of interest.

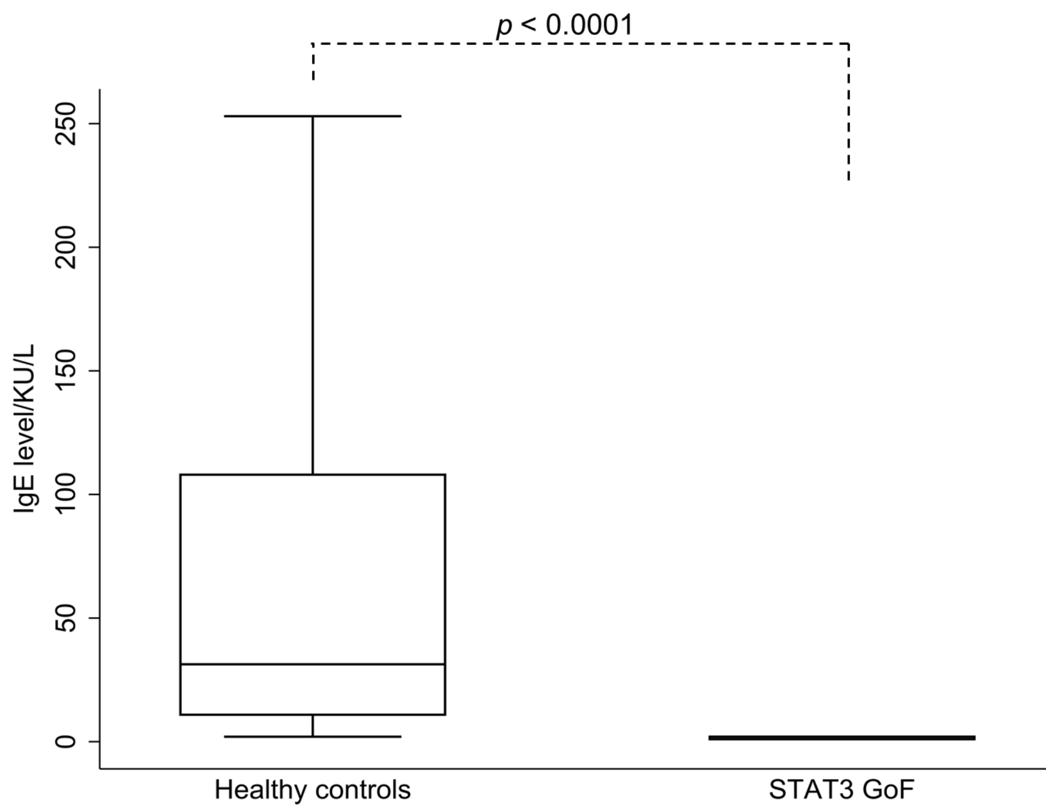


Figure 1: Boxplot of serum IgE concentrations in healthy controls and patients with *STAT3* GOF mutations. *P* value determined using the Mann-Whitney test.

References

1. Flanagan SE, Haapaniemi E, Russell MA, Caswell R, Lango Allen H, De Franco E, et al. Activating germline mutations in STAT3 cause early-onset multi-organ autoimmune disease. Nat Genet 2014;46:812– 4.

2. Haapaniemi EM, Kaustio M, Rajala HL, van Adrichem AJ, Kainulainen L, Glumoff V, et al. Autoimmunity, hypogammaglobulinemia, lymphoproliferation, and mycobacterial disease in patients with activating mutations in STAT3. Blood 2015;125:639 – 48.

3. Milner JD, Vogel TP, Forbes L, Ma CA, Stray-Pedersen A, Niemela JE, et al. Early-onset lymphoproliferation and autoimmunity caused by germline STAT3 gain-of function mutations. Blood 2015;125:591–9.

4. Mogensen TH. STAT3 and the hyper-IgE syndrome: clinical presentation, genetic origin, pathogenesis, novel findings and remaining uncertainties. JAKSTAT 2013;2:e23435.

5. Martins TB, Bandhauer ME, Bunker AM, Roberts WL, Hill HR. New childhood and adult reference ntervals for total IgE. J Allergy Clin Immunol 2014;133:589 – 91.

Conclusions

Identifying monogenic autoimmunity can lead to improved treatment for patients and gives clinicians and families knowledge on prognosis and recurrence risk. Discovering new causes of monogenic autoimmune diabetes can also give insights into beta-cell autoimmunity that could have wider implications for patients with polygenic type 1 diabetes.

This section of this thesis will summarise the main findings of each chapter, give the impact of the results and suggest future avenues of research in monogenic autoimmunity.

CHAPTER 1 - A TYPE 1 DIABETES GENETIC RISK SCORE CAN DISCRIMINATE MONOGENIC AUTOIMMUNITY WITH DIABETES FROM EARLY ONSET CLUSTERING OF POLYGENIC AUTOIMMUNITY WITH DIABETES

Conclusions

We showed that a genetic risk score using the top 10 risk alleles for type 1 diabetes (T1D-GRS) could discriminate monogenic autoimmunity from patients who are likely to have polygenic clustering of diabetes and additional autoimmunity in whom a known monogenic cause was excluded. Identifying monogenic autoimmunity from the more common polygenic clustering of autoimmune diseases is a challenge as there is significant clinical overlap between these groups. Markers for autoimmune diabetes are useful for identifying non-autoimmune monogenic diabetes from type 1 diabetes, however islet autoantibodies (e.g. anti-glutamic acid decarboxylase antibody) are present in both monogenic and polygenic autoimmune diabetes, age of onset can be

similar, and insulin is usually required in full replacement doses for both groups. We showed that the T1D-GRS was a similar discriminator to age of diabetes diagnosis (ROC-AUC 0.80 vs 0.79) however when combined, discrimination improved beyond either alone (ROC-AUC 0.88, p=0.04). Specific clinical features such as coeliac disease or autoimmune enteropathy showed different prevalence in those with monogenic autoimmunity compared to those with unknown aetiology, but as these features were present in both groups they could not be used alone to guide testing or gene discovery.

This study also provides evidence that the polygenic risk of developing diabetes, including that from the strongest predisposing HLA DR3 and DR4 haplotypes, does not influence the phenotype of patients with monogenic autoimmune disease. All the patients with monogenic autoimmunity had early-onset diabetes (diagnosed <5 years), however they had a similar range of T1D-GRSs as the population of non-diabetic controls. Our data also suggests there are novel monogenic aetiologies to discover in our cohort as there was a small enrichment of patients without monogenic disease in the lowest quartile of the type 1 diabetes controls, where most (79%) known monogenic autoimmunity was found.

Impact of findings

This study provided a genetic diagnosis to 37 families, informing the families and clinicians on prognosis and recurrence risk which ranges from <1% for de novo *STAT3* mutations to 50% for male offspring of *FOXP3* mutation carriers.

It is important to identify monogenic autoimmunity as many forms genetic subtypes are suitable for targeted therapy. For example, abatacept can be used to treat patients with biallelic *LRBA* mutations [27] and anti-IL6 antibody therapy with tociluzumab for patients with GOF STAT3 mutations [22].

Whilst there is some overlap in the range of T1D-GRS scores in individuals with polygenic and monogenic autoimmunity, which prevents this from being used as an exclusion test, the T1D-GRS can help to prioritise patients for routine genetic testing. This is important as it will help to prevent expensive genetic testing being undertaken on individuals who are most likely to have polygenic disease and will also preclude any anxiety experienced by families regarding the possibility of their child having an inherited disease.

The T1D-GRS may prove useful in the selection of patients for gene discovery, particularly those with a score below the 25th centile of T1D controls. Where patients have novel or very rare variants of uncertain significance the T1D-GRS could assist with variant classification by suggesting the most likely aetiology.

Future directions

One future direction for this cohort of patients is to perform gene discovery in those without a causative variant in the genes tested and who have a low T1D-GRS (<25th centile of T1D controls). The ideal strategy would be to perform whole genome sequencing to a high depth as this will capture >98% of the genome and allow for both coding variants and non-coding regulatory variants to be identified. The strategy of gene discovery studies will depend on family structure, with outbred patients (defined as having a coefficient of inbreeding [F] <0.0156 [28]) sequenced as trios to look initially for *de novo* or compound heterozygous variants and patients who are the result of consanguineous union (F > 0.0156) sequenced as singletons to look initially for homozygous variants. These new genetic causes may be a novel aetiological gene or mutations in as yet undefined intronic/regulatory regions. The power to identify new genetic causes will rely on the number of families sequenced, as confirming a new genetic cause depends

on identifying variants in the same gene/regulatory region in multiple unrelated pedigrees.

Fine mapping of the HLA region could provide additional information on the genetic risk in patients without a known genetic cause, as has been evidenced by studies into coeliac disease [29]. This may also identify rare haplotypes that infer high risk and underlie the early-onset of diabetes and autoimmunity in patients without a confirmed monogenic cause. Furthermore, many loci that infer risk for multiple autoimmune diseases were not included in this study [30], and a genotyping assay that captures these pleiotropic loci may give better discrimination as it could identify patients whose disease phenotype does not fit their genetic predisposition. In addition, combining the T1D-GRS with genetic risk scores for other autoimmune diseases such as thyroid disease [31] and coeliac disease [32] could identify patients with a low T1D-GRS but whose non-diabetic disease manifestations fit with a polygenic aetiology. This could enable a more granular and individualised genetic risk to be ascertained, and could also enable assessment of genetic risk in patients without diabetes but with multiple early-onset autoimmune diseases.

CHAPTER 2 – GENOTYPE AND CLINICAL PHENOTYPE DO NOT PREDICT PROGNOSIS IN IPEX SYNDROME

Conclusions

We sought to identify genetic or clinical characteristics in patients with Immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome to identify correlation with prognosis. We did not identify evidence of

phenotype-genotype relationships in IPEX syndrome; those with missense variants did not differ in their prognosis to those with protein truncating variants expected to result in the complete loss of FOXP3 protein. Moreover, individuals with the same missense mutation had highly variable phenotypes, even within the same family, and one patient with a protein truncating variant expected to result in the loss of FOXP3 survived to adulthood with a milder clinical course. The presenting feature (either enteropathy or diabetes) did also not predict a different prognosis or disease course in our large cohort of patients.

Medical management for IPEX is challenging and the only curative therapy, haematopoietic stem cell transplantation (HSCT), has high mortality risk. Whilst many patients with IPEX syndrome die in infancy, some patients have a milder clinical course, even if they have the same mutation as those with severe forms. We showed that decisions on medical management cannot be based on presentation or genotype and must be based on individual clinical need. We also showed that while recurrence risk can be predicted by genetic testing, phenotype cannot.

Furthermore, panel testing of genes with similar phenotypes is identifying more individuals with atypical clinical disease and is highlighting the true spectrum of IPEX. Understanding what causes this variability in phenotype could allow for better selection of patients for HSCT by identifying which patients are likely to progress quickly to severe disease and which patients are likely to have a milder clinical course where the risk of HSCT would be unlikely to outweigh the prognosis.

Impact of findings

The 48 diagnoses provided in this study enabled prenatal diagnosis in 2 families and facilitated HSCT in 4 patients. Furthermore, it gave information for families and clinicians on recurrence risk; where the *FOXP3* variant was carried by the unaffected mother each offspring has a 50% chance of inheriting the variant, with females being unaffected carriers and males being affected. We have studied the largest collection of families with IPEX syndrome to date. Even in this large cohort, we could not find evidence that presenting feature or genotype affected prognosis, suggesting that they do not have a role in disease progression. This paves the way for further studies into patients with IPEX syndrome to elucidate the cause of the highly variable phenotypes that are observed.

Our study will also increase knowledge and recognition of the milder forms of IPEX, which were previously thought to be atypical rare forms. We suggest that IPEX is a more variable clinical disorder than previously understood and the criteria for testing of *FOXP3* could be widened to include males with isolated early-onset diabetes, isolated autoimmune enteropathy or other autoimmunity in early childhood. Further recognition of atypical cases may allow for further study of the determinants of variability. We have identified the oldest surviving male with a *FOXP3* mutation (current age 61 years) which may act to reassure families and clinicians that IPEX syndrome is not necessarily life limiting, particularly if the variant is hypomorphic, though this should be treated with caution due to the wide variability in prognosis.

Future directions

Publication of this work will disseminate the findings and show that, at least in this cohort, genotype and presentation do not dictate prognosis. Furthermore, 17 of the variants identified in our cohort are novel and their publication will assist in the classification of *FOXP3* variants. Another key direction of research in IPEX

syndrome is to identify the genetic characteristics of these patients that may be involved in determining phenotype. For example, full HLA typing for these patients should be undertaken, as the risk of developing autoimmune diabetes is strongly associated with class II HLA alleles (particularly HLA DR4 and DR3) [33]. Furthermore, strongly protective haplotypes exist (e.g. HLA DRB1*15) that may influence the likelihood of the development of diabetes in these individuals [34]. Fine mapping of the HLA would be most desirable to identify rare alleles that may be having an effect, as has been shown for coeliac disease [29]. New long read sequencing technologies are enabling improved analysis and phasing of the highly polymorphic HLA region, and these would be ideal to study this cohort [35]. There are also >50 loci outside the HLA region that are associated with type 1 diabetes, and genotyping of these may provide additional insights [36].

Histopathology of patients with autoimmune enteropathy has also shown that some individuals have overexpression of HLA DR molecules on enterocytes and inappropriate expression of class II HLA on crypt epithelia [37, 38]. Determining the regulatory networks that lead to this inappropriate expression may also identify hallmark genetic features that influence phenotype in IPEX syndrome.

Functional work to assess the expression of FOXP3 in the individual with a protein truncating *FOXP3* variant but a milder disease course would also be desirable. If, as predicted, he does not have expression of FOXP3, immunophenotyping to look for Treg-like lymphocytes ($CD4^{+}CD25^{+}CD127^{low}$) [39] or atypical lymphocyte profiles would be useful to understand his milder phenotype. Indeed, immunophenotyping of all patients with hemizygous FOXP3 mutations may give insight into the causes of their variable phenotypes.

Longitudinal study or patients with IPEX would also be useful to further understand key clinical features that may be associated with a milder course, and

it would allow for prospective measurement of autoantibodies and immunophenotyping which, unlike genetics, are dynamic. This would be difficult for this very-rare disease as patients will be located throughout the world, however through collaboration with other international groups it would be possible to follow patients through life.

We were not able to look in detail at geographic or socio-economic factors that may be influencing disease phenotype in patients with IPEX syndrome. It is well established that the proportion of specific autoimmune diseases varies by country and that incidence is increasing in many [40]. This is not explained by genetic variation alone and therefore looking at the autoimmune manifestations in patients with IPEX syndrome and correlating this to their country of origin may give insights into possible environmental factors that influence phenotype.

CHAPTER 3 - RECESSIVELY INHERITED *LRBA* MUTATIONS CAUSE AUTOIMMUNITY PRESENTING AS NEONATAL DIABETES

Conclusions

We used exome sequencing for a patient with autoimmune lymphoproliferative disease and neonatal diabetes (NDM; diagnosed at 7 weeks) to look for novel causes of NDM and identified compound heterozygous mutations in *LRBA,* confirming the role of *LRBA* in neonatal diabetes. We then used targeted NGS to screen an additional 170 probands in whom the 24 known causes of NDM had been ruled out. This identified a further 8 probands and family member testing identified one affected sibling. Clustering of early-onset diabetes and additional autoimmunity is usually polygenic, however identifying monogenic autoimmunity

can facilitate personalised medicine. This is particularly true for patients with biallelic disease causing variants in *LRBA*, who are amenable to therapy with Abatacept which replaces the lost signalling from CTLA-4 that underlies their disease.

Seven of the patients we report are consanguineous and 6 of these had additional autoimmune disease. In one patient, NDM was the only clinical feature at 2 years of age. We showed that, in our cohort, biallelic LRBA mutations have a minimum prevalence of 0.6% (9/1561) in patients diagnosed with diabetes before 12 months, and of 6.3% (4/63) in patients who are the result of consanguineous union diagnosed with diabetes between 6-12 months. Combined selection criteria of consanguinity and autoimmune disease gave the highest pick up rate, with 6/17 (35%) of patients found to have recessively inherited *LRBA* mutations.

Impact of findings

Our study led to a genetic diagnosis for 10 patients with early-onset autoimmunity. This also informed the families and clinicians of recurrence risk (25% to siblings <1% to offspring of patient). Identifying patients with recessively inherited *LRBA* mutations can facilitate targeted therapy. It has been shown that treatment with abatacept, an immunoglobulin-CTLA-4 mimetic, can improve symptoms in patients with *LRBA* mutations [27]. LRBA post-translationally regulates CTLA-4, a potent suppressive receptor, and abatacept replaces the lost CTLA-4 signalling in these individuals. Patients with *LRBA* mutations are also amenable to haematopoietic stem cell transplantation, and identifying these patients early, at the onset of disease, could allow for the search for a matched donor to begin before the disease progresses. None of our patients were treated with abatacept. Three patients died before a genetic diagnosis was made and the

cost of abatacept may have been prohibitive as many of our patients were from developing countries.

This study confirms recessively inherited *LRBA* mutations as a cause of neonatal diabetes. It is now included on the Exeter Molecular Genetics laboratory's custom targeted NGS panel meaning diagnoses are happening soon after presentation for patients with neonatal diabetes. We have also shown that recessively inherited *LRBA* mutations are a relatively common cause of diabetes and autoimmunity in our cohort of patients diagnosed before 12 months, and testing of *LRBA* should be considered in consanguineous patients with early-onset autoimmunity. We have increased the number of genetic causes of neonatal diabetes to 25, and causes of early-onset autoimmunity that incudes neonatal diabetes to 4. As one patient had isolated diabetes at the age of 2 years, *LRBA* should be included on genetic testing panels for neonatal diabetes. Furthermore, as *LRBA* is a highly polymorphic 58 exon gene, analysis by targeted NGS is preferable as it will require less precious DNA from these patients and rationalise data analysis.

Future directions

Assessment of the factors influencing the variability in phenotype observed, both in our cohort and in previous reports, is warranted. All our patients had protein truncating variants that would be predicted to result in the complete loss of LRBA by nonsense mediated decay. Despite this, the phenotype varied wildly with some individuals surviving to adulthood while others died in infancy. Furthermore, only 11/57 (19%) of the previously reported patients have diabetes. While the high proportion of individuals with diabetes in our cohort represents referral bias, identifying the factors that determine whether patients with biallelic *LRBA* mutations develop diabetes will give insights into the mechanisms of beta-cell

autoimmunity that could have implications for patients with type 1 diabetes. Type 1 diabetes has been classically thought of as a single aetiology, however there is growing evidence for heterogeneity [41]. If immunophenotyping of prospective cases of LRBA deficiency was performed in parallel with patients with newly diagnosed early-onset type 1 diabetes this could find heterogeneity in type 1 diabetes. If some patients with type 1 diabetes display a similar immunophenotype to those with *LRBA* mutations, they may be amenable to therapy with abatacept to counter the autoimmune response to the beta-cells.

Most patients with early onset diabetes and additional autoimmunity did not have a mutation in *LRBA* or the other 24 known causes of NDM (11/17 consanguineous and 23/25 non-consanguineous patients). Further study of these patients to characterise the aetiology of their disease is warranted. Initially this could involve undertaking testing of the type 1 diabetes genetic risk score as employed in chapter 1. Whole genome sequencing to look for novel genetic aetiologies in those with low polygenic risk for type 1 diabetes could then be undertaken. In combination with immunophenotyping this could seek novel mutations in regulatory regions of the genome. Measurement of intracellular LRBA or cell-surface CTLA-4 expression could distinguish patients likely to have deep-intronic regulatory mutations around *LRBA*. Classifying these variants remains a challenge and analysis in combination with functional data would enable a guided approach.

Longitudinal follow up of these patients may impart further knowledge of the disease course and prognosis for patients with recessively inherited *LRBA* mutations. At the time of publication none of our patients had undergone treatment with abatacept or HSCT, but follow up could enable further assessment of the efficacy of these treatments if they are employed. It is not known if early

intervention with abatacept or transplantation could improve the diabetes and lower insulin requirement in patients with biallelic *LRBA* mutations, and follow up of patients with diabetes undergoing treatment with abatacept could give new insights.

CHAPTER 4 - LOW IgE IS A USEFUL TOOL TO IDENTIFY *STAT3* GAIN OF FUNCTION MUTATIONS

Conclusions

We hypothesised that patients with gain-of-function (GOF) *STAT3* mutations would have the reciprocal immunoglobulin phenotype of those with hyper-IgE syndrome (HIES) caused by loss of function (LOF) *STAT3* mutations. All our patients (n=6) had serum IgE levels below the lower limit of the reference range (2KU/L; range 0.7-2KU/L). This is striking as all patients were below the 1st centile of healthy individuals, and the likelihood that this is by chance is therefore extremely small. We showed that 2KU/L had a specificity of 100% (95% CI: 54.1 – 100) and sensitivity of 97.2% (95% CI: 96.2-97.9) to identify *STAT3* GOF.

We also tested serum IgE levels in our patients with hemizygous *FOXP3* mutations (chapter 4a) and showed that the 13/16 (81%) had raised serum IgE, in keeping with previous reports. The main differential diagnosis for non-consanguineous males with early-onset multiple autoimmunity is *STAT3* GOF mutations or hemizygous *FOXP3* mutations, and the levels in our patients were significantly different (p=0.002) suggesting testing IgE could have utility to differentiate these phenotypically similar patients.

Impact of findings

This study provides a method for identifying patients suitable for *STAT3* genetic testing that is affordable and is readily available. It could therefore be used to screen patients before sending samples for genetic testing. It would also be useful to assess the pathogenicity of missense variants in *STAT3* [11, 21, 22, 42]. For example, both disorders are associated with increased infections and atopic dermatitis. Measurement of Serum IgE could therefore be useful to characterise the nature of the variants identified without the need for time consuming functional studies. This would give knowledge on disease course, as patients with HIES do not develop organ specific autoimmunity whilst those with *STAT3* GOF do.

Furthermore, GOF *STAT3* mutations are seen in malignancies (particularly large granular lymphocytic [LGL] leukaemia [43] and it may be that patients with *STAT3* GOF have a higher rate of LGL. Identifying and accurately characterising variants in *STAT3* would therefore allow for monitoring for autoimmunity and potential malignancies. In patients without a coding mutation in *FOXP3* or *STAT3*, raised serum IgE may suggest that there are uncharacterised intronic variants affecting *FOXP3* and enable identification of novel regulatory loci.

Since the publication of our manuscript, a response has been published [23]. This was a larger study of 23 individuals with *STAT3* GOF mutations. The authors showed that low IgE is less useful to identify patients with *STAT3* GOF mutations than our data suggested as they identified 6 patients with serum IgE levels above 2KU/L. When combined with our patients IgE <2KU/L had a sensitivity of 0.69 [95%CI 0.49-0.84] which is too low to guide genetic testing. Measurement of serum IgE to assist with the interpretation of novel missense variants in *STAT3* is still warranted as most patients with GOF have low IgE, and most with LOF have high IgE.

Future directions

To further assess the usefulness of IgE for identifying STAT3 GOF mutations study of additional patients is warranted as the numbers are still low (n=29). The 6 patients reported with IgE levels above 2KU/L may represent an atypical patient group or have variants which affect *STAT3* function in a way that does not reduce IgE levels. The variants identified in the patients were not reported in the publication by Tangye *et al*, therefore further collaboration would be beneficial to correlate specific mutations with IgE levels. Functional work may also be beneficial to determine if the mechanism of increased STAT3 activation differs between some patients accounting for the diverse effects on IgE levels. It may also be beneficial to perform a sensitivity/specificity analysis of serum IgE level's usefulness to identify patients with IPEX syndrome caused by hemizygous *FOXP3* mutations from those with *STAT3* GOF. Assessment of serum IgE in other forms of monogenic autoimmunity may identify common pathways of IgE regulation and improve knowledge of the complex nature of STAT3 signalling.

Final remarks

The work that is collated within this thesis has focussed on the identification of patients with autoimmune diabetes as part of a syndrome of monogenic autoimmunity, and factors that influence the phenotypic variability observed in the known causes. To this end, it has identified biallelic variants in *LRBA* as a cause of autoimmunity that may present as neonatal or early-onset diabetes (chapter 3); shown that a type 1 diabetes genetic risk score can be used to discriminate monogenic autoimmune diabetes from polygenic clustering of diabetes and additional autoimmunity (chapter 1); that the polygenic risk loci, including the established HLA DR3 and DR4 alleles, do not determine if patients with monogenic autoimmunity develop diabetes (chapter 1); and that genotype or presenting feature does not predict prognosis in patients with IPEX syndrome (chapter 2).

The first step towards the work that has been compiled in this thesis was to identify the cohort of patients to study. This was less straightforward than initially thought; all of the clinical information regarding autoimmune diseases for these individuals, other than their diabetes phenotype, was stored either as free text in a database or was within scanned clinical notes stored for each patient. In order to improve this moving forward, I have added new fields to the existing database in order to easily identify historic cases and accurately record autoimmune diseases in new referrals. Furthermore, I have systematically stored the clinical information for these patients in a sister database that is set up to record immunological data. This has been invaluable in forming a resource for my on-going work in gene discovery for monogenic diabetes. As most of the historic cases were referred for diabetes testing, clinical information on their additional autoimmunity was often not recorded in the original referral but was provided in

follow up correspondence. In order to improve this for future referrals I generated a bespoke autoimmunity request form (appendix 2) for prospective cases, which is already being used by clinicians to refer new patients from across the globe.

Initially, testing for monogenic autoimmunity in our laboratory was sporadic, with only the *FOXP3* and *STAT3* genes tested for referrals where there was a strong suspicion of monogenic autoimmunity. Early in my PhD studies I added several other monogenic autoimmunity genes to our targeted NGS panel (for example *LRBA*, which I confirmed was a cause of autoimmune neonatal diabetes during my PhD [Chapter 3]), and I have continued to add new confirmed aetiological genes or likely candidates with each new iteration of the panel. This has been important not only to test all genes at once and increase our diagnostic yield, but also in identifying patients who have been referred with early-onset diabetes which it emerged was the first feature of monogenic autoimmunity.

The work detailed in chapter 1 (A Type 1 diabetes genetic risk score can discriminate monogenic autoimmunity with diabetes from early onset clustering of polygenic autoimmunity with diabetes) has also enabled me to develop a testing pipeline for monogenic autoimmunity and gene discovery (figure 1), as it showed that the genetic risk score could separate monogenic autoimmunity from polygenic clustering of diabetes and additional autoimmunity. This has so far proved fruitful, as we are able to triage samples into their most likely aetiology and easily select samples for gene discovery based on not having a causative variant in a known gene and having a low polygenic risk for type 1 diabetes. This has led to two new putative disease genes; replication studies and functional work are on-going (Johnson *et al.* unpublished).

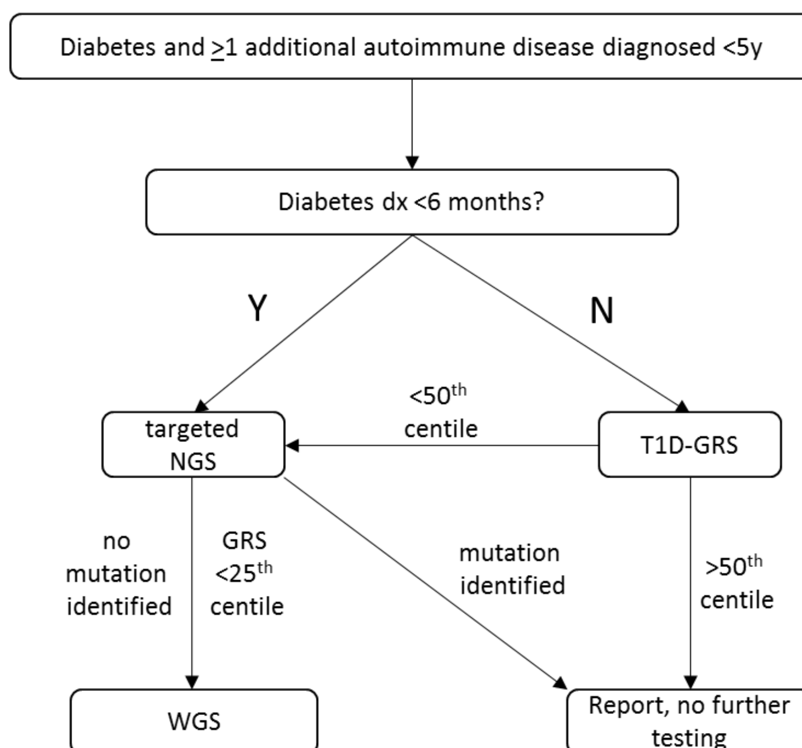


Figure 1: Testing flowchart for monogenic autoimmunity referrals as informed by work in this thesis. NGS – Next generation sequencing. WGS – Whole genome sequencing. T1D-GRS – type 1 diabetes genetic risk score. Dx – diagnosed.

Identifying monogenic causes of autoimmune diabetes has wider implications for patients with polygenic type 1 diabetes. There is increasing recognition that type 1 diabetes is likely to be a common endpoint rather than a single aetiology and that significant heterogeneity exists between patients. Evidence for this includes the variation in the preclinical stage of disease (i.e. the time from seroconversion to clinical onset) and histological evidence from new onset T1D pancreata that distinct groups can be defined by the proportion of infiltrating $CD20^{+}$ B cells ($CD20_{hi}$ or $CD20_{lo}$) that correlate with age of onset. Furthermore, studies of individuals with type 1 diabetes have suggested that a defect in the regulatory T cell compartment be partly responsible for the breakdown in tolerance to the pancreatic β-cells. Many of the disorders described within this thesis result from reduced function or number of regulatory T-cells (e.g. IPEX syndrome caused by hemizygous *FOXP3* mutations, CVID-8 caused by recessively inherited *LRBA* mutations and Immunodeficiency 41 caused by recessively inherited mutations in *IL2RA*).

It may therefore be that there is overlap between the monogenic causes of autoimmune diabetes described and discussed in this thesis and some of the subgroups of type 1 diabetes. This could be assessed by immunophenotyping both those with confirmed monogenic aetiology and clinically defined type 1 diabetes to look for similarities. If phenotypic overlap is found, patients with new onset type 1 diabetes could have trials of therapies that have shown efficacy in their counterpart monogenic subtype. For example, if some patients showed an immunophenotype similar to patients with LRBA deficiency (Chapter 3), abatacept may be an effective therapy to maintain beta cell mass.

Monogenic autoimmunity represents a unique opportunity to study ‘human knockouts’. While animal models (e.g. mice, zebrafish and drosophila) have been

invaluable to study the functional consequences of mutations in a single gene, they are all evolutionarily separated from humans and therefore have key differences in their genetic make-up and physiology. This has meant that in many cases, the phenotype observed in humans is not replicated in the model organism, for example *LRBA* knockout mice do not show any overt immune disease. The ability to study the tissues and cells of patients with monogenic autoimmune disease will therefore offer truer insight into the role of these genes in the human immune system.

These patients could also be used to generate a biobank resource accessible by researchers around the world who are studying the pathway or gene involved. Immune diseases are perhaps ideal for this; the primary tissue in monogenic autoimmunity is the lymphocytes within blood, which is easily obtainable and less invasive to biopsy than other tissues (for example the pancreas for the study of monogenic diabetes). Established technologies to isolate and study viable primary blood mononuclear cells (PBMCs) and intact RNA, and indeed store them long-term, mean the development of a monogenic autoimmunity biobank would be feasible. The uses for these samples are diverse, and could include benchmarking of assays, functional work on cell lines, or simply a discrete genetic phenotype to compare polygenic diseases against.

Identifying further single gene defects in the immune system will also provide further knowledge of the adaptive immune system. There are over 1500 genes that have a role in the function of the immune system (www.immport.org), representing 10-15% of all human genes. For many of these genes, the true function of the gene is not known. Characterising human ‘knockouts’ or ‘knock-ins’ offers the opportunity to study the consequence of a genes’ loss and therefore to deduce it’s normal function. Examples of this include the identification that

LRBA mutations cause CVID-8, which led to functional work to determine it has an essential role as a regulator of CTLA-4 (introduction part 1A and chapter 3). Known immune genes may also be shown to have pleiotropic effects that were previously unrecognised, for example CASP8 (introduction part 1B) was not known to be important for embryonic development prior to it's implication in Mendelian disease.

The case made for low IgE as a sensitive and specific biomarker of *STAT3* GOF in chapter 4 of this thesis has now been refuted. As more patients are identified for new monogenic forms of autoimmunity, the phenotype invariably widens and original hallmarks become less prevalent. This is evidenced by the original report of *STAT3* GOF mutations having a high prevalence of neonatal diabetes (4/5 individuals), now it is known that only approximately 30% of patients with GOF mutations in *STAT3* develop diabetes [11, 21, 22]. This progress to refute or confirm a finding with additional evidence is the very nature of science, and myself and the co-authors express gratitude to Tangye *et al* for publishing their findings. What this work does highlight however is the need to identify cheap biomarkers to prioritise genetic testing in individuals with suspected monogenic autoimmunity as the cost of genetic screening if prohibitive for many individuals. It is therefore important that the scientific community continues to investigate potential biomarkers for monogenic autoimmunity which will help to prioritise genetic testing.

There are still approximately 60 patients within our cohort who have early-onset diabetes and additional autoimmunity without a known monogenic cause. Many of these are likely to harbour novel monogenic causes of autoimmunity, and this cohort is now primed for gene discovery. Identifying novel causes of monogenic

autoimmunity will give insight into the complex nature of the adaptive immune system and could give new insights into beta-cell autoimmunity which will have relevance for the millions around the world with type 1 diabetes. Furthermore, in the age of personalised medicine, finding new monogenic causes of disease could lead to new treatments tailored to the underlying molecular defect.

For patients with confirmed monogenic autoimmunity, further study is warranted to determine the factors underlying the high level of variability in clinical presentation and disease course. Identifying the underlying reason why some patients develop diabetes while others, some with identical variants, do not, may hold the key to understanding heterogeneity in polygenic type 1 diabetes. This could enable new treatment avenues targeting the specific defect underlying the development of islet autoimmunity.

The data presented within this thesis has been presented at international conferences to disseminate findings rapidly, and much of it has been published in peer-reviewed journals. This has contributed to the knowledge base in monogenic autoimmunity and autoimmune diabetes research, and fuelled additional research and collaboration in the field.

References

[1] Waldmann TA, Iio A, Ogawa M, McIntyre OR, Strober W (1976) The metabolism of IgE. Studies in normal individuals and in a patient with IgE myeloma. Journal of immunology (Baltimore, Md : 1950) 117: 1139-1144

[2] Yazdanbakhsh M, van den Biggelaar A, Maizels RM (2001) Th2 responses without atopy: immunoregulation in chronic helminth infections and reduced allergic disease. Trends in immunology 22: 372-377

[3] Minegishi Y, Saito M, Tsuchiya S, et al. (2007) Dominant-negative mutations in the DNA-binding domain of STAT3 cause hyper-IgE syndrome. Nature 448: 1058-1062

[4] Wu LC, Zarrin AA (2014) The production and regulation of IgE by the immune system. Nature reviews Immunology 14: 247-259

[5] Akula S, Mohammadamin S, Hellman L (2014) Fc receptors for immunoglobulins and their appearance during vertebrate evolution. PloS one 9: e96903

[6] Amarasekera M (2011) Immunoglobulin E in health and disease. Asia Pacific allergy 1: 12-15

[7] Greenberger PA, Bush RK, Demain JG, Luong A, Slavin RG, Knutsen AP (2014) Allergic bronchopulmonary aspergillosis. The journal of allergy and clinical immunology In practice 2: 703-708

[8] Diaz RA, Charles Z, George E, Adler AI (2013) NICE guidance on omalizumab for severe asthma. The Lancet Respiratory medicine 1: 189-190

[9] Freeman AF, Holland SM (2008) The Hyper IgE Syndromes. Immunology and allergy clinics of North America 28: 277-viii

[10] Zhang Q, Davis JC, Lamborn IT, et al. (2009) Combined immunodeficiency associated with DOCK8 mutations. The New England journal of medicine 361: 2046-2055

[11] Flanagan SE, Haapaniemi E, Russell MA, et al. (2014) Activating germline mutations in STAT3 cause early-onset multi-organ autoimmune disease. Nature genetics 46: 812-814

[12] Amberger JS, Bocchini CA, Schiettecatte F, Scott AF, Hamosh A (2015) OMIM.org: Online Mendelian Inheritance in Man (OMIM(R)), an online catalog of human genes and genetic disorders. Nucleic acids research 43: D789-798

[13] Hennezel E, Bin Dhuban K, Torgerson T, Piccirillo C (2012) The immunogenetics of immune dysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome. Journal of Medical Genetics 49: 291

[14] Akira S (1999) Functional roles of STAT family proteins: lessons from knockout mice. Stem cells (Dayton, Ohio) 17: 138-146

[15] O'Shea JJ, Schwartz DM, Villarino AV, Gadina M, McInnes IB, Laurence A (2015) The JAK-STAT pathway: impact on human disease and therapeutic intervention. Annual review of medicine 66: 311-328

[16] Ozaki K, Spolski R, Feng CG, et al. (2002) A critical role for IL-21 in regulating immunoglobulin production. Science (New York, NY) 298: 1630-1634

[17] Avery DT, Ma CS, Bryant VL, et al. (2008) STAT3 is required for IL-21-induced secretion of IgE from human naive B cells. Blood 112: 1784-1793

[18] Saito M, Nagasawa M, Takada H, et al. (2011) Defective IL-10 signaling in hyper-IgE syndrome results in impaired generation of tolerogenic dendritic cells and induced regulatory T cells. The Journal of experimental medicine 208: 235-249

[19] Punnonen J, de Waal Malefyt R, van Vlasselaer P, Gauchat JF, de Vries JE (1993) IL-10 and viral IL-10 prevent IL-4-induced IgE synthesis by inhibiting the accessory cell function of monocytes. Journal of immunology (Baltimore, Md : 1950) 151: 1280-1289

[20] Palomares O, Martin-Fontecha M, Lauener R, et al. (2014) Regulatory T cells and immune regulation of allergic diseases: roles of IL-10 and TGF-beta. Genes and immunity 15: 511-520

[21] Haapaniemi EM, Kaustio M, Rajala HL, et al. (2015) Autoimmunity, hypogammaglobulinemia, lymphoproliferation, and mycobacterial disease in patients with activating mutations in STAT3. Blood 125: 639-648

[22] Milner JD, Vogel TP, Forbes L, et al. (2015) Early-onset lymphoproliferation and autoimmunity caused by germline STAT3 gain-of-function mutations. Blood 125: 591-599

[23] Tangye SG, Forbes LR, Leiding J, et al. (2017) Low IgE Is Insufficiently Sensitive to Guide Genetic Testing of STAT3 Gain-of-Function Mutations. Clinical chemistry 63: 1539-1540

[24] Flanagan SE, Haapaniemi E, Russell MA, et al. (2014) Activating germline mutations in STAT3 cause early-onset multi-organ autoimmune disease. Nat Genet 46: 812-814

[25] Mogensen TH (2013) STAT3 and the Hyper-IgE syndrome: Clinical presentation, genetic origin, pathogenesis, novel findings and remaining uncertainties. JAKSTAT 2: e23435

[26] Martins TB, Bandhauer ME, Bunker AM, Roberts WL, Hill HR (2014) New childhood and adult reference intervals for total IgE. The Journal of allergy and clinical immunology 133: 589-591

[27] Tomer Y, Dolan LM, Kahaly G, et al. (2015) Genome wide identification of new genes and pathways in patients with both autoimmune thyroiditis and type 1 diabetes. J Autoimmun 60: 32-39

[28] Bittles A (2001) Consanguinity and its relevance to clinical genetics. Clinical genetics 60: 89-98

[29] Gutierrez-Achury J, Zhernakova A, Pulit SL, et al. (2015) Fine mapping in the MHC region accounts for 18% additional genetic risk for celiac disease. Nature genetics 47: 577-578

[30] Li YR, Li J, Zhao SD, et al. (2015) Meta-analysis of shared genetic architecture across ten pediatric autoimmune diseases. Nat Med 21: 1018-1027

[31] Schultheiss UT, Teumer A, Medici M, et al. (2015) A genetic risk score for thyroid peroxidase antibodies associates with clinical thyroid disease in community-based populations. The Journal of clinical endocrinology and metabolism 100: E799-807

[32] Abraham G, Tye-Din JA, Bhalala OG, Kowalczyk A, Zobel J, Inouye M (2014) Accurate and robust genomic prediction of celiac disease using statistical learning. PLoS genetics 10: e1004137

[33] Nerup J, Platz P, Andersen OO, et al. (1974) HL-A antigens and diabetes mellitus. Lancet (London, England) 2: 864-866

[34] Erlich H, Valdes AM, Noble J, et al. (2008) HLA DR-DQ Haplotypes and Genotypes and Type 1 Diabetes Risk. Diabetes 57: 1084

[35] Liu C, Xiao F, Hoisington-Lopez J, et al. (2017) Accurate typing of class I human leukocyte antigen by Oxford nanopore sequencing. bioRxiv

[36] Noble JA (2015) Immunogenetics of type 1 diabetes: A comprehensive review. Journal of Autoimmunity 64: 101-112

[37] Cuenod B, Brousse N, Goulet O, et al. (1990) Classification of intractable diarrhea in infancy using clinical and immunohistological criteria. Gastroenterology 99: 1037-1043

[38] Mirakian R, Hill S, Richardson A, Milla PJ, Walker-Smith JA, Bottazzo GF (1988) HLA product expression and lymphocyte subpopulations in jejunum biopsies of children with idiopathic protracted diarrhoea and enterocyte autoantibodies. J Autoimmun 1: 263-277

[39] Otsubo K, Kanegane H, Kamachi Y, et al. (2011) Identification of FOXP3-negative regulatory T-like (CD4(+)CD25(+)CD127(low)) cells in patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome. Clinical immunology (Orlando, Fla) 141: 111-120

[40] Egro FM (2013) Why is type 1 diabetes increasing? Journal of molecular endocrinology 51: R1-13

[41] Arif S, Leete P, Nguyen V, et al. (2014) Blood and islet phenotypes indicate immunological heterogeneity in type 1 diabetes. Diabetes 63: 3835-3845

[42] Freeman AF, Holland SM (2008) The hyper-IgE syndromes. Immunology and allergy clinics of North America 28: 277-291, viii

[43] Rajala HL, Porkka K, Maciejewski JP, Loughran TP, Jr., Mustjoki S (2014) Uncovering the pathogenesis of large granular lymphocytic leukemia-novel STAT3 and STAT5b mutations. Annals of medicine 46: 114-122

APPENDIX 1

Reference sequence transcripts and genes in targeted pancreatic panel

v5.1

GENE	REFSEQ ID
MODY	
ABCC8	NM_001287174
CEL	NM_001807
CISD2	NM_001008388
GATA4	NM_002052
GATA6	NM_005257
GCK	NM_000162
HNF1A	NM_000545
HNF1B	NM_000458
HNF4A	NM_175914
INS	NM_001185098
INSR	NM_000208
KCNJ11	NM_000525
LMNA	NM_170707
mtDNA_3243	NC_012920
NEUROD1	NM_002500
PAX6	NM_001604
PCBD1	NM_000281
PDX1	NM_000209
PLIN1	NM_002666
POLD1	NM_002691
PPARG	NM_015869
RFX6	NM_173560
TRMT10A	NM_001134665
WFS1	NM_006005
ZFP57	NM_001109809
Neonatal Diabetes	
ABCC8	NM_001287174
BSCL2	NM_032667
CISD2	NM_001008388
EIF2AK3	NM_004836
FOXP3	NM_014009
GATA4	NM_002052
GATA6	NM_005257
GCK	NM_000162
GLIS3	NM_001042413
HNF1B	NM_000458
IER3IP1	NM_016097
IL2RA	NM_000417

INS	NM_001185098
INSR	NM_000208
LRBA	NM_006726
KCNJ11	NM_000525
MNX1	NM_005515
NEUROD1	NM_002500
NEUROG3	NM_020999
NKX2-2	NM_002509
PDX1	NM_000209
PTF1A	NM_178161
C10orf115	NR_103721
RFX6	NM_173560
SLC19A2	NM_006996
SLC2A2	NM_000340
STAT3	NM_139276
WFS1	NM_006005
ZFP57	NM_001109809
Early-onset autoimmunity with diabetes	
AIRE	NM000383.3
CD274	NM_014143
FOXP3	NM_014009
IL2RA	NM_000417
ITCH	NM_001257138
LRBA	NM_001199282
STAT1	NM_007315
STAT3	NM_139276
STAT5B	NM_012448

APPENDIX 2

PLEASE RETURN THIS FORM WITH EDTA BLOOD OR DNA WHEN GENETIC TESTING IS REQUESTED

Genetic testing for early-onset multiple autoimmune disease

Genetic testing is provided free of charge for any patient diagnosed with diabetes and ≥1 other autoimmune disorders before 5 years.

Samples must be labelled with name and date of birth, please send either
(1) Our preferred option is 3-5 mls blood taken in tubes containing EDTA and transported fresh (not frozen) at room temperature to arrive in the UK within 5 days. Blood samples should be sent in leak-proof packaging and include absorbent material to absorb any leakage OR
(2) Send 5-10 micrograms of DNA (to allow repeats) at room temperature. Again please make sure the tube is very securely sealed.

Please include samples from both parents whenever possible – whether affected or unaffected.

Please fill in this form electronically, e-mail to Matthew Johnson (mj318@exeter.ac.uk) and send a printed copy with samples to: Prof Sian Ellard, Department of Molecular Genetics, RILD Level 3, Royal Devon and Exeter NHS Foundation Trust, Barrack Road, Exeter, EX2 5DW, UK

For clinical advice please contact Prof Andrew Hattersley by e-mail a.t.hattersley@exeter.ac.uk or telephone +44 1392 408260

Patient details

SURNAME:	CLINICIAN NAME:
FORENAME:	CLINICIAN E-MAIL ADDRESS FOR REPORT:
D.O.B. (DD/MM/YYYY):	HOSPITAL:
NHS/CHI NUMBER (for UK patients):	
GENDER:	CITY
ETHNIC ORIGIN:	COUNTRY

Parent details

MOTHER'S SURNAME:	MOTHER'S FORENAME:	MOTHER'S D.O.B.:
FATHER'S SURNAME:	FATHER'S FORENAME:	FATHER'S D.O.B.:

Clinical information

PRESENTING FEATURE:	BIRTH WEIGHT (g):		CURRENT WEIGHT:	
DATE OF DIAGNOSIS (DD/MM/YYYY):	GESTATION (WEEKS):		CURRENT HEIGHT:	
DIABETIC?	INITIAL TREATMENT:	CURRENT TREATMENT:	GAD ANTIBODIES TESTED?	IA2 ANTIBODIES TESTED?
DATE OF DIAGNOSIS (DD/MM/YYYY):	DOSE:	DOSE:	GAD TITRE:	IA2 TITRE:
THYROID DYSFUNCTION?	TREATMENT:	TPO ANTIBODY MEASURED?	Tg ANTIBODY MEASURED?	TR ANTIBODY MEASURED?
DATE OF DIAGNOSIS (DD/MM/YYYY):	DOSE:	TPO Ab TITRE:	Tg Ab TITRE:	TR Ab TITRE:
GASTROINTESTINAL SYMPTOMS (GIVE TYPE)?	TREATMENT:	ENTERIC PROTEIN LOSS?	tTG ANTIBODY TESTED?	AE ANTIBODY MEASURED?
DATE OF DIAGNOSIS (DD/MM/YYYY):	DOSE:	RESPONSE TO TREATMENT?	tTG Ab TITRE:	AE Ab TITRE:
HAEMATOLOGICAL SYMPTOMS (GIVE TYPE)?	IMMUNOGLOBULINS MEASURED?		LYMPHOCYTE PROFILE MEASURED?	
	IgA LEVEL (NORMAL RANGE):	IgE LEVEL (NORMAL RANGE):	IF SO, PLEASE PROVIDE DETAILS:	
DATE OF DIAGNOSIS (DD/MM/YYYY):	IgG LEVEL (NORMAL RANGE):	IgM LEVEL (NORMAL RANGE):		
ADRENAL INSUFFICIENCY?	DERMATOLOGICAL DISORDERS (GIVE TYPE)?		ANY KNOWN ALLERGIES?	
ARTHRITIS?	HISTORY OF RECURRENT INFECTIONS?		CHRONIC MUCOCUTANEOUS CANDIDIASIS?	
DEVELOPMENTAL DELAY?	DELAYED PUBERTY?		FACIAL DYSMORPHISM?	
ANY OTHER FURTHER DETAILS/OTHER (A SEPARATE DOCUMENT WITH FULL DETAILS OF ANY ADDITIONAL MEDICAL PROBLEMS WOULD BE VERY HELPFUL):				

Family history

ARE PARENTS RELATED? IF YES, HOW?:
AFFECTED FATHER? (AGE DIAGNOSED, AUTOIMMUNE FEATURES, TREATMENT):

AFFECTED MOTHER? (AGE DIAGNOSED, AUTOIMMUNE FEATURES, TREATMENT):
AFFECTED SIBLING(S)? (AGE DIAGNOSED, AUTOIMMUNE FEATURES, TREATMENT):
OTHER FAMILY MEMBERS AFFECTED? (A PEDIGREE SHOWING AGE AT DIAGNOSIS, AUTOIMMUNE FEATURES AND CURRENT TREATMENT OF AFFECTED FAMILY MEMBERS WOULD BE VERY HELPFUL):
IF SAMPLES FROM OTHER FAMILY MEMBERS HAVE BEEN SENT PREVIOUSLY PLEASE GIVE DETAILS:
IF A MUTATION HAS ALREADY BEEN IDENTIFIED IN A FAMILY MEMBER PLEASE GIVE DETAILS: Gene Mutation Name and date of birth of relative with mutation: Relationship to this person

Consent

1. I understand that my sample will be used only for diagnostic and research purposes relevant to myself and others in my family. Please Tick ☐
2. I also consent for my sample to be used for future research into all forms of genetic diabetes and other beta cell conditions, whether or not it is of direct clinical benefit to me. Please Tick: Yes ☐ No ☐
3. I am also happy to be contacted about research into genetic diabetes and you may contact me directly at:

   Name Address Telephone E-mail

Signed by patient/ guardian/advocate: ……………………………………. **Date: ……………..………**

For more information (and patient information sheets) please see www.diabetesgenes.org/content/genetic-beta-cell-research-bank