This electronic thesis or dissertation has been downloaded from Explore Bristol Research, http://research-information.bristol.ac.uk

Author:
Fareed, Rana Y Y

Title:
The changing frequency of Type 1 diabetes-associated genes over time

Insights into how our changing environment influences risk

General rights
Access to the thesis is subject to the Creative Commons Attribution - NonCommercial-No Derivatives 4.0 International Public License. A copy of this may be found at https://creativecommons.org/licenses/by-nc-nd/4.0/legalcode This license sets out your rights and the restrictions that apply to your access to the thesis so it is important you read this before proceeding.

Take down policy
Some pages of this thesis may have been removed for copyright restrictions prior to having it been deposited in Explore Bristol Research. However, if you have discovered material within the thesis that you consider to be unlawful e.g. breaches of copyright (either yours or that of a third party) or any other law, including but not limited to those relating to patent, trademark, confidentiality, data protection, obscenity, defamation, libel, then please contact collections-metadata@bristol.ac.uk and include the following information in your message:

- Your contact details
- Bibliographic details for the item, including a URL
- An outline nature of the complaint

Your claim will be investigated and, where appropriate, the item in question will be removed from public view as soon as possible.

The changing frequency of Type 1 diabetes-associated genes over time: Insights into how our changing environment influences risk

Rana Fareed

A dissertation submitted to the University of Bristol in accordance with the requirements for the award of the degree MD in the Faculty of Health Sciences

Bristol Medical School -Translational Health Sciences submitted June 2020

Words: 50798

Abstract

Type 1 diabetes (T1D) is a chronic autoimmune disease. Both genetic and environmental risk factors contribute to its development. The incidence of the condition is increasing at a rate of 3% per year in most westernised populations. Counterintuitively, while the incidence has been increasing, the proportions of individuals positive for high-risk Human leucocyte antigen (*HLA)* class II genes have been decreasing. This might suggest an environmental impact on changing incidence. It is, however, unknown whether this change has continued into the 21st century. In addition, a panel of non-*HLA* genes also contribute to the risk of T1D, but their frequencies over time have not been monitored. The aim of this study is to explore 1) whether *HLA* class II frequency continues to change over time, 2) the changes in non-*HLA* SNP frequencies over time and 3) whether a report of non-*HLA* SNPs associated with the development of islet autoimmunity, but not diabetes, can be replicated in this study and if the dynamics over time have changed for these SNPs

Methods

Two populations were available for this study, individuals with diabetes participating in the long-running Bart's Oxford study (BOX) recruiting since 1985 and the "Golden Years" cohort (GY) diagnosed between 1922 and 1948. *HLA* class II genotyping was carried out using well-established PCR-SSP. Non-*HLA* SNPs associated with T1D were analysed using Taqman genotyping. Autoantibodies to glutamic acid decarboxylase (GAD), insulin, insulinoma-associated protein-2 (IA-2), IA-2β and Zinc transporter 8 (ZnT8) were measured using radio-binding assays. Descriptive statistics and chi-square for trend were used to analyse changes to genotype frequency over time while chi-square analysis and logistic regression were used to test for associations between the SNPs and islet autoantibodies.

Results

HLA class II data from 1737 cases diagnosed with diabetes under the age of 21 years between 1985 and 2015 were analysed, and results do not support a change in *HLA* class II frequency over this time span. Genotyping of 19 non-*HLA* T1D associated SNPs in DNA samples from 1992 BOX and 285 GY participants did not provide evidence for changes in frequency over time. Serum collected a median of one day from diagnosis (range -61 to 90 days) was available from 550 people with diabetes (median age at diagnosis 10.9, range 0.7 to 21 years) participating in the BOX study. ZnT8A, IA-2A and IA-2βA were more common ($p<0.001$) in newly-diagnosed patients homozygous for *FCRL3* rs3761959 CC, compared with other genotypes. Furthermore, all 20 individuals homozygous for the disease-associated allele of *RELA* had GAD autoantibodies, compared with 79% of those without this allele ($p=0.055$). Logistic regression showed that *FCRL3* was associated with IA-2A, ZnT8A, and IA-2βA ($p<0.002$ for all), independently of age at diagnosis, sex, and *HLA* class II genotype. In addition to *FCRL3* and *RELA*, the *LPP* GG genotype had the highest association with GADA positivity ($p=0.018$) and positive association with IA-2βA ($p=0.036$), independent of age at diagnosis, sex, and *HLA* class II genotypes.

Conclusion

This study has shown for the first time that the well-documented change in *HLA* class II frequency over time is specific for *HLA* class II and not shared by non-*HLA* loci associated with T1D. There is no evidence that *HLA* class II frequency is continuing to decrease in the twenty-first century, but a larger study incorporating data from other long-term longitudinal studies would be beneficial. Associations of three non-*HLA* SNPs with islet autoimmunity was confirmed in this study.

Dedication

To my reason to be, the stars that brighten my life, mum and dad, my husband Al, and our Zain, I dedicate this work.

A special dedication to the Memory of Alistair Williams (1959-2020)

Acknowledgements

I would like to thank my supervisors, Professor Kathleen Gillespie, and Dr Alistair Williams for your continuous mentorship, reassurance, and support, and for believing in me.

A big thank-you to Georgina Mortimer for your help, support, and advice inside and outside the laboratory, and a big thank-you to Dr Anna Long for always finding the time to accommodate me and answer my questions.

Thank-you Claire and Sian and thank-you to the Autoantibody lab for lending me a helping hand. I would also like to thank the Diabetes and Metabolism, and Technical Support teams, for making me feel like a part of your family.

Last but not least, a special thank-you to my parents and my husband. I would not have made it without your love, support, understanding and patience.

Author's declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: .... DATE: ....

Contribution statement: I contributed to the *HLA* genotyping of recent T1D cases and analysed the data. In total I genotyped 19 SNPs in the Golden Years cohort and 4 SNPs in the full T1D BOX cohort, and several other SNPs in a subset of BOX. I analysed all available data. I also genotyped the 3 SNPs associated with islet autoimmunity in BOX cases where "at-diagnosis" islet autoantibody data were available for me to analyse. I prepared an ethics application to collect more samples from the GY population.

Table of Contents

Abstract 1

Dedication 3

Acknowledgements 4

Author's declaration 5

Table of Figures 13

List of Tables 17

Abbreviations 22

Chapter 1 Introduction.. 25

1.1 Definition of Diabetes 26

1.2 Epidemiology of T1D 28

1.3 The pathogenesis of T1D: islet autoimmunity.............................. 30

1.4 Risk factors of T1D.. 33

1.4.1 Environmental factors 33

1.4.1.1 Some environmental factors associated with type 1 diabetes 34

1.5 Hypotheses to explain environmental impacts on genetic background 36

1.6 Clinical trials 39

1.7 Genetics of T1D 40

1.7.1 Twin studies 40

1.7.2 Genetic susceptibility before GWAS (Genome-Wide Association Studies) 41

1.7.3 After GWAS 52

1.8 Islet Autoantibodies 63

1.9 Interactions of genes and islet autoantibodies 77

1.9.1 *HLA* and islet autoantibodies 77

1.9.2 T1D non-*HLA* genes and islet autoantibody associations 78

1.10 Rationale 81

1.11 Hypothesis and specific aims 81

Chapter 2 Materials and Methods 83

2.1 Study Populations: 84

2.2 Genetic samples and methods 85

2.2.1 Whole Genome Amplification steps: 86

2.2.2 *HLA* genotyping 88

2.2.2.1 *HLA* class II genotyping procedure 89

2.2.3 SNP genotyping 93

2.2.3.1 TaqMan genotyping steps 93

2.2.3.2 *CCR-5* rs333 genotyping steps..98

2.3 Islet Autoantibody samples and methods 100

2.3.1 Subjects.. 100

2.3.2 Methods... 101

2.3.3 Genetic analysis 103

2.3.4 Statistical Analysis.. 103

Chapter 3 Analysis of *HLA* class II risk over time in cases participating in the Bart's Oxford Study (1985-2015) 105

3.1 Introduction 106

3.2 Materials and Methods 107

3.2.1 Population examined 107

3.2.2 Data analysis 108

3.3 Results 108

3.3.1 *HLA* frequencies in BOX probands by date at diagnosis 110

3.3.2 Reanalysis of Golden Years data to include cases diagnosed under age 21 years 112

3.3.3 The effect of age-at-onset on *HLA*-mediated risk in BOX 113

3.4 Discussion 114

Chapter 4 Analysis of non-*HLA* risk in the Golden Years cohort (1922-48) compared with the Bart's Oxford Cohort (1985-2015) 116

4.1 Introduction 117

4.2 Methods and data analysis 118

4.3 Results 118

4.3.1 Non-*HLA* SNPs analyses in BOX and GY 118

4.3.2 Allele frequencies in BOX and GY 121

4.3.3 Gender in BOX and GY 124

4.4 The power of the study and future recruitment 126

4.5 Discussion 127

Chapter 5 Analysis of associations between non-*HLA* SNPs and Islet autoantibodies at diagnosis 130

5.1 Introduction 131

5.2 Methods 132

5.3 Results **135**

5.3.1 Newly-diagnosed BOX subjects; the relationship between *FCRL3* genotypes and islet autoantibodies **135**

5.3.2 Newly-diagnosed BOX subjects; the relationship between *RELA*/*FIBP* genotypes and islet autoantibodies **139**

5.3.3 Newly-diagnosed BOX subjects; the relationship between *LPP* genotypes and islet autoantibodies **140**

5.3.4 Newly-diagnosed BOX subjects; T1D non-*HLA* SNPs and islet autoantibodies **141**

Chapter 6 General Discussion.. **149**

6.1 *HLA* associations with T1D over time... **150**

6.2 Non-*HLA* risk over time **153**

6.3 Possible environmental determinants of type 1 diabetes **154**

6.4 Islet autoantibody associations with SNPs.................................... **157**

6.5 Strengths and weaknesses **160**

6.6 Future directions for T1D prevention **163**

6.7 Conclusion... **165**

References... **166**

Appendix **201**

A. Non-*HLA* SNPs in T1D patients diagnosed under 21 years old **202**

B. Non-*HLA* SNPs in under 5 and 10 years old children **206**

C. Non-*HLA* SNPs and islet autoantibodies associations at diagnosis **213**

D. Golden Years dataset **219**

E. BOX Study dataset **220**

Table of Figures

Figure 1.1 Global incidence and prevalence of T1D in children under the age of 15………………………………………………………. 28

Figure 1.2 Immunohistochemistry of a pancreatic islet in type 1 diabetes………………………………………………………………….. 32

Figure 1.3 Susceptibility and progression to T1D ……………………………. 33

Figure 1.4 The model of the "Threshold Hypothesis"……………………….. 37

Figure 1.5 The *HLA* region on Chromosome 6………………………………… 42

Figure 1.6 Structural composition of MHC class I and class II heterodimers…………………………………………………………… 44

Figure 1.7 *HLA* class II genotypes in BOX and GY……………………………. 46

Figure 1.8 Timeline of discovery of genes associated with type 1 diabetes…………………………………………………………… 49

Figure 1.9 The most common genes that are associated with risk of developing T1D…………………………………………………… 62

Figure 1.10 An antigen-antibody complex……………………………………… 63

Figure 1.11 Pancreatic islet autoantigens……………………………………… 65

Figure 1.12 Crystal structure of the GAD molecule………………………… 70

Figure 1.13 Crystal structure of insulinoma-associated protein-2 (IA-2) autoantigen……………………………………………………. 74

Figure 2.1 Map showing the BOX study region……………………………… 85

Figure 2.2 Genotypes as "called" by the qPCR machine software……… 97

Figure 2.3 *CCR-5* alleles………………………………………………………………. 99

Figure 3.1 A representative gel showing the results from an individual with the highest-risk *HLA* class II genotype…… 108

Figure 3.2 *HLA* class II genotype frequencies for 60 T1D cases tested.. 110

Figure 4.1 Genotypes by Gender in BOX and GY……………………………. 125

Figure 5.1 *FCRL3* genotypes and islet autoantibodies………………………. 136

Figure 5.2 The odds ratios of association between *FCLR3* genotypes and islet autoantibodies……………………………………………….. 138

Figure 5.3 *RELA*/*FIBP* genotypes and GADA…………………………………… 139

Figure 5.4 *LPP* genotypes and islet autoantibodies………………………….. 140

Figure 5.5 *BACH2* genotypes and GADA………………………………………….. 142

Figure 5.6 *CCR-5* genotypes and GADA……………………………………..…… 143

Figure 5.7 *CTLA-4* genotypes and IA-2βA……………………………………..… 144

Figure 5.8 *IL2* genotypes and IAA………………………………………….………. 145

Figure 6.1 The potential for linked analyses across results chapters…. 163

Figure A.1 T1D risk genotypes in BOX and GY………………………………… 202

Figure A.2 Risk allele frequencies in BOX and GY…………………………… 203

Figure A.3 Non-*HLA* risk genotypes in T1D GY and BOX children under the age of 10 years……………………………………………… 212

List of Tables

Table 1.1: Studies of *HLA* class II genotypes over time…………………. 48

Table 1.2: T1D associated SNPs: The SNPs which will be followed up in this study………………………………………………………. 61

Table 1.2: Independent predictors of progression to T1D in children with multiple islet autoantibodies…………………. 67

Table 1.3: Subclasses of islet autoantibodies……………………………… 69

Table 2.1: *HLA DRB1* primer sequences in *HLA* class II genotyping… 90

Table 2.2: *HLA DQB1* primer sequences in *HLA* class II genotyping.. 91

Table 2.3a: SNP probe sequences with the alleles detected………… 94

Table 2.3b: SNP probe sequences with the alleles detected………… 95

Table 2.4: The characteristics of newly-diagnosed BOX participants………………………………………………………………. 101

Table 3.1: ***HLA*** **class II analysis…………………………………………………… 109**

Table 3.2: ***HLA*** **in BOX Study Cases…………………………………………….. 111**

Table 3.3: ***HLA*** **comparison over time as previously published……… 112**

Table 3.4: Comparison of ***HLA*** **genotypes in under 21 years old……. 112**

Table 3.5: ***HLA*** **genotypes comparison in under 5 years old…………. 113**

Table 4.1a: SNP genotypes in BOX and GY………………………………… 119

Table 4.1b: SNP genotypes in BOX and GY………………………………… 120

Table 4.2a: Allele frequencies in BOX and GY…………………………….. 122

Table 4.2b: Allele frequencies in BOX and GY…………………………….. 123

Table 5.1: The logistic regression models of ***FCRL3*** **association**

with a) IA-2, b) IA-2β and c) ZnT8 autoantibodies………… 133

Table 5.2: The logistic regression model of *LPP* association with a) IA-2 and b) IA-2β autoantibodies………………………………… 134

Table 5.3: T1D non-*HLA* SNPs and islet autoantibodies…………………. 141

Table A.1a Genotypes by gender in BOX and GY…………………………… 204

Table A.1b Genotypes by Gender in BOX and GY…………………………. 205

Table A.2 GY children diagnosed with T1D under the age of 10 years……………………………………………………………………… 206

Table A.3 GY children (1985-1995) diagnosed with T1D under the age of 10 years……………………………………………………….. 207

Table A.4 BOX children (1995-2005) diagnosed with T1D under the age of 10 years……………………………………………………… 208

Table A.5 GY children diagnosed with T1D under the age of 5 years……………………………………………………………………… 209

Table A.6 BOX children (1985-1995) diagnosed with T1D under the age of 5 years……………………………………………………….. 210

Table A.7 BOX children (1995-2005) diagnosed with T1D under the age of 5 years……………………………………………………….. 211

Table A.8 *FCRL3* genotypes and IA-2A………………………………………… 213

Table A.9 *FCRL3* genotypes and IA-2βA……………………………………. 213

Table A.10 *FCRL3* genotypes and ZnT8A…………………………………….. 214

Table A.11 *RELA* genotypes and GADA……………………………………….. 214

Table A.12 *LPP* genotypes and GADA………………………………………….. 215

Table A.13 *LPP* genotypes and IA-2βA……………………………………….. 215

Table A.14 *BACH2* genotypes and GADA…………………………………… 216

Table A.15 *CCR-5* genotypes and GADA……………………………………… 216

Table A.16 *UBASH3A* genotypes and GADA……………………………….. 217

Table A.17 *CTLA-4* genotypes and IA-2βA…………………………………… 217

Table A.18 *CTLA-4* genotypes and IAA……………………………………….. 218

Table A.19 *IL2* genotypes and IAA……………………………………………… 218

Table A.20 A representation of the Golden Years dataset……………. 219

Table A.21 A representation of the BOX study dataset………………… 220

Abbreviations

APCs	Antigen-presenting cells
APS	Autoimmune polyendocrinopathy syndrome
BACH2	Basic leucine zipper transcription factor 2 gene
BOX	Bart's Oxford family study
CCR-5	C-C chemokine receptor type 5 gene
CLEC16A	C-type lectin domain family 16, member A gene
COBL	Cordon-Bleu WH2 Repeat Protein gene
CTLA-4	Cytotoxic T-lymphocyte associated protein 4 gene
CTSH	Cathepsin H gene
DAISY	Diabetes Autoimmunity Study in the Young
DASP	Diabetes Autoantibody Standardization Program
DIPP	Type 1 Diabetes Prediction and Prevention study
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
EGFR	Epidermal Growth Factor Receptor
ERBB3	V-ERB-B2 Erythroblastic leukaemia viral oncogene homolog 3 gene
FCRL3	Fc receptor-like protein 3
FIBP	FGF1 intracellular binding protein
GADA	Glutamic Acid Decarboxylase Autoantibodies
GLIS3	GLIS family zinc finger 3 gene
GWAS	Genome-Wide Association Studies
GY	Golden Years study

HLA	Human Leukocyte Antigens
IAA	Insulin Autoantibodies
IA-2A	Tyrosine phosphatase-related insulinoma-associated 2 autoantibodies
IFIH1	Interferon-induced with helicase C domain 1 gene
IFN	Interferon IFN
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL2	Interleukin 2 gene
IL-10	Interleukin 10 gene
IL2RA	Interleukin 2 receptor alpha gene
IL18RAP	Interleukin 18 Receptor Accessory Protein gene
INS	Insulin gene
LPP	Lipoma Preferred Partner
LYP	Lymphoid protein tyrosine
MHC	Major Histocompatibility Complex
MODY	Maturity Onset Diabetes of the Young
NK	Natural Killer
NOD	Non-obese diabetic
PTP	Protein tyrosine phosphatase
PTPN2	Protein tyrosine phosphatase non-receptor type 2 gene
PTPN22	Protein tyrosine phosphatase non-receptor type 22 gene
RA	Rheumatoid Arthritis
RELA	RELA proto-oncogene, NF-kB subunit

RGS1	Regulator of G protein signalling 1 gene
RNA	Ribonucleic acid
SH2B3	Lymphocyte Adaptor Protein gene
SLC30A8	Solute Carrier Family 30 Member 8
SLE	Systemic Lupus Erythematosus
SNPs	Single nucleotide polymorphisms
TEDDY	The Environmental Determinants of Diabetes in the Young
T1D	Type 1 diabetes
T2D	Type 2 diabetes
T1DGC	Type 1 Diabetes Genetics Consortium
T1D-GRS	Type 1 Diabetes Genetic Risk Score
UBASH3A	Ubiquitin-associated and SH3 domain-containing protein A gene
ZnT8A	Zinc transporter 8 autoantibodies

Chapter 1

Introduction

1.1 Definition of Diabetes

Diabetes mellitus (DM) is a chronic metabolic disorder that is defined according to the World Health Organization (WHO) as “a condition primarily defined by the level of hyperglycaemia giving rise to risk of microvascular damage (retinopathy, nephropathy and neuropathy)”. DM is characterised by elevated plasma glucose levels due to abnormalities in insulin production, secretion, and function (American Diabetes, 2014).

Diabetes is diagnosed when a fasting plasma glucose level is ≥ 7mmol/L (126mg/dl) or a venous plasma glucose level is ≥ 11.1 mmol/L (200mg/dl), or the glycated haemoglobin reading (HbA1c) is ≥ 6.5% (World Health Association (WHO), 2016).

Diabetes is a progressive disease, and one of the major causes of morbidity and mortality worldwide. Globally, diabetes contributed to the deaths of 3.7 million people in 2012 alone, around half of which were due to poorly controlled blood glucose. Furthermore, diabetes caused premature deaths of approximately 1.6 million people who died under the age of 70. It has been estimated that 171 million people in the world had diabetes in the year 2000, a figure that is predicted to increase to 366 million by 2030 (Wild *et al*., 2004; WHO, 2016). In the UK, one in fifteen people is living with diabetes. In 2019, it was reported that 3.8 million people were diagnosed with diabetes, compared to 1.4 million in 1996 (Diabetes UK report, 2019).

Regarding complications, diabetes results in microvascular, macrovascular, and nerve damage if the glucose level is not tightly controlled. This means that diabetics are at a high risk of cardiovascular and cerebrovascular events, blindness, renal failure, and amputations. Individuals with type one diabetes have between a 3.5-fold and a 4.5-fold higher risk of a cerebrovascular and a cardiovascular event than people without diabetes (Diabetes UK

report, 2019). Diabetes is regarded as one of the main causes of preventable visual impairment and blindness (Kohner *et al*., 1996). It is estimated that after twenty years of living with diabetes, the majority will develop retinopathies. When it comes to the effect of diabetes on the kidneys, people living with diabetes are at a higher risk of renal dysfunction than the general population; one in five will have some form of renal dialysis or transplant during their lifetime. As for neuropathic sequelae, it is estimated that 24 amputations per day result from feet neuropathy and ulceration (Holman *et al*., 2012; Diabetes UK report, 2019).

DM is clinically divided into a number of types depending on diagnostic criteria, aetiology and genetics (Kharroubi and Darwish, 2015). The most frequent forms of DM are type 2 diabetes (T2D) which accounts for about 90% of patients diagnosed with diabetes (Diabetes UK report, 2019) and type 1 diabetes (T1D) which represents approximately 8-9%, while the remaining 1-2% consists of other forms of diabetes (Maahs *et al*., 2010; Diabetes UK report, 2019). In an attempt to achieve tailored management for diabetics to minimise complications, Ahlqvist *et al*. (2018) investigated the classification of diabetes (Ahlqvist *et al*., 2018). They identified five diabetes subgroups that were significantly different in terms of genetics, disease progression and risk of complications, where the insulin-deficient group of patients was at the highest risk of developing retinopathy whereas the insulin-resistant group had the highest risk of nephropathy, although this remains to be replicated and widely adopted.

Around 36,000 children and young people under the age of 19 have diabetes in the UK. Approximately 90% have T1D, followed by early-onset T2D, Maturity Onset Diabetes of the Young (MODY), and other rarer forms of diabetes. Annually, more than 3000 children between the ages of ten and fourteen are diagnosed with diabetes (Diabetes UK report, 2019).

In addition to its impact on health, diabetes has an economic impact as well. Currently, diabetes costs the NHS £315 every second. This translates to £10 billion per year or ten per cent of the NHS total budget (Hex *et al*., 2012).

1.2 Epidemiology of T1D

Epidemiologically, the prevalence of T1D was reported to roughly follow a north-south gradient globally (Onkamo *et al*., 1999). Countries at higher latitudes and with limited hours of sunlight had higher incidence rates of T1D, as in Finland that accounted for the highest incidence of 57.6/100 000/year (Patterson *et al*., 2009) compared with countries of much lower incidences such as Japan 1.6/100 000/year and China

0.1/100 000/year (Kawasaki and Eguchi 2004; Soltesz *et al*., 2007; Islam *et al*., 2014; Chen *et al*., 2017). A map of the global incidence of T1D in children under the age of 15 years is shown in **Figure 1.1**.

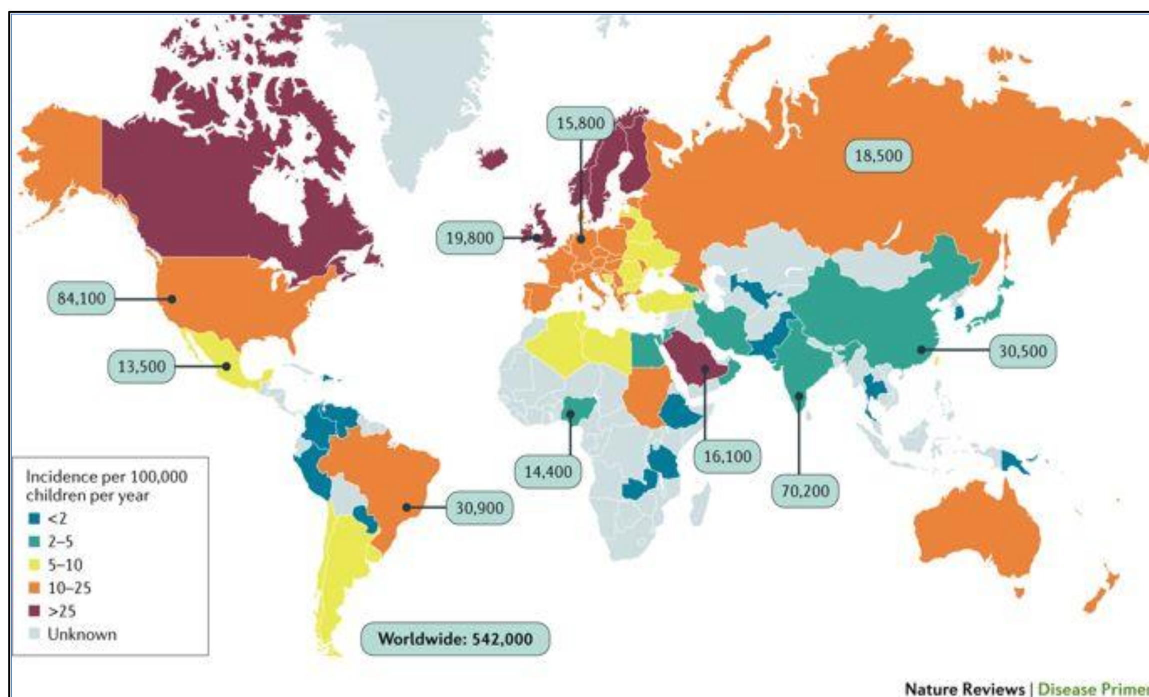

Figure 1.1: *Global incidence and prevalence of T1D in children under the age of 15: This figure of the global incidence and prevalence of type 1 diabetes is presented with permission from the authors (Katsarou et al., 2017). Incidence data of T1D in children are colour-coded; maroon red for highest (>25 per 100,000 per year); blue for lowest (<2 per 100,000 per year); grey for unknown. Data from the International Diabetes Federation (http://www.diabetesatlas.org/ across-the-globe.html).*

A seasonal variation in developing T1D has been observed as those born in Spring were more likely to have T1D (Kahn *et al*., 2009); this may reflect environmental effects.

Although it can happen at any age, T1D is considered the commonest form of diabetes in childhood worldwide. The condition is most often diagnosed in children and adults under the age of thirty years with a peak age of diagnosis of 9-14 years (Diabetes UK report, 2019). During recent decades, an annual increase of 3-5% (Onkamo *et al*., 1999) in the incidence of T1D has been observed. There appears to be a left shift in the age at-diagnosis over time with an increasing number of children under the age of five (Harjutsalo *et al*., 2008) being diagnosed mainly in westernised countries (Gale, 2002; Pundziute-Lycka *et al*., 2002). There

are suggestions that this may have started to plateau more recently in Norway, Finland, Sweden, and the UK; however, the overall trend is that of a 3% annual rise in T1D cases (Harjutsalo *et al*., 2013; Skrivarhaug *et al*., 2014; Patterson *et al*., 2019). T1D tends to affect males more than females in comparison with other autoimmune diseases where there is usually a female preponderance (Gale and Gillespie, 2001; Ostman *et al*., 2008).

1.3 The pathogenesis of T1D: islet autoimmunity

Immune tolerance: Positive and negative selection of T-cells in the thymus

Type 1 diabetes is recognised as a failure of self-tolerance to islet antigens. The immune system has highly regulated pathways to recognise self-peptides including islet peptides. Immature T-cells (thymocytes) are produced in the bone marrow. On relocation to the thymus and rearrangement of the T-cell-receptor (TCR) genes, the immature thymocyte expresses CD4 and CD8. These cells are first subjected to positive selection. Cells where the T-cell receptor can bind with reasonable affinity to either class I or class II Major histocompatibility (MHC) molecules survive this selection; the remainder undergo apoptosis and die. The MHC/HLA molecules are effectively the plate that serve up peptides to the immune system. The role of the positive selection step is to prevent the production of T cells that will not bind to any MHC complex present, regardless of the peptide bound.

Cells surviving positive selection are subjected to a second step, negative selection. Here, T cells that bind with high affinity to MHC (HLA in humans) complexes bound to self-peptides expressed on the surfaces of antigen-presenting cells in the thymus undergo apoptosis or are otherwise suppressed. Those that do not bind with avidity to the HLA complex complete

development and become mature cytotoxic T cells which express only CD8 or helper T cells which express only CD4. The negative selection step leads to self tolerance; cells that bind an MHC-self-peptide complex are removed from the T-cell population. The HLA is introduced in more detail in section 1.7.2.

Immune tolerance: B cells

Similar mechanisms apply to developing B cells, suppressing B cells that express antibodies that interact strongly with self-antigens. B-cell development is tightly regulated, including the induction of B-cell memory and antibody-secreting plasmablasts and plasma cells. Immune tolerance breach is signalled by islet autoantigen-specific B lymphocytes. T-B lymphocyte interactions that lead to expansion of pathogenic T cells underlie T1D development. Infiltration of the pancreatic islets by $CD45^{+}$cells and cytotoxic T-cells signifies insulitis- inflammation of the pancreatic islets- which is a hallmark in T1D pathogenesis as shown in **Figure 1.2** (Ye *et al.*, 2014).

The timing and the exact mechanism that initiates the process of autoimmunity is still poorly understood. T1D is a multifactorial condition where islet autoimmunity is precipitated in individuals above a certain threshold of exposure to risk gene combinations and environmental factors, in addition to epigenetic mechanisms, and their combined impact on the immune system to trigger islet autoimmunity and the subsequent development of T1D (Cudworth *et al*., 1979; Tuomilehto-Wolf and Tuomilehto, 1991; Dahlquist and Mustonen, 1994; Todd, 2010). While the genes underlying genetic susceptibility for type 1 diabetes are increasingly well understood, the environmental factors have remained elusive (Sheehan *et al*., 2020). After the initial trigger, the autoimmune response occurs but progression rates

until the symptoms of T1D become clinically established are variable (Gardner *et al*., 1999; Long *et al*., 2018).

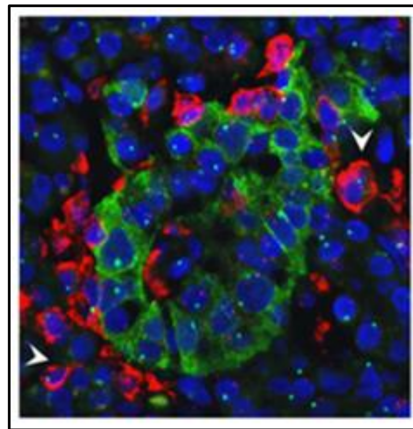

Figure 1.2: *Immunohistochemistry of a pancreatic islet in type 1 diabetes: Insulitis in a section of the pancreatic islets of Langerhans in T1D. The infiltrating CD45+ leucocytes (red) target the islets that are positive for insulin (green) (Ye et al., 2014).*

When approximately 70% (Butler *et al*., 2007) of functioning beta cells are lost through targeted killing of beta cells as shown in **Figure 1.2**, insulin levels are gradually reduced until insulin deficiency ensues as described in the Eisenbarth model (Eisenbarth, 1986) which has been updated by others (Insel and Dunne, 2016) and is shown in **Figure 1.3**. Lack of insulin leads to hyperglycemia that results in classical symptoms, including polydipsia, polyuria, and polyphagia. Only then a clinical diagnosis of T1D is made, mandating a life-long treatment with insulin (DiMeglio *et al*., 2018).

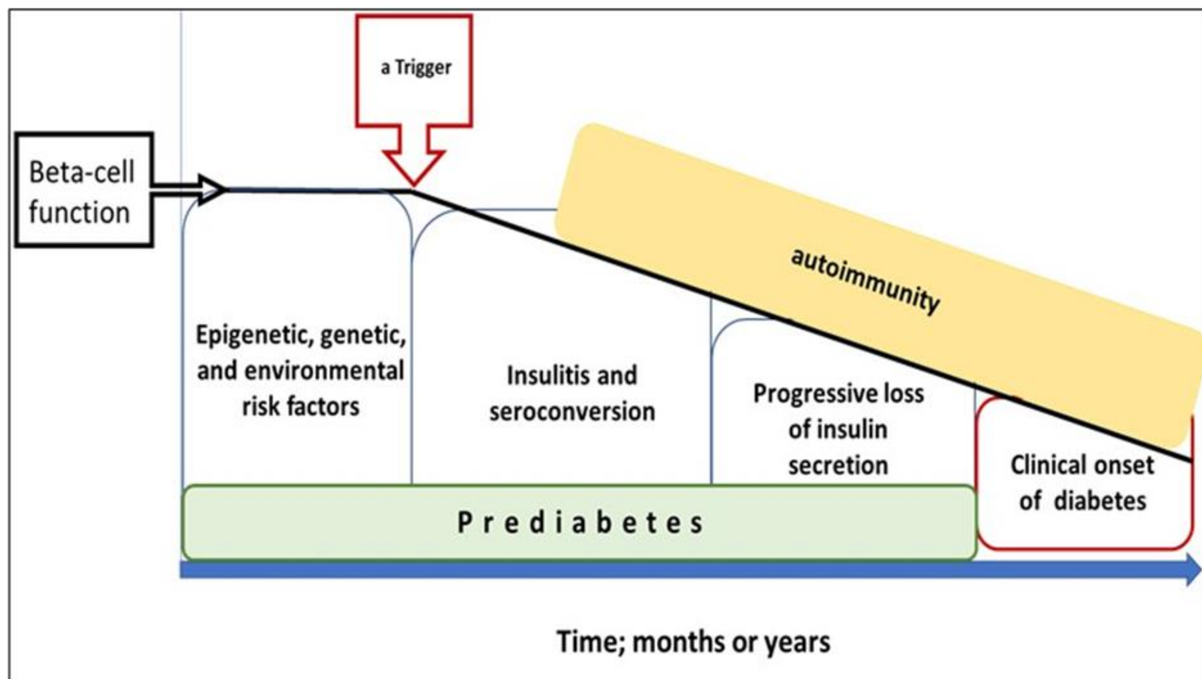

Figure 1.3: *Susceptibility and progression to T1D: The combined effects of genetic susceptibility, epigenetic, and environmental factors on triggering autoimmunity against pancreatic beta-cells; insulitis and appearance of autoantibodies. This preludes the ongoing destruction of functioning beta cells over a variable period of time until metabolic changes are evident; when insulin loss becomes so extensive that endogenous insulin secretion can no longer suppress glucose levels, resulting in the onset of clinical T1D (Adapted from Eisenbarth, 1986).*

1.4 Risk factors of T1D

1.4.1 Environmental factors

To date, no single environmental factor can be confidently identified as causative in T1D. It has been suggested that possibly a single factor or numerous elusive factors could exert effects in a "hit and run" manner, that would cause an insult to the immune system without leaving a trace (van Belle *et al*., 2011).

1.4.1.1 Some environmental factors associated with type 1 diabetes

In search of the possible environmental triggers, studies have suggested viruses as potential candidates. Enteroviruses (Coxsackie B virus) were investigated in animals (Coppieters *et al*., 2012) and thought to contribute to T1D risk in humans (Hyoty, 2002; Stene and Rewers, 2012; Morgan and Richardson, 2014). Krogvold *et al*. (2015) reported the presence of persistent low viral RNA in pancreatic samples collected a few weeks after onset in living newly-diagnosed T1D patients (Krogvold *et al*., 2015). In Philadelphia, a surge in T1D cases was observed two years after an outbreak of Measles (Lipman *et al*., 2002). Exposure to congenital rubella was thought to be related to early-onset T1D in children (Ginsberg-Fellner *et al*., 1985), but no robust association was confirmed (Gale, 2008). In Finland, Ekman *et al*. (2019) investigated the association of Cytomegalovirus (CMV) with T1D in the Type 1 Diabetes Prediction and Prevention (DIPP) study group (https://clinicaltrials.gov/ct2/show/NCT03269084), where 356 children under the age of two years with high-risk *HLA* class II genotypes and persistent autoantibodies were tested for IgG antibodies specific for CMV. They found that CMV infection in young children with persistent autoimmunity delayed the onset of clinical diabetes (Ekman *et al*., 2019), a similar protective role of early CMV infection against coeliac disease was reported (Plot *et al*., 2009; Jansen *et al*., 2016). Interestingly, children under the age of five who had a history of upper respiratory viral infection during infancy had a greater risk of developing T1D (Beyerlein *et al*., 2016). Overall, many viruses have been investigated, but no single virus has been identified as causative of T1D (Hara *et al*., 2013; Rodriguez-Calvo *et al*., 2016).

Diet was also investigated, including gluten (Hummel *et al*., 2002; Pastore *et al*., 2003), vitamin D (Pani *et al*., 2000; Nejentsev *et al*., 2004), and cow's milk intake in infancy (Dahlquist

et al., 1990; Virtanen *et al*., 1998; Virtanen *et al*., 2000), but the overall findings are controversial. BABYDIAB is a study which recruited newborns of parents with T1D and followed them up for the presence of markers of islet autoimmunity including autoantibody testing. Children were sampled at birth, then at nine months, two years, then every three years till the age of eleven years (Hummel *et al*., 2004). They reported that infants younger than three months who consumed gluten-containing food and carried high-risk *HLA* class II genotypes *DR3-DQ2*/*DR4-DQ8*, were at high risk of developing islet autoantibodies which predict future T1D (Ziegler *et al*., 2003). On the other hand, in Sweden, the ABIS (All Babies in Southeast Sweden) study which recruited newborn children from the general population and their mothers (1997-1999), found that gluten introduction in late infancy increased the risk of islet autoimmunity in two-and-a-half-year-old children. Additionally, ABIS investigators observed that weaning from breast milk at or before two months of age and having cow's milk introduced early also preceded autoantibodies development (Ludvigsson *et al*., 2002; Wahlberg *et al*., 2006). This was not replicated in other studies (Couper *et al*., 1999; Norris *et al*., 2003; Ziegler *et al*., 2003; Virtanen *et al*., 2011). The DAISY (Diabetes Autoimmunity Study in the Young) study was a birth cohort started in 1993 recruiting almost 2000 young children who were either at risk of T1D based on population screening by *HLA* genotype or first-degree relatives of T1D patients in Denver. All were followed up for a mean of four years and serially tested for islet autoantibodies. The DAISY study reported that children younger than four months and older than seven months when they had cereals -defined as gluten-containing and gluten-free cereals including rice milk, were at higher risk of developing insulin autoantibodies and subsequently at higher risk of T1D (Norris *et al*., 2003).

With regard to chemicals, the use of nitrosamines was linked to T1D risk (Helgason and Jonasson, 1981). Among medications, antibiotics were of interest as their introduction and

usage were on the rise alongside the rise in T1D incidence in the last few decades. Animal studies on non-obese diabetic (NOD) mice and biobreeding diabetes-prone rats suggested that antibiotics in early life protect against T1D (Brugman *et al*., 2006; Hansen *et al*., 2012). Controversially, Candon *et al*. (2015) reported that male pups of NOD mice given antibiotics from conception till 40 weeks postnatally had a higher incidence of diabetes (Candon *et al*., 2015). In humans, however, that was not the case. A population of T1D Danish children (n = 1578) who were born (1997-2012) were studied to determine if antibiotics had any role in T1D, but no link was found between the two (Mikkelsen *et al*., 2017).

Effects of maternal weight on T1D were also investigated (McKinney *et al*., 1997; Vlajinac *et al*., 2006). It was reported that maternal obesity and gestational weight gain of fifteen kilograms or higher increased the risk of developing persistent islet autoimmunity in genetically susceptible children (Rasmussen *et al*., 2009). In a population-based Swedish study, high maternal body mass index (BMI) in early pregnancy was associated with an increased risk of T1D in children of non –diabetic parents (Hussen *et al*., 2015).

1.5 Hypotheses to explain environmental impacts on genetic background

Theories and models such as the hygiene hypothesis, molecular mimicry, the threshold hypothesis, the accelerator, the overload and other hypotheses have been proposed to help explain aspects of the pathogenesis of T1D. The hygiene hypothesis postulates under-exposure to pathogens early in life affects the normal maturation of the immune system and increases the risk of allergic and autoimmune diseases (Bach, 2002; Gale, 2002; Bach, 2005). Molecular mimicry is where a structural resemblance between a pathogenic antigen and self-

antigen causes the body to attack itself along with the pathogen (Lis *et al*., 2012). Both were suggested in an attempt to understand the mechanisms of environmental impact on T1D.

The threshold hypothesis as shown in **Figure 1.4** was introduced as an algorithmic explanatory model of T1D development based on gene-environment complex interactions in relation to a calculated disease threshold. The model was based on odds ratios of both genetic and environmental factors plotted on a y-axis curve, where at an odds ratio of one a flat line of risk for both genes and environment is present. On the x-axis, arrows represent additional genetic or environmental effects (Wasserfall *et al*., 2011).

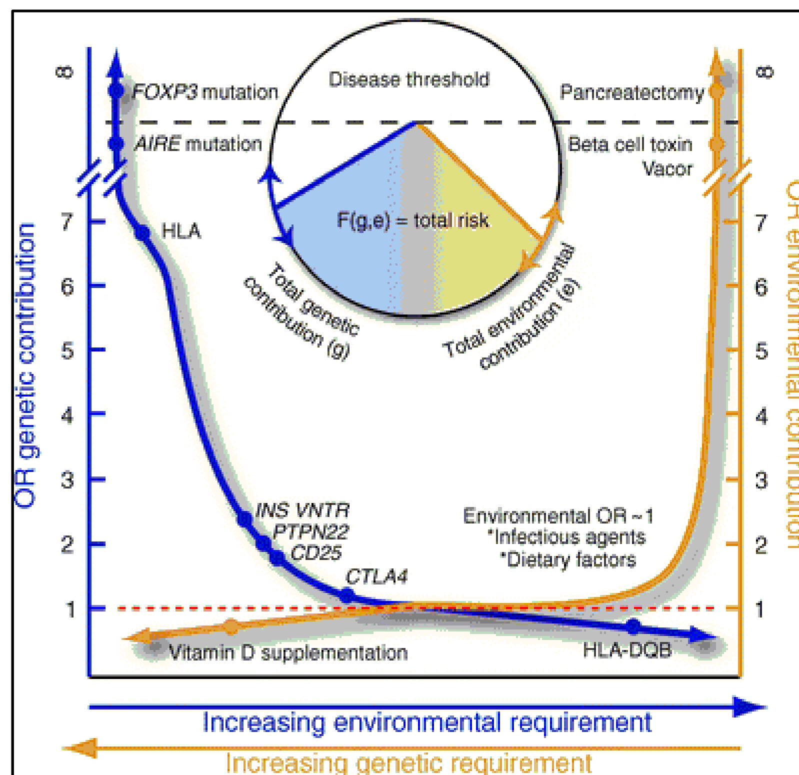

Figure 1.4: *The model of the "Threshold Hypothesis": Genetic factors represented by blue and environmental factors represented by orange colour. Odds ratios of genes and environmental factors ranked on the y-axis. An odds ratio of 1 represents a flatline of risk below which either extra genetic or environmental load (arrows on x-axis) is required to complement the total environmental or genetic risk; respectively, to reach hypothesised disease threshold (Wasserfall et al., 2011).*

The Accelerator Hypothesis was proposed to look into the effect of central obesity on diabetes as a whole although genetic studies do not support this hypothesis, suggesting that both T1D and T2D were essentially one disease, where T1D is an accelerated form of T2D, and that three accelerating factors; the rate of beta-cell apoptosis (an intrinsic factor), insulin resistance, and beta-cell autoimmunity (both are extrinsic factors), would determine the development of either a clinical T1D or T2D "picture" based on their interactions, and the presence or absence of autoimmunity (Wilkin, 2001; Wilkin, 2008). However, a series of studies (Couper *et al*., 2009; Lamb *et al*., 2009; Libman *et al*., 2011) led to controversial results regarding the association of weight gain, length, and growth rate (how quickly weight and length or height gain may happen) of young children, with the risk of development of autoimmunity. Recently, a study by Yassouridis *et al.* (2017) reported that in children of non-diabetic mothers, a rapid increase in BMI potentiated the risk of developing islet autoantibodies (Yassouridis *et al*., 2017).

The overload hypothesis was proposed (Dahlquist, 2006) to focus on the plausible effects of various environmental factors –other than obesity- on overloading the pancreatic beta cells after, rather than at initiation of autoimmunity, by increasing insulin demand and accelerating beta-cell death and more rapid progression to clinical symptoms. Infections, major stressful events, overfeeding and cold climate were suggested as risk factors (Hagglof *et al*., 1991; Dahlquist, 2006; Islam *et al*., 2014).

The search for environmental determinants underlying type 1 diabetes has slowed in recent years with the major focus being the Environmental Determinants of Diabetes in the Young (TEDDY study) which has been designed with sufficient statistical power to identify true effects. The study has followed from birth 8676 children with T1D risk *HLA* class II *DR-DQ*

genotypes in the USA, Finland, Germany, and Sweden. To date the study has evaluated a number of candidate environmental triggers, including diet (Beyerlein *et al.*, 2015), infections (Lynch *et al.*, 2018), probiotics (Yang *et al*., 2017) and microbiome (Stewart *et al.*, 2018) but as yet many investigations have not supported the identification of individual factors leading to an increasing number of investigators hypothesising that multiple environmental factors work together to precipitate T1D (Rewers *et al.*, 2018).

1.6 Clinical trials

Extensive clinical trials have been carried out and are ongoing to test therapies to slow or prevent the development of T1D (Herold *et al*., 2005). Examples of these trials are The European Nicotinamide Diabetes Intervention Trial (ENDIT) which tested nicotinamide (Gale *et al*., 2004), and the Diabetes Prevention Trial – type 1 (DPT-1) which used injectable and oral insulin (Diabetes Prevention Trial-Type 1 Diabetes Study 2002). Both groups recruited first-degree relatives of T1D but neither proved successful. However, such trials showed the need for international partnership as an advantage in T1D trials, giving rise to TrialNet. TrialNet (www.diabetestrialnet.org), an international collaborative network, has emerged to identify global networks of individuals with T1D and 'at-risk' relatives for inclusion in clinical trials. TrialNet recruited first-degree relatives of T1D patients for a CTLA-4 Immunoglobulin (Abatacept) trial (Orban *et al*., 2011) and an Anti-CD3 monoclonal antibody (Teplizumab) trial that targets T-cells (Skyler, 2013). Both trials showed initial promise in preserving insulin production in newly-diagnosed individuals. A trial targeting B-cells using Rituximab in newly-diagnosed T1D patients, delayed the decline in insulin production by approximately eight months (Pescovitz *et al*., 2014). Current plans are ongoing to use combined therapies to

delay or prevent T1D. Recently, TrialNet reported that Teplizumab slowed down the onset of T1D, as of seventy-six at-risk individuals recruited, 43% of those who had the medication developed T1D compared to 72% of those who had a placebo. Moreover, the Teplizumab group took a median of forty-eight months to progress to diabetes, while the median duration was almost half for the placebo group to reach onset of disease (Herold *et al*., 2019). Type 1 diabetes research is now entering an era of focus on combinations of immunotherapy to test in individuals identified as "at risk" using combinations of measurements of genetic risk and islet autoimmunity. These are described in more detail in the next sections.

1.7 Genetics of T1D

1.7.1 Twin studies

T1D is a polygenic disease that does not follow a simple mode of inheritance. Twin studies showed that concordance rates in monozygotic twins are around 30–50% (Olmos *et al*., 1988; Kyvik *et al*., 1995; Hyttinen *et al*., 2003). In monozygotic twins younger than five, concordance rate goes up to around 85% never reaching 100% (Redondo *et al*., 2008). These findings suggest that T1D is not a result of genetics alone and that the environment has an important role in the pathogenesis of T1D (Petersen *et al*., 1997). It was found that in monozygotic twin pairs age-at-onset of T1D in one twin impacts risk in the second twin. If the first twin is diagnosed at less than 24 years of age, the second twin has a 38% risk of developing T1D. This risk is reduced to 6% when the first twin is diagnosed over 24 years (Redondo *et al*., 2008). In families of individuals with T1D, first-degree relatives are at a higher risk of developing T1D compared with the general population risk (0.4%) (Gillespie *et al*., 2002). The genetic risk is >

1% when the mother has diabetes, rising to more than 3% when the father has diabetes, and to 6% when the sibling has diabetes (Risch, 1987; Warram *et al*., 1988; Mehers and Gillespie, 2008). The reason for these differences has not yet been explained.

1.7.2 Genetic susceptibility before GWAS (Genome-Wide Association Studies)

Twin studies have shown that around half of risk associated with T1D is genetic. Before the era of Genome-Wide Association Studies (GWAS), only six genes/gene regions were reported to be associated with the risk of developing T1D -with the *HLA* region alone contributing approximately 50% of risk, and these remain among the most closely associated with disease. The most recent GWAS analysis, however, published in 2021 identified 152 regions of the genome outside the *HLA* as associated with T1D at genomewide significance (Robertson *et al*., 2021). In this introduction, only the genes associated with type 1 diabetes which have been robustly replicated in multiple studies will be described and these will subsequently form part of the research studies described in this thesis. Non-*HLA* regions will be investigated by the most closely associated single nucleotide polymorphisms (SNPs) which have been tested in multiple cohorts to allow comparison of data regardless of whether or not these SNPs are potentially causal (have a direct effect on function).

There are also three genes which have been associated with islet autoimmunity but not with clinical T1D which is intriguing. These three additional SNPs will also be described and included in the analysis.

***HLA* genes:** the main genetic region associated with T1D with closest association with class II- and to a lesser extent class I- molecules) as shown in **Figure 1.5**.

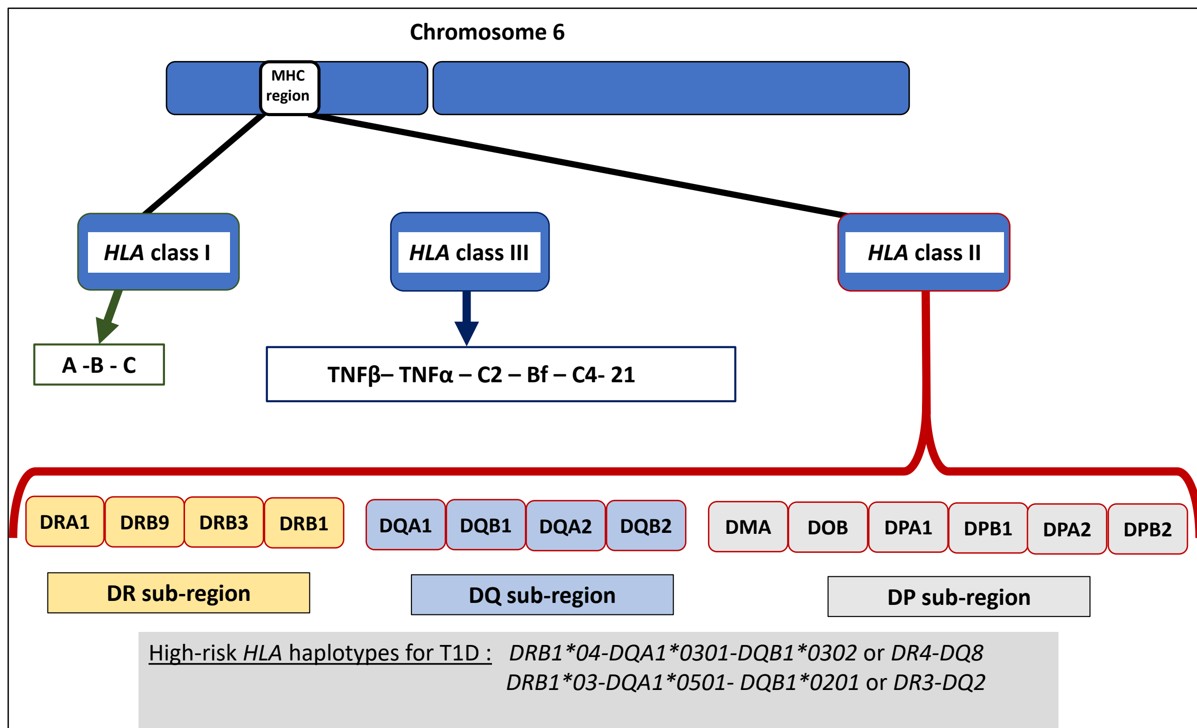

Figure 1.5: *The HLA region on Chromosome 6: Adapted from Gillespie, 2006. Three classes of HLA are identified. Class I has A, B, C regions. Class II has DR, DQ and DP regions. Class III encodes complement system components.*

HLA class II molecules are expressed on dendritic cells, macrophages, B lymphocytes and thymus epithelium (professional antigen-presenting cells or APCs) and present extracellular antigens to $CD4^+$ T-cells while class I molecules are found on nucleated cells and present intracellular antigens to $CD8^+$ T-cells (Bakay *et al*., 2013). *HLA* are Located on the short arm of chromosome 6 (6p21.3), and were first linked to T1D in the 1970s (Nerup *et al*., 1974). Structurally, both molecules have alpha chains; however, only the alpha chain is polymorphic in MHC class I molecule, and in addition to the alpha polypeptide chain, it has a beta-

microglobulin chain not coded by the *HLA* gene region. Class II molecules have both alpha and beta polypeptide chains and do not have a beta-microglobulin chain as shown schematically **in Figure 1.6** (Burrows *et al*., 2006; Mohan and Unanue, 2012).

HLA genes are highly polymorphic conferring both susceptibility to and protection from T1D. *HLA* class II *DRB1*03-DQB1*0201* (*DR3-DQ2*) and *DRB1*04-DQB1*0302* (*DR4-DQ8*) are the two main haplotypes that are associated with increased risk of T1D (Noble *et al*., 1996; Hirschhorn, 2003; Larsen and Alper, 2004; Lambert *et al.*, 2004). One or both these haplotypes are found in approximately 90% of children diagnosed with T1D (Devendra and Eisenbarth, 2003). The genotype *DR4-DQ8/DR3-DQ2* confers the highest risk for developing T1D early in life (Caillat-Zucman *et al*., 1992), and around 5% of children with this genotype will develop islet autoimmunity and T1D by the age of 15 years, compared with only 0.3% in the general population (Nokoff *et al*., 2012). The haplotype *DRB1*15-DQB1*0602* (*DR15-DQ6*) is protective against T1D and is found in less than 1% of type 1 diabetic children and adults compared with more than 20% of the general population (Gillespie *et al*., 2006; Thomas *et al*., 2021); hence it decreases risk of developing type 1 diabetes. *HLA* risk and susceptibility genotypes were assigned a hierarchy of risk (Lambert *et al*., 2004).

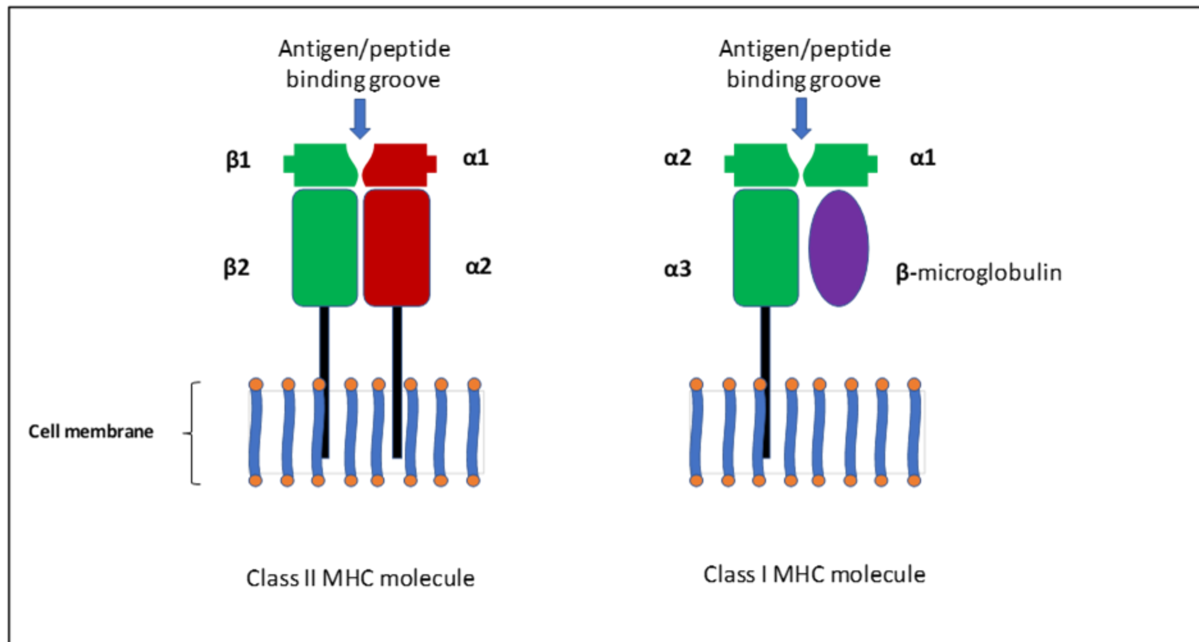

Figure 1.6: *Structural composition of MHC class I and class II heterodimers: Class II alpha and beta chains where α2 and β2 are anchored in the cell membrance and α1 and β1 form the binding groove for peptides/antigens (usually 12-20 amino acids long). Class I molecules have a polypeptide alpha chain where α3 is anchored in the cell membrane with a beta macroglobulin molecule. α1 and α2 fold to provide the binding site for peptides (usually 8-10 amino acids long but sometimes longer) (Burrows et al., 2006; Mohan and Unanue 2012).*

A recent review by Dendrou and colleagues has carefully reviewed current understanding of immune surveillance by HLA molecules and how *HLA* variation at the *HLA* region contributes to type 1 diabetes and other autoimmune diseases. They reflect that while GWAS have progressed data on susceptibility to and protection from disease, much remains to be elucidated about the antigens and TCRs with the disease-associated complexes (Dendrou *et al.*, 2018).

With the accelerated rise in the number of T1D cases in recent decades in comparison to the first half of the 20th century (Onkamo *et al*., 1999; Green *et al*., 2001; Gale, 2002), studies looked into the distribution of *HLA* class II genotypes in search for clues behind the reported increased incidence. In Finland, a comparison between children diagnosed with T1D in the

1960s and mid-1980s had different *HLA* patterns. Those diagnosed in the 1960s had *HLA-DR3* more often than those diagnosed later at the same age (Kontiainen *et al*., 1988). Furthermore, Kontiainen *et al*. (1988) also reported that the association of *HLA* class II *DR3* with class I *B8* and *HLA* class II *DR4* with *HLA* class I *B5* almost doubled in those diagnosed after the 1960s. As all children were from the same population of Helsinki, questions arose regarding environmental attribution to the increasing incidence of T1D. Later, Hermann *et al*. (2003) reported that Finnish children (n=367) diagnosed with T1D between 1939 and 1965 had a higher frequency of high-risk *HLA* class II genotypes compared to those (n=736) diagnosed between 1990 and 2001. They also observed that genotypes conferring protection against T1D including *HLA* class II *DR15-DQ6* were lower in children diagnosed before 1965 (Hermann *et al*., 2003). In the UK, comparing two cohorts, the Golden Years (GY) population that represents T1D patients diagnosed between 1922 and 1948 and the Bart's Oxford (BOX) prospective family study recruiting T1D children diagnosed under the age of 15 years and their families from 1985 to date, it was found that the frequency of the high-risk *HLA* class II genotypes *DR3-DQ2/DR4-DQ8* was significantly decreased from 47% in the GY cohort (n=194) to 35% in age-matched BOX participants (n=1150) diagnosed between 1985 and 2002 as shown in **Figure 1.7** (Gillespie *et al*., 2004). Moreover, a higher proportion of BOX participants had intermediate-risk genotypes *DR3-DQ2*/X and *DR4-DQ8*/X.

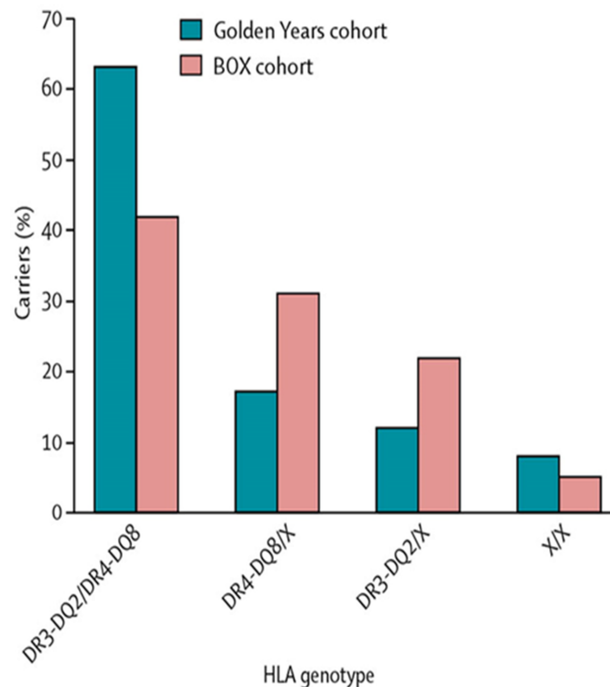

Figure 1.7*: HLA class II genotypes in BOX and GY: A decrease in the frequency of high-risk HLA class II genotypes (DR3-DQ2/DR4-DQ8) in age-matched BOX participants (pink) diagnosed between 1895 and 2003 compared with the GY cohort (turquoise) diagnosed between 1922 and 1948. The frequency of the intermediate-risk genotypes was higher in BOX (Gillespie et al., 2004).*

These findings supported the hypothesis that increased environmental pressure contributes to the recent increase in T1D (Hermann *et al*., 2003; Gillespie *et al*., 2004). Fourlanos *et al*. (2008) studied the genetic pattern in Australian T1D patients (n=462) diagnosed under the age of 18 years between 1950 and 2005. They found that the highest-risk *DR3*/*DR4* genotype decreased in frequency by 51% (p< 0.0001) over time but did not change in incidence when analysed according to the age of onset over five decades, while lower-risk genotypes were increasing in frequency over time, reinforcing the earlier findings in Finland and the UK (Fourlanos *et al*., 2008). Additionally, they reported that recently diagnosed patients had a lower age of onset associated with intermediate and low-risk *HLA* class II genotypes (Fourlanos *et al*., 2008). In the same year in Colorado, Vehik *et al*. (2008), analysed the genetic

pattern in T1D children under the age of 17 years who were diagnosed between 1978 and 2004. The frequency of the high-risk genotype *DR3-DQ2*/*DR4-DQ8* was lower in children (n=264) diagnosed after 2002 compared to those (n=244) diagnosed before 1988, and this difference was mainly seen in children aged 5 to 9 years (Vehik *et al*., 2008). Steck *et al*. (2011) studied two large groups who were diagnosed with T1D at the age of 18 or younger over a span of forty years (1965-2006); the Type 1 Diabetes Genetics Consortium (T1DGC) (n= 4,075) (http://www.t1dgc.org) and the Barbara Davis Center (BDC) (n=1,675). They showed that the proportion of high-risk *HLA* class II genotype was getting lower over time in both groups in a stepwise manner, with a significant decrease among T1DGC children diagnosed at 5 years or younger (p= 0.004) after 1995 compared to before 1985. This was accompanied by an increase in intermediate and low-risk genotypes among children diagnosed after 1985 in the two groups (Steck *et al*., 2011). All these studies, as outlined in **Table 1.1**, suggested that the observed rapid rise in the number of intermediate and low-risk *HLA* class II genotypes in cases diagnosed recently potentially stems from the impact of a diabetogenic environment, that had slowly and progressively lowered the age of disease onset.

Country of study	Year of diagnosis	Subjects	*HLA* genotypes changes over time
Finland	1939-1965 1990-2001	Finnish children (n=367) Finnish children (n=736)	A dcrease in high-risk *HLA* genotype proportion from 25% in 1960s to 18% in 1990s and protective *HLA* genotypes icluding *DR15-DQ6* were lower in 1960s
U.K.	1922-1948 1985-2002	GY cohort (n=194) BOX (n=1150) diagnosed ≤15 years	A decrease in the high-risk *HLA-DR3-DQ2 /DR4-DQ8* genotype from GY 47% to BOX 35% and an increase of intermediate-risk genotypes *DR3-DQ2 /X* and *DR4-DQ8 /X* in BOX
Australia	1950-2005	Australian children (n=462) diagnosed ≤18 years	A decrease in the high-risk *HLA* genotype from 79% in 1950-1969 to 28% in 2000–2005 while lower-risk *HLA* genotypes were increasing in frequency with a lower age-at-onset over time
USA	1978-2004	Children (n=244) diagnosed before 1988 and children (n=264) diagnosed after 2002 ≤ 17 years	The frequency of high-risk *HLA* genotype *DR3-DQ2 /DR4-DQ8* was lower in children diagnosed after 2002 maily in children aged 5-9 years
USA	1965-2006 1965-2008	T1DGC cohort (n=4075) BDC cohort (n=1675) diagnosed ≤18 years	A decrease in the highest-risk *HLA -DR3 /4-DQB1*0302* genotype with an increase in intermediate and low-risk *HLA* genotypes mainly in children diagnosed ≤ 5 years over time

Table 4.1: *Studies of HLA class II genotypes over time: The studies that observed changes in distribution of HLA class II genotypes; reduction in high-risk genotypes and increase in intermediate and low-risk genotypes frequencies among T1D patients diagnosed over the last few decades compared to those diagnosed in the first half of the 20th century.*

The importance of the *HLA* in susceptibility to T1D cannot be overstated but non-*HLA* genes and gene combinations can also contribute to risk, especially in individuals with low-risk *HLA* combinations – in this thesis the key genes are described in the order they were identified as risk genes and this is shown schematically in **Figure 1.8**; the genes are described in the order they were identified in the subsequent section.

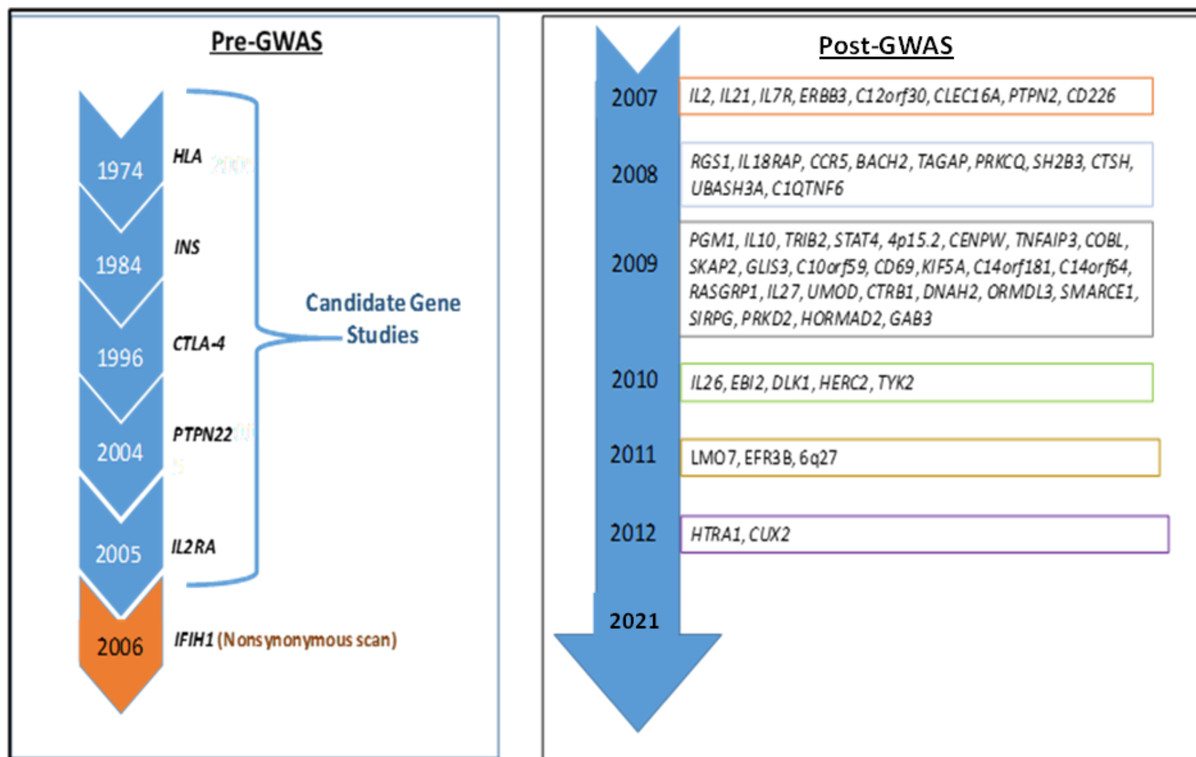

Figure 1.8: *Timeline of discovery of genes associated with type 1 diabetes: Between 1974 and the advent of GWAS only six genes were associated with T1D while >50 were identified between 2007 and 2020. Adapted from (Bakey et al., 2013).*

Insulin gene (*INS*): In 1983, the second genetic locus that accounts for around 10% of genetic susceptibility to T1D was reported (Bell *et al*., 1984). *INS* codes for insulin and is located on the short arm of chromosome 11 (11p15). In the promoter of *INS*, there is a variable number tandem repeat sequence (VNTR). This VNTR is classified according to the number of DNA base-pair (bp) repeats into three main types: the short VNTR (Class I) has 30-70bp repeats. The rare intermediate (Class II) has, on average 85bp repeats (Bennett and Todd, 1996), and the long VNTR (Class III) has 120 – 170bp repeats. Classes, I and III are associated with different and opposing levels of insulin expression in both the thymus and the pancreas (Kennedy *et al*., 1995; Pugliese *et al*., 1997; Pugliese, 1998; Vafiadis *et al*., 2001). The class I VNTR is

associated with risk of T1D with lower levels of (pro) insulin expression in the thymus. While class III is protective (Bennett *et al*., 1996) and it is associated with a higher level of (pro) insulin expression in the thymus, promoting self-tolerance and elimination of auto-reactive T-cells against insulin (Nakayama *et al*., 2005). Insulin is also an autoantigen in the pancreas against which insulin autoantibodies are directed (Palmer *et al*., 1983; Knip *et al*., 2005). It was reported that *INS* had a weak association with developing persistent autoimmunity (Steck *et al*., 2009).

Cytotoxic T-lymphocyte associated protein 4 gene (*CTLA-4*): codes for a glycoprotein that is expressed by activated T-cells, located on the long arm of chromosome 2 (2q33). It was initially reported in 1996 to be associated with T1D (Nistico *et al*., 1996) in addition to other autoimmune diseases such as Graves disease (Ueda *et al*., 2003), rheumatoid arthritis (Gregersen *et al*., 2009) and coeliac disease (Holopainen *et al*., 1999). It acts as a down-regulator of T-cell activation (Walunas *et al*., 1996). Gerold and colleagues generated a transgenic non-obese diabetic (NOD) mouse that lacks the soluble splice variant of *CTLA-4* (sCTLA-4) and found that silencing the soluble variant may act in favour of autoimmunity enhancing the progression rate to T1D (Gerold *et al*., 2011).

Protein tyrosine phosphatase non-receptor type 22 gene (*PTPN22*): a fourth genetic locus was linked to T1D in 2004 (Bottini *et al*., 2004). It is located on the short arm of chromosome 1 (1p13), and influences T-cell activation by downregulating T-cell receptor signalling. *PTPN22* codes for LYP (lymphoid protein tyrosine) kinase, which is an intracellular protein that binds Csk (Tyrosine-protein kinase) leading to decreased T-cell activation. A gain-of-function

mutation in *PTPN22* is thought to make autoreactive T-cells escape thymic negative selection, thus enhancing autoimmunity and T1D (Cerosaletti and Buckner, 2012). It was reported that a C1858T SNP is associated with an amino acid change of an arginine at position 620 to a tryptophan (Begovich *et al*., 2004; Bottini *et al*., 2004; Vang *et al*., 2005). In addition to T1D, the Trp620 variant is associated with a number of autoimmune diseases including rheumatoid arthritis (RA) (Begovich *et al*., 2004), systemic lupus erythematosus (SLE) (Kyogoku *et al*., 2004), Graves' thyroiditis (Smyth *et al*., 2004), and Addison disease (Vang *et al*., 2005). In the DAISY cohort, carriers of *PTPN22* R620W T allele developed persistent autoimmunity independent of their *HLA* risk genotype (Steck *et al*., 2009).

Interleukin 2 receptor alpha gene (*IL2RA*) or CD25: is located on the short arm of chromosome 10 (10p15) and codes for the high-affinity alpha subunit (CD25) of the *IL2* (Interleukin 2) receptor complex (Fichna *et al*., 2012). The association was discovered in 2005 and was firmly associated with T1D in 2007 (Qu *et al*., 2007). The *IL2* receptor is implicated in both effector T-cells and regulatory T-cells activity. It is postulated that reduced expression of *IL2RA* in those at risk of T1D affects the role of regulatory T-cells that counteract autoimmunity (Garg *et al*., 2012; Cerosaletti *et al*., 2013).

Interferon-induced with helicase C domain 1 gene (*IFIH1*): also known as melanoma differentiation-associated gene 5 (MDA-5), is located on the long arm of chromosome 2 (2q24), and was reported to be associated with T1D in 2006 (Smyth *et al*., 2006). It codes for a cytoplasmic helicase MDA5 that identifies viral dsRNA in infected cells triggering an immune response (Kumar *et al*., 2009). It was, therefore, thought that beta-cell death could result from

the interaction of enteroviruses with *IFIH1* in the pancreas (von Herrath, 2009) offering a potential link between genes and environmental triggers for the first time. Nejentsev and colleagues added support for the importance of *IFIH1* in the pathogenesis of T1D when they reported that four *IFIH1* rare variants were protective from T1D, as they negatively affected IFIH1 protein activity. Therefore, they concluded that understanding *IFIH1* actions may contribute to unravelling the mechanism of the interplay between the genes and environmental factors -in this case viruses- in T1D (Nejentsev *et al*., 2009).

1.7.3 After GWAS

With the emergence of GWAS in 2007 (www.ebi.ac.uk/gwas), it became possible to identify accurately the majority of the common genetic variants in the human genome that are associated with distinct phenotypes. That was accomplished by using a minimal set of single nucleotide polymorphisms (SNPs) in large populations via high throughput array analysis at a lower cost (Bennett and Todd, 1996; Smyth *et al*., 2008). By the year 2009, forty genetic locations had been reported to be associated with T1D (Barrett *et al*., 2009). By 2012, that figure increased to identify around 60 genes or loci associated with T1D (Bakay *et al*., 2013). Some of the genes identified have a clear role in the process of autoimmunity, while the function of some other genes and their contribution to the pathogenesis of T1D remains unclear.

C-type lectin domain family 16, member A gene (*CLEC16A* or *KIAA0350*): is located on the short arm of chromosome 16 (16p13), and expressed by immune cells (Hakonarson *et al*., 2007; Todd *et al*., 2007; Wellcome Trust Case Control, 2007).

CLEC16A was also found to be associated with multiple sclerosis in addition to T1D (International Multiple Sclerosis Genetics, Hafler *et al*., 2007; Wellcome Trust Case Control, 2007; Zoledziewska *et al*., 2009). Generally, C-lectin proteins have anti-inflammatory properties, but the function of *CLEC16A* encoded protein in T1D is under investigation in several laboratories.

Protein tyrosine phosphatase non-receptor type 2 gene (*PTPN2*): also known as (*TC-PTP* or *PTP-S2*), it is located on the short arm of chromosome 18 (18p11). Its mode of action is similar to *PTPN22* as a negative regulator of signalling pathway (Cerosaletti and Buckner, 2012). *PTPN2* also influences beta-cell death mediated by type I and II interferon (IFN) (Moore *et al*., 2009), and was linked to T1D risk (Todd *et al*., 2007). It was reported that *PTPN2* influences *IL2* action in T1D, but the exact mechanism is still unknown (Yamanouchi *et al*., 2007; Tang *et al*., 2008).

Interleukin 2 gene (*IL2*): located on the long arm of chromosome 4 (4q27), *IL2* codes for a cytokine that is required in B-cells in addition to T-cell homeostasis due to its role in maintaining regulatory T-cell proliferation and growth (Malek and Castro, 2010). Reduced *IL2* signalling may favour effector T-cell activity in T1D (Hulme *et al*., 2012). Due to its importance in the process of autoimmunity, IL2 protein therapeutic trials were used in cancer, but challenges of proper dosing and timing arose. However, this did not prevent such trials in T1D by (Rosenzwajg *et al*., 2014). A low dose of IL2 (0.33–1 MIU/day) over the course of five days was investigated. The results were a dose-dependent regulatory T-cell activation without activating effector T-cells and NK (Natural Killer) cells. However, no significant changes in

glucose level or C-peptide production were observed (Rosenzwajg *et al*., 2014; Yang *et al*., 2015).

Interleukin 10 gene (*IL-10*): is located on the long arm of chromosome 1 (1q32), and codes for a cytokine IL-10 or (human cytokine synthesis inhibitory factor (CSIF)) that is characterised by pleiotropic effects in inflammatory responses and immune regulation. The IL-10 protein enhances CD25 expression on regulatory T-cells, and it was identified as a candidate gene for T1D in 2009 (Barrett *et al*., 2009). Xu *et al.* (2015) reported that intraperitoneal injection of *IL-10*-carrying adenovirus, prevented T1D development in female non-obese diabetic (NOD) mice (Xu *et al*., 2015).

Interleukin 18 Receptor Accessory Protein gene (*IL18RAP*): is located on the long arm of chromosome 2 (2q12), *IL18RAP* codes for a protein that is an accessory subunit of the IL18 receptor complex. This protein helps the binding of the pro-inflammatory IL18 cytokine to its receptor. *IL18RAP* susceptibility allele for T1D is inversely associated with coeliac disease (Smyth *et al*., 2008).

Cordon-Bleu WH2 Repeat Protein gene (*COBL*): is located on the short arm of chromosome 7 (7p12), and codes for an actin-binding protein that plays an important part in neural morphogenesis (Ahuja *et al*., 2007). Abnormal *COBL* expression was associated with abnormalities in dendrite and axon production (Renault *et al*., 2008). Achenbach and colleagues reported that *COBL* is associated with T1D without significant effect on the rate

of progression of the disease (Achenbach *et al*., 2013). Additionally, *COBL* is one of the few genes shared between T1D and T2D (Fortune *et al*., 2015).

Lymphocyte Adaptor Protein gene (*SH2B3*): codes for a protein, which is a member of the SH2B3 adaptor family. This protein is necessary for haematopoiesis and acts as a negative regulator of cytokine signalling. *SH2B3* is located on the long arm of chromosome 12 (12q24), and is linked to T1D (Smyth *et al*., 2008).

GLIS family zinc finger 3 gene (*GLIS3*): is a member of the GLI-similar zinc finger protein family, located on the short arm of chromosome 9 (9p24.2). *GLIS3* codes for a nuclear protein that is both an activator and repressor of transcription. *GLIS3* is associated with T1D (Awata *et al*., 2013; Nogueira *et al*., 2013; Wen and Yang, 2017) as well as T2D; however, the T1D-risk variant is thought to be protective in T2D (Barker *et al*., 2011; Morris *et al*., 2012; Yang and Chan, 2016). GLIS3 protein is expressed by the pancreas where it influences pancreatic beta-cell development and insulin release (Nogueira *et al*., 2013) and by other body organs like the thyroid (Lichti-Kaiser *et al*., 2014). Rare disease-causing variants or mutations in this gene are associated with the development of neonatal diabetes mellitus and congenital hypothyroidism syndrome (NDH syndrome) (Senee *et al*., 2006; Dimitri *et al*., 2015).

Regulator of G protein signalling 1 gene (*RGS1*): is mapped to the long arm of chromosome 1 (1q31), and is involved in B-cell activation and proliferation (Hong *et al*., 1993). The *RGS1* gene is one of the shared genetic loci between T1D and coeliac disease (Smyth *et al*., 2008).

C-C chemokine receptor type 5 gene (*CCR-5*) or CD195: is located on the short arm of chromosome 3 (3p21), this gene codes for a member of the beta chemokine-receptor family (chemoattractant molecules) that is expressed on immune cells. CCR-5 protein plays a role in directed migration of immune cells and is thought to be involved in granulocyte differentiation and proliferation (Luster, 1998). Additionally, this protein facilitates HIV infection of host T-cells. Mutations in the *CCR-5* gene in the form of genetic deletion of 32 bp (Delta 32) provides homozygous carriers of this mutation with immunity against HIV -1 (Liu *et al*., 1996). Interestingly, male Delta 32- carriers have been reported to be at a greater risk of developing diabetic nephropathy (Mlynarski *et al*., 2005).

Basic leucine zipper transcription factor 2 gene (*BACH2*): is a protein-coding gene located on the long arm of chromosome 6 (6q15). The encoded protein regulates cellular differentiation and function of regulatory T-cells and B-cells (Kometani *et al*., 2013; Roychoudhuri *et al*., 2013). *BACH2* is associated with cancers including leukaemia and lymphoma (Ono *et al*., 2007; Ichikawa *et al*., 2014) and was reported as one of the genetic loci associated with T1D by GWAS (Grant *et al*., 2009). A plausible mechanism of *BACH2* involvement in T1D pathogenesis was that inhibiting the expression of *BACH2* product could help accelerate β-cell death by cytokines-mediated apoptosis. Furthermore, the *BACH2* disease allele was thought to negatively impact regulatory T-cells function, facilitating an autoimmune response (Marroqui *et al*., 2014; Onuma *et al*., 2019).

V-ERB-B2 Erythroblastic leukaemia viral oncogene homolog 3 gene (*ERBB3*): is located on the long arm of chromosome 12 (12q13), and codes for a member of the epidermal growth

factor receptor (EGFR) family of receptor tyrosine kinases. The encoded protein forms heterodimers with other EGFR proteins leading to cell differentiation or proliferation. *ERBB3* is reportedly associated with different types of cancer like colon and breast cancers (Jeong *et al*., 2006; Mujoo *et al*., 2014). *ERBB3* in T1D is thought to be associated with APCs function (Wang *et al*., 2010), as well as beta-cell function and apoptosis (Kaur *et al*., 2016).

Cathepsin H gene (*CTSH*): is located on the long arm of chromosome 15 (15q24), and codes for a member of the peptidase C1 family, a lysosomal cysteine proteinase that is involved in lysosomal proteins degradation. It was reported that *CTSH* gene-encoded protein is highly expressed in progressive prostatic malignancy (Jevnikar *et al*., 2013). *CTSH* is associated with T1D (Cooper *et al*., 2008). It was found that *CTSH* affects beta-cell function, and the rate of progression of T1D in newly-diagnosed cases (Floyel *et al*., 2014).

Ubiquitin-associated and SH3 domain-containing protein A gene (*UBASH3A*): was mapped to the long arm of chromosome 21 (21q22.3) as a T1D risk gene by the T1DGC (Concannon *et al*., 2008). The encoded protein is a member of the T-cell ubiquitin ligand (TULA) family, which negatively regulate T-cell signalling. In the DAISY study, they found that a variant of *UBASH3A* was associated with the development of insulin autoantibodies and T1D risk in children with *HLA-DR3/4*, *DQB1*0302* genotype (Johnson *et al*., 2012).

Common SNPs in non-*HLA* genes and *HLA* variants shown to be associated with type 1 diabetes are summarised in **Table 1.2**. A more detailed table in chapter 2, section 2.2.3.1 provides more detail on the sequences of the variants.

Chr	**Gene associated with T1D**	**Name**	**Associated SNP to be studied in this project**	**Position relative to gene and Functional significance**
1	*IL10*	Interleukein 10 gene	rs3024505	Located in an intergenic region proximal to the 3'UTR. High potential for containing regulatory sequences but not proven; in perfect linkage with other polymorphisms located within the *IL-10* gene making causal links difficult (Franke *et al*., 2008).
1	*PTPN22*	Protein tyrosine phosphatase non receptor type 22 gene	r6679677	A missense mutation (R620W) on *PTPN22* which encodes lymphoid-tyrosine phosphatase (LYP). Some evidence that the mutation attenuates proximal T and BCR signalling (see section 1.7.3 on *PTPN22*).
1	*RGS1*	Regulator of G protein signalling 1 gene	rs2816316	Intron variant. Function unknown.
2	*CTLA-4*	Cytotoxic T lymphocyte Associated Protein 4 gene	rs3087243	Downstream_transcript_variant. Also known in the literature as the CT60 G>A.
2	*IFIH1*	Interferon induced with helicase C domain 1 gene	rs2111485	Located in the 23.5 kb intergenic region between *FAP* and *IFIH1* within an H3K4me1 chromatin mark in peripheral blood mononuclear cells, indicative of an active/poised enhancer (Hon *et al.,* 2009). Single-nucleotide polymorphism (SNP) rs2111485 alters a predicted binding site for transcription factor AP-1 and corresponds to an expression quantitative trait locus, the protective allele reducing expression of

				IFIH1 RNA in monocytes stimulated with interferon-gamma, though not unstimulated monocytes (Bentham *et al.*, 2015).
2	*IL18RAP*	Interleukin 18 receptor accessory protein gene	rs917997	Upon IL-12 and IL-18 treatment, peripheral blood mononuclear cells from subjects carrying susceptibility alleles at rs917997 produced higher levels of IFNγ than those with protective genotypes. Additionally, the SNP modified IL18RAP surface protein expression by NK cells and gene expression in activated T-cells. Taken together, these data suggest that the disease-associated rs917997 allele G permits hyperresponsiveness to IL-18 (Myhr *et al.,* 2013).
3	*CCR-5/CD195*	C-C chemokine receptor type 5 gene	No SNP available - deletion detection	The chemokine receptor CCR-5 is an important co-receptor for cell fusion. A 32-bp deletion of the *CCR-5* gene, leading to complete absence of functional CCR-5 expression, has been associated with resistance to human immunodeficiency virus (HIV) infection in homozygotes and slower HIV disease progression in heterozygotes (Pasi *et al.,* 2000).
4	*IL2*	Interleukin 2 gene	rs4505848	Intron variant. Adjacent to *KIAA1109* and *IL21*. No functional effects reported.
6	*BACH2*	Basic leucine zipper transcription factor 2 gene	rs11755527	Intron Variant. No known fuctional effects.
6	*HLA*	Human leucocyte antigen genes	*HLA* not SNP variants (*DRB1/DQB1*)	Encode cell-surface proteins responsible for the regulation of the immune system. See section 1.7.2.
7	*COBL*	Cordon-Bleu WH2 Repeat protein gene	rs4948088	Intergenic SNP. No potential function effects reported.

9	*GLIS3*	GLIS family zinc finger 3 gene	rs7020673	Duarte and colleagues explored the ENCODE database (https://genome.ucsc.edu) for potential functional evidence and found ChiP-Seq base evidence of CTCF (CCCTC-binding factor) and CEBPB (CCAAT/enhancer-binding protein beta) transcription factor binding sites (TFBS) overlapping with the position of this SNP (Duarte *et al*., 2017).
10	*IL2RA/CD95*	Interleukin 2 receptor alpha gene	rs12251307	Intergenic SNP. No reported functional effects.
11	*INS*	Insulin gene	rs689	VNTR in insulin promoter associated with opposing levels of insulin transcription in the pancreas and thymus. See section 1.7.3 on the insulin gene.
12	*ERBB3*	V-Erb-B2 Erythroblastic leukaemia viral oncogene homologue 3 gene	rs2292239	Intron variant. No reported functional effects.
12	*SH2B3*	Lymphocyte Adaptor Protein gene	rs3194504	Also known as R262W this variant lies in exon 2 of *SH2B3* and results in an amino acid substitution of arginine to tryptophan. This may be functionally relevant because it is located in the LNK-family pleckstrin homology (PH) domain of the LNK molecule 2 but there is a paucity of data on functional aspects of this SNP (Lavrikova *et al*., *2011)*.
15	*CTSH*	Cathepsin H gene	rs3825932	The risk variant rs3825932 in the candidate gene cathepsin H (*CTSH)* predicts β-cell function in both model systems and human T1D. Higher CTSH protein expression in β-cells may protect against immune-mediated damage and preserve β-cell function, thereby representing a possible therapeutic target (Floyel *et al*., 2014).

16	*CLEC16A/KIAA0350*	C type lectin domain family 16, member A gene	rs12935413	This SNP is an intron variant and lies adjacent to *CLEC16A/KIAA0350, IL2* and *IL21* so individuals effects are difficult to discriminate.
18	*PTPN2*	Protein tyrosine phosphatase non receptor type 2 gene	rs1893217	The single nucleotide polymorphism (SNP) rs1893217 in *PTPN2* results in a dysfunctional PTPN2 protein and exists in perfect linkage disequilibrium with *PTPN2* SNP rs2542151 (Spalinger *et al*., 2016).
21	*UBASH3A*	Ubiquitin-associated and SH3 domain-containing protein A gene	rs9976767	Located in the intronic region between the exons 5 and 6, rs9976767 could be affecting the expression of different UBASH3A isoforms (Diaz-Gallo *et al.*, 2013).

***Table 1.2**: T1D associated SNPs: The SNPs which will be followed up in this study. The sequences are described in detail in chapter 2 (Table 2.3).*

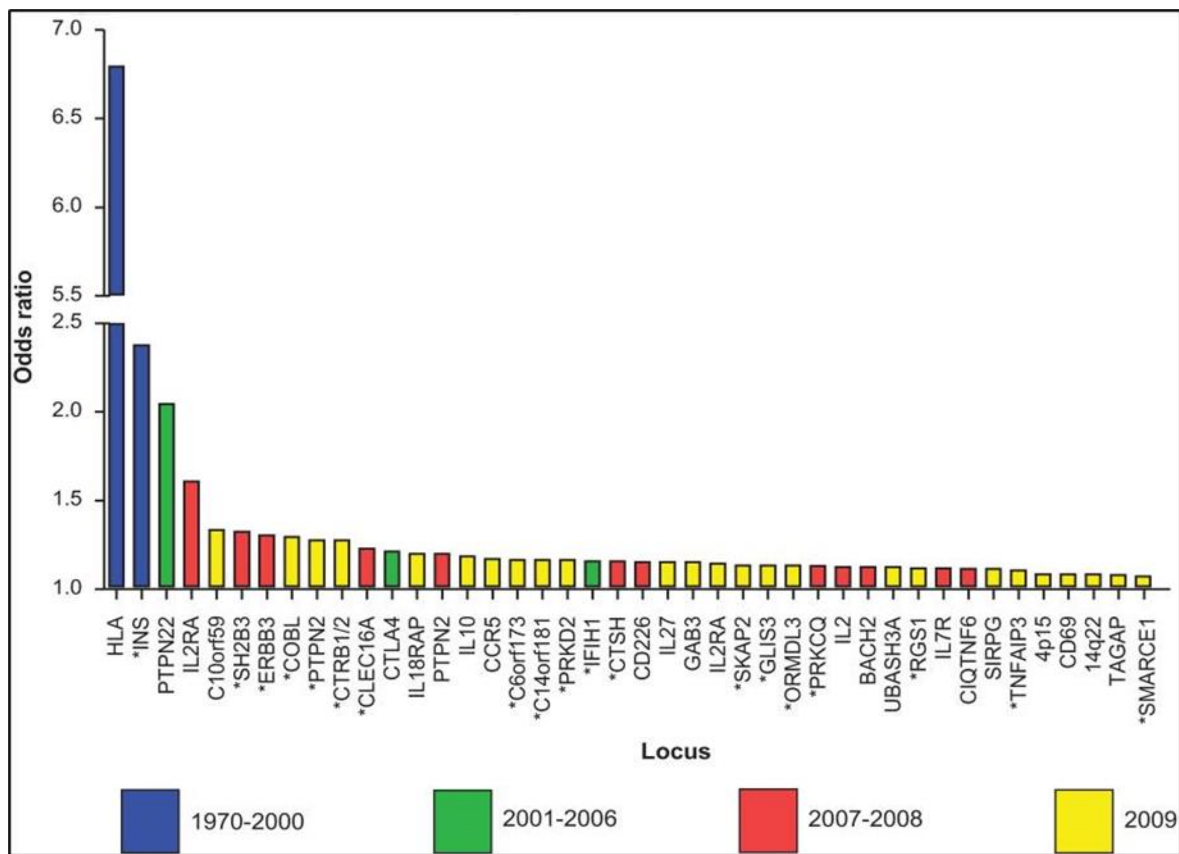

Figure 1.9*: The most common genes that are associated with risk of developing T1D: Arranged sequentially from highest to lowest based on their odds ratios. HLA variants have the highest odds ratio of association with T1D risk at around (6.7), followed by INS (2.4), PTPN22 (2.1), IL2RA (1.6). The remainder are arranged between 1 to 1.5 (Pociot et al., 2010). Twenty of the 42 genes shown in this figure have been robustly repeated in multiple studies and were, therefore, selected for analysis in this project.*

Each of the SNPs identified before and after GWAS has an impact on developing T1D, albeit in many cases the biological mechanisms underlying this are not fully understood. The effect on risk is variable, as shown by the odds ratios as shown in **Figure 1.9**. *HLA* have the highest impact with an odds ratio of more than 6.5; followed by *INS* at around 2.4. The remainder falls between 2 and 1; their individual effect is minimal in comparison with *HLA* (Pociot *et al*., 2010). Recognising their minor effects are still important, it was reported that prediction of T1D could be improved when the effect of risk SNPs is added to *HLA* and is superior to prediction relying on the effect of *HLA* alone (Steck *et al*., 2014; Winkler *et al*., 2014).

1.8 Islet Autoantibodies

Islet autoantibodies have been referred to several times in this introduction already because they are currently the most important biomarkers to predict future T1D.

When the body is exposed to an antigen, a cascade of T and B-cell activity ensues as described in section 1.3 . Plasma cells - that originate from B lymphocytes - produce antibodies. In T1D, these antibodies are not thought to be pathogenic, but they can be detected in the periphery and act as a marker for ongoing islet autoimmunity. Growth factors help B-cells to produce IgM antibodies after initial exposure to the antigen. Upon further or sustained exposure to the same antigen, IgG antibodies are produced that are refined over time, resulting in antibodies with high-antigenic affinity, and are specific for the antigens, forming antibody-antigen complexes as shown in **Figure 1.10** (Burnet, 1961; Wabl *et al*., 1999).

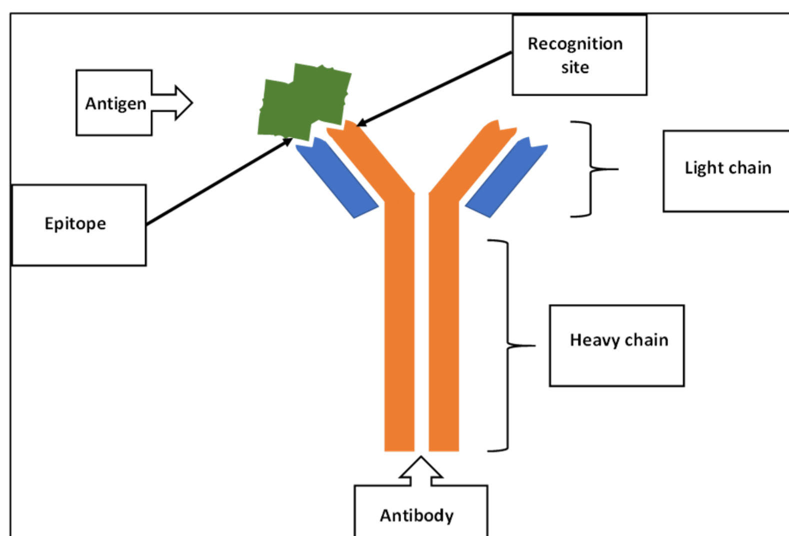

Figure 1.10: *An antigen-antibody complex: The antibody is composed of a pair of light (represented by blue) and heavy (represented by orange) chains of amino acid residues. The recognition site of an antibody or paratope is the part where an antibody binds an antigen, The epitope is the part of the antigen that the antibody is bound to (Male, 2014).*

The presence of islet cell antibodies (ICA) was first detected in the pre-diabetes phase in 1981 (Gorsuch *et al*., 1981); however, initially their disease specificity was low as only 40% of first degree relatives who had ICA developed T1D (Bonifacio *et al*., 1990).

It became clear that several "biochemical" antibodies contribute to ICA, and the use of these has largely replaced ICA over the years, as these newer autoantibody combinations showed comparable sensitivity and significantly better specificity than ICA (Bingley *et al*., 1994).

Moreover, the use of different protocols by different laboratories led to controversies over islet autoantibody measurement. The latter issue was eventually overcome by the development of the Diabetes Autoantibody Standardization Program (DASP), known today as the Islet Autoantibody Standardization Program (IASP), that comprises a number of international laboratories working together to improve assay performance and agree on standard measurements of autoantibodies (Bingley *et al*., 2003). To date, radio-binding, bridge-ELISA (enzyme-linked immunosorbent assay), and electrochemiluminescence (ECL) assays have emerged as the gold standards for measuring antibodies (Schmidli *et al*., 1994; Brooking *et al*., 2003; Miao *et al*., 2013; Liberati *et al*., 2018).

During the pre-diabetes stage, four major primary autoantibodies can be detected against islet autoantigens in the pancreas (Knip, 2002). These include autoantibodies to: glutamic acid decarboxylase (GADA) (Baekkeskov *et al*., 1990), insulin (IAA) (Palmer *et al*., 1983; Holmberg *et al*., 2006), protein tyrosine phosphatase-related insulinoma-associated 2 (IA-2A) (Verge *et al*., 1998), and Zinc transporter 8 (ZnT8A) (Wenzlau *et al*., 2007). The autoantigens that are targeted by these autoantibodies are all found within or in the proximity of insulin secretory vesicles in the pancreatic beta cells (Reetz *et al*., 1991; Solimena *et al*., 1996; Wenzlau *et al*., 2007) and **Figure 1.11**. More recently, an additional islet autoantibody to Tetraspanin-7

autoantigen has been described, but few data are available on its frequency (McLaughlin *et al.*, 2016).

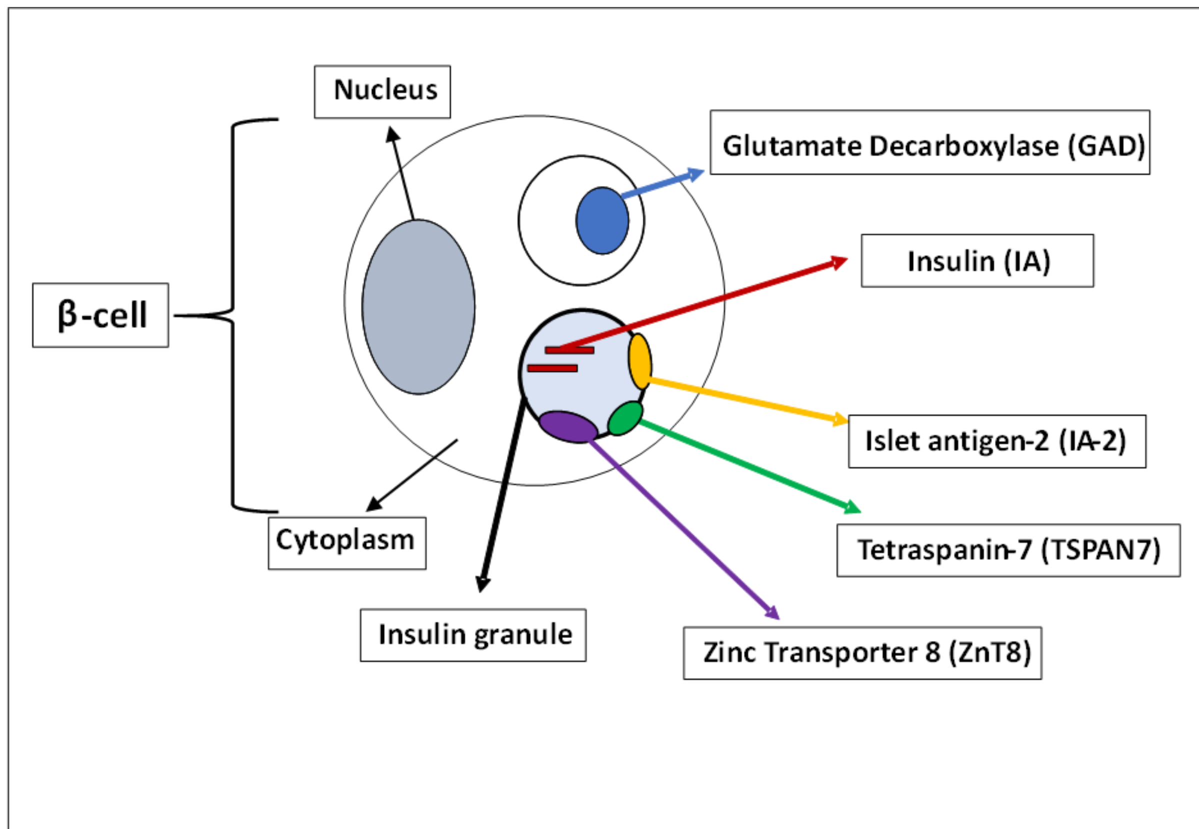

Figure 1.11: *Pancreatic islet autoantigens: The islet autoantigens GAD, Insulin, IA-2, ZnT8 and TSPAN7 in the pancreatic β -cell against which autoantibodies in T1D are directed. Insulin, IA-2, ZnT8 and TSPAN7 are located in the insulin granule while GAD is found in the synaptic-like microvesicle.*

Detection of autoantibodies defines the T1D prodrome and can be used to stratify the risk of progression to clinical diabetes (Bosi *et al.*, 2017). Autoantibodies to islet antigens often develop sequentially (Yu *et al.*, 1996), as insulin autoantibodies were commonly the first to be detected in genetically susceptible children, from the age of 6 months onwards (Hummel *et al.*, 2004), while GADA, IA-2A, IA-2βA, and ZnT8A tend to develop later (Achenbach *et al.*, 2009).

The presence of at least two autoantibodies in first-degree relatives of T1D probands is predictive of developing T1D (Ziegler *et al*., 2013) and the higher the number of autoantibodies detected, the higher the likelihood of progression to T1D (Bingley *et al*., 1994; Pihoker *et al*., 2005; Ziegler and Nepom, 2010). In addition to the number of autoantibodies, seroconversion detected before the age of 3 years; female gender; and the presence of high-risk *HLA* genotype *DR3*/*DR4-DQ8*, each independently increased the risk of progression to T1D (Ziegler *et al*., 2013; Knip *et al*., 2016) **and Table 1.3**.

Variable	10-year risk % (95% CI)	Bivariable Hazard Ratio (95% CI)	*P* Value	Multivariable Hazard Ratio (95% CI)[a]	*P* Value
Serocoversion age, y					
<3	74.9 (69.7-80.1)	1.72 (1.36-2.17)	<.001	1.65 (1.30-2.09)	<.001
≥3	60.9 (51.5-70.3)	1 [Reference]		1 [Reference]	
HLA genotype					
DR3/DR4-DQ8	76.6 (69.2-84.0)	1.40 (1.12-1.73)	0.003	1.35 (1.09-1.68)	0.007
Other	66.2 (60.2-72.2)	1 [Reference]		1 [Reference]	
Sex					
Girls	74.8 (68.0-81.6)	1.30 (1.05-1.60)	0.02	1.28 (1.04-1.58)	0.02
Boys	65.7 (59.3-72.1)	1 [Reference]		1 [Reference]	
No. of autoantibodies					
Three	72.1 (66.5-77.7)	1.27 (1.02-1.59)	0.04	1.19 (0.95-1.49)	0.14
Two	65.1 (56.3-73.9)	1 [Reference]		1 [Reference]	

[a] Multivariable hazard ratio includes all listed variables (seroconversion age, *HLA* genotype, sex, and number of autoantibodies).

Table 1.5: *Independent predictors of progression to T1D in children with multiple islet autoantibodies: A multivariable Cox proportional hazards regression model showed faster progression to T1D in children with multiple islet autoantibodies who had seroconversion before the age of 3 years (hazard ratio [HR], 1.65 [95% CI, 1.30-2.09; P < .001]; 10-year risk, 74.9% [95% CI, 69.7%-80.1%]); HLA genotype DR3/DR4-DQ8 (HR, 1.35 [95% CI, 1.09-1.68; P=.007]; 10-year risk, 76.6% [95% CI, 69.2%-84%]); and for girls (HR, 1.28 [95% CI, 1.04-1.58; P=.02];10-year risk, 74.8% [95% CI, 68.0%-81.6%]). Adapted from (Ziegler et al., 2013).*

The autoimmune process usually starts by recognising one autoantigen and then spreads to involve other islet autoantigens. Individuals were least likely to develop T1D if they persistently had only one autoantibody. The mechanism underlying epitope spreading is not fully understood in humans (Naserke *et al*., 1998; Ziegler *et al*., 1999; Brooks-Worrell *et al*., 2001; Barker *et al*., 2004; Ziegler *et al*., 2013). Looking into what could affect epitope spreading in the NOD mouse model (Kikutani and Makino, 1992; Bach, 1994; Delovitch and

Singh, 1997), it was suggested that B-cells, as antigen-presenting cells are intrinsically able to process and present certain antigens (Dai *et al*., 2005). It is also worth noting that NOD mice lacking B-cells did not develop diabetes (Noorchashm *et al*., 1999). However, the B-cells in transgenic mice were not essential to develop T1D, and in human studies, one person developed T1D despite being B-cell deficient. These inconsistent findings led to a delay in examining the role of B-cells in T1D (Holz *et al*., 2000; Martin *et al*., 2001; Bloem and Roep, 2017) which has been reversed since the description of a large number of B-cells in pancreases from early-onset T1D (Leete *et al*., 2016).

The DIPP study followed children in Finland with T1D *HLA-DR-DQ* risk haplotypes for two decades from birth (https://clinicaltrials.gov/ct2/show/NCT03269084). Looking into T1D autoantibodies, the study reported that among children who had detectable autoantibodies, those with certain subclasses of autoantibodies progressed to diabetes (Hoppu *et al*., 2004; Hoppu *et al*., 2006; Ronkainen *et al*., 2006), while other isotypes conferred relative protection

from T1D as shown in **Table 1.4** (Achenbach *et al*., 2004; Knip *et al*., 2016). It is now increasingly accepted that IgG is the predominant islet autoantibody subclass.

Autoantibody	**Reference**	**Progressors**	**Non-progressors**
Insulin autoantibodies (IAA)	(Hoppu *et al* ., 2004)	IgG1, IgG3	Weak IgG3
Islet antigen 2 antibodies (IA-2A)	(Hoppu *et al* ., 2006)	Weak IgE	Strong IgE
Glutamic acid decarboxylase antibodies (GADA)	(Ronkainen *et al* ., 2006)	Weak IgG4	Boosting IgG4

Table 1.6: *Subclasses of islet autoantibodies: Genetically susceptible Finnish children that progressed to develop T1D had certain subclasses of islet autoantibodies; IAA (IgG1 and IgG3 subclasses), IA-2 (weak IgE subclass), and GADA (weak IgG4 subclass). Those who did not progress to TID had different subclasses; IAA (weak IgG3 subclass), IA-2 (strong IgE subclass), and GADA (boosting IgG4 subclass) (Hoppu et al., 2004; Hoppu et al., 2006; Ronkainen et al., 2006).*

It is worth noting that after the diagnosis of T1D, the number and titre of autoantibodies at onset generally decreased with time. However, they can still persist for decades, which is mainly the case for GADA and IA-2A, followed by ZnT8A (Williams *et al*., 2016).

GADA

GADA are directed against the autoantigen glutamic acid decarboxylase 65 (GAD65) which is an enzyme involved in the synthesis of GABA (gamma-aminobutyric acid); a major inhibitory neurotransmitter in the CNS (Central Nervous System). The autoantibodies tend to target epitopes in three domains of the GAD molecule, namely; the NH2 terminal domain, the middle pyridoxal-5'-phosphate (PLP)-binding domain, and the COOH terminal domain as shown by the crystal structure in **Figure 1.12** (Bonifacio *et al*., 2000; Piquer *et al*., 2005).

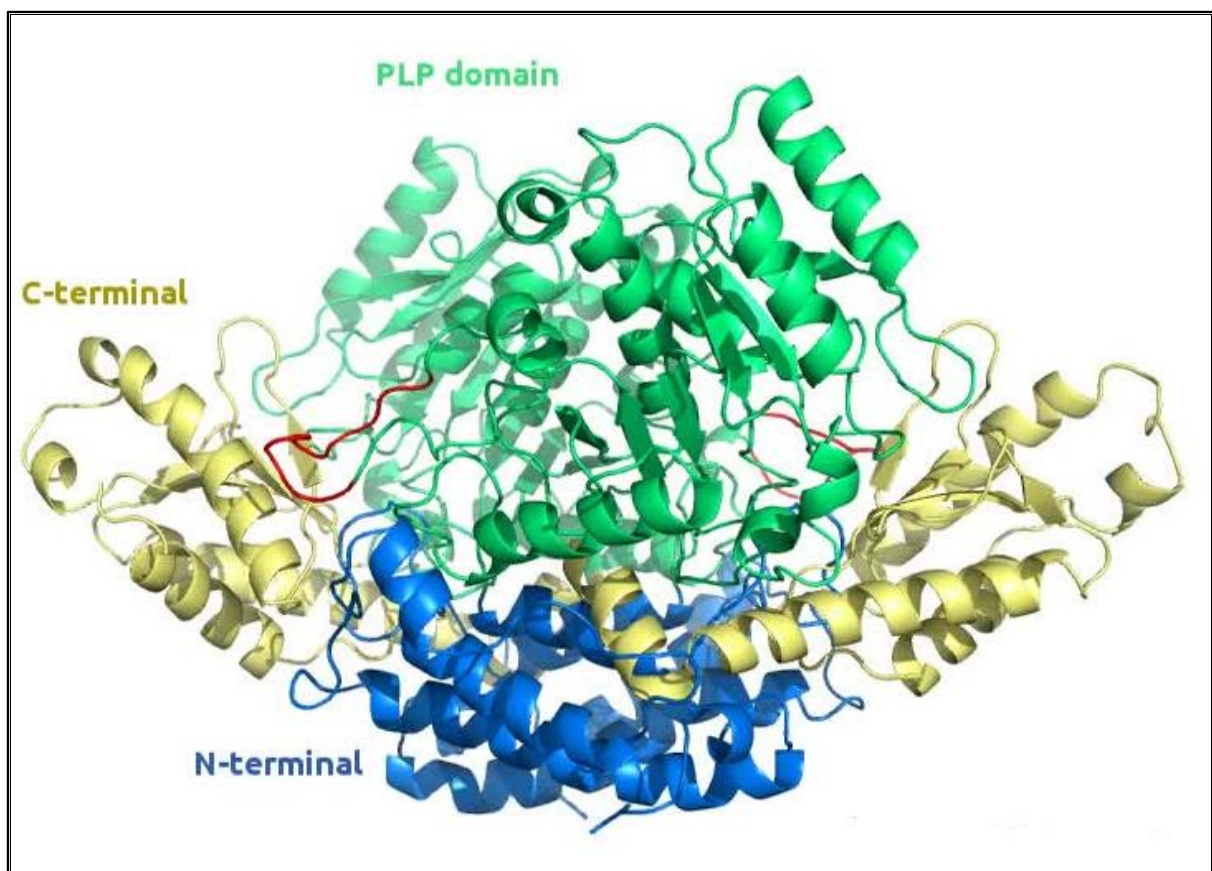

Figure 1.12: *Crystal structure of the GAD molecule: The three main domains; NH2 terminal domain, middle PLP domain, and COOH terminal domain; against which GAD autoantibodies are directed (Kass et al., 2014).*

GAD autoantigens were first linked to a rare neurological condition called stiff-man syndrome and to T1D. The antigen was found to be highly expressed in both brain cells and pancreatic

beta cells based on rat tissue analysis (Baekkeskov *et al*., 1990). In humans, GAD65 autoantigen is mainly found on the cytoplasmic membrane side of synaptic-like microvesicles in pancreatic beta-cells (Mally *et al*., 1996) and is highly expressed by the pancreas. An isoform of GAD65; GAD67, may also be bound by GADA and is found to be expressed by human thymic cells which are APCs crucial to the recognition of self or autoantigens and development of immune tolerance by the thymus (Gotter *et al*., 2004).

In addition to T1D, GADA were associated with other diseases like cerebellar ataxia and autoimmune polyendocrinopathy syndrome (APS) (Saiz *et al*., 2008; McKeon *et al*., 2012). In APS, where multiple organs are attacked by the immune system, GADA were found to be more associated with gastrointestinal damage rather than with diabetes (Soderbergh *et al*., 2004).

Development of GADA was strongly associated with T1D high-risk *HLA* class II haplotype *DR3-DQ2* (Knip *et al*., 2002). As discussed earlier, GADA can develop years before the onset of clinical diabetes (Bingley *et al*., 2001) and they were detected in up to 80% of T1D patients. The TEDDY study applied frequent islet autoantibody testing where GADA were commonly found from the age of 2-3 years and were detected in half the children followed up following seroconversion (Krischer *et al*., 2017; Krischer *et al*., 2019). Additionally, GAD autoantibodies had a tendency to persist for years after diagnosis (Nokoff *et al*., 2012).

Although GADA are detected in a majority of T1D patients, not all people who are positive for GADA go on to develop T1D. To detect those who are going to progress more specifically, the use of truncated GAD antigen was applied to GADA measurement. Although this has already been utilised in measuring IA-2 and ZnT8 autoantibodies (Payton *et al*., 1995; Wenzlau *et al*., 2007), use of truncated antigen was not a common practice in GADA assays. Using N-terminally truncated GAD65 radiolabel antigen instead of the full-length GAD in radio-binding

assays showed significant improvement in detecting first-degree relatives who were at high risk to progress to diabetes, without losing assay sensitivity compared to using the full-length GAD antigen (Williams *et al*., 2015; Williams *et al*., 2015).

IAA

In humans, insulin is mainly expressed by the pancreas and to a lesser extent by the epithelial cells in the thymus (Pugliese *et al*., 1997; Vafiadis *et al*., 1997).

Autoantibodies against insulin were the first autoantibodies to be detected very early in life (Kimpimäki *et al*., 2001), but their prevelance and levels decline being detected in only half of those who were “at-risk” aged 15-21 years (Vardi *et al*., 1988; Krischer *et al*., 2015).

Autoantigens and hence epitopes targeted by IAA were represented by using proinsulin, insulin analogues and animal recombinants including porcine, sheep, and fish, all used in competition studies (Achenbach *et al*., 2004). In BABYDIAB, children who developed high-affinity IAA early on were reactive against proinsulin and were more likely to develop other autoantibodies and progress to T1D. It is worth noting that high affinity-IAA binding was associated with changes within A chain- amino acids 8 to 13- of the insulin molecule and with carriers of *HLA DRB1*04*, in contrast to low-affinity IAA that were not reactive to proinsulin and were associated with changes in the B chain and absent *HLA DRB1*04.* Additionally, children with low-affinity IAA, i.e. a limited IgG response, did not seroconvert and did not develop T1D, indicating that IAA affinity could serve as a predictor of future T1D development (Castano *et al*., 1993; Brooks-Worrell *et al*., 1999; Achenbach *et al*., 2004).

Proinsulin, mainly secreted by beta-cells, is regarded as the primary target for IAA, as loss of tolerance to proinsulin was common at onset in young children with high IAA frequency who rapidly developed T1D (Parikka *et al*., 2012; Ziegler *et al*., 2012). Furthermore, self-reactive T-cells targeting proinsulin were detected in people who were in the pre-diabetes phase and in those who had recent-onset T1D. In animal studies, proinsulin specific T-cells were detected in transgenic mice that expressed the high T1D genetic risk factor *HLA-DR4* (Fugger *et al*., 1994; Rudy *et al*., 1995; Congia *et al*., 1998; Dubois-LaForgue *et al*., 1999; Arif *et al*., 2004; Durinovic-Bello *et al*., 2004).

Shortly after diagnosis, insulin autoantibodies are masked by antibodies induced by exogenous insulin treatment, making it difficult to measure IAA after approximately two weeks of insulin therapy (Baxter *et al*., 1976; Vahasalo, 1992; Brooks-Worrell *et al*., 1999; Nokoff *et al*., 2012).

The strong association between IAA and the high T1D genetic risk *HLA DR4-DQ8* haplotype has been reported by several studies (Knip *et al*., 2002; Giannopoulou *et al*., 2015) and those who expressed *HLA-DR4* were found to have the highest levels of insulin autoantibodies (Eisenbarth *et al*., 1992).

IA-2A

The IA-2 autoantigen is a receptor-type protein tyrosine phosphatase (PTP) like protein that is located in the membrane of insulin secretory granule (Solimena *et al*., 1996). Structurally, it is comprised of three components; an ectodomain, a short cytoplasmic juxtamembrane (JM), and a PTP-like cytoplasmic domain as shown in **Figure 1.13**.

IA-2β is an isoform of IA-2 that shares a lot of structural similarities with IA-2 at the PTP-like domain (Kim *et al*., 2007; Lampasona and Liberati, 2016). IA-2 plays a role in regulating insulin secretory granule's function, insulin secretion and in beta cell proliferation (Trajkovski *et al*., 2004; Mziaut *et al*., 2008; Trajkovski *et al*., 2008). IA-2 is expressed by many tissues in rodents (Solimena *et al*., 1996; Takeyama *et al*., 2009). In humans, it is mainly expressed by the

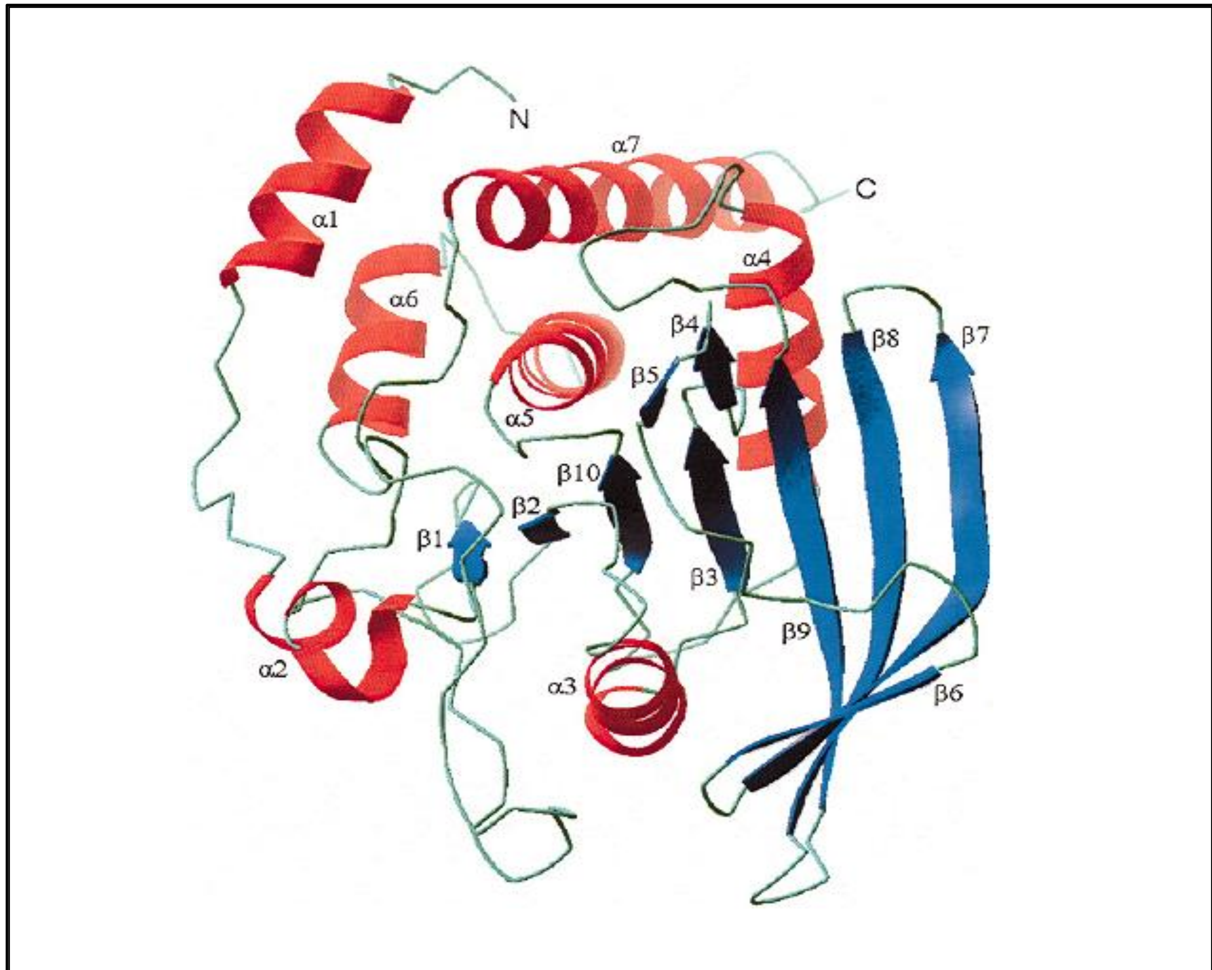

Figure 1.13: *Crystal structure of Insulinoma-associated protein-2 (IA-2) autoantigen: Illustration of crystal structure of insulinoma-associated protein-2 (IA-2) autoantigen (Kim et al., 2007).*

pancreatic islet cells (Solimena *et al*., 1996) and to a lesser extent by the spleen and thymus where they could play a role in the regulation of self-reactive immune cells (Diez *et al*., 2001).

IA-2A tend to target epitopes in the JM and PTP-like cytoplasmic domains of the IA-2 antigen at the onset of T1D (Lampasona *et al*., 1996; Bearzatto *et al*., 2002; Dromey *et al*., 2004; Mziaut *et al*., 2008). Interestingly, binding of antibodies to those two domains showed the epitopes to be closely associated within the structure of IA-2, which may shed some light on the mechanism of the variable spreading of the autoimmune process from one epitope to another (McLaughlin *et al*., 2015).

Genetically speaking, Like IAA, IA-2A development is strongly associated with the *HLA DR4-DQ8* haplotype (Giannopoulou *et al*., 2015). Autoantibodies targeting IA-2 autoantigen could be detected ten years after diagnosis, and sometimes they could persist for even longer (Nokoff *et al*., 2012). Autoantibodies to IA-2 (Decochez *et al*., 2002) and its homologue protein IA-2β are associated with a more aggressive autoimmune response, and enhanced progression to clinical diabetes (Achenbach *et al*., 2008).

ZnT8A (Zinc transporter 8 autoantibodies)

Pancreatic β-cells have the highest quantities of zinc in the body, and this is tightly regulated. Insulin is thought to be bound to zinc and stored in the insulin secretory granules. In response to specific stimuli such as hyperglycaemia, insulin is secreted along with zinc which is considered to play a crucial role in insulin synthesis, storage and release (Emdin *et al*., 1980). An important regulator of zinc abundance in the beta-cell is ZnT8 (Zinc transporter 8) protein, a beta-cell specific member of the *SLC30* (solute carrier) subfamily of the CDF (cation diffusion facilitator) family. This transmembrane protein family shares a distinct structure of six transmembrane domains, and a histidine-rich intracellular loop between helices IV and V. ZnT8 is mainly expressed by beta-cells (Chimienti *et al*., 2006; Lemaire *et al*., 2012), and to a

lesser extent by alpha cells, and other tissues like the retina, adipocytes, and some lymphocytes (Chimienti *et al*., 2006; Deniro and Al-Mohanna, 2012). ZnT8 protein forms a homodimer that has a cytoplasmic COOH terminal domain and NH2 terminal domain. Epitopes in the COOH terminal domain were recognised by a majority of ZnT8 autoantibodies in patients with ZnT8A, whereas epitopes in NH2 terminal domain were identified by only a small fraction of ZnT8 autoantibodies (Wenzlau *et al*., 2008).

The epitope mainly targeted by ZnT8A is strongly associated with a single amino acid change at position 325, resulting in either arginine (R), tryptophan (W) or glutamine (Q), as determined by genetic polymorphisms in its coding gene, *SLC30A8* gene (Kawasaki *et al*., 2008; Wenzlau *et al*., 2008). The autoantibodies to the 325 epitope are highly specific to the *SCL30A8* genotype of the individual, such that those homozygous for the most common *SLC30A8* alleles do not recognise antigens that contain other amino acids at that position. Assays for ZnT8 are, therefore, designed to measure antibodies to ZnT8-325R and ZnT8-325W variants separately or together using dimeric (R-W) antigens.

SLC30A8 genetic variants at the level of amino acid 325 in the COOH terminal domain were associated with defects in insulin maturation, glucose metabolism and increased risk of developing T2D (Kirchhoff *et al*., 2008; Xu *et al*., 2011; Shan *et al*., 2014). ZnT8A were detected as early as 9 months in newly-diagnosed children with T1D who were followed up from birth in the BABYDIAB study (Hummel and Ziegler, 2011). When they were 5 years old, approximately 50% of those with ZnT8A developed T1D. ZnT8A were found in 70% of patients at the onset of T1D in comparison with 2-3% in T2D patients (Wenzlau *et al*., 2007).

1.9 Interactions of genes and islet autoantibodies

It is vital to distinguish the risk for islet autoimmunity from that for T1D, as mechanisms of interactions between the genes on the one hand, and interactions between the genes and the environmental factors, on the other hand, could be quite different in how they potentially initiate autoimmunity versus epitope spreading, and subsequent progression to overt diabetes (Steck *et al*., 2009).

1.9.1 *HLA* and islet autoantibodies

It is age-dependent, but up to 40% of T1D patients have the highest-risk *HLA* class II genotype *DR3-DQ2/DR4-DQ8* compared with only 3% of the general population. Only a small fraction 1:15 of those with T1D genetic susceptibility will develop the condition (Kimpimäki *et al*., 2001; Rewers *et al*., 1996). Furthermore, around 90% of newly-diagnosed cases have no family history of T1D (Veijola *et al*., 1996).

As discussed previously, those who carry *HLA-DR4* are at increased risk for developing autoimmunity to insulin and IA-2 while GADA is associated with *HLA-DR3* (Regnell and Lernmark, 2017).

The order and timing of seroconversion could, therefore, be influenced by *HLA* class II genotype. Carriers of the *HLA-DR4-DQ8* genotype are more likely to have multiple autoantibodies, mainly IAA and IA-2A and their median time of progression to clinical diabetes is two years. On the other hand, *HLA-DR3-DQ2* carriers were highly associated with developing GADA and slower progression to T1D (Krischer *et al*., 2017).

1.9.2 T1D non-*HLA* genes and islet autoantibody associations

Comparison of antibody profiles over time has shown that higher numbers of recently diagnosed BOX patients developed four autoantibodies at diagnosis, as well as having higher levels of IA-2βA (Long *et al*., 2012). However, while T1D GWAS have led to an explosion of information about autoimmunity, little is known about the genetic determinants of islet autoimmunity. In a genome-wide study (Brorsson *et al*., 2015), novel associations between islet autoantibodies and non-*HLA* genes *FCRL3*, *RELA*/*FIBP*, and *LPP* that are associated with autoimmune diseases but not with T1D risk were reported. These significant associations between genetic and autoimmune markers were found close to a diagnosis of T1D, the median age at diagnosis was nine years, and the median disease duration at blood sampling was seven years, with 25% of samples taken within three years.

***FCRL3* (Fc receptor-like protein 3):** *FCRL3* is located on the long arm of chromosome 1 (1q23), and codes for an fc-like receptor glycoprotein which is a member of the immunoglobulin receptor superfamily. The encoded protein is strongly expressed by B-cells, NK cells and to a lesser extent by T-cells (Swainson *et al*., 2010). *FCRL3* is mainly expressed in secondary immune organs, such as the lymph nodes and the spleen, but rarely expressed in the bone marrow and the thymus (Jin *et al*., 2015). *FCRL3* has immunoreceptor-tyrosine activation motifs and immunoreceptor-tyrosine inhibitory motifs in its cytoplasmic domain (Pawlowicz *et al*., 2016). It is thought to play a role in reduced B-cell signalling (Yang *et al*., 2013). Kochi *et al*. (2009) reported that *FCRL3* could regulate B-cell signalling through its ability to activate and/or inhibit tyrosine-protein kinase, which was displayed when an FCRL3 chimeric protein bound to B-cell receptors caused the phosphorylation of tyrosine-based motifs in the

cytoplasmic domain (Kochi *et al*., 2009). In addition to its effect on B-cells, *FCRL3* may also influence T-cell function. *FCRL3* was reported to be expressed by a subset of dysfunctional regulatory T-cells that are non-responsive to antigens and less capable of opposing the proliferation of effector T-cells (Nagata *et al*., 2009; Swainson *et al*., 2010). *FCRL3* genetic variants were associated with autoimmune diseases such as systemic lupus erythematosus (Kochi *et al*., 2005), Graves' disease (Khong *et al*., 2016) and rheumatoid arthritis (Lin *et al*., 2016). It is postulated that *FCRL3* over-expression is associated with loss of self-tolerance and development of autoimmunity, being attributed to a genetic variant 169 C>T in the promoter region of *FCRL3* that increases binding to NFκB transcription factor, resulting in increased *FCRL3* expression (Kochi *et al*., 2005; Jin *et al*., 2015).

***RELA/FIBP* locus**

***RELA* (RELA proto-oncogene, NF-kB subunit):** *RELA* is also known as Nuclear Factor Of Kappa Light Polypeptide Gene Enhancer In B-Cells 3 (http://www.genecards.org/cgi-bin/carddisp.pl?gene=RELA). It is located on the long arm of chromosome 11 (11q13.1), and coding for a (Transcription factor p65) protein NF-kappa-B, which is a pleiotropic transcription factor that plays a role in activating transcription of certain genes. It is found in almost all cell types and is involved in inflammation, immunity, differentiation, cell growth, tumorigenesis, and apoptosis. Upon activation, NF-kappa-B translocates to the nucleus where its NFKB1 form creates a complex with the *RELA* gene product (https://www.ncbi.nlm.nih.gov/gene/5970), (http://www.uniprot.org/uniprot/Q04206).

FIBP: known as FGF1 intracellular binding protein, it codes for acidic fibroblast growth factor. The encoded intra-cellular binding protein is thought to play a role in cellular mitogenesis and cellular differentiation (https://www.ncbi.nlm.nih.gov/gene/9158). It is reported to be associated with Thauvin-Robinet-Faivre syndrome; an autosomal recessive syndrome that is characterized by developmental delay, learning disabilities and congenital anomalies (Thauvin-Robinet *et al*., 2016). *RELA/FIBP* was reported to be associated with Crohn's disease (Jostins *et al*., 2012).

***LPP* (Lipoma Preferred Partner):** also known as LIM domain-containing preferred translocation partner in lipoma, a novel member of the LIM protein gene family (http://www.genenames.org/cgi-bin/gene_symbol_report?match=LPP). *LPP* is located on the long arm of chromosome 3 (3q27.3-q28). The encoded protein is proline-rich with a leucine zipper motif in its amino-terminal region and three LIM domains that facilitate protein-protein interactions (Petit *et al*., 1996). It plays a role in cell-cell adhesion and cell motility which makes it a potential novel link between cellular surface events and changes in gene expression, additionally it is thought to act as a transcriptional co-activator (Grunewald *et al*., 2009). Initially, *LPP* was reported to be associated with the risk of benign and malignant tumours in humans (Grunewald *et al*., 2009). Other studies showed that *LPP* was linked to risk of developing coeliac disease in Caucasians in two meta-analyses by (Almeida *et al*., 2014; Huang *et al*., 2017), and in a study by (Izzo *et al*., 2011), and was reported to be expressed by the pancreatic islets (Kutlu *et al*., 2009) and the small intestine (Hunt *et al*., 2008).

1.10 Rationale

The frequency of high-risk *HLA* class II genotypes is decreasing while the incidence of T1D has increased over recent decades (Gillespie *et al*., 2004; Fourlanos *et al*., 2008; Vehik *et al*., 2008; Steck *et al*., 2011). The rapid rate of change suggests an increase in pressure from the environment in individuals with low and moderate genetic susceptibility (Hermann *et al*., 2003), but this environmental pressure may also increase the penetrance of non-*HLA* risk variants where increasing numbers of individuals with low and moderate genetic risk account for new cases of T1D. This is an area of type 1 diabetes research which has become less popular over the last decades perhaps because few natural history studies recruit for long enough to allow changes in gene frequencies to be monitored over decades. The Bristol based Bart's Oxford study which is described in detail in chapter 2; section 2.1. is unique in continuously recruiting since 1985, therefore, facilitating this novel study.

1.11 Hypothesis and specific aims

Hypothesis: That the decrease in *HLA*-mediated susceptibility over time in individuals with T1D is associated with a concomitant increase in non-*HLA* genetic susceptibility variants.

Aim 1: To determine whether *HLA* class II susceptibility is continuing to decrease by analysing risk genotype frequencies over the last thirty years in participants in the population-based Bart's Oxford (BOX) Family study.

Aim 2: To determine whether the frequency of non-*HLA* susceptibility variants has altered over time.

Aim 3: To determine genetic associations between islet autoantibodies and non-*HLA* genetic determinants in the Bart's Oxford family study.

Aims 1 and 2 are directly linked; they both focus on analysing gene frequencies over time. Aim 1 focuses on analysing *HLA* class II data over the time the population-based BOX study has been recruiting while in Aim 2, the analysis involves comparison of non-*HLA* T1D associated gene frequencies between an early type 1 diabetes cohort – the Golden years cohort diagnosed between 1922 and 1948 and the BOX cohort recruited from 1985. Aim 3 is less directly linked and was not part of the original study. The analysis of non-*HLA* SNPs associated with islet autoantibodies was suggested by first-year reviewers Professors Julian Shield and Pat Kehoe. The results proved interesting and were added as an additional chapter but the effects of sampling over time could not be analysed because all the islet autoantibody data analysed were collected over a relatively short period between 1985 and 2002. A Venn diagram has been incorporated into chapter 6 to further clarify linkages between the aims and the data generated in this thesis.

Chapter 2

Materials and Methods

2.1 Study Populations:

1) The Golden Years cohort (GY): represents people from a UK national registry (n=400) who were diagnosed with T1D between 1922 and 1948. They had been awarded the Alan Nabarro Medal by Diabetes UK as they survived T1D for more than 50 years from diagnosis. They were characterized by low insulin requirements, normal body weight, high HDL, and history of parental longevity. With regard to the complications of diabetes, 43% of the GY cohort developed diabetic retinopathy but neither diabetic nephropathy nor large vessel disease was evident among those patients (Bain *et al*., 2003).

   There were 376 genetic samples available in total from the GY cohort with 285 from individuals diagnosed under the age of 21 years.

2) The Bart's Oxford cohort (BOX): is a prospective, population-based family study which has been recruiting patients diagnosed with T1D under the age of 21 years and their families in the BOX region as outlined in **Figure 2.1** since 1985. Paediatricians and physicians who were interested in diabetes in this region initiated the study, and it was initially designed to monitor T1D incidence in children and to predict those at risk of future diabetes (Gardner *et al*., 1997; Bingley *et al*., 1999). To date 5,078 families have been referred, >4,000 recruited and of these 55% remain under long term follow-up.

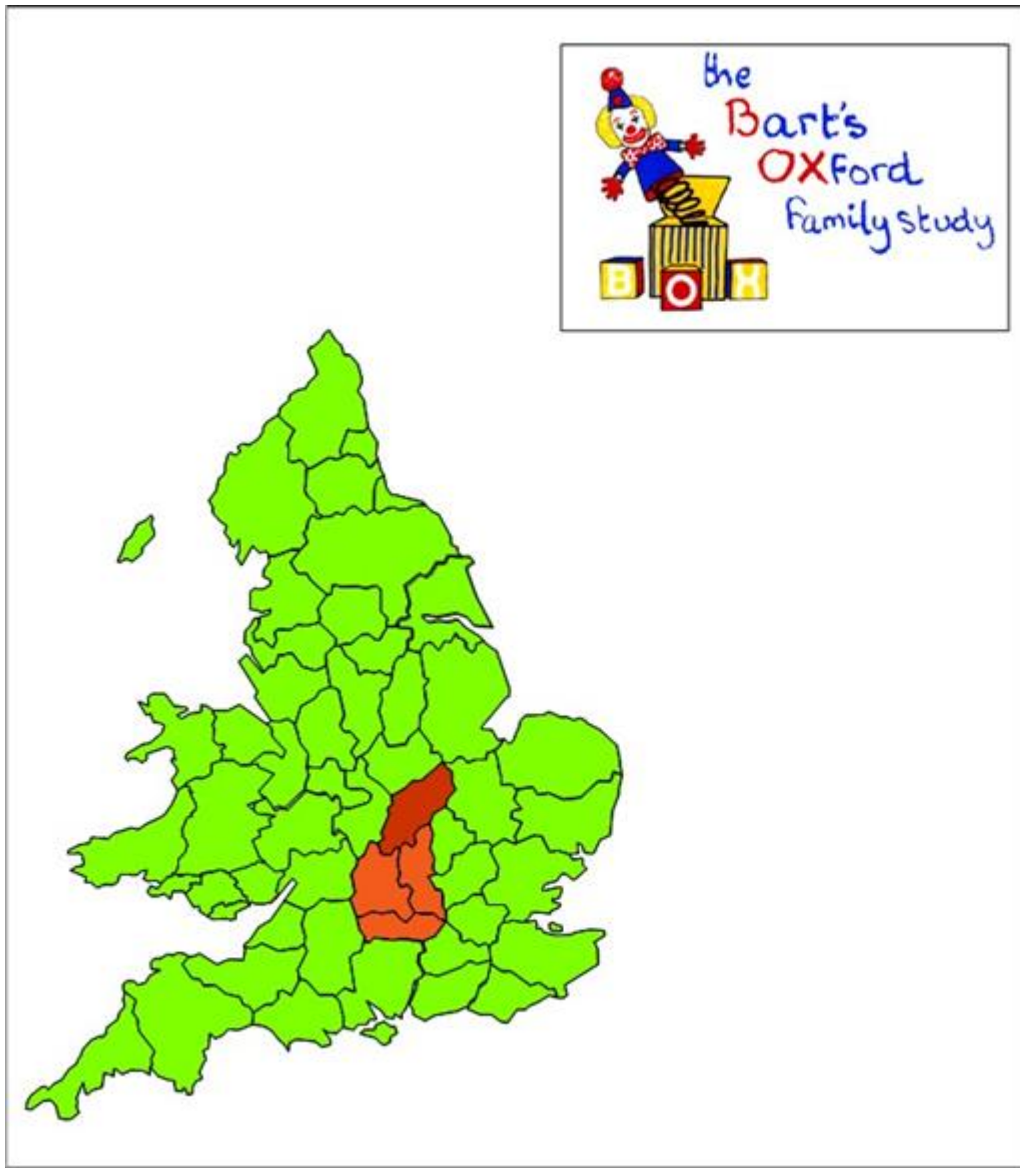

Figure 2.1: *Map showing the BOX study region: The former Oxfordshire Authority Health Region where the Bart's Oxford (BOX) family study participants are recruited.*

2.2 Genetic samples and methods

All chemicals were purchased from Sigma-Aldrich Ltd (Gillingham, UK) unless otherwise stated.

Genetic samples were obtained in the form of whole blood samples collected by local nurses or as mouth swabs. Kits for mouth swabs collection were sent via post to consented BOX

participants. In addition to leaflets, the participants could access video links demonstrating sample collection on the BOX study webpage.

(http://www.bristol.ac.uk/translational-health-sciences/research/diabetes/research/box/new-samples-and-video-guides/)

Mouth swab samples were returned by post to the laboratory for DNA extraction. All samples either whole blood or mouth brush were extracted using a traditional phenol-chloroform extraction method. DNA was quantified using a measurement of OD260 and quality controlled by measurement of OD260/280 ratios (Gillespie *et al*., 2000) and where necessary underwent whole genome amplification (WGA) following manufacturer's instructions (GenomiPhi V2 DNA Amplification Kit; GE Healthcare Life Sciences). DNA was stored at -20°C.

<u>2.2.1 Whole Genome Amplification steps:</u>

Before genotyping, native DNA samples were whole genome amplified in order to generate sufficient DNA for multiple tests. This approach had previously been validated previously in the laboratory and been shown to produce 100% concordant results between amplified and native samples. The amplification would also help preserve the native DNA for use in future experiments. The steps of WGA are explained using GenomiPhi V2 DNA Amplification Kit (GE Healthcare Life Sciences). The four reaction components of the GenomiPhi V2 DNA Amplification Kit ; enzyme mix, reaction buffer, sample buffer, and 10ng/µl Lambda positive DNA control usually stored at - 80°C were thawed on ice. The native 100ng/µl DNA samples are stored at -80 and the samples to be amplified were thawed on ice then centrifuged briefly. A total of 94 native DNA samples and two controls can be processed at a time. On ice, an aliquot of 1µl per well of native 100ng/µl DNA was plated into a 96-well PCR plate. Two wells

were spared for 1µl of 10ng/µl DNA per well of the positive control and 1µl of ddH_2O as the negative control. Then the sample buffer was added to the plate as 9µl per well, with DNA and sample buffer mix as 10µl per well. The plate was sealed with an adhesive thermal-seal and incubated for three minutes at 95°C in the PCR machine (BIO-RAD T100™, OX5 1GE, UK) and afterwards was cooled on ice. Then a mixture of 9µl reaction buffer and 1µl V2 enzyme was added per well to the plate. The resultant mixture of 20µl per well was further incubated for one and a half hours at 30°C, followed by ten minutes at 65°C resulting in amplified DNA of 20µl per well. To test whether the amplification had been successful, a new 96-well PCR plate was prepared with an aliquot of 1µl per well of amplified DNA and 5µl per well of density dye Sucrose Creosol Red (SCR; 60% sucrose, 40% ddH_2O and 1mM creosol red). For a large 2% agarose gel preparation; 8g agarose powder dissolved in 400ml 1X Tris-borate -EDTA (TBE) buffer -2g agarose powder and 100ml TBE buffer for a small gel- were added into a microwave-safe glass container with a capacity of 1 litre or more. The mixture was microwaved on full-power for 3 to 4 minutes with intermittent shaking before setting in a preformed cast, making sure it would not overheat by observing bubbles formation. Eye, hand, and body protection was used at all times to minimize the risk of burns and possible spillage. Then under running tap water, the mixture was carefully cooled till hand hot with a gentle swirling motion. A 16µl Midori Green dye -4 µl for a small gel- was added, and the product was poured into gel-mould, combs placed, and the gel left to set for 30 to 40 minutes -10 minutes for a small gel. The combs were removed once the gel was set, then placed in the electrophoresis tub so that the wells were immersed with TBE buffer for better band separation of the primers and the DNA product.The amplification product and SCR mix was loaded onto the gel (containing 0.04% (w/v) midori green (Geneflow, MG 04) to visualise DNA prior to electrophoresis) for 140 minutes at 100V -40 minutes at 120V for a small gel. Band

separation from the cathode to the anode was observed as DNA is negatively-charged. The 1X TBE buffer solution (pH 8.3) consisted of deionized water containing 90mM Tris borate and 2mM EDTA. The gel was imaged using a ChemiDoc™ MP System imager (BIO-RAD, 1708280) to examine if the sample DNA had amplified; the positive and negative controls were also checked .

The samples with successful amplification (usually all samples) were selected and aliquoted from the first plate into 0.5ml sarstedt tubes. Each tube would have a total of 49µl WGA DNA comprising 19µl of amplified DNA and 30µl Tris-EDTA (TE) buffer to solubilize and preserve the DNA from degradation. The WGA DNA tubes were stored at -20 °C.

2.2.2 *HLA* genotyping

HLA genotyping of BOX DNA samples collected between 1985 and 2015 was performed prior to this project; however, DNA samples collected from new BOX recruits since, were genotyped for *HLA DQB1* and *DRB1*. Analysis of *HLA* class II was carried out by Polymerase Chain Reaction-Sequence Specific Primers (PCR-SSP) (Bunce *et al*., 1995). The resultant PCR products were separated using 2% agarose gel-electrophoresis. To categorize *HLA* class II risk, the heterozygous diplotype *HLA DRB1*03-DQA1*0501-DRB1*0201* (*DR3-DQ2*)/*DRB1*04-DQA1*0301-DRB1*0302* (*DR4-DQ8*) was defined as high-risk whereas *DR3-DQ2/X* or *DR4-DQ8/X* diplotypes were intermediate-risk (including when *DR3-DQ2* and *DR4-DQ8* were homozygous) and all the other combinations were considered low-risk (*X/X*) (Gillespie *et al*., 2000; Gillespie *et al*., 2004; Lambert *et al*., 2004). In a linear regression analysis in Chapter 5, this nomenclature was further simplified as outlined in section 5.1.

2.2.2.1 *HLA* class II genotyping procedure

Personal protective equipment was put on before commencing the experiment. The experiment was carried out on ice to keep reaction materials at 4°C to prevent degradation until they were placed in a PCR machine. A total of 2163 BOX probands and 285 GY were genotyped for *HLA DRB1* and *HLA DQB1* prior to this study. In this study, 60 new BOX samples were genotyped for *HLA* class II *DQB1* and *DRB1*. Six WGA DNA samples can be genotyped for *HLA* class II *DQB1* and *DRB1* on a 96-well plate each time. Pre-aliquoted sequence-specific primer mixes for *HLA DRB1* and *DQB1* as shown in **Tables 2.1** and **2.2** were stored in the fridge in tubes and numbered accordingly. The primer sequences, concentrations, combinations and a detailed protocol of how they were prepared has been published previously (Gillespie *et al*., 2000; Aitken *et al*., 2016).

Primer number	Gene	Primer Name	Sequence (5'-3')
1	*DR01*	5'01 3'047 3'048	TTG TGG CAG CTT AAG TTT GAA T CTG CAC TGT GAA GCT CTC AC CTG CAC TGT GAA GCT CTC CA
2	*DR15*	5'02 3'01	TCC TGT GGC AGC CTA AGA G CCG CGC CTG CTC CAG GAT
3	*DR16*	5'02 3'02	TCC TGT GGC AGC CTA AGA G AGG TGT CCA CCG CGG CG
4	*DR03*	DR3 F DR3 R	GTT TCT TGG AGT ACT CTA CGT C TGC AGT AGT TGT CCA CCC G
5	*DR04*	5'04 3'047 3'048	GTT TCT TGG AGC AGG TTA AAC A CTG CAC TGT GAA GCT CTC AC CTG CAC TGT GAA GCT CTC CA
6	*DR11*	5'05 3'06	GTT TCT TGG AGT ACT CTA CGT C CTG GCT GTT CCA GTA CTC CT
7	*DR12*	5'08 3'08	AGT ACT CTA CGG GTG AGT GTT CAC TGT GAA GCT CTC CAC AG
8	*DR13*	5'03 5'05 3'10 3'045 3'17	TAC TTC CAT AAC CAG GAG GAG A GTT TCT TGG AGT ACT CTA CGT C CCC GCT CGT CTT CCA GGA T TGT TCC AGT ACT CGG CGC T CCC GCC TGT CTT CCA GGA A
9	*DR14*	5'05 5'08 3'11	GTT TCT TGG AGT ACT CTA CGT C AGT ACT CTA CGG GTG AGT GTT TCT GCA ATA GGT GTC CAC CT
10	*DR07*	5'07 3'079	CCT GTG GCA GGG TAA GTA TA CCC GTA GTT GTG TCT GCA CAC
11	*DR08*	5'08 3'045 3'18	AGT ACT CTA CGG GTG AGT GTT TGT TCC AGT ACT CGG CGC T GCT GTT CCA GTA CTC GGC AT
12	*DR09*	5'09 3'079	GTT TCT TGA AGC AGG ATA AGT TT CCC GTA GTT GTG TCT GCA CAC
13	*DR10*	5'10 3'047	CGG TTG CTG GAA AGA CGC G CTG CAC TGT GAA GCT CTC AC
14	*DR52*	5'52.1 5'52.2 3'13 3'14	TTT CTT GGA GCT GCG TAA GTC GTT TCT TGG AGC TGC TTA AGT C CTG TTC CAG GAC TCG GCC A GCT GTT CCA GTA CTC GGC AT
15	*DR53*	5'53 3'048	GAG CGA GTG TGG AAC CTG A CTG CAC TGT GAA GCT CTC CA
	Control Control	5'C 3'C	TGC CAA GTG GAG CAC CCA A GCA TCT TGC TCT GTG CAG AT

Table 2.1: *HLA DRB1 primer sequences in HLA class II genotyping.*

Primer number	Variant	Primer Name	Sequence (5'-3')
1	2	*DQB1*02* F *DQB1*02* R	GTGCGTCTTGTGAGCAGAAG GTAGTTGTGTCTGCACACCC
2	201	*DQB1*0201* F *DQB1*0201* R	GTCCGGTGGTTTCGGAATGA TGCTCTGGGCAGATTCAGAT
3	301	*DQB1*0301/0304* F *DQB1*0301/0304* R	GACGGAGCGCGTGCGTTA CGTGCGGAGCTCCAACTG
4	302	*DQB1*0302* F *DQB1*0302* R	GTGCGTCTTGTGACCAGATA CTGTTCCAGTACTCGGCGG
5	303	*DQB1*0303* F *DQB1*0303* R	GACCGAGCGCGTGCGTCT CTGTTCCAGTACTCGGCGT
6	304	*DQB1*0304* F *DQB1*0304* R	TTTCGTGCTCCAGTTTAAGGC TGGCTGTTCCAGTACTCGGCGG
7	305	*DQB1*0305* F *DQB1*0305* R	GCTACTTCACCAACGGGACC TGCACACCGTGTCCAACTC
8	307	*DQB1*0307* F *DQB1*0307* R	CCCGCAGAGGATTTCGTGTA CCCCAGCGGCGTCACCA
9	4	*DQB1*0401/0402* F *DQB1*0401/0402* R	CTACTTCACCAACGGGACC TGGTAGTTGTGTCTGCATACG
10	501	*DQB1*0501* F *DQB1*0501* R	ACGGAGCGCGTGCGGGG GCTGTTCCAGTACTCGGCAA
11	502	*DQB1*0502* F *DQB1*0502* R	TGCGGGGTGTGACCAGAC TGTTCCAGTACTCGGCGCT
12	503	*DQB1*0503* F *DQB1*0503* R	TGCGGGGTGTGACCAGAC GCGGCGTCACCGCCCGA
13	601	*DQB1*0601* F *DQB1*0601* R	TTTCGTGCTCCAGTTTAAGGC CCGCGGAACGCCAGCTC
14	602	*DQB1*0602/10/13* F *DQB1*0602/10/13* R	CCCGCAGAGGATTTCGTGTT CCTGCGGCGTCACCGCG
15	0603/7	*DQB1*0603/7* F *DQB1*0603/7* R	GGAGCGCGTGCGTCTTGTA GCTGTTCCAGTACTCGGCAT
16	0603-5/7-9/12	*DQB1*0603/8/12* F *DQB1*0603/8/12* R	GGAGCGCGTGCGTCTTGTA AACTCCGCCCGGGTCCC
17	0603/8/12	*DQB1*0603-05/07-09/12* F *DQB1*0603-05/07-09/12* R	GGAGCGCGTGCGTCTTGTA TGCACACCGTGTCCAACTC
Control	HgH	HgH F HgH R	ACCAGCTCAAGGATCCCAA CACCCATTACCCAAGAGCTTA

Table 2.2: *HLA DQB1 primer sequences in HLA class II genotyping.*

WGA DNA samples stored at -20°C were thawed on ice, then centrifuged and vortexed briefly to ensure homogeneity. GoTaq® G2 Flexi DNA Polymerase (10,000u) (Promega, www.promega.co.uk) was used in PCR reactions which also included the following PCR reagents: 5X Green GoTaq® Flexi Buffer, 5X Colourless GoTaq® Flexi Buffer and 25mM magnesium chloride (MgCl2). Deoxyribonucleotide triphosphates (dNTPs 1mM) were purchased from Sigma and all stored at-20°C. The $MgCl_2$, dNTPs and buffer were thawed on ice and vortexed to mix. The primers were also defrosted in batches and mixed thoroughly with a multichannel pipette. The primers were plated as 5µl per well into two 96-well high-profile non-skirted PCR plates on ice, following a plate map for six samples. For each of the six DNA samples, an Eppendorf tube sized 1.5ml was prepared. Each of the six reaction tubes was vortexed briefly for proper mixing of reagents. A total of six reagents; purified water 46.4µl, Green GoTaq buffer 68µl, 25mM MgCl2 20.4µl, dNTPs 1mM 10.2µl, WGA DNA 20.4µl, and DNA polymerase GoTaq enzyme 1.7µl were added into each Eppendorf tube. Following a plate map, an aliquot of 5µl per well of the reaction mix was added to the 5µl primer mix. The plates were securely sealed with an adhesive thermal-seal to prevent product evaporation during PCR thermo-cycling. A stored programme for *HLA* class II *DQB* and *DR* analysis was utilised. Briefly, this was a Touchdown PCR with an initial one-minute denaturation at 96°C; the 5 cycles of 96°C for 25 seconds; 70°C for 50 seconds and 72°C for 45 seconds followed by 21 cycles where the annealing temperature was reduced to 65°C and a final 4 cycles where the annealing temperature was reduced to 55°C and the extension temperature extended to 120 seconds. Finally, after the thermo-cycling was complete, the DNA amplification was stored at 4°C prior to gel-electrophoresis.

2.2.3 SNP genotyping

Genotyping for 22 single nucleotide polymorphisms (SNPs) was performed using WGA DNA samples from the GY (n= 285) and BOX cases (n= 1992). Both cohorts were age-matched for those diagnosed with T1D under the age of 21 years. SNP genotyping was done by using TaqMan® Universal Master Mix II assay (Applied Biosystems). Of the 22 SNPs, *FCRL3*, *RELA* and *LPP* were associated with the risk of islet autoimmunity as previously explained in section 1.9.2 and will be explained in more detail in chapter 5; section 5.3.

2.2.3.1 TaqMan genotyping steps

A wide range of Taqman SNP genotyping kits are available from ThermoFisher Scientific (https://www.thermofisher.com). The genotyping was carried out using TaqMan® Universal Master Mix II on a Real-time PCR (qPCR) machine using Step One software v2.2.2 (Applied Biosystems), with the exception for *CCR-5* SNP rs333 where deletion genotyping was performed as described in section 2.2.3.2. A list of the non-*HLA* SNPs that were genotyped in this project and their probes' sequences are shown in **Tables 2.3a and 2.3b**.

SNP	Allele detected	SNP probe context sequence (VIC/FAM)
rs6679677	A	ACAAGGTCTGAATCCTTGCTCCCAA[T]CAATAATCTGTGATCTTAAGCAATT
PTPN22	C	ACAAGGTCTGAATCCTTGCTCCCAA[G]CAATAATCTGTGATCTTAAGCAATT
rs2816316	A	ATCCTATCTAAAACAACAGACAACT[T]AATATTCCTCCACAACAGGAGGGAG
RGS1	C	ATCCTATCTAAAACAACAGACAACT[C]AATATTCCTCCACAACAGGAGGGAG
rs2292239	G	AATAGTGAAGAGACTTTTGAATCTA[C]AGGGCAGCACTTAAGGGATCTAGGG
ERBB3	T	AATAGTGAAGAGACTTTTGAATCTA[A]AGGGCAGCACTTAAGGGATCTAGGG
rs3184504	C	TTGCTCCAGCATCCAGGAGGTCCGG[G]GGTGCACACGGCTTGAGATGCCTGA
SH2B3	T	TTGCTCCAGCATCCAGGAGGTCCGG[A]GGTGCACACGGCTTGAGATGCCTGA
rs4948088	A	CAAGACATGAGCTAGTCTTGGGATA[T]CCACCTCTGCTGCCAGGCCAAAAAG
COBL	C	CAAGACATGAGCTAGTCTTGGGATA[G]CCACCTCTGCTGCCAGGCCAAAAAG
rs12935413 *CLEC16A / KIAA0350*	A	AGAATCTCAGACAGGTTACACTGTC[T]GAGGATTGAACCGAGGCATATGTTG
	G	AGAATCTCAGACAGGTTACACTGTC[C]GAGGATTGAACCGAGGCATATGTTG
rs1893217	A	TCACTTGTCACCATTCCTAGGGACA[T]AGGTAGAGGAAGAAGAGTGTATCTG
PTPN2	G	TCACTTGTCACCATTCCTAGGGACA[C]AGGTAGAGGAAGAAGAGTGTATCTG
rs11755527	C	AAGGGCTGGGAAAGAAAGGAGAAAG[G]AAAGTATGGACAACTGGGTGGTGCT
BACH2	G	AAGGGCTGGGAAAGAAAGGAGAAAG[C]AAAGTATGGACAACTGGGTGGTGCT
rs3024505	A	GGGCTGCCCAGGCAGAGCGTGAGGG[T]GACTAGTGTTTACTCAGCTCATTTT
IL-10	G	GGGCTGCCCAGGCAGAGCGTGAGGG[C]GACTAGTGTTTACTCAGCTCATTTT
rs917997	C	ATAGATAATGCTAGAACCAAGCTAT[G]CAGATGTCCAGCGTTTTGACCTTAG
IL18RAP	T	ATAGATAATGCTAGAACCAAGCTAT[A]CAGATGTCCAGCGTTTTGACCTTAG
rs3087243	A	TCTTCACCACTATTTGGGATATAAC[T]TGGGTTAACACAGACATAGCAGTCC
CTLA-4	G	TCTTCACCACTATTTGGGATATAAC[C]TGGGTTAACACAGACATAGCAGTCC

Table 2.3a: *SNP probe sequences with the alleles detected: The disease associated variant highlighted in red, the VIC labelled probe is in blue (coded allele 1 by the StepOne Plus PCR software); the FAM labelled probe is in green (coded allele 2 by the StepOne Plus PCR software). The deletion polymorphism CCR-5 is not described here. Details can be found in section 2.2.3.2.*

SNP	Allele detected	SNP probe context sequence (VIC/FAM)
rs4505848 *IL-2*	A	TGTTCTAGGAATGGTGCTAGTTGCT**[T]**TAGATAGAGATGATGTAGCATCTGT
	G	TGTTCTAGGAATGGTGCTAGTTGCT**[C]**TAGATAGAGATGATGTAGCATCTGT
rs7020673 *GLIS3*	C	TGATAATGGCTACAGATTGCTGGAG**[G]**AAATTCAGGACCTTCAGGAATACAC
	G	TGATAATGGCTACAGATTGCTGGAG**[C]**AAATTCAGGACCTTCAGGAATACAC
rs12251307 *IL2RA*	C	CGAAGTAGCTAACGTGTTGGAAGTC**[G]**CCATAAGGCACATGAGCTGCAGAGA
	T	CGAAGTAGCTAACGTGTTGGAAGTC**[A]**CCATAAGGCACATGAGCTGCAGAGA
rs3825932 *CTSH*	C	GGACAATTGACTACCAGTTTGCCTC**[G]**GGAGAGATTATTCTGGGGCCAGAAT
	T	GGACAATTGACTACCAGTTTGCCTC**[A]**GGAGAGATTATTCTGGGGCCAGAAT
rs689 *INS*	A	CATGGCAGAAGGACAGTGATCTGGG**[T]**GACAGGCAGGGCTGAGGCAGGCTGA
	T	CATGGCAGAAGGACAGTGATCTGGG**[A]**GACAGGCAGGGCTGAGGCAGGCTGA
rs2111485 *IFIH1*	T	GCATGGGGTCATAAATATAAAGCCT**[A]**GAAGGGTGGAATTTCCCTGAGGAA
	C	GCATGGGGTCATAAATATAAAGCCT**[G]**GAAGGGTGGAATTTCCCTGAGGAA
rs9976767 *UBASH3A*	A	TCTTTCCTACTTCCTGGAAAAATCT**[T]**CAGGGATCCAGCCATCTCCTTTATG
	G	TCTTTCCTACTTCCTGGAAAAATCT**[C]**CAGGGATCCAGCCATCTCCTTTATG
rs3761959 *FCRL3*	C	TTCCTTCCTGTAGTCTGAGGAATAC**[G]**GGTAATGTAGGGAGAAAAAAAAAAA
	T	TTCCTTCCTGTAGTCTGAGGAATAC**[A]**GGTAATGTAGGGAGAAAAAAAAAAA
rs568617 *RELA*	C	TCCGAGTTCTGGCCTCTCTCTGCCT**[G]**AAGTCACTGTGTGACGTGTCTCA AG
	T	TCCGAGTTCTGGCCTCTCTCTGCCT**[A]**AAGTCACTGTGTGACGTGTCTCA AG
rs1464510 *LPP*	G	ATGTGATTCTGAAACTGATTTGAAA**[C]**AGCCTGGTTCATTTTTACTGTGTTGC
	T	ATGTGATTCTGAAACTGATTTGAAA**[A]**AGCCTGGTTCATTTTTACTGTGTTGC

***Table 2.3b**: SNP probe sequences with the alleles detected: The disease associated variant highlighted in red, the VIC labelled probe is in blue (coded allele 1 by the StepOne Plus PCR software); the FAM labelled probe is in green (coded allele 2 by the StepOne Plus PCR software). The last three SNPs of FCRL3, RELA and LPP are not associated with the risk of T1D but with islet autoimmunity as explained in section 1.9.2 and in chapter 5. Alleles marked in red are the risk alleles for T1D.*

A total of 376 GY samples were genotyped for 16 T1D SNPs before age-matching and 1380 BOX samples were genotyped for *UBASH3A* in this study. The remainder of the SNP genotyping had been completed prior to this study. To put the experiments in context, the GY 376 samples required five 96-well plates to genotype each of the 16 SNPs, and BOX 1380 samples were genotyped on fifteen 96-well plates. Where necessary, samples were retyped

when samples did not amplify successfully in the first run. The failure rate was approximately 1%. For each SNP, positive controls for each homozygous and heterozygous genotype were available and added to each plate. Additionally, a fourth control of ultra-pure water was used as a negative control to determine if there was any contamination.

Personal protective equipment was worn as per laboratory protocols. For each plate a total of 92 WGA DNA samples were thawed on ice, then centrifuged and vortexed briefly. The samples were plated on ice into a 96-well optically-clear plate, sparing the last four wells for homozygous and heterozygous positive controls 11, 12, and 22 (allele 1 is the VIC labelled allele for each SNP and allele 2 is the FAM labelled probe) as positive controls and the last well for ultra-pure water as the negative control. The remainder of the experiments were carried out at room temperature. Briefly for each plate (and following manufacturer's instructions where concentrations of reagents were not clear); the ultrapure water and Taqman mastermix were removed from storage at 4°C, swirled gently and then 550µl and 600µl respectively were added to a 2ml eppendorf tube. . The probe (15µl) -usually stored at -20°C, was then added to the mix. The SNP probe is light-sensitive, and care was taken to minimize exposure to light. The main reaction mixture tube was briefly centrifuged and vortexed. An aliquot of 11.65µl per well was plated into the 96-wells. The plate was sealed with an optical adhesive seal, then spun briefly with a plate spinner. Finally, the plate was placed in a qPCR machine for analysis.

The STEPONE software uses the known positive controls to cluster homozygotes for allele 1, homozygotes for allele 2 and heterozygotes as shown in **Figure 2.2**.

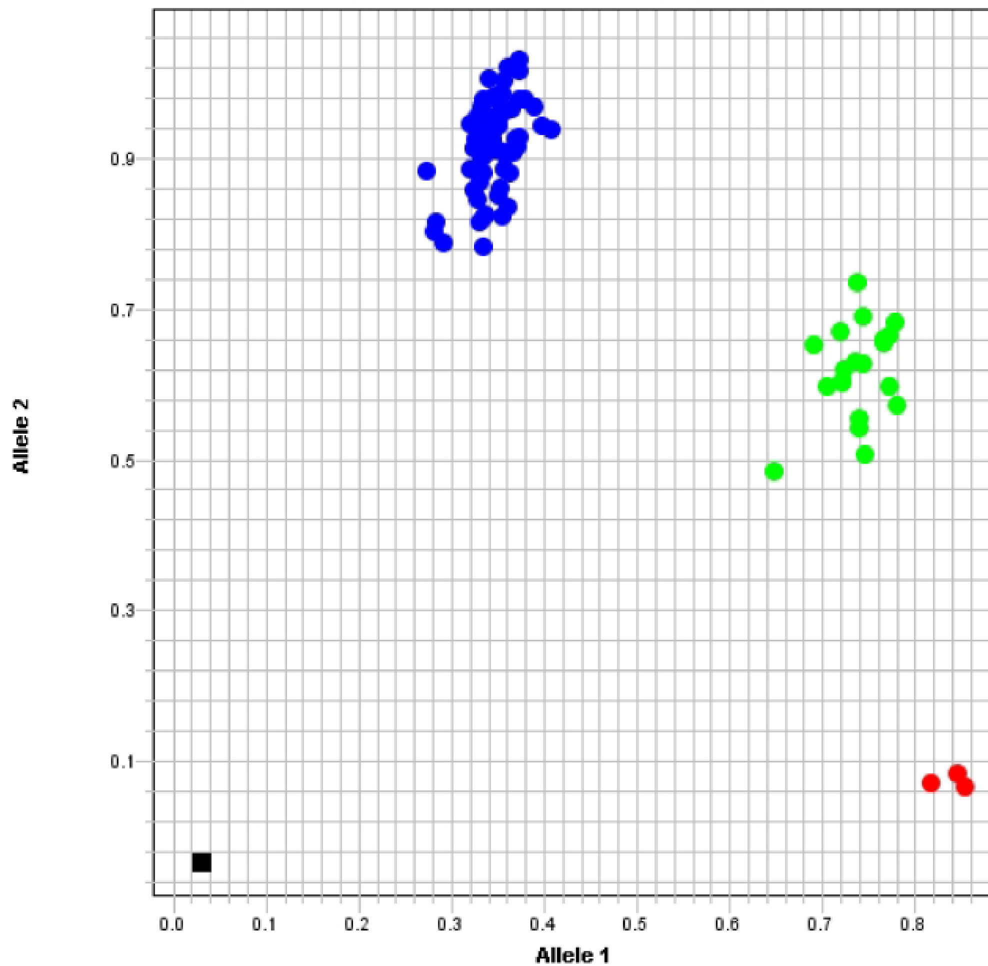

Figure 2.2: *Genotypes as "called" by the qPCR machine software: The black square is the negative control; the cluster of red dots is the number of subjects having two copies of allele 1 (genotype 11), the cluster of blue dots represents the subjects with two copies of allele 2 (genotype 22), and the green cluster of dots shows those with one copy of allele 1 and 2 (genotype 12).*

2.2.3.2 *CCR-5* rs333 genotyping steps

Genotyping of *CCR-5* rs333 SNP was carried out on a BIO-RAD **T100** Thermal™ Cycler using GoTaq® G2 Flexi DNA Polymerase (10,000u) (Promega) . The resultant PCR product was then size-separated using 2.5% agarose gel-electrophoresis to separate the alleles. The gel was read on a gel-imager as described in section 2.2.1. A total of 376 WGA genetic samples of the GY cohort were available for *CCR-5* genotyping, while 1992 BOX genetic samples had been genotyped prior to this study.

Personal protective equipment were put on before starting and experiments were carried out on ice. Unless otherwise stated reagents for this assay were from Promega.

Briefly an individual PCR reaction mix included 0.6 µl of WGA DNA; 2.5 µl of forward primer (*CCR-5* F 5'-GATAGGTACCTGGCTGTCGTCCAT-3') and reverse primer (*CCR-5* R 5'-ACCAGCCCCAAGATGACTATCT-3') at 20pmol/µl, 1.45µl ddH_2O, 0.6µl MgCl2 (25mM), 0.3µl dNTP (1mM); 2µl GoTaq Green buffer and 0.05µl of GoTaq Green DNA polymerase. No DNA sample was added to the last well where ddH_2O was used as a negative control. The plate was sealed with an adhesive thermal-seal, and amplified in a PCR machine following a pre-stored *CCR-5* genotyping programme.

A 2.5% agarose gel-electrophoresis was carried out (similar to the method described in section 2.2.1). Briefly, 10g agarose powder was added to 400ml TBE buffer for large 2.5% agarose gels and 2.5g agarose powder in 100ml for small 2.5% agarose gels. The remainder of the experiment was carried out as described in section 2.2.1 to determine *CCR-5* genotypes as shown in **Figure 2.3**.

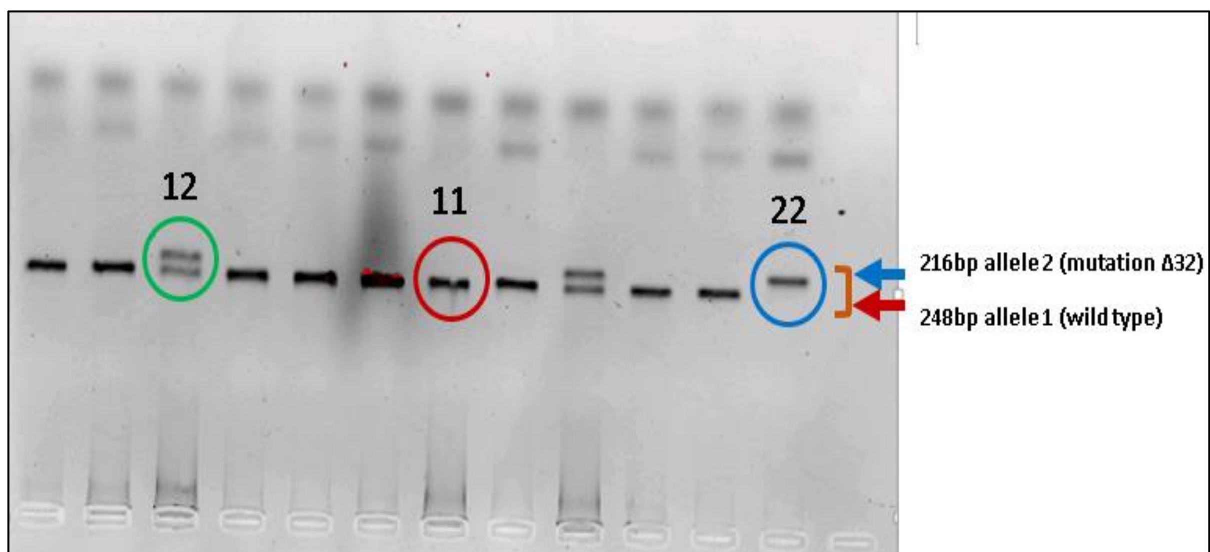

Figure 2.3: *CCR-5 alleles: A CCR-5 gel-image of the results obtained by agarose gel-electrophoresis showing a 32bp difference between allele one and allele two. Allele 1 (wild type) is associated with risk of development of T1D.*

Scoring genotype data: All genetic results were scored first by the person carrying out the experiment and then second scored by a colleague to ensure high quality results. Any queries were repeated but repeats are consistently below 3%.

2.3 Islet Autoantibody samples and methods

2.3.1 Subjects

Islet autoantibody data were available on a group of newly-diagnosed BOX children (n=613) (Long *et al*., 2012). The participants at sample collection were under the age of 21 years. The median age of diagnosis was 10.9 years, and the range was 0.7 to 21 years. A genetic sample was available for analysis on 552 (90%) of participants. Serum and DNA samples had been collected from the consented participants within a median of one day from the onset of T1D, and a range of -61 to 90 days. The patients were divided according to date of diagnosis into four quartiles, as shown in **Table 2.4**.

Factor	Overall	Quartile 1	Quartile 2	Quartile 3	Quartile 4	*P*
BOX subjects	613 (34.0)	153 (30.2)	154 (37.1)	153 (37.4)	153 (28.6)	
Minimum date of diagnosis	July 1985	July 1985	April 1990	February 1994	July 1997	NA
Maximum date of diagnosis	February 2002	March 1990	February 1994	June 1997	February 2002	NA
Age (years)	10.9 (0.7-20.8)	10.9 (0.7-20.7)	10.9 (1.3-20.6)	10.5 (1.3-20.8)	11.3 (1.5-17.4)	0.411
Disease duration (days)*	1 (-60 to 90)	3 (-61 to 90)	1 (-9 to 88)	1 (-9 to 90)	1 (-21 to 89)	0.078
Sex (male)	346 (56.4)	90 (58.8)	83 (53.9)	88 (57.5)	85 (55.5)	0.799
HLA class II						
DR3-DQ2/DR4-DQ8	225 (36.7)	57 (37.3)	55 (35.7)	55 (35.9)	58 (37.9)	0.848
DR4-DQ8/DR4-DQ8	30 (4.9)	3 (2.0)	9 (5.8)	6 (3.9)	12 (7.8)	0.043
DR3-DQ2/DR3-DQ2	49 (8.0)	13 (8.5)	14 (9.1)	11 (7.2)	11 (7.2)	0.560
DR4-DQ8/X	143 (23.3)	36 (23.5)	38 (24.7)	35 (22.9)	34 (22.2)	0.628
DR3-DQ2/X	98 (16.0)	24 (15.7)	23 (14.9)	29 (19.0)	22 (14.4)	0.981
X/X	68 (11.1)	20 (13.1)	15 (9.7)	17 (11.1)	16 (10.5)	0.578

Table 2.4: *The characteristics of newly-diagnosed BOX participants (Long et al., 2012): The total number was (n=613), of whom 346 (56.4%) were males. All participants were diagnosed between July 1985 and February 2002. The overall age range in years was 10.9 (0.7-20.7). The median of T1D duration was 1 day with a range of -61 to 90. The majority were carriers of at least one of the two HLA class II DR3-DQ2 and DR4-DQ8 haplotypes (36.7%), while only 4.9% had DR4-DQ8/DR4-DQ8.*

2.3.2 Methods

Radio-binding assays were performed to measure autoantibodies against insulin, GAD, IA-2, IA-2β, and ZnT8 antigens. This work was completed by other members of the laboratory.

Regarding insulin autoantibodies, samples that were collected within a fortnight from the onset of disease were used for IAA measurement. Samples collected beyond this period would result in false-positive results, as antibodies against exogenous insulin rather than endogenous insulin would be detected after initiating insulin treatment (Long et al., 2012).

Antibodies to GAD, Insulin, IA-2 and where appropriate IAA were measured using radio-binding assays. These assays are well described in the literature for IAA (Williams *et al*., 1997), IA-2A, (Williams *et al*., 2008), GADA (Williams *et al*., 2015) and ZnT8A (Long *et al*., 2012) and as these data were provided by other laboratory members, the methods are, therefore, only

described briefly here. In principle the antigen is radiolabelled either by iodination or in vitro transcription/translation. The radiolabel is added to serum from individuals of interest. The antigen/autoantibody complex is separated using Poly A Sepharose, washed and the level of antibody measured in a beta or gamma counter as appropriate. Autoantibodies to insulin, full-length GAD65, and intracytoplasmic (606–979) or juxtamembrane (609–631) regions of IA-2 were measured. IA-2βA and ZnT8A were assayed using similar protocols with plasmids encoding the protein tyrosine phosphatase domain of IA-2β (723–1015) or C-terminus of ZnT8 (268–369, 325R, or 325W) provided by Dr. Vito Lampasona. ZnT8A were determined by combining results from separate assays for autoantibodies recognising either arginine (ZnT8RA) or tryptophan (ZnT8WA) at position 325. IAA, GADA, IA-2βA, and ZnT8A results were expressed in arbitrary units and IA-2A in DK units/mL derived from standard curves, with extrapolation of values above the top standard. JMA results were calculated as an index.

Thresholds for IAA, GADA, and IA-2A positivity were set at the 97.5th percentile of sera from 2,860 schoolchildren (Bingley *et al*., 1997). The threshold for ZnT8A measurement was based on the 97.5th percentile of 523 schoolchild sera (Long *et al*., 2012). Thresholds for IA-2βA were set at the mean ± 3 SDs of 270 schoolchild sera (Long *et al*., 2012; Long *et al*., 2012).

When expressing results of autoantibody measurements, arbitrary units were used to express the results for IAA, GADA, ZnT8A, and IA-2βA (Williams *et al*., 2008), whereas DK units/ml were used to express for IA-2A results (Long *et al*., 2012).

Assay performance was based on the outcomes of the Diabetes Autoantibody Standardization Program (DASP). DASP was established as a means to evaluate and determine the performance of islet autoantibody assays used in different laboratories. A total of 60 laboratories across 18 countries took part in DASP (Lampasona *et al*., 2011)

2.3.3 Genetic analysis

As explained earlier in "Genetic samples and methods" section, native DNA samples were collected, whole-genome amplified, and *HLA* class II and SNP genotyping were performed.

Three additional *non-HLA* SNPs *FCRL3* rs3761959, *RELA*/*FIBP* rs568617, and *LPP* rs1464510 known to be associated with islet autoimmunity, but not T1D, were genotyped as part of the aims of this MD in 376 GY before age-matching, and 1400 BOX genetic samples, adding to 19 previously genotyped SNPs using a TaqMan protocol as previously described in section 2.2.3.1. *HLA* class II genotypes and a total of 22 SNPs were analysed in relation to four islet autoantibodies.

2.3.4 Statistical Analysis

Genetic data represent categorical variables and as such were described as frequencies while comparisons were conducted using chi-square and chi-square for trend as appropriate. Bonferroni correction for multiple comparisons was not applied as it is considered overly conservative but care was taken only to apply statistical tests where data were sufficient. P values <0.05 were considered significant.

Regarding statistical analyses of islet autoantibody associations with non-*HLA* SNPs, a chi-square univariate analysis was done to test the significance of the interaction of each of the three non-*HLA* SNPs *FCRL3*, *RELA*, and *LPP* with IAA, GADA, IA-2A, IA-2βA, and ZnT8A. Associations with a p<0.05 were deemed significant and were further tested in multivariate logistic regression analyses to examine the degree of the potential influence of other variables

on these genetic-autoantibody associations as shown in **Tables 5.1 and 5.2** in the methods section of chapter 5. All calculations were performed using SPSS (IBM SPSS Statistics 23.0).

Chapter 3

Analysis of *HLA* class II risk over time in cases participating in the Bart's Oxford Study (1985-2015)

3.1 Introduction

Comparison of the *HLA* class II frequencies between cases with T1D diagnosed before 1948 and after 1985 showed a reduction in high-risk genotypes and an increase in intermediate-risk profiles in several different studies including BOX (Hermann *et al*., 2003; Gillespie *et al*., 2004; Fourlanos *et al.,* 2008). This change in *HLA*-mediated risk occurred over a period when the increase in the incidence of T1D was well documented (Onkamo *et al*., 1999; Green *et al*., 2001; Gale, 2002). Data regarding changes in the incidence of T1D in the early 21st century are less clear with some studies suggesting that there may be ongoing increases in some areas while high incidence countries such as Finland may have reached a plateau (Harjutsalo *et al*., 2013; Skrivarhaug *et al*., 2014; Patterson *et al*., 2019). In particular, the EuroDiab Ace study (Patterson *et al*., 2019) showed that “Despite reductions in the rate of increase in some high-risk countries, the pooled estimate across centres continues to show a 3.4% increase per annum in incidence rate, suggesting a doubling in incidence rate within approximately 20 years in Europe”.

One of the advantages of the Bart’s Oxford family study is that it is population-based making it an ideal study in which to monitor changes over time. In this chapter, the aim was to examine *HLA* class II data over the time since 1985 that the study has been recruiting in order to address the hypothesis “that *HLA* risk for T1D continues to decrease over time”.

3.2 Materials and Methods

As described in chapter 2; section 2.2.2.1, *HLA* class II *DRB1* and *DQA1* genotyping was carried on whole genome amplified DNA from cases with type 1 diabetes participating in the BOX study. The genotyping method was learned

and then 60 samples were fully genotyped to ensure understanding of the data to be analysed in order to test the hypothesis that *HLA* class II frequencies were continuing to change over time.

3.2.1 Population examined

The BOX Genetic Results Database contains *HLA* class II data on all study participants who have provided samples either mouth brush or venepuncture samples for analysis of *HLA* class II *HLA DRB1-DQB1* by PCR-SSP (Gillespie *et al*., 2000). These data were selected to identify all probands with a result. Family members who also had diabetes were removed from the analysis to avoid biasing the data. Data were analysed in 5-year bands, between 01/01/1985 and 31/12/2014. Individual genotypes were coded as “High-risk” if they carried both risk haplotypes (*HLA DRB1*03-DQB1*0201*/ *HLA DRB1*04-DQB1*0302); “*Medium-risk” if an individual was positive for only one risk haplotype and another non-risk haplotype and “Low-risk” if *HLA DRB1*03-DQB1*0201* and *HLA DRB1*04-DQB1*0302* were absent. The protective haplotype *HLA DRB1*15-DQB1*0602* was not analysed separately due to its low frequency in BOX cases (<1%).

3.2.2 Data analysis

Data frequencies were converted to percentages and compared by date of diagnosis in year groups. Differences were calculated by chi-square test for trend with $p<0.05$ considered significant.

3.3 Results

A typical gel is shown in **Figure 3.1**. The PCR products for PCR mixes *DRB1 1-15* and *DQB1 1-17* underwent agarose gel-electrophoresis as described in section 2.2.2.1.

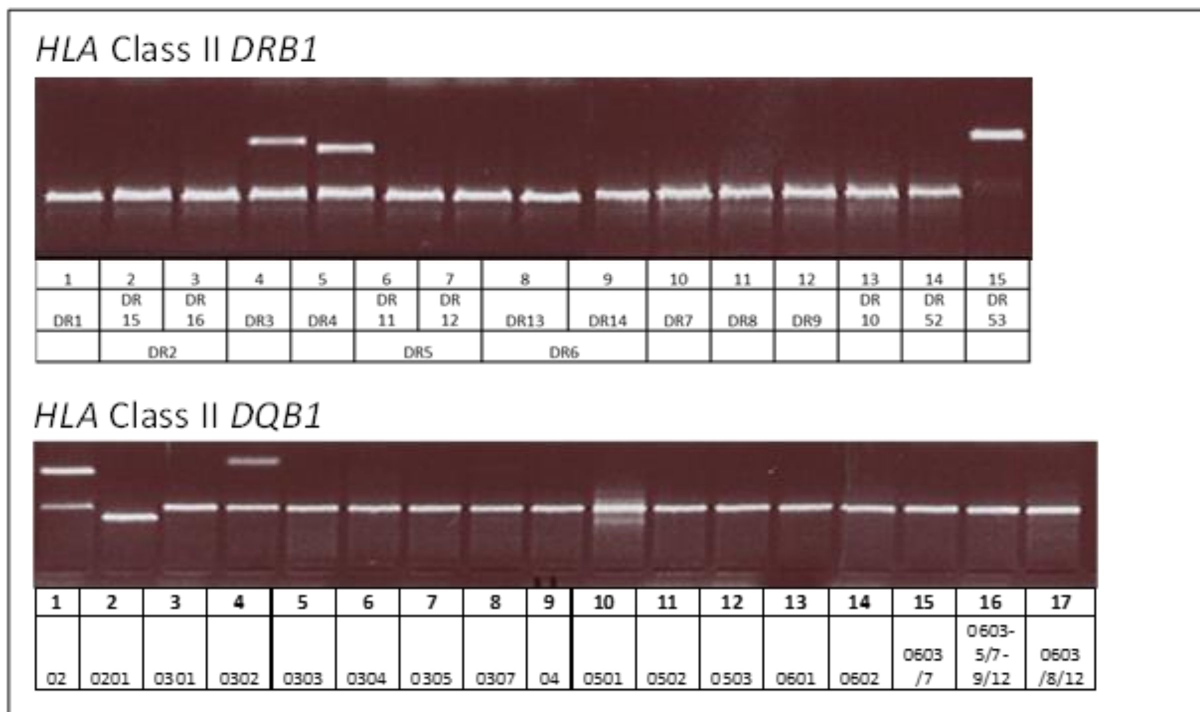

Figure 3.1: *A representative gel showing the results from an individual with the highest-risk HLA class II genotype (HLA-DRB1*03, HLA-DRB1*04 and HLA-DQB1*0201, HLA-DQB1*0302). The constant band running at higher molecular weight -closer to the bottom of the image- is a control band which should amplify in every individual. This is commonly a non-polymorphic region of the gene for Human Growth Hormone (sequences on Tables 2.1 and 2.2 in chapter 2). Occasionally other controls are used if the allele specific variant is a similar size to the control band. The allele specific bands can be uncoded using the matrix incorporated with the gel image.*

Table 3.1 shows the first 10 results obtained while learning the *HLA* class II genotyping procedure.

Sample ID	*DRB1* Allele 1	*DRB1* Allele 1	*DQB1* Allele 1	*DQB1* Allele 1	DR/DQ genotype
1	04	04	0302	0302	DR4-DQ8/DR4-DQ8
2	03	04	0201	0302	DR3-DQ2/DR4-DQ8
3	01	01	0501	0501	X/X
4	03	04	0201	0302	DR3-DQ2/DR4-DQ8
5	03	04	0201	0302	DR3-DQ2/DR4-DQ8
6	01	04	0501	0302	DR4-DQ8/X
7	03	04	0201	0302	DR3-DQ2/DR4-DQ8
8	04	04	0302	0302	X/X
9	04	12	0301	allele not detected	DR4-DQ8/X
10	03	04	0201	0302	DR3-DQ2/DR4-DQ8

Table 3.1: *HLA class II analysis: Five individuals were positive for the highest risk genotype DR3-DQ2/DR4-DQ8, two were negative for HLA class II risk (X/X) and the remainder had intermediate-risk (either homozygous or heterozygous for DR3-DQ2 or DR4-DQ8).*

When the 60 available DNA samples had been *HLA* class II genotyped (including the ten samples described above), the results were collated in a graph as shown in **Figure 3.2**.

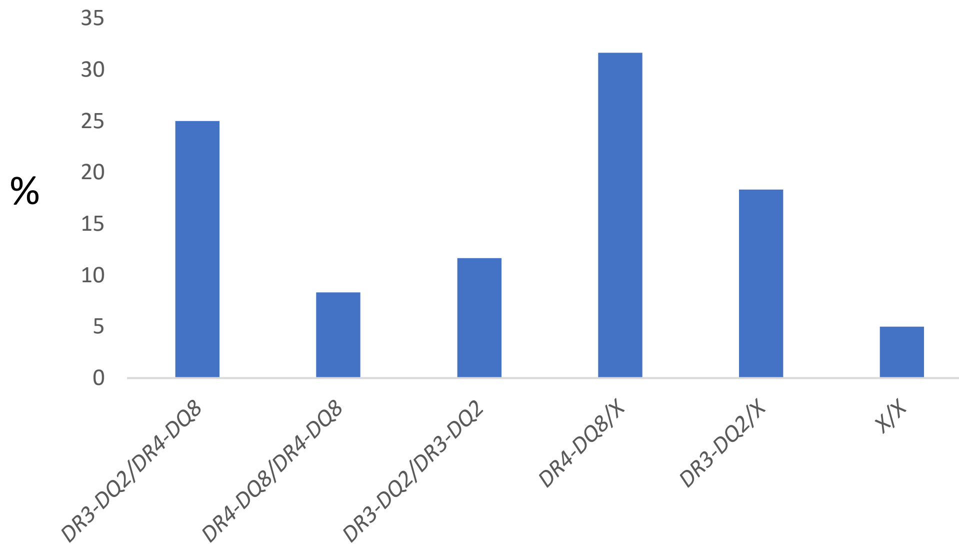

Figure 3.2: *HLA class II genotype frequencies for 60 T1D cases tested.*

Once the *HLA* genotyping technique had been mastered, the data available in the BOX ACCESS database were analysed. Some time was spent checking the data where some individuals had incomplete data. Where it was possible, their data were completed and updated. Only the proband from each family was included in the analysis.

3.3.1 *HLA* frequencies in BOX probands by date at diagnosis

Data with complete *HLA* class II genotypic data, date of birth and date of diagnosis were selected for further analysis. Ultimately, results on 1737 probands were analysed. Each *HLA* genotype was coded as high, medium and low risk as outlined in the methods and **Table 3.1**. Age at diagnosis was calculated by subtracting date at diagnosis from date of birth. Analysis was carried out in EXCEL. The results obtained when the frequency of *HLA* risk genotypes were analysed in 5-year groupings by date of diagnosis of diabetes are shown in **Table 3.2**.

Date of Diagnosis	High-risk *HLA* n	%	Intermediate-risk *HLA* n	%	Low-risk *HLA* n	%
1985-1990	100	33	164	55	37	12
1990-1995	137	34	222	56	39	10
1995-2000	171	37	258	55	37	8
2000-2005	75	28	185	60	33	12
2005-2010	44	29	91	59	19	12
2010-2015	44	30	77	53	24	17
Total	**571**	**33**	**977**	**56**	**189**	**11**

Table 3.2: *HLA in BOX Study Cases: HLA risk genotypes by date of diagnosis in 5 year bands from 1985 to 2015. In the row entitled "total", the frequency of n is the total n but for the % columns, the average is given because the total would not be helpful.*

These data did not support the hypothesis of decreases in the high-risk *HLA* over time ($p>0.05$; chi-square for trend) and suggest that there have been minimal changes to *HLA* high-risk frequencies over the decades the BOX study has been recruiting although the numbers of samples from 2000 onwards were relatively low compared to those before 2000 and this will be discussed in more detail in section 3.4. The data analysed here involved children with diabetes diagnosed under the age of 21 years while the original study focused on children under the age of 15 years (Gillespie *et al*., 2004). In the published study three age-matched individuals from BOX were compared with each GY participant as shown below on **Table 3.3**. Therefore, there were 194 individuals in the group diagnosed between 1922 and 1948 and 582 in the 1985-2002 group.

Date of Diagnosis	High-risk *HLA* n	%	Intermediate-risk *HLA* n	%	Low-risk *HLA* n	%
1922-1948 n=194	92	47	85	44	17	9
1985-2002 n=582	206	35	320	55	56	10

Table 3.3: *HLA comparison over time as previously published (Gillespie et al., 2004).*

3.3.2 Reanalysis of Golden Years data to include cases diagnosed under age 21 years

HLA risk is known to decrease with increasing age at onset. The original GY data were, therefore, reanalysed using data from 278 participants diagnosed under the age of 21 years and compared with the expanded BOX *HLA* dataset for probands diagnosed under age 21 analysed in this study. The results are shown in **Table 3.4**.

Date of Diagnosis	High-risk *HLA* n	%	Intermediate-risk *HLA* n	%	Low-risk *HLA* n	%
1922-1948 n=278	109	39	148	53	21	8
1985-2015 n=1737	571	33	977	56	189	11

Table 3.4*: Comparison of HLA genotypes in under 21 years old: HLA Comparison of GY and BOX cases diagnosed with T1D under the age of 21 years.*

These data suggest the difference in *HLA* susceptibility can be still observed in the enlarged datasets spread across a wider age group (p<0.01; chi-squared for trend analysis), but this may also dilute the differences observed in younger age groups.

3.3.3 The effect of age-at-onset on *HLA*-mediated risk in BOX

As the greatest effect on genotype over time was previously observed in those diagnosed under the age of 5 years, this subgroup was examined in isolation in the BOX study **as shown in Table 3.5**.

Date of Diagnosis	High-risk *HLA* n	%	Intermediate-risk *HLA* n	%	Low-risk *HLA*	%
1985-1990	28	57	18	37	3	6
1990-1995	38	38	52	52	10	10
1995-2000	35	44	40	50	5	6
2000-2005	12	25	34	69	3	6
2005-2010	9	30	18	60	3	10
2010-2015	13	41	17	53	2	6
Total	**135**	**35**	**179**	**38**	**26**	**27**

Table 3.5: *HLA genotypes comparison in under 5 years old: HLA high, intermediate, and low-risk genotypes in children diagnosed under the age of 5 years.*

The results in those diagnosed under the age of 5 years may suggest a decrease in high-risk *HLA* frequencies over time. The numbers of samples available after 2000 in the under age 5 group make it difficult to feel confident in the results, and a larger analysis focused in this age group is warranted.

3.4 Discussion

A decrease over time in the *HLA*-mediated risk profile for T1D is well described (Hermann *et al*., 2003; Gillespie *et al.*, 2004; Fourlanos *et al*., 2008), but the data are often derived from different cohorts recruited decades apart. The hypothesis that *HLA* susceptibility is continuing to decrease over time in a unique, continuously recruiting population-based longitudinal T1D cohort was tested in this chapter. There was no evidence for an ongoing decrease within the BOX study, but this result should be interpreted cautiously because there are several limitations to this study.

Firstly, the numbers of cases diagnosed after 2002 were fewer than those diagnosed before. This is because the funding stream for the BOX study changed in 2002. Prior to this date, nurse fieldworkers used to visit families at home to collect venepuncture blood samples for genetic studies. The study continued to recruit after 2002, but all data were collected by questionnaire. In 2015, the study was refunded to collect DNA from families recruited since 2002. This is still ongoing, and, therefore, there were fewer *HLA* results in the later part of the cohort. The longer aim, therefore, would be to repeat this analysis when numbers of samples have increased and include samples collected between 2015 and 2020 which are increasing rapidly. One focus as a result of this study is the collection of samples from children diagnosed with T1D under the age of 5 years as this is likely to be where the greatest environmental pressure will be observed.

As the Bart's Oxford study has been running for longer than any other type 1 diabetes cohort, there are no equivalent recent studies in the literature to compare these data. Interestingly, however, we were able to replicate and expand the comparison between *HLA* class II risk-genotypes in the Golden Years cohort compared with BOX probands from 1985 to 2015 rather

than 1985 to 2002. This strengthens our earlier data as well as studies from our colleagues in the type 1 diabetes research community (Hermann *et al*., 2003; Fourlanos *et al*., 2008).

Recent studies of incidence (Patterson *et al*., 2019; Gartner *et al*., 2020), however, suggest that increasing incidence has plateaued in many Westernised countries -although increases in developing countries continue. It may, therefore, be true that although the BOX sample numbers after 2000 are a limitation, high-risk *HLA* frequencies may be stabilising.

Although the aim of this study is to examine *HLA* frequencies in a single cohort, cross sectional studies are possible. One possible future analysis is to apply for access to the latest GWAS data (Robertson *et al.,* 2021), where data from >22,000 participants with T1D were analysed who had been diagnosed under the age of 15 years between the years of 1995 and 2015. Given the size of this early onset population and the availability of *HLA* (imputed from SNPs) and non-*HLA* SNPs, it may provide an additional source of data. This approach also has potential limitations because while BOX is population-based, individuals with T1D in the GWAS analysis come from different ethnic backgrounds, including European, African and East Asian populations. Whether *HLA* frequencies in T1D are stabilising over time, therefore, remains to be defined but the data analysed here will contribute to a larger future analysis.

As described in the introduction, *HLA* only accounts for half of genetic susceptibility to T1D. In the study outlined in the next chapter, non-*HLA* SNPs associated with T1D will be tested and analysed to determine whether any differences in frequencies can be observed over time in the same samples analysed in this chapter for *HLA*.

Chapter 4

Analysis of non-*HLA* risk in the Golden Years cohort (1922-48) compared with the Bart's Oxford Cohort (1985-2015)

4.1 Introduction

There is agreement among T1D longitudinal studies that the increases observed in incidence are related to the observation that more people with only one *HLA* risk haplotype are developing the condition (Onkamo *et al*., 1999; Green *et al*., 2001; Gale and Gillespie, 2001; Hermann *et al*., 2003; Gillespie *et al*., 2004; Fourlanos *et al*., 2008). Non-*HLA* risk has not been assessed. It is possible that non-*HLA* determinants of risk are increasing over time as *HLA*-mediated risk decreases. In this chapter the hypothesis that non-*HLA* risk is increasing over time in T1D cases will be tested.
The aim of the studies in this chapter, therefore, was to genotype all available samples from the GY and BOX populations for 19 genetic variants robustly associated with T1D in multiple studies as described in sections 1.7.2 and 1.7.3 to determine whether there is any evidence of dynamic change over time.

Most T1D associated SNPs lie close to, or within immune response genes as shown in section 1.7.2 and 1.7.3 in the introduction. Two genes where we hypothesized that SNPs might have changed over the time were:

1. The *IFIH1* gene which is an RNA helicase that activates type I interferon signals in response to viral infection (Kumar *et al*., 2009; von Herrath, 2009; Nejentsev *et al*., 2009; Bentham *et al.*, 2015).
2. The *GLIS3* gene which is expressed in the pancreas and unlike other T1D associated genes, there are increasing data suggesting associations with T2D as well (Wen and Yang, 2017). This may, therefore, be a scenario where increasing BMI over time -which increases risk of T2D- could be having direct effects on T1D risk.

4.2 Methods and data analysis

In this chapter, 19 non-*HLA* SNPs known through GWAS and earlier studies to be associated with type 1 diabetes were analysed as described in chapter 2. Briefly, DNA samples from participants in the Golden Years and BOX cohorts were genotyped. Data were collated and analysed and described as frequencies while comparisons were conducted using chi-square and chi-square for trend as appropriate. As these analyses included multiple comparisons for 19 different variants caution was applied to examine data before deciding whether to proceed with Bonferroni correction as effect sizes would be diminished 19 fold.

4.3 Results

4.3.1 Non-*HLA* SNPs analyses in BOX and GY

Genotyping of 19 non-*HLA* SNPs in BOX probands (n=1992), and GY cases (n=285) under the age of 21 years at diagnosis of T1D was completed as outlined in chapter 2 and the results are shown in tabular form in **Table 4.1 a and b**. and graphically in Appendix **Figure A.1**. Overall, few convincing changes over time were observed between the two cohorts. It is encouraging for two cohorts recruited in different ways over a wide time span – the SNP frequency was, unexpectedly, consistent overall with p>0.05 for all. These data suggest that non-*HLA* SNPs have not changed over time in the way that the *HLA* have. There were some very subtle changes in 10 out of the 19 genes as shown in **Table 4.1 a and b**, but none reached statistical significance even before Bonferroni correction for multiple comparisons.

Taqman Probe	Allele 1/Allele 2	Genotype	No. BOX patients	% BOX patients	No. GY patients	% GY patients
rs11755527 *BACH2*	C/G	C/C	455	26%	55	21%
		C/G	861	49%	143	54%
		G/G	452	26%	67	25%
			1768		265	
rs333 *CCR-5*	WT/Δ32	WT/WT	1387	79%	210	80%
		WT/Δ32	355	20%	47	18%
		Δ32/Δ32	19	1%	4	2%
			1761		261	
rs12935413 *CLEC16A/KIAA0350*	A/G	A/A	168	9%	31	12%
		A/G	767	43%	97	37%
		G/G	839	47%	136	52%
			1774		264	
rs4948088 *COBL*	A/C	A/A	1	0%	0	0%
		A/C	97	5%	17	6%
		C/C	1679	94%	251	94%
			1777		268	
rs3087243 *CTLA-4*	A/G	A/A	301	17%	50	19%
		A/G	822	46%	125	47%
		G/G	652	37%	92	34%
			1775		267	
rs3825932 *CTSH*	C/T	C/C	866	49%	133	51%
		C/T	745	42%	111	43%
		T/T	161	9%	17	7%
			1772		261	
rs2292239 *ERBB3*	G/T = A/G	G/G	652	37%	85	32%
		G/T	826	47%	136	52%
		T/T	290	16%	43	16%
			1768		264	
rs7020673 *GLIS3*	C/G	C/C	379	21%	64	24%
		C/G	868	49%	130	49%
		G/G	530	30%	71	27%
			1777		265	
rs2111485 *IFIH1*	C/T = A/G	C/C	246	14%	28	10%
		C/T	823	46%	126	46%
		T/T	705	40%	117	43%
			1774		271	

Table 4.1a: *SNP genotypes in BOX and GY: The numbers and the percentages of the three genotypes 11, 12, and 22 in BOX and age-matched GY under the age of 21 years. The risk genotypes of the 19 non-HLA SNPs that are associated with T1D are highlighted in purple. The subtle changes in genotypes distribution in 10 out of 19 genes are highlighted in orange (p>0.05).*

Taqman Probe	Allele 1/Allele 2	Genotype	No. BOX patients	% BOX patients	No. GY patients	% GY patients
rs4505848 *IL2*	A/G	A/A	676	38%	93	36%
		A/G	847	48%	126	49%
		G/G	255	14%	37	14%
			1778		256	
rs12251307 *IL2RA*	C/T	C/C	1452	82%	215	81%
		C/T	302	17%	49	19%
		T/T	17	1%	0	0%
			1771		264	
rs3024505 *IL-10*	A/G	A/A	36	2%	5	2%
		A/G	437	25%	65	25%
		G/G	1293	73%	192	73%
			1766		262	
rs917997 *IL18RAP*	A/G = C/T	C/C	1069	60%	156	59%
		C/T	608	34%	92	35%
		T/T	100	6%	17	6%
			1777		265	
rs689 *INS*	A/T	A/A	70	4%	6	2%
		A/T	430	24%	58	22%
		T/T	1270	72%	202	76%
			1770		266	
rs1893217 *PTPN2*	A/G	A/A	1090	61%	171	67%
		A/G	599	34%	75	30%
		G/G	87	5%	8	3%
			1776		254	
rs6679677 *PTPN22*	A/C	A/A	59	3%	13	5%
		A/C	488	27%	75	28%
		C/C	1230	69%	178	67%
			1777		266	
rs2816316 *RGS1*	A/G	A/A	1236	70%	195	74%
		A/G	490	28%	63	24%
		G/G	48	3%	7	3%
			1774		265	
rs3184504 *SH2B3*	C/T = A/G	C/C	374	21%	52	20%
		C/T	866	49%	124	48%
		T/T	530	30%	81	32%
			1770		257	
rs9976767 *UBASH3A*	A/G	A/A	447	26%	80	30%
		A/G	820	48%	135	50%
		G/G	437	26%	53	20%
			1704		268	

Table 4.1b: *SNP genotypes in BOX and GY: The numbers and the percentages of the three genotypes 11, 12, and 22 in BOX and age-matched GY under the age of 21 years. The risk genotypes of the 19 non-HLA SNPs that are associated with T1D are highlighted in purple. The subtle changes in genotypes distribution in 10 out of 19 genes are highlighted in orange (p>0.05).*

The raw data examining the frequency of SNP genotype by age (under 5 years and 10 years) and by decade of diagnosis (1985-1995 and 1995-2005) are shown in the Appendix in **Tables A.2** to **A.7** but these analyses did not provide any further insights or statistical support for changes in trends over time for non-*HLA* SNPs. In Appendix **Figure A.3**, the data in the under 10s by decade of diagnosis is shown but again there is no very convincing and statistically robust evidence of a particular trend over time in children diagnosed early in life. However, candidates for further study in larger numbers are *CCR-5*, *SH2B3*, *RGS1*, *CTLA-4* and *IFIH1*.

4.3.2 Allele frequencies in BOX and GY

Examination of genotype can miss subtle effects on allele frequencies. These were, therefore, examined for the 19 non-*HLA* SNPs of T1D in patients under the age of 21 years in BOX and age-matched GY, and the results are shown in **Table 4.2 a and b** and graphically in Appendix **Figure A.2**. Overall, as observed for genotypes over time, the allele frequencies were remarkably consistent with several small changes, none of which reached statistical significance even before Bonferroni correction for multiple comparisons. Of the 19 SNPs, similar to the genotypic data, *UBASH3A* on chromosome 21 had a 5% increase in the frequency of its risk allele G, from 45% in GY to 50% in BOX.

Taqman Probe	Allele 1/Allele 2	BOX Allele 1/2 frequency	GY Allele 1/2 frequency
rs11755527 *BACH2*	C/G	0.5	0.5
		0.5	0.5
rs333 *CCR-5*	WT/Δ32	0.9	0.9
		0.1	0.1
rs12935413 *CLEC16A/KIAA0350*	A/G	0.3	0.3
		0.7	0.7
rs4948088 *COBL*	A/C	0.0	0.0
		1.0	1.0
rs3087243 *CTLA-4*	A/G	0.4	0.4
		0.6	0.6
rs3825932 *CTSH*	C/T	0.7	0.7
		0.3	0.3
rs2292239 *ERBB3*	G/T = A/G	0.6	0.6
		0.4	0.4
rs7020673 *GLIS3*	C/G	0.5	0.5
		0.5	0.5

Table 4.2a: *Allele frequencies in BOX and GY: Allele frequencies for 19 non-HLA SNPs analysed in BOX and age-matched GY under the age of 21 years.*

Taqman Probe	Allele 1/Allele 2	BOX Allele 1/2 frequency	GY Allele 1/2 frequency
rs2111485 *IFIH1*	C/T = A/G	0.4	0.3
		0.6	0.7
rs4505848 *IL2*	A/G	0.6	0.6
		0.4	0.4
rs12251307 *IL2RA*	C/T	0.9	0.9
		0.1	0.1
rs3024505 *IL-10*	A/G	0.1	0.1
		0.9	0.9
rs917997 *IL18RAP*	A/G = C/T	0.8	0.8
		0.2	0.2
rs689 *INS*	A/T	0.2	0.1
		0.8	0.9
rs1893217 *PTPN2*	A/G	0.8	0.8
		0.2	0.2
rs6679677 *PTPN22*	A/C	0.2	0.2
		0.8	0.8
rs2816316 *RGS1*	A/G	0.8	0.9
		0.2	0.1
rs3184504 *SH2B3*	C/T = A/G	0.5	0.4
		0.5	0.6
rs9976767 *UBASH3A*	A/G	0.5	0.6
		0.5	0.4

Table 4.2b*: Allele frequencies in BOX and GY: Allele frequencies for 19 non-HLA SNPs analysed in BOX and age-matched GY under the age of 21 years.*

4.3.3 Gender in BOX and GY

Distribution of genotypes of 19 non-*HLA* SNPs based on gender in BOX probands and GY who were age-matched under 21 years old is shown in **Figure 4.1** and in tabular form in Appendix **Table A.1 a and b**. Comparisons between males in GY (n=148; 52%), and BOX males (n=1106; 56%) showed subtle differences in 7 of the 19 genes studied, indicating that there are no striking gender-related differences and this was supported by none of these comparisons reaching statistical significance even before Bonferroni correction.

It is worth noting that the gender patterns for *UBASH3A*, where risk appears higher for females in both cohorts but larger cohorts, particularly of GY participants, are required to address this question with confidence.

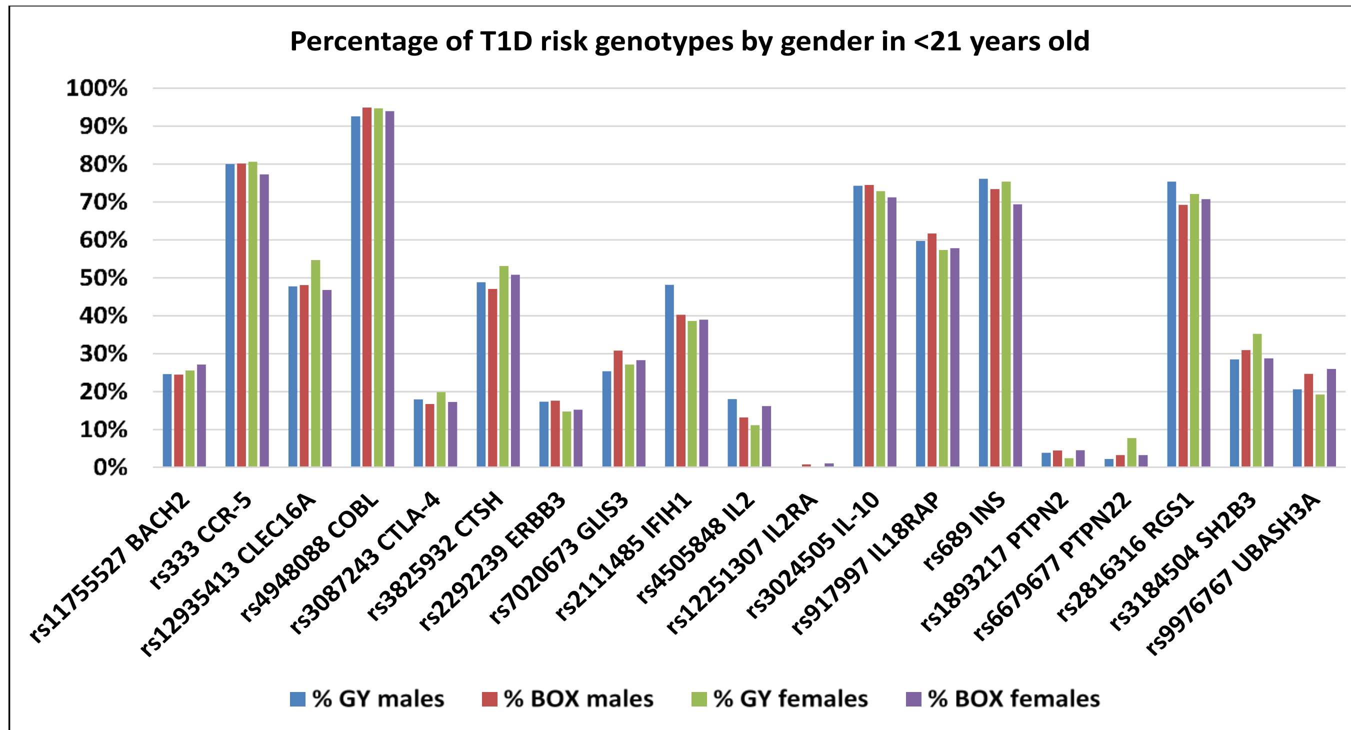

Figure 4.1: *Genotypes by Gender in BOX and GY: The distribution of high-risk genotypes in 19 non-HLA T1D associated genes in individuals diagnosed under the age of 21 years in both cohorts.*

4.4 The power of the study and future recruitment

While the results generated in this study suggest that non-*HLA* SNPs are not changing dramatically over time, the size and, therefore, power of the study was addressed to calculate how many GY participants would be needed to have a fully powered study to address the questions asked in this chapter.

Power calculations were carried out to demonstrate the current power of the study to detect a difference at the significance of 0.05. With the available sample size of BOX (1992) and GY (285) after age-matching for SNP analysis, the study overall achieved 56% power at 0.05 level of significance. Calculations showed that doubling the BOX sample size would modestly increase the power of the study to 59%. On the other hand, doubling the GY sample size would further increase the power to 76% for the study to detect a difference at 0.05 significance.

In order to reach a power of 90% at a significance of 0.05, the study would be required to recruit thousands of participants for both GY and BOX. Recruitment to BOX continues but not to the GY population.

The GY cohort could be expanded because the number of people living with T1D for more than 50 years is growing all the time. Diabetes UK agreed to advertise a new study to target the GY population in the UK if an ethically approved study was established.

As part of this project, a protocol was prepared to submit an IRAS application for ethical approval to collect GY individuals nationally. The study is on hold at the moment pending funding which will be applied for later this year.

4.5 Discussion

This study analysing 19 different variants suggests that the decrease observed in *HLA* susceptibility to T1D over time is not happening or is not happening dramatically for the non-*HLA* SNPs associated with T1D. There are, however, some limitations of the study, the most important being the size of the GY cohort. Efforts will be made in future to increase the number of GY participants. Individuals diagnosed with T1D at the ages 0-21 years from 1960 onwards will be in their early 70s or younger and may be willing to participate. This would also allow us to examine the genetic profile over time of individuals who have survived diabetes for more than 50 years.

As a model for how several of the larger changes observed could have a functional impact if replicated in larger numbers, some of the larger changes -although not statistically proven- were considered. The risk genotype of *UBASH3A* was 20% in the GY and higher at 26% in BOX. The T1D *UBASH3A* susceptibility allele is reportedly associated with reduced expression of *IL2* that is needed by the regulatory T-cells to combat autoimmunity (Ge *et al*., 2017; Todd, 2018). That may suggest that BOX patients have a greater disturbance in their regulatory T-cells function and a higher potential for autoimmunity than the GY patients. Interestingly, in ongoing studies of BOX Slow Progressors to T1D (Long *et al*., 2018; Hanna *et al*., 2020) and (Boldison *et al*. Diabetologia in press) show that individuals with multiple islet autoantibodies who do not develop T1D within the expected time frames have enhanced regulatory T-cell numbers. The BOX cases who do develop diabetes may indeed, therefore, have regulatory dysfunction and this is under follow up in slow vs rapid progressors to T1D.

The *CLEC16A* risk genotype frequency was 52% in GY and lower at 47% in BOX. *CLEC16A* is thought to play a role in regulating β-cell mitochondrial autophagy -known as mitophagy; an essential cellular mechanism that removes defective mitochondria, hence maintaining healthy functioning cells. The T1D risk allele of *CLEC16A* is linked to impaired β-cells secretion of insulin, perhaps by compromising mitophagy (Pearson *et al*., 2018; Gingerich *et al*., 2020). It is difficult to interpret the lower risk frequency in BOX patients, and it may imply that current T1D patients have fewer defective β-cells which is unlikely given the increasing incidence of the condition. Similar to *CLEC16A*, the risk genotypes of both *INS* and *RGS1* were 4% lower in BOX 72% and 70% than in the GY 76% and 74% respectively, in patients diagnosed under the age of 21 years. *RGS1* is associated with B-cell proliferation. One of the suggested mechanisms of *RGS1* risk in T1D is based on NOD mice, where *RGS1*-lacking mice had reduced numbers of follicular helper T-cells that potentiate the antibody production ability of B-cells. Furthermore, follicular helper T-cells were reportedly increased close to T1D onset in humans (Caballero-Franco and Kissler, 2016; Heuts *et al*., 2017; Parkkola *et al*., 2017). As *RGS1* risk is declining in BOX patients, it would be interesting to find out if the repertoire of the follicular helper T-cells is affected. The *INS* risk allele in T1D is associated with a low level of insulin mRNA in the thymus; where high expression is required to educate the immune system to tolerate self-antigens and banish auto-reactive T-cells (Pugliese, 1997; Pugliese, 1998). Low expression allows anti-insulin reactive T lymphocytes to escape thymic filtration, consequently amassing a systemic autoimmune response and risk of *T1D* (Ramos-Lopez *et al*., 2008). The pattern of changes in *CLEC16A, RGS1* and *INS* genetic risk, while not significant, do follow the same pattern as those observed in *HLA*; newer T1D cases had lower genetic risk. Furthermore, *INS* is one of the strongest genetic associations after *HLA* in terms of genetic impact (Bell *et al*., 1984). It is possible that the environment is modulating the risk distribution

of *INS* as it is with *HLA*. *INS* may be particularly important because insulin autoantibodies often appear first in the pathogenesis of T1D, and insulin is considered to be the primary antigen (Knip *et al*., 2005; Parikka *et al*., 2012; Ziegler *et al*., 2012). However, the underlying mechanism of how the environment might impact risk requires examination. One possible mechanism would be through altered methylation of the *INS* gene (Pugliese, 1998; Yang *et al*., 2011).

The two genes hypothesised in the introduction to this chapter *IFIH1* and *GLIS3*, where changes in frequencies over time might relate to changes in viral or metabolic triggers, respectively, over time were not supported by the data. For *IFIH1* the frequency of the risk genotype increased from 40% in the GY cohort to 43% in the BOX cohort while for *GLIS3* the frequency descreased by 3% over time. A future aim will be to test the apriori hypothesis that *IFIH1* risk is increasing over time in a larger analysis over an extended period of time.

Another approach to answering the question addressed in this and the previous chapter would be to use the *HLA* and non-*HLA* data generated to compile a cumulative genetic risk score (GRS) which could be analysed over time. This is an increasingly popular strategy in type 1 diabetes genetics (Ferrat *et al*., 2020). The samples analysed in this study have been re-quantified by the genetics team in the laboratory as some date back to 1998 and will be tested for the latest T1D GRS in collaboration with the exeter team (Ferrat *et al*., 2020). Where possible the data generated in this project will be integrated into this GRS. More broadly, as the GRS become more prevalent in clinical practice, these data may increasingly become available through the National Pediatric Diabetes Audit (National Paediatric Diabetes Audit - NHS Digital).

Chapter 5

Analysis of associations between non-*HLA* SNPs and Islet autoantibodies at diagnosis

5.1 Introduction

During the pre-diabetes phase, islet autoantibodies are detected to GAD, insulin, IA-2 and ZnT8. It is well established that antibodies to GAD are associated with *HLA DR3*, and IAA and IA-2A are associated with *HLA DR4* with no very convincing genetic associations for ZnT8A, although they are negatively associated with *HLA *A24* (Long *et al*., 2013; Ye *et al*., 2015). Recent genome-wide studies showed associations between SNPs in *FCRL3* on chromosome 1 and at the *RELA/FIBP* locus on chromosome 11 with IA-2A and ZnT8A positivity, following the onset of T1D (Brorsson *et al*., 2015). Intriguingly, unlike the SNPs analysed in chapter 3, these SNPs are reported to be associated with islet autoimmunity but not T1D (Brorsson *et al*., 2015). The aim of this study was to investigate:

1. whether islet autoantibodies were associated with *FCRL3* rs3761959, the *RELA/FIBP* locus rs568617 and *LPP* rs1464510 at diagnosis of T1D to determine whether the results observed by Brorsson and colleagues could be replicated.
2. An additional hypothesis generating aim was to explore whether any of the additional 19 SNPs associated with T1D might provide preliminary evidence for novel associations between islet autoantibodies and non-*HLA* genes. Although approximately 600 newly-diagnosed samples is a relatively small cohort, few studies have samples close to diagnosis, including before insulin treatment which allows insulin autoantibodies to be analysed.

5.2 Methods

As outlined in section 2.3.4, univariate analysis was by chi-square testing. Multivariate logistic regression analyses were used to control for confounders and examined the degree of the potential influence of disease characteristics on genetic-autoantibody associations. The variables that were included in the logistic regression model were *HLA* class II genotypes; age at onset of diagnosis with T1D; gender; and *FCRL3*, *RELA*, and *LPP* genotypes. As outlined in chapter 2; section 2.2.2, in this analysis *HLA* genotypes were categorised in a short hand form to make the modelling more efficient. Therefore only the *HLA DQ* allele was used to donate the risk groups as follows: 2/2 = (*DR3-DQ2*)/(*DR3-DQ2*), 2/8= (*DR3-DQ2*)/ (*DR4-DQ8*), 2/X = (*DR3-DQ2*)/X, 8/8 = (*DR4-DQ8)/* (*DR4-DQ8),* 8/X = (*DR4-DQ8*)/X, and X/X where X/X was the reference category with no risk *HLA* haplotypes. Age at-onset was divided into four age-groups coded as 0-4, 5-9, 10-14, and 15-20. Those with age group 0-4 years represented the reference category. Furthermore, males were the reference category for gender comparisons, coded as 1 and females as 2 while for SNPs genotypes 11, 12, and 22, 22 was the reference category for *FCRL3*, *RELA* and *LPP* genotypes comparisons. The logistic regression models of SNPs and autoantibody associations with significance $p < 0.05$ are shown in **Tables 5.1 to 5.2**. These data are an inherent part of the methods and are, therefore, included here. All calculations were performed using SPSS (IBM SPSS Statistics 23.0).

a)

Variables	Significance	Odds ratio	95% C.I.for Odds ratio
***HLA* class II genotype**	0.000		
DR3-DQ2/DR3-DQ2	0.000	0.114	(0.044-0.298)
DR3-DQ2/DR4-DQ8	0.473	0.750	(0.342-1.645)
DR3-DQ2/X	0.028	0.388	(0.167-0.903)
DR4-DQ8/DR4-DQ8	0.115	5.506	(0.661-45.848)
DR4-DQ8/X	0.496	1.354	(0.566-3.242)
Age in years	0.913		
5 to 9	0.929	0.967	(0.462-2.023)
10 to14	0.667	0.860	(0.434-1.707)
15 to 20	0.552	0.779	(0.342-1.774)
Gender (Female)	0.123	1.429	(0.908-2.248)
rs3761959 *FCRL3* genotypes	0.000		
Genotype 11	0.000	3.566	(1.835-6.931)
Genotype 12	0.332	1.300	(0.765-2.209)

b)

Variables	Significance	Odds ratio	95% C.I.for Odds ratio
***HLA* class II genotype**	0.000		
DR3-DQ2/DR3-DQ2	0.003	0.272	(0.116-0.638)
DR3-DQ2/DR4-DQ8	0.382	1.327	(0.704-2.502)
DR3-DQ2/X	0.146	0.593	(0.293-1.200)
DR4-DQ8/DR4-DQ8	0.021	4.721	(1.259-17.705)
DR4-DQ8/X	0.090	1.814	(0.911-3.615)
Age in years	0.910		
5 to 9	0.765	0.907	(0.479-1.718)
10 to14	0.575	0.844	(0.465-1.529)
15 to 20	0.501	0.780	(0.379-1.606)
Gender (Female)	0.228	1.273	(0.860-1.886)
rs3761959 *FCRL3* genotypes	0.000		
Genotype 11	0.000	3.012	(1.731-5.241)
Genotype 12	0.125	1.455	(0.902-2.349)

c)

Variables	Significance	Odds ratio	95% C.I.for Odds ratio
***HLA* class II genotype**	0.552		
DR3-DQ2/DR3-DQ2	0.780	0.882	(0.366-2.126)
DR3-DQ2/DR4-DQ8	0.567	0.826	(0.431-1.586)
DR3-DQ2/X	0.312	1.498	(0.684-3.279)
DR4-DQ8/DR4-DQ8	0.922	0.951	(0.354-2.561)
DR4-DQ8/X	0.783	1.103	(0.550-2.211)
Age in years	0.001		
5 to 9	0.000	3.375	(1.829-6.230)
10 to14	0.018	1.926	(1.120-3.313)
15 to 20	0.016	2.355	(1.171-4.739)
Gender (Female)	0.178	1.309	(0.885-1.935)
rs3761959 *FCRL3* genotypes	0.000		
Genotype 11	0.001	2.642	(1.490-4.682)
Genotype 12	0.835	0.950	(0.590-1.532)

Table 5.1: *The logistic regression models of FCRL3 association with **a)** IA-2, **b)** IA-2β and **c)** ZnT8 autoantibodies: Reference categories are not shown in the model. Model variables are highlighted in yellow: HLA class II genotype (reference category is X/X); age at the onset of T1D (reference category is 0-4 years); gender (reference category is male); FCLR3 genotypes (reference category is genotype 22). A p-value of <0.05 is regarded as significant.*

a)

Variables	Significance	Odds ratio	95% C.I.for Odds ratio
***HLA* class II genotype**	0.211		
DR3-DQ2/DR3-DQ2	0.468	1.616	(0.442-5.907)
DR3-DQ2/DR4-DQ8	0.283	0.642	(0.285-1.442)
DR3-DQ2/X	0.502	1.408	(0.518-3.826)
DR4-DQ8/DR4-DQ8	0.837	0.884	(0.271-2.879)
DR4-DQ8/X	0.333	0.657	(0.281-1.537)
Age in years	0.000		
5 to 9	0.472	1.249	(0.681-2.291)
10 to14	0.000	3.230	(1.742-5.989)
15 to 20	0.002	4.175	(1.719-10.138)
Gender (Female)	0.131	0.703	(0.445-1.111)
rs1464510 *LPP* genotypes	0.057		
Genotype 11	0.017	2.341	(1.166-4.701)
Genotype 12	0.347	1.274	(0.770-2.108)

b)

Variables	Significance	Odds ratio	95% C.I.for Odds ratio
***HLA* class II genotype**	0.000		
DR3-DQ2/DR3-DQ2	0.001	0.239	(0.101-0.566)
DR3-DQ2/DR4-DQ8	0.487	1.255	(0.661-2.384)
DR3-DQ2/X	0.124	0.574	(0.283-1.165)
DR4-DQ8/DR4-DQ8	0.022	4.699	(1.251-17.654)
DR4-DQ8/X	0.060	1.966	(0.971-3.981)
Age in years	0.915		
5 to 9	0.972	0.989	(0.520-1.880)
10 to14	0.622	0.861	(0.474-1.564)
15 to 20	0.649	0.847	(0.413-1.735)
Gender (Female)	0.188	1.305	(0.878-1.939)
rs1464510 *LPP* genotypes	0.008		
Genotype 11	0.003	2.301	(1.316-4.025)
Genotype 12	0.018	1.728	(1.098-2.717)

Table 5.2: *The logistic regression model of LPP association with* ***a)*** *IA-2 and* ***b)*** *IA-2β autoantibodies: Reference categories are not shown in the model. Model variables are highlighted in yellow: HLA class II genotype (reference category is X/X); age at the onset of T1D (reference category is 0-4 years); gender (reference category is male); LPP genotypes (reference category is genotype 22). A p-value of <0.05 is regarded as significant.*

5.3 Results

5.3.1 Newly-diagnosed BOX subjects; the relationship between *FCRL3* genotypes and islet autoantibodies

The possible association of *FCRL3* with islet autoantibodies IA-2A, IA-2βA, ZnT8A, GADA and IAA, was investigated in 552 BOX participants with new T1D onset and DNA available for testing. The data are shown below as graphs and in tabular form in Appendix **Tables A.8-A.10**.

IA-2A

Of 552, 435 (78%) were positive for IA-2A at diagnosis and carriers of the CC or 11 genotype of *FCRL3* (n=151; 89%) were strongly associated with IA-2A at onset ($p<0.001$) as shown in **Figure 5.1**.

IA-2βA

Furthermore, 374 (68%) of BOX patients were positive for IA-2βA. The strongest association ($p<0.001$) with IA-2βA positivity at onset was accounted for by 135 (79%) carriers of the CC or 11 genotype of *FCRL3* as shown in **Figure 5.1**.

ZnT8A

Finally, *FCRL3* was associated with ZnT8A positivity at disease onset in 386 (70%) of 552 newly-diagnosed BOX probands. *FCRL3* genotype CC or 11 was significantly ($p<0.001$) associated with positivity for ZnT8A at clinical onset in 140 (82%) of BOX patients compared to the CT (12) and TT (22) genotypes as shown in **Figure 5.1**.

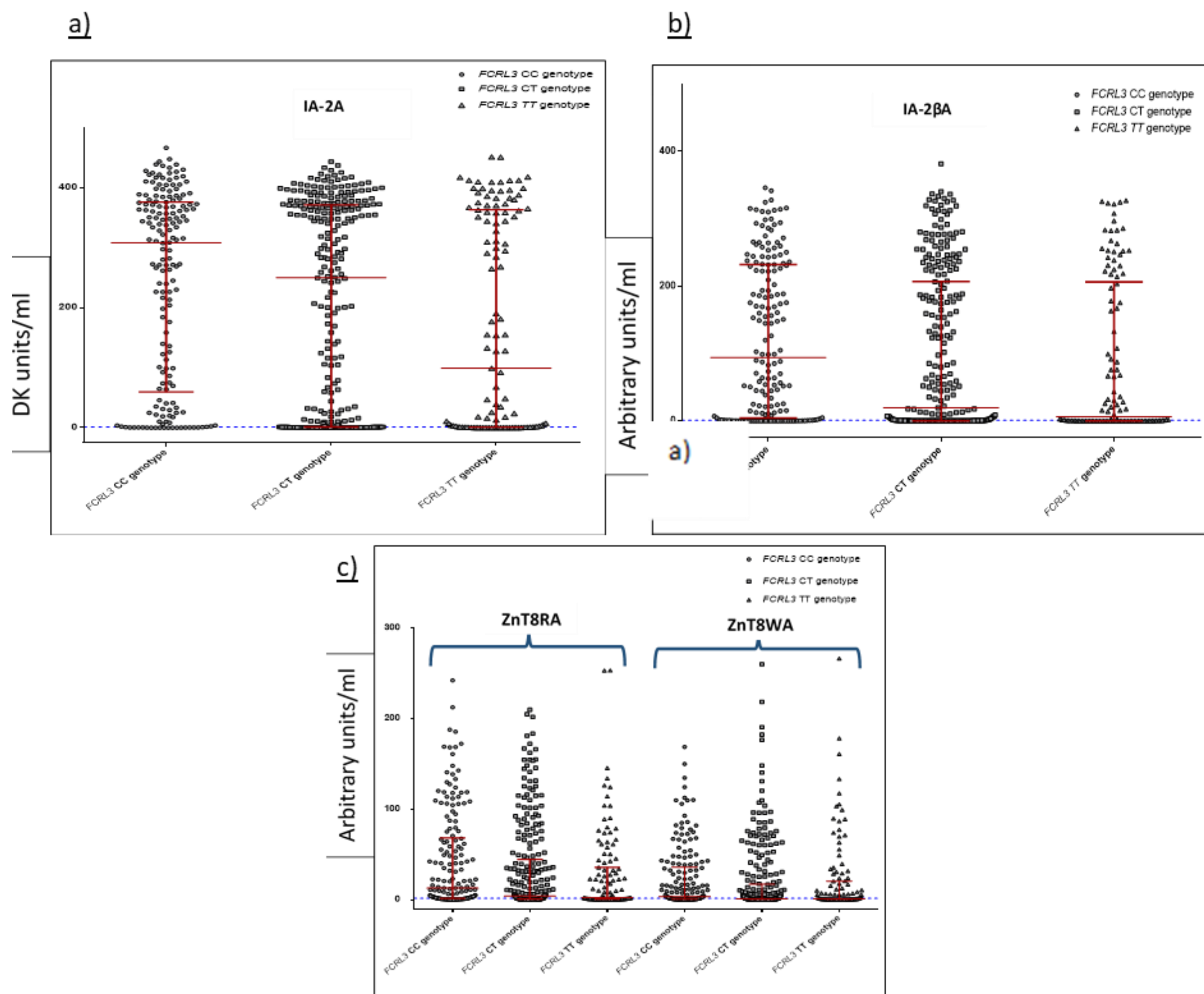

Figure 5.1: *FCRL3 genotypes and islet autoantibodies: a)IA-2A, b) IA-2βA and c) ZnT8RA/ZnT8RWA prevalence according to FCRL3 genotypes in BOX participants at diagnosis of T1D (*p<0,001 CC vs CT/TT). The blue dotted line represents the threshold of autoantibody positivity. The red lines represent the median and interquartile range (IQR).*

Islet autoantibodies IA-2A, IA-2βA and ZnT8A, appeared to be significantly more common among carriers of the CC genotype of *FCRL3* rs3761959 (p<0.001), when compared to the carriers of the genotypes CT and TT as shown in **Figure 5.1** parts **a), b)** and **c)** respectively.

In order to show the relative size of the effects of islet autoantibody associations with *FCLR3*, the odds ratios of association were compared as shown in **Figure 5.2**. The CC genotype odds ratio of association with the risk of IA-2A was 3, approximately 2.5 for the risk of both IA-2βA and ZnT8A, and 1 with the risk of GADA**.** Both TT and CT genotypes had an odds ratio of 1 with GADA, IA-2A, IA-2βA and ZnT8A. No association was found between *FCRL3* and GADA or *FCRL3* and IAA, also shown in **Figure 5.2**.

Furthermore, logistic regression showed that the *FCRL3* CC genotype association with the autoantibodies IA-2A, IA-2βA, and ZnT8A ($p<0.002$ for all) was significantly independent of age at diagnosis; sex; and *HLA* class II genotype as previously explained in section 5.2 and **Table 5.1**.

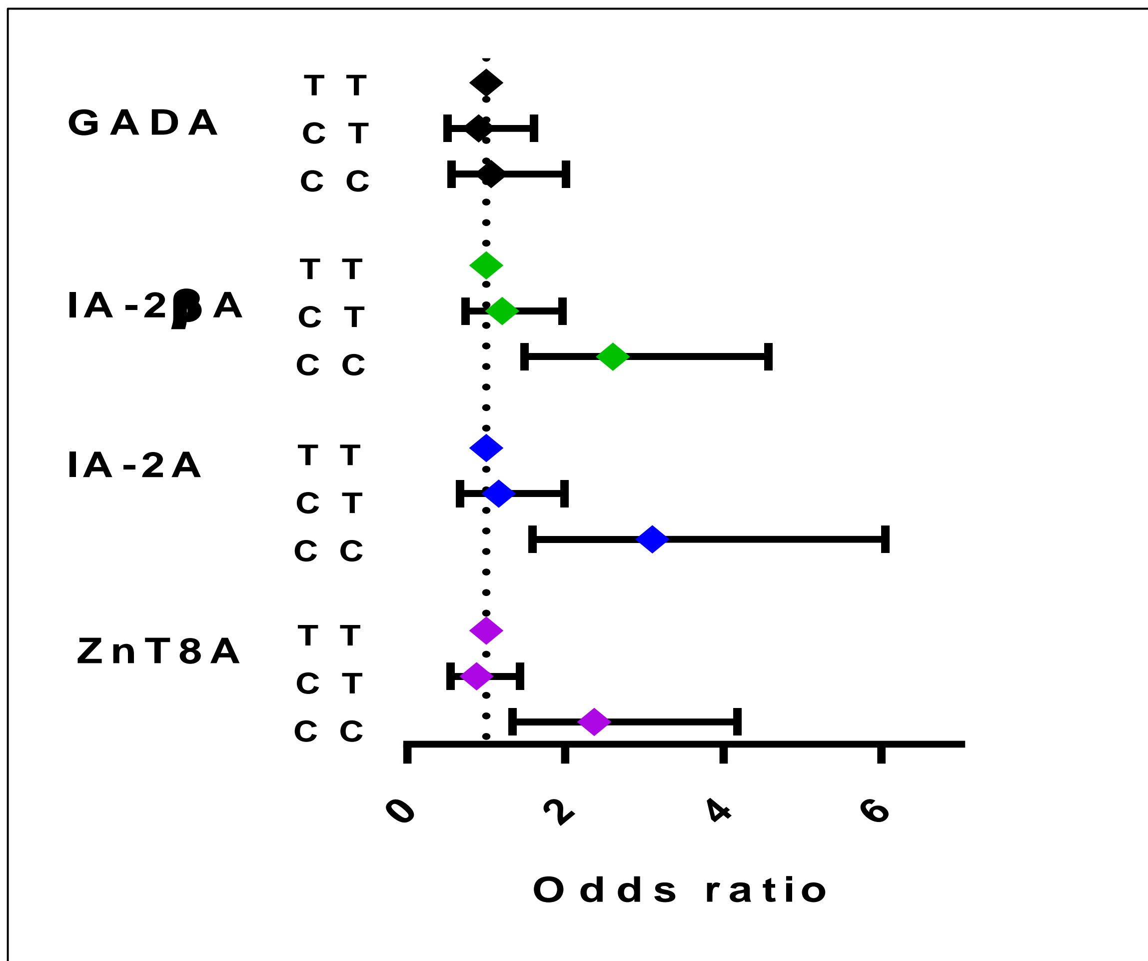

Figure 5.2: *The odds ratios of association between FCLR3 genotypes and islet autoantibodies: The CC genotype of FCLR3 is significantly associated with IA-2A, IA2βA and ZnT8A but not GADA.*

5.3.2 Newly-diagnosed BOX subjects; the relationship between *RELA/FIBP* genotypes and islet autoantibodies

Of 548 newly-diagnosed BOX patients, 436 (80%) were positive for GADA. Testing for an association of *RELA* genotypes with GADA positivity showed that all 20 carriers of the TT genotype had a 100% association with GADA at a borderline significance of p=0.055. Regarding the genotypes CC and CT, 278 (80%) of CC and 138 (77%) of CT carriers were associated with GADA positivity at diagnosis, as shown in **Figure 5.3**, and in tabular form in Appendix **Table A.11**.

No association was found between *RELA* and IA-2A, ZnT8A, or IA-2βA in BOX participants at disease onset.

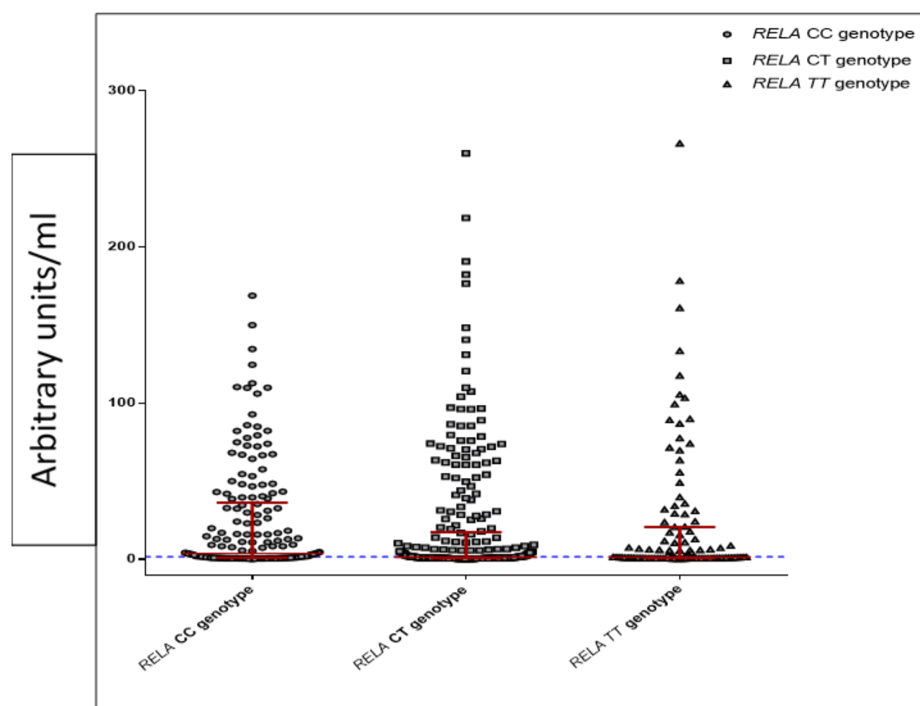

Figure 5.3: *RELA/FIBP genotypes and GADA: GADA prevalence according to RELA/FIBP locus genotypes in BOX participants as diagnosis of T1D (p=0.055 TT vs CT/TT). The blue dotted line represents the threshold of autoantibody positivity. The red lines represent the median and interquartile range (IQR).*

5.3.3 Newly-diagnosed BOX subjects; the relationship between *LPP* genotypes and islet autoantibodies

LPP was associated with GADA positivity in 442 (80%) out of 551 BOX patients at the onset. Of 129 carriers of the GG or 11 genotype, 114 (88%) were positive for GADA (p=0.018) compared to 216 (79%) of GT and 112 (75%) of TT genotypes as shown in **Figure 5.4 a**.

Furthermore, 371 (68%) of 544 newly-diagnosed BOX patients were positive for IA-2βA when tested for association with *LPP*. Around 74% of 128 carriers of the GG or 11 genotype were significantly positive for IA-2βA (p=0.036) compared to 70% of GT and 60% of TT genotypes, as demonstrated in **Figure 5.4 b**. The data on *LPP* genotype frequency and GADA/IA-2βA positivity are shown in tabular form in Appendix **Tables A.12** and **A.13**, respectively.

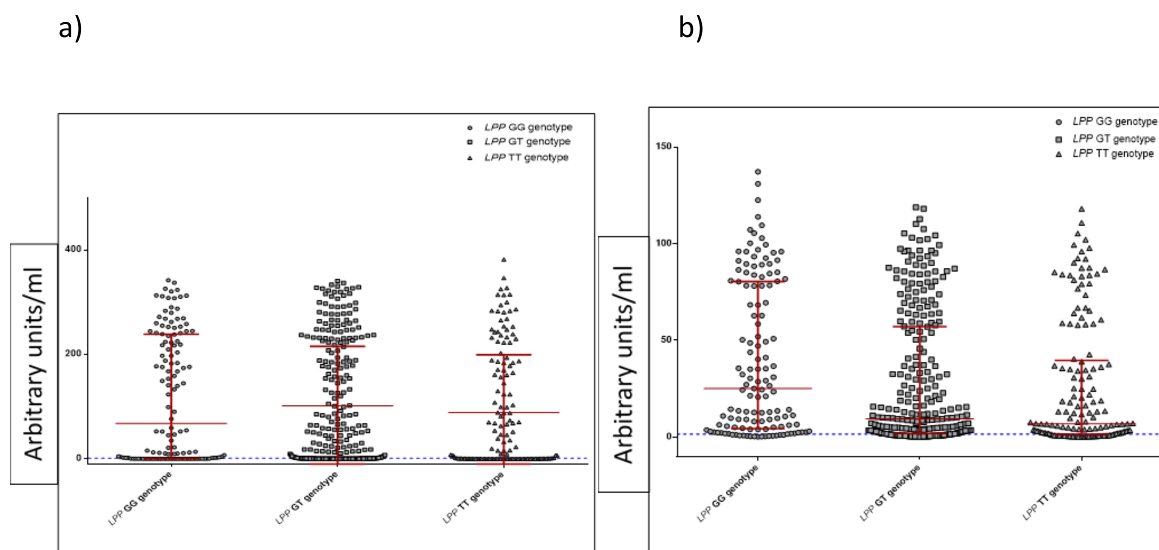

Figure 5.4: *LPP genotypes and islet autoantibodies: a) GADA and b) IA-2βA prevalence according to LPP genotype in BOX participants at diagnosis of T1D (p=0.018 GG vs GT/TT for GADA and p=0.036 GG vs GT/TT for IA-2βA). The blue dotted line represents the threshold of autoantibody positivity. The red lines represent the median and interquartile range (IQR).*

5.3.4 Newly-diagnosed BOX subjects; T1D non-*HLA* SNPs and islet autoantibodies

As a hypothesis-generating aim linking the non-*HLA* SNP data generated in chapter 4 through analysis of a subset of BOX genotypes generated for individuals on whom islet autoantibody data were available at diagnosis of T1D, a different approach was used to examine associations between all 19 T1D non-*HLA* SNPs and GADA, IAA, IA-2A, IA-2βA, and

ZnT8A in 552 newly-diagnosed BOX participants. The T1D high-risk genotypes of 5 of the 19 SNPs showed associations with islet autoantibodies, as shown in **Table 5.3**.

GADA	IA2-βA	IAA
BACH2 (p=0.028)	*CTLA-4* (p=0.023)	*IL2* (p=0.028)
CCR-5 (p=0.029)		*CTLA-4* (p=0.059)
UBASH3A (p=0.055)		

Table 5.3: *T1D non-HLA SNPs and islet autoantibodies: Uncorrected p-values are shown for the T1D associated SNPs associated with islet autoantibodies. BACH2 and CCR-5 are associated with GADA while UBASH3A has a borderline association with GADA. CTLA-4 is associated with IA-2βA and a borderline association with IAA. Finally, IL2 is associated with IAA at the onset of diagnosis in newly-diagnosed BOX patients under the age of 21 years.*

GADA were associated with 3 genes *BACH2*, *CCR-5*, and *UBASH3A* shown in tabular form in Appendix **Tables A.14-A.16**. Newly-diagnosed BOX carriers of the *BACH2* GG risk-genotype were more likely to develop GADA (p_{uncorr}=0.028) as shown in **Figure 5.5**.

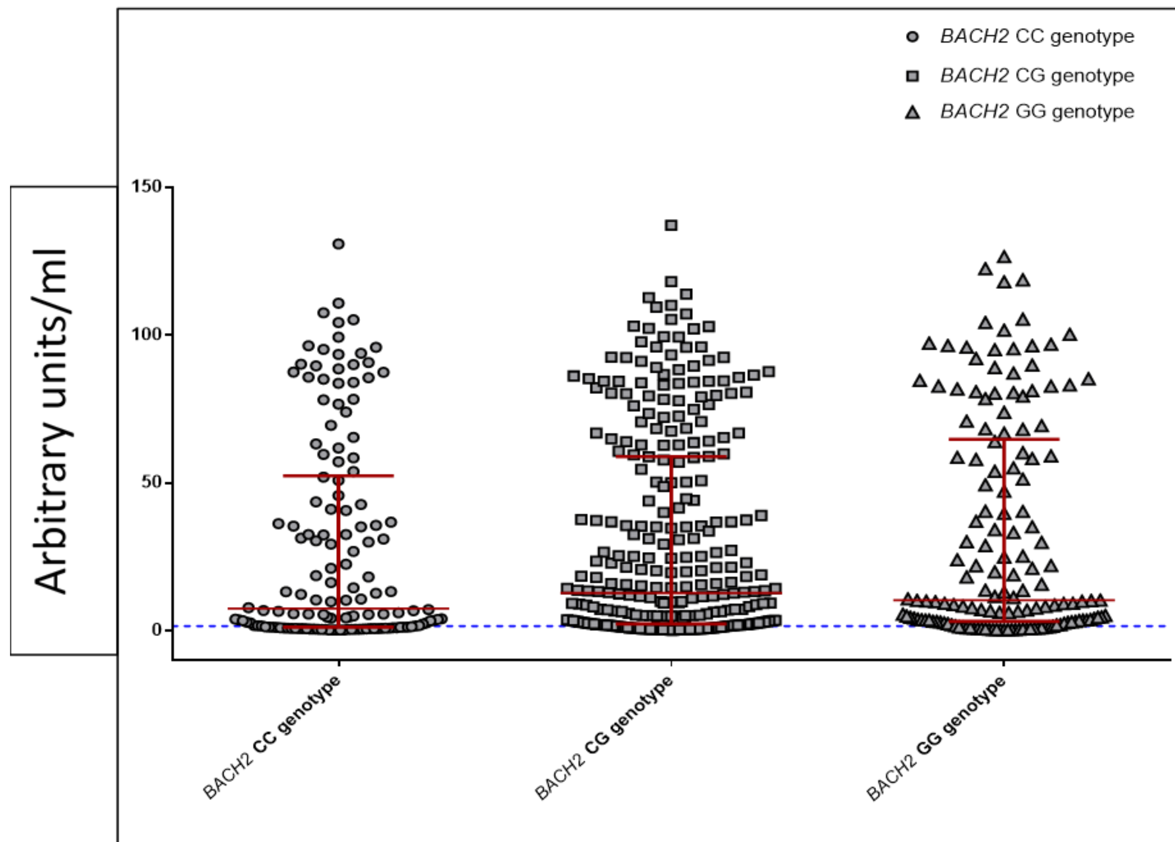

Figure 5.5: *BACH2 genotypes and GADA: GADA prevalence according to BACH2 genotypes in BOX participants at diagnosis of T1D (P uncorr=0.028 GG vs CC/CG). GG is the risk genotype for T1D. The blue dotted line represents the threshold of autoantibody positivity. The red lines represent the median and interquartile range (IQR).*

Furthermore, GADA positivity was linked to *CCR-5* wild type homozygotes risk-genotype (p_{uncorr}=0.029) as shown in **Figure 5.6**. A borderline association (p_{uncorr}= 0.055) between the *UBASH3A* risk genotype GG and GADA positivity was also noticed in BOX probands at disease onset.

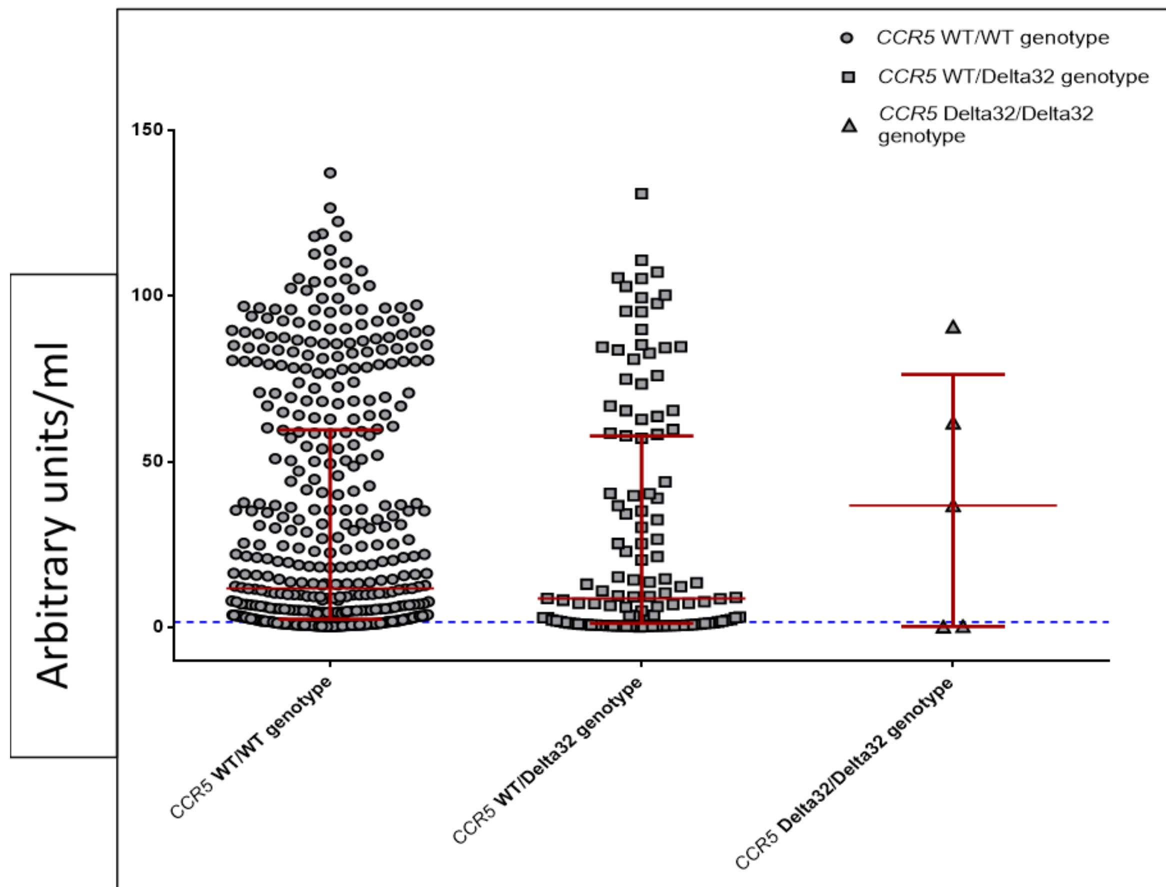

Figure 5.6: *CCR-5 genotypes and GADA: GADA prevalence according to CCR-5 genotypes in BOX participants at diagnosis of T1D (P uncorr=0.029 WT/WT vs WT/delta 32 and delta32 homozygotes). WT/WT is the risk genotype for T1D. The blue dotted line represents the threshold of autoantibody positivity. The red lines represent the median and interquartile range (IQR).*

The AA risk genotype of *CTLA-4* showed a positive association with risk of IA-2βA (p_{uncorr}=0.023) as in **Figure 5.7** and in tabular form Appendix **Table A.17**, while the *CTLA-4* GG genotype had a borderline association with IAA (p_{uncorr}=0.059) shown in tabular form in Appendix **Table A.18**.

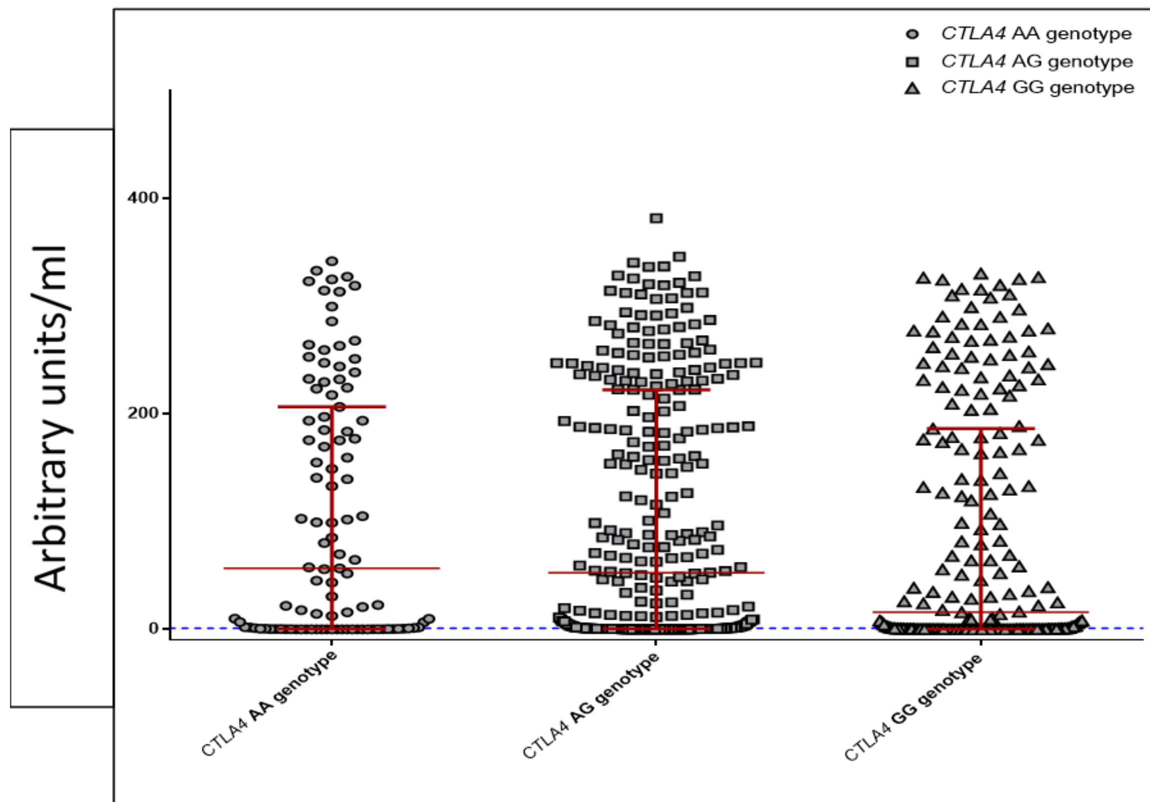

Figure 5.7: *CTLA-4 genotypes and IA-2βA: IA-2βA prevalence according to CTLA-4 genotypes in BOX participants at diagnosis of T1D (Puncorr=0.023 AA vs AG/GG). AA is the risk genotype for T1D. The blue dotted line represents the threshold of autoantibody positivity. The red lines represent the median and interquartile range (IQR).*

Finally, newly-diagnosed BOX carriers of *IL2* risk genotype GG were positively associated with risk of IAA (p_{uncorr}=0.028) as shown in **Figure 5.8** and in tabular form Appendix **Table A.19**.

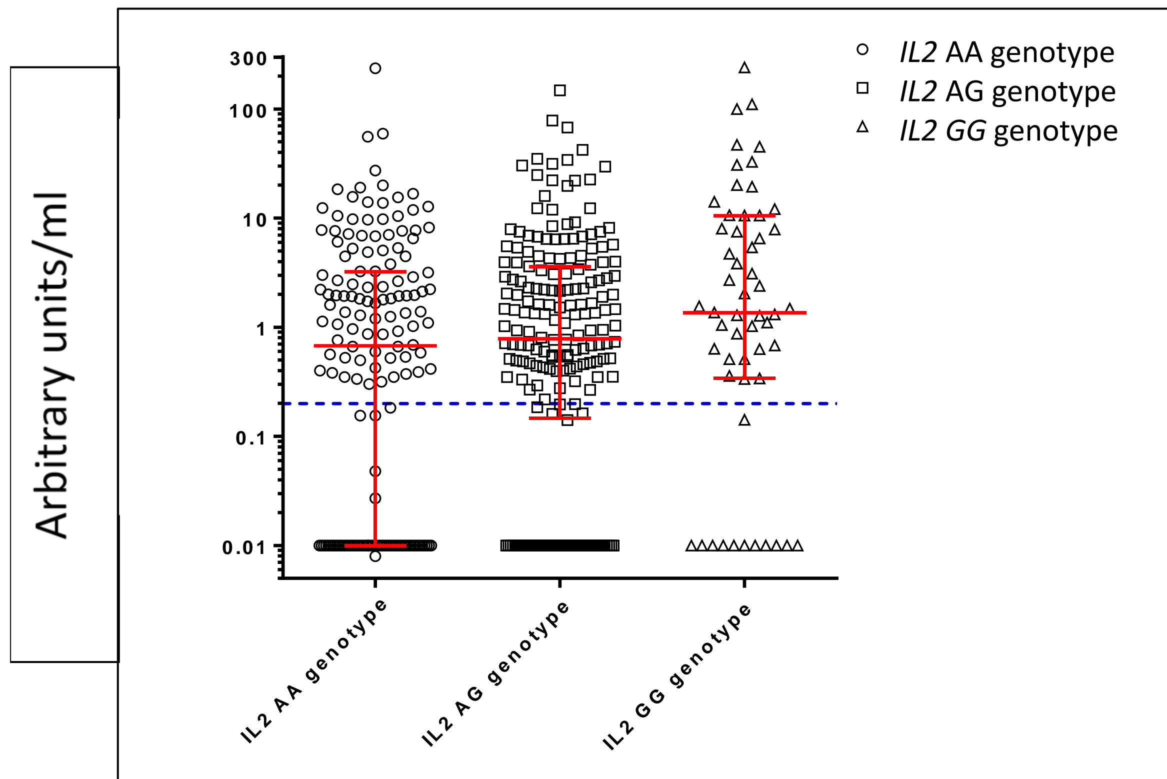

Figure 5.8: *IL2 genotypes and IAA: IAA Prevalence according to IL2 genotypes in BOX participants at diagnosis of T1D (Puncorr=0.028 GG vs AA/AG). GG is the risk genotype for T1D. The blue dotted line represents the threshold of autoantibody positivity. The red lines represent the median and interquartile range (IQR).*

However, the significance will be lost for the observed associations between T1D non-*HLA* SNPs and autoantibodies after correction for multiple analyses by applying Bonferroni correction.

5.4 Discussion

The results described in the first part of this chapter confirm the importance of *FCRL3*, *RELA*, and *LPP* in islet autoimmunity. Although they have not been identified as risk-genes for T1D (Brorsson *et al*., 2015; Howson *et al*., 2012; Rich and Concannon, 2015), they have, however, been associated with other autoimmune diseases including, Graves' disease, coeliac disease, and Crohn's disease (Jostins *et al*., 2012; Almeida *et al*., 2014; Khong *et al*., 2016; Huang *et al*., 2017)**.** Generally, the autoantibodies of immune diseases are associated with disease-causing genes. However, some genes are associated with the autoantibodies of a particular condition without a direct link to the disease itself. An example is *IFIH1*- a T1D risk gene and candidate for possible environmental effects because it is a viral response gene - is associated with gastric parietal cell autoantibodies (PCA) but not with autoimmune gastritis (Brorsson *et al*., 2015; Rich and Concannon, 2015). A possible explanation is that autoimmune diseases share genetic loci, and common biological pathways (Cotsapas *et al*., 2011; Richard-Miceli and Criswell, 2012; Rich and Concannon, 2015;). Furthermore, the genes of autoimmune diseases are linked to T-cells, B-cells, and innate immunity (Cotsapas *et al*., 2011; Rich and Concannon, 2015). *FCRL3* is associated with B-cell function (Kochi *et al*., 2005; Swainson *et al*., 2010; Yang *et al*., 2013; Jin *et al*., 2015) while *RELA* is linked to helper T-cell function (Bettelli *et al*., 2005). Additionally, *LPP* is expressed by the pancreas and the gut and possibly implicated in affecting gut permeability, and predisposition to inflammation (Hunt *et al*., 2008; Kutlu *et al*., 2009; Brorsson *et al*., 2015).

Overall, a key finding in this study is the confirmation and extension of the reported associations (Brorosson *et al*., 2015) between *FCLR3*, *RELA* and *LPP* with islet autoantibodies in well-characterised samples at diagnosis of diabetes. These data add to the understudied

concept that the genetic determinants of the humoral immune response can be separated from associations with clinical onset. This leads to the possibility that the same may be true for T-cell responses but there are no published data to support this, perhaps because T-cell responses are labour intensive and not usually analysed in sufficient numbers for robust genetic analysis.

There have been no follow up publications on *FCLR3*/*RELA*/*LPP* associations with islet autoimmunity since 2015 and the data generated here will, therefore, make an important addition to the literature on this subject when published (manuscript in preparation).

There may be an association between antibody level and the SNPs tested as well as antibody prevalence. This is noted particularly for *RELA/FIBP* genotypes and GADA as shown in **Figure 5.3** and for *FCRL3* genotypes and ZnT8RA as shown in **Figure 5.1** but this is less obvious for ZnT8WA which makes the observation difficult to explain. This will be looked at in more detail in future studies. The reason for caution is that level and severity of autoimmunity do not always correlate for islet autoantibodies.

In the hypothesis-generating approach to examine all 19 non-*HLA* SNPs associated with T1D, preliminary evidence was found associating *BACH2*, *CCR-5,* and *UBASH3A* with GADA. *CTLA-4* was associated with IA-2βA and IAA while *IL2* was associated with IAA. These are interesting data which make biological sense, but a more statistically robust study is required with larger numbers of individuals with islet autoantibody data at diagnosis. For instance the potential association between *UBASH3A* and GADA is noteworthy for the Bristol Diabetes Group where

they have previously reported high levels of GADA in children with Down Syndrome and diabetes (Gillespie *et al.*, 2006; Gillespie *et al.*, 2013). *UBASH3A* is on chromosome 21, it is, therefore, possible that three copies of the risk variant in children with Down Syndrome may be linked to increased GADA. This hypothesis is now under investigation by another PhD student.

The preliminary association between *CTLA-4* and IA-2βA will be followed up in children at risk of T1D because the presence of autoantibodies to the β isoform of IA-2A is associated with rapid progression to diabetes (Achenbach *et al*., 2008). A recent review by Shapiro and colleagues delved into the effects of several T1D associated genes including *UBASH3A* and *CTLA-4* (Shapiro *et al*., 2021) examining the contribution of each variant on risk and progression in both humans and in mouse models of type 1 diabetes where functional effects are easier to determine.

The *IL2* association with IAA is potentially of particular importance as a low dose IL2 clinical trial is ongoing currently in the UK (ITAD – T1DUK (type1diabetesresearch.org.uk)) with an ultimate focus on prevention in young children in whom IAA is the most common islet autoantibody (Zeigler *et al*., 2013).

There is, therefore, a major drive to collect "at-diagnosis" sera from BOX study participants at clinical presentation and before insulin treatment to follow up the associations between *CTLA4*, *UBASH3A* and *IL2* described in this chapter.

Chapter 6

General Discussion

6.1 *HLA* associations with T1D over time

The overall aim of this project was to study the dynamics of genetic susceptibility to T1D over the last 100 years. This project revisited an area where there had been intense scrutiny in the first decade of the twenty-first century – the changing dynamics of *HLA*-mediated susceptibility over time in individuals with T1D. The central hypothesis in chapter 3 tested whether a decrease in the highest-risk genotypes could be observed in a population-based study recruiting since 1985. The results failed to provide convincing evidence that this trend is continuing, but there are some limitations to the study, which reduce confidence in the results and support additional analysis. More than twice as many samples were available before 2000 compared with after that date. This is because of a funding pause in 2002 for sample collection for the BOX study, although in 2015, funding was provided to collect samples retrospectively. Unfortunately, the relatively low number of recent samples collected makes the results difficult to interpret when subdivided into 5-year bands. Since completion of this project, an additional 297 samples from cases have been collected and are undergoing DNA extraction, and *HLA* genotyping and an updated analysis will hopefully answer the question addressed in chapter 3. It is notable that no other longitudinal studies have followed up the changing dynamics of genetic susceptibility over time making comparisons with published studies difficult. The dearth of published data in this area, even from cross-sectional studies, might suggest that other research groups are also observing unclear trends.

Increased numbers in the BOX study did confirm the change over time between the current population and the GY cohort. This leads to questions regarding the environmental pressure that could cause the highest-risk haplotype to decrease in frequency within a few decades.

One interpretation may be that the GY cohort is different because it is a population of survivors, but the trend over time has been observed in many other populations (Hermann *et al*., 2003; Fourlanos *et al*., 2008; Vehik *et al*., 2008; Steck *et al*., 2011). Those with one single risk haplotype are now the majority among individuals in the general population with T1D. That may result in different islet autoantibody patterns as *HLA DR4-DQ8* is known to be associated with IAA and IA-2A, both associated with more rapid progression than GADA which is associated with *HLA DR3-DQ2* (Eisenbarth *et al*., 1992; Knip *et al*., 2002; Achenbach *et al*., 2008; Giannopoulou *et al*., 2015; Regnell and Lernmark, 2017).

There is increasing evidence that there may be different environmental drivers for GADA autoimmunity compared with insulin and IA-2 autoimmunity. In the TEDDY study where high-risk children have been studied from birth, they have shown that insulin autoimmunity appears first in some children and GADA first in others (Krischer *et al*., 2019). While both often appear in the first two years of life, insulin autoimmunity occurs earlier, focusing attention on early environmental determinants. One example is that children born by caesarean section are at increased risk (Bonifacio *et al*., 2011) and this is thought to be caused by resulting differences in gut microbiome development and longitudinal studies are ongoing (Stewart *et al*., 2018).

An important area which has not been examined over time is the frequency of *HLA* class I genes. As there are clear patterns of linkage disequilibrium between *HLA* class I and II haplotypes, one might expect that there will be differences and this is a potential future direction for this study.

HLA class I A, B, and C genetic loci have also been implicated in the pathogenesis of T1D (Fennessy *et al*., 1994; Honeyman *et al*., 1995; Nakanishi *et al*., 1999; Noble *et al*., 2002; Robles *et al*., 2002). HLA class I proteins help present self-antigens to cytotoxic $CD8^+$ T lymphocytes, and self-reactive cells would be deleted, suggesting a role in educating T-cells as part of self-tolerance induction. Therefore, disruption of *HLA* class I function could trigger an autoimmune reaction. Studies showed that *HLA* class I alleles could also affect progression to T1D independently from *HLA* class II (Noble *et al*., 2002; Tait *et al*., 2003; Valdes *et al*., 2005; Nejentsev *et al*., 2007; Howson *et al*., 2009). Interestingly, Fennessy *et al*. (1994) found that most of the Finnish T1D children without class II high-risk genotype *DR3/DR4* had a higher frequency of *HLA-A* genes compared to controls (Fennessy *et al*., 1994). Among relatives of T1D patients with high-risk *HLA* class II haplotypes, DAISY reported that the majority of siblings of T1D children (n=693) who carried class I *HLA-A* seroconverted by the age of 2-3 years. Those with antibody positivity had a higher percentage of *HLA-A* than autoantibody negative controls suggesting that *HLA* class I genes potentially accelerate islet autoantibody appearance and persistence (Robles *et al*., 2002). In animal studies, a transgenic model of NOD mice carriers of human class I *HLA-A2* progressed rapidly to T1D in comparison with non-transgenic mice (Yoon *et al*., 1998; Robles *et al*., 2002; DiLorenzo and Serreze, 2005). Collectively, these studies point out the significance of *HLA* class I in driving the autoimmune process that predisposes to T1D. Given the complexity of the MHC region, further research is required to stratify disease susceptibility of class I molecules independently from the impact of *HLA* class II and to examine effects over time.

6.2 Non-*HLA* risk over time

It is well recognised that *HLA* have the main genetic impact in T1D. However, studies uncovered more than 60 genes that are linked to T1D on a genome-wide scale (Bakay *et al*., 2013). Although their effects are less than the *HLA*, they are of importance as T1D is polygenic; hence, full details of the genetic complexity of T1D are required to better understand the pathogenesis. Additionally, it is not unrealistic to assume that a diabetogenic environment would affect T1D associated genes- *HLA* and *non-HLA*- alike. Therefore, in addition to *HLA*, the study analysed 19 non-*HLA* genetic regions associated with risk of T1D development (Bakay *et al*., 2013).

SNP analyses between the two age-matched cohorts did not provide robust evidence for major changes in the risk patterns of some SNPs genotypes over time. Indeed one might have predicted that a UK-wide population of survivors with diabetes diagnosed between 1922 and 1948 and a population of all cases from a localised region might have resulted in genotypic differences that were not necessarily related to dynamic changes over time. The very consistent SNP genotype frequencies observed -even in a study with limited power- suggest that non-*HLA* are not changing in response to environmental influences. As *HLA* class II are the "dinner plate" which serve the antigen to the immune system, the results of this study can cautiously be interpreted as indicating that immune changes affecting antigen presentation are driving the reported changes over time.

Regarding gender comparisons of the genetic distribution of the non-*HLA* genes between GY and BOX, some changes were noticed in males, others in females, and some were observed in both. In males, the biggest change was in *IFIH1* as its high-risk genotype was lower in BOX males 40% than GY males 48%. *IFIH1* plays a role in inducing an antiviral immune response, serving as a potential link in the genetic and environmental interaction. The *IFIH1* T1D high-risk allele was suggested to trigger an exaggerated antiviral response that consequently propels an autoimmune reaction. Recently, *IFIH1* was linked to seasonal variation of T1D, where the carriers of the high-risk alleles were more likely diagnosed in summer (Dou *et al*., 2017; Jermendy *et al*., 2018). Replication studies would be required to help understand such observations.

6.3 Possible environmental determinants of type 1 diabetes

The possibility of changes to the gut microbiome was raised in section 6.1. There are around a thousand species of bacteria living in our gastrointestinal tract, existing in an ecosystem that is influenced by numerous factors such as mode of delivery of the newborn, diet, water pH, medications, and geographical location (Zheng *et al*., 2018). The composition of these bacteria is thought to play a vital role in the development of the newborn's immune system (Heavey, 1999; Bjorksten *et al*., 2001; Clarke *et al*., 2010). A newborn's gut is sterile at birth, and shortly afterwards, bacterial colonisation happens, mainly by flora acquired from the mother (Biasucci *et al*., 2008). It was reported that newborns delivered vaginally had a gut microbiome related to their mothers' vaginal commensals, while the gut microbiome of newborns born by cesarean section was similar to commensals on their mothers' skin (Dominguez-Bello *et al*., 2010). Because T1D is a disease with immunological dysregulation,

studies looked into intestinal microflora as a possible contributor (Alkanani *et al*., 2015). The TEDDY study was established to investigate the environmental factors and risk of progression to T1D. The study screened newborns recruited from the general population and those with a family history of T1D who had high T1D genetic risk. Those children were followed up from birth until the age of 15 years (Group 2008). TEDDY reported regional variations in terms of bacterial composition and diversity. Less diverse microflora was found in Colorado and Finland, while Sweden, a neighbouring country of Finland, showed a microbiome composition closer to Washington (Kemppainen *et al*., 2015). Recently, a metagenomes study by TEDDY reported that bacteria that produce short-chain fatty acids, such as butyrate, could protect against autoimmunity (Vatanen *et al*., 2018). The dynamics of the gut microbiome and its possible role in promoting autoimmunity is a complex area that requires more research.

Rate of caesarean section is just one factor that has changed over time, and it is likely that many different environmental determinants have also altered. Apart from the gut microbiome, another major focus is epigenetic changes to the genome in response to environment.

Epigenetics is also commonly suggested as a mechanism whereby the environment can act directly on gene function. The term was coined in 1942 by Conrad Waddington as the ''the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being.' (Waddington, 1968; Waddington, 2012). Epigenetics comprises the study of all the changes that modify a genetic expression; hence the function of a gene, on a molecular level without affecting the gene sequence. While some epigenetic changes are heritable and can contribute to gene regulation (Bird, 2007; Ledford, 2008;

Berger *et al*., 2009; Allis and Jenuwein 2016), it is clear that in the early embryonic period, the genome undergoes demethylation and a brand-new re-methylation occurs which is modulated by environmental factors (Groom *et al*., 2011). Examples of epigenetic mechanisms include DNA methylation, where a covalent methyl group is added to the DNA structure, thereby affecting the silencing of a gene. Another mechanism is histone modification which is a vital component of the cell's chromatin that result in alterations in gene expression, and finally are the short non-coding RNA transcripts (ncRNAs) that influence gene expression (Li, 2002; Petronis, 2010; Cao, 2014; Santosh *et al*., 2015). In T1D, a genome-wide analysis of DNA-methylation data from pancreatic islets (n= 89 donors) showed that DNA-methylation was associated with particular T1D genotypes for *HLA* and *INS.* Methylation quantitative trait loci (mQTL) were significantly enriched in the *HLA* region (Olsson *et al*., 2014). Additionally, risk of T1D was influenced by an increase in DNA-methylation reducing *INS* gene expression in humans (Pugliese, 1998; Yang *et al*., 2011) and animal models (Rui *et al*., 2016). Until recently, methylation studies have been carried out after T1D onset, making interpretation of data difficult because hyperglycaemia may influence methylation. The DAISY study examined peripheral blood samples taken from early life to onset of disease from 87 T1D patients and matching-controls. They detected differences in methylation patterns that remained comparable from birth until after seroconversion before progressing to T1D (Johnson *et al*., 2020). A recent study showed an association between DNA methylation and rs689 in the insulin gene before the onset of T1D, when islet autoantibodies are present, suggesting methylation may have a role in the relationship between INS variation and T1D development. (Carry *et al*., 2020).

One future study extending the work carried out in this thesis is to examine genome-wide methylation status in GY and BOX participants. In a first step a colleague examined samples from the Avon Longitudinal Study of Parents and Children (ALSPAC) study (Ye *et al*., 2018) and provided evidence that at 5 loci, *ITGB3BP, AFF3, PTPN2, CTSH and CTLA4*, DNA methylation is potentially mediating the genetic risk of T1D mainly by influencing local gene expression. These data were replicated in the BOX cohort (Ye *et al*., 2018) and these loci are, therefore, a particular focus for future studies.

6.4 Islet autoantibody associations with SNPs

Specific genotypes, in particular *HLA* genotypes, increase the likelihood of the appearance of certain autoantibodies. As described in section 6.1, *HLA DR3* appears to be associated with GADA, while IAA and IA-2A are found in increased frequency in carriers of *HLA DR4* (Krischer *et al*., 2017; Regnell and Lernmark, 2017). Brorsson *et al*. (2015) reported that genes other than *HLA* possibly influence the pattern of islet autoantibody appearance in T1D patients (Brorsson *et al*., 2015), as previously suggested by (Plagnol *et al*., 2011; Howson *et al*., 2012) in genome-wide studies. Interestingly, three non-*HLA* SNPs *FCRL3*, *RELA*, and *LPP* were shown to be associated with T1D autoantibodies, but not with T1D. Following up on that observation, *FCRL3*, *RELA*, and *LPP* were analysed in BOX for possible association with islet autoantibodies. The Brorsson study was replicated in that *FCRL3* was shown to have a significant association with IA-2A, IA-2βA, and ZnT8A in newly-diagnosed BOX patients ($p<0.001$) close to disease onset. On the other hand, *FCRL3* had no association with GADA. Furthermore, age of onset, gender, and *HLA* class II did not affect this association when logistic regression was applied ($p<0.002$). It is worth noting that IA-2 and IA-2β autoantibodies are associated with

accelerated progression to clinical onset of T1D (Achenbach *et al*., 2008). Additionally, the FCRL3 protein is expressed on B lymphocytes (Swainson *et al*., 2010), which ultimately differentiate into antibody-producing Plasma cells. Immunological tolerance of B-cells is thought to be compromised by *FCRL3,* perhaps by blocking B-cell receptor signalling, prompting autoantibody production (Kochi *et al*., 2005; Kochi *et al*., 2009). In autoimmune diseases, patients with risk genotypes for *FCRL3* have reportedly inflated numbers of autoantibodies (Kochi *et al*., 2005; Umemura *et al*., 2006). Although *FCRL3* is not a risk gene for T1D per se, but for other autoimmune diseases, and it is possible to assume *FCRL3* association with IA-2A and IA-2βA could act as a predictor of an aggressive autoimmune response that speeds up the onset of T1D.

The genetic region *RELA*/*FIBP* was associated with GADA in recently diagnosed BOX participants. All patients harbouring the genotype TT of *FIBP* SNP rs568617 had GADA at disease onset, and the association was marginally significant. The locus is reportedly associated with the positivity for IA-2A by (Brorsson *et al*., 2015); however, this was not replicated in BOX. Furthermore, Brorsson *et al*. (2015) did not find an association with GADA at diagnosis either. As the number of BOX patients with the TT genotype showing GADA positivity was limited to 20, it is possible that a larger sample size would show stronger association with GADA, and perhaps uncover an association with IA-2A as previously reported (Brorsson *et al*., 2015). However, it should be noted that the median duration of diabetes at sampling was 7 years in the Brorsson study, compared with only 1 day in BOX and this could have influenced the results. In the literature, the *RELA*/*FIBP* region is associated with risk of

inflammatory bowel disease (Jostins *et al*., 2012; Liu *et al*., 2015; Ellinghaus *et al*., 2016). However, knowledge about the locus's association with autoimmunity in T1D is scarce.

Another gene associated with GADA is *LPP*. The Brorsson study also reported that the association in individuals with T1D was apparent in cases three years after the onset of T1D while our data showed the association was much earlier in BOX, at disease onset (Brorsson *et al*., 2015).

LPP is associated with autoimmune thyroid disease (Cooper *et al*., 2012), vitiligo (Jin *et al*., 2010), and coeliac disease, and it is strongly expressed in the gut (Hunt *et al*., 2008; Lettre and Rioux, 2008; Izzo *et al*., 2011). Although the pancreas expresses *LPP* (Kutlu *et al*., 2009), its function appears to be linked to islet autoantibodies rather than T1D itself. This study also showed an association of *LPP* with IA-2βA in newly-diagnosed BOX at clinical onset (p=0.036). While our data suggest that *LPP* could be linked to an aggressive immune response and acceleration to T1D onset as suggested by the association with IA-2βA (Achenbach *et al*., 2008), although these antibodies were not measured in the Brorsson study. Instead, their finding of delayed *LPP* and GADA association years after diagnosis, implied a link to persistence of the immune response. While there is an accumulating evidence for a role of the gut microbiome and intestinal permeability in T1D (Bosi *et al*., 2006; Brown *et al*., 2011; Maffeis *et al*., 2016), and a shared autoimmune basis with coeliac disease (Smyth *et al*., 2008; Almeida *et al*., 2014; Huang *et al*., 2017), that is insufficient to clarify the function of *LPP* in T1D autoimmunity. This study adds novel data to the intriguing associations between genes and islet autoimmunity which are not associated with clinical T1D.

In a hypothesis-generating approach, 19 additional T1D risk genes were interrogated for possible associations with autoantibodies in newly-diagnosed BOX patients. GADA, IA-2βA, or IAA had associations with four of the genes *BACH2*, *CCR-5*, *CTLA-4*, and *IL2* at clinical onset. Uncorrected p-values are shown because statistical significance would be lost after correcting for 19 analyses for each of the islet autoantibodies if the conservative Bonferroni test had been applied. The small sample size and the rarity of GWAS in studies of islet autoimmunity drive the need for robust replication studies in larger populations.

6.5 Strengths and weaknesses

As indicated in the sections above, the studies described in this thesis have some weaknesses. The two cohorts are different in terms of survival. The GY cohort is comprised of patients who were diagnosed with T1D and survived its complications for more than 50 years. BOX, on the other hand, is a prospective family study, ongoing for more than 35 years where none of the participants has achieved 50 years of T1D survival yet. Indeed, it would be interesting to compare the two cohorts when BOX eventually has a group of survivors after another 15 years. Furthermore, BOX started in the 1980s as a regional study in the Oxfordshire region. Although it has expanded since then, a more representative cohort of the general UK population would be ideal for comparing with the GY population.

Another key factor is the power of the genetic comparison between the GY and BOX populations. The GY population is underpowered but is the only currently available resource. The low number of the GY participants (n=376), where only 285 have genetic data after age-matching for those diagnosed under 21 years, is a major contributor in lowering the power.

The current statistical power of the study at p=0.05 is 56%. Doubling the number of BOX participants would increase the power slightly to 59% at p=0.05 while doubling the number of the GY would increase the power to 76% in order to detect a difference at a significance of 0.05. Generally, in genetics, the power needs to be 90% at 0.05 significance, so the current study would require a sample size of thousands to be recruited in GY and BOX. BOX recruitment is set to continue, and Diabetes UK data indicate the number of individuals obtaining the Nabarro medal after surviving T1D for 50 years is increasing annually. An ethics application was, therefore, prepared, including the protocol and IRAS documents for the Genetics of Longstanding Diabetes "GoLD study" to allow recruitment of an expanded GY population and analysis by the cumulative GRS. This study will be part of a planned grant application to Diabetes UK.

In terms of genetic change over time, dynamic changes in *HLA* genetic risk are too rapid to happen on their own without external influence. Studies long suggested the potential effect of the environment in forcing genetic change (Hermann *et al*., 2003; Gillespie *et al*., 2004; Fourlanos *et al*., 2008; Vehik *et al*., 2008; Steck *et al*., 2011). However, it is not yet understood how and when this environmental pressure is exerted. Although a number of environmental influencers were of interest, such as viruses, diet, geography, and others, no direct causal link has been found to cause T1D (Dahlquist *et al*., 1990; Virtanen *et al*., 1998; Pani *et al*., 2000; Virtanen *et al*., 2000; Hummel *et al*., 2002; Pastore *et al*., 2003; Nejentsev *et al*., 2004; Hara *et al*., 2013; Rodriguez-Calvo *et al*., 2016). The change in *HLA* trend is a clue that the environment is modifying the genetic risk of T1D, perhaps by increasing the penetrance of lower-risk genotypes in recently diagnosed patients.

The data generated in the individual chapters in this thesis are not always straightforward and **Figure 6.1** shows where data have been linked and explains why if this has not happened. In particular, the original plan for this thesis was to generate a Genetic Risk Score (GRS) from the *HLA* and non-*HLA* data analysed in this study. However, it became increasingly clear that multiple T1D genetic risk scores have been created and they are severely limited in that they all incorporate different combinations of SNPs, few have *HLA* data as in the current study, and do not allow data to be compared. Through collaboration in the UK Type 1 Diabetes consortium it was agreed that T1D populations in the UK would all use the well-established Exeter GRS (Ferrat *et al.*, 2020) which is now also used clinically by the NHS. BOX DNA samples have been sent to Exeter for this analysis. It is also planned to analyse the GY DNA samples in the same way. The only possible disadvantage of using a GRS is that strong effects in individual genes may be missed. However, this study suggests that changes in variation frequency over time are largely restricted to the *HLA* region. When the data from Exeter are complete, a comparison of GRS including *HLA* variants vs a GRS alone will be carried out.

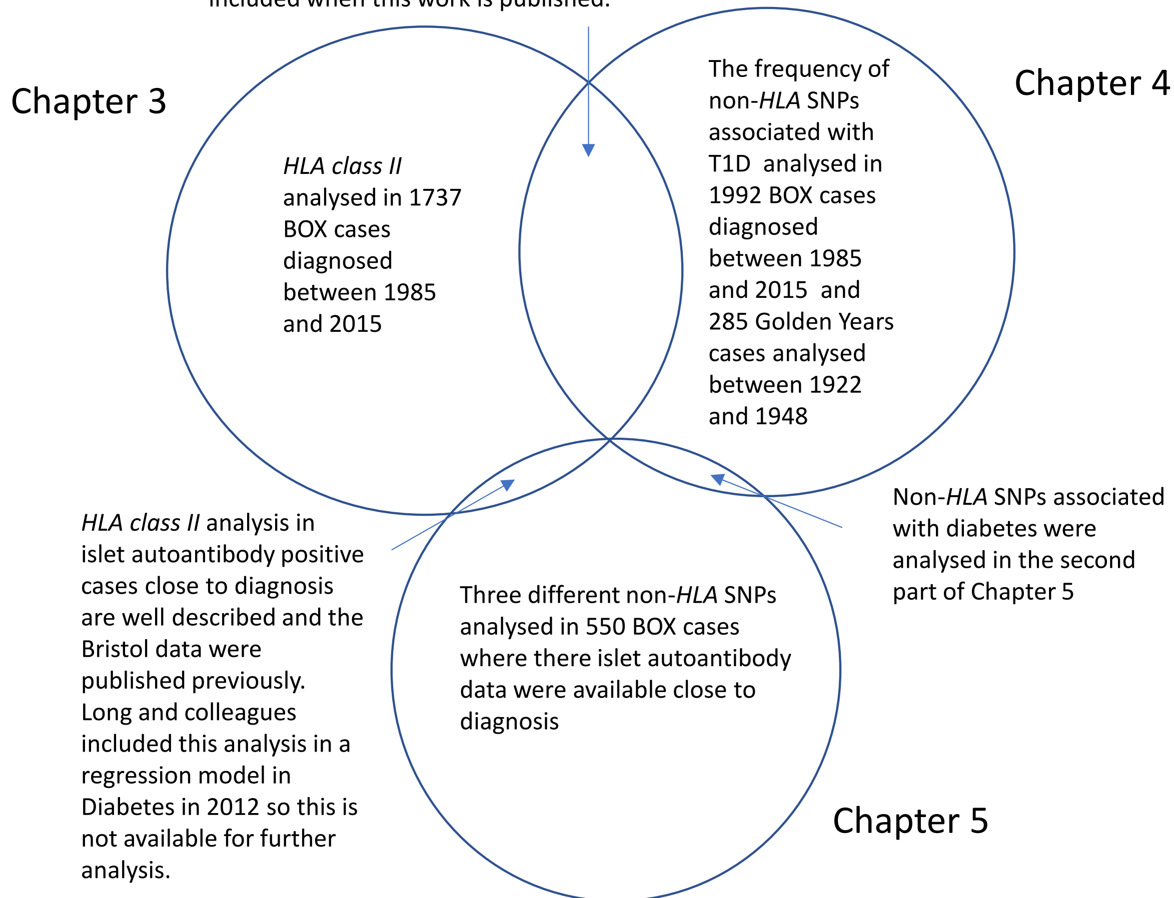

Figure 6.1: *The potential for linked analyses across results chapters: Explaining where linked analysis has not been completed in the current project and the status of ongoing studies using data generated in this project.*

6.6 Future directions for T1D prevention

Currently, clinical trials recruit relatives of T1D patients who are deemed “at-risk” based mainly on their *HLA* class II genetic risk and autoantibody profile (Orban *et al*., 2011; Skyler, 2013; Pescovitz *et al*., 2014) and recently immunotherapy was shown to delay the onset of diabetes by an average of 2 years in an “at-risk” population (Herold *et al*., 2019). Although

HLA are strongly associated with T1D, only approximately 1 in 12 people carrying the highest-risk genotype develop T1D (Rewers *et al*., 1996; Kimpimäki *et al*., 2001). Islet autoantibodies, on the other hand, are a better marker of a pre-diabetes period for clinical interventions to be tested (Pihoker *et al*., 2005; Ziegler and Nepom, 2010; Ziegler *et al*., 2013; Knip *et al*., 2016). However, they are still not used routinely in clinical practice compared with coeliac tissue transglutaminase autoantibodies (Pihoker *et al*., 2005; Volta *et al*., 2008; Bingley, 2010; Volta *et al*., 2010; Caio *et al*., 2019). Islet autoantibodies detected before the age of 6 months are unreliable as they may be maternally derived (Stanley *et al*., 2004). Thereafter, serial measurements of autoantibodies in plasma are required to detect positivity (Ziegler *et al*., 2013; Krischer *et al*., 2015). Once seroconversion is detected, it is not a guarantee of autoantibody persistence. Furthermore, some people have positive autoantibodies for many years, but they do not necessarily develop diabetes (Long *et al*., 2018; Jacobsen *et al*., 2020; So *et al*., 2020). As one's gene sequences do not change as autoantibodies might do, efforts to combine genetic susceptibility and autoantibodies as well as incorporating family history are increasing (Ferrat *et al.*, 2020). These approaches will be how people at risk of type 1 diabetes are identified in the general population in the future, and one missing piece of the jig-saw remains the genes that drive autoantibody responses. The study described here provides additional data to the field and highlights the importance of incorporating *FCLR3* and *LPP* in particular into the GRS for T1D. The other aspect of clinical importance is that the current GRS is based on *HLA* class II *DR3-DQ2*/*DR4-DQ8* carrying the highest risk but data described here show that the frequency of *HLA* susceptibility genes must be monitored over time in order to remain relevant for clinical usefulness.

T1D is challenging, incurable, and exhausting for patients and their families. Research to identify at-risk people for early intervention is relentless. This study sought to improve the

early selection of individuals at risk of T1D vetted by a better predictive genetic and antibody tests. The holy grail remains perfecting a clinically applicable genetic and autoimmune profile that stratifies people by risk for trials before the development of T1D (Herold *et al*., 2019; Jacobsen *et al*., 2020; Sims *et al*., 2020).

6.7 Conclusion

This study examined the recent patterns of genetic susceptibility to T1D over time. There was no convincing proof that *HLA* susceptibility continues to decrease or that non-*HLA* genes are important players in changing susceptibility and changing incidence of T1D over-time. The need for additional samples from individuals who developed T1D before the 1980s was highlighted, and an ethics protocol to do this was prepared. Some unexpected associations of non-*HLA* SNPs with islet autoimmunity were replicated in individuals at diagnosis in the BOX study. Overall, this project has prepared the way to establish an improved study to examine genetic susceptibility to T1D over time and highlighted the importance of some poorly understood genetic drivers of islet autoimmunity.

References

Achenbach, P., E. Bonifacio, A. J. Williams, A. G. Ziegler, E. A. Gale, P. J. Bingley and E. Group (2008). "Autoantibodies to IA-2beta improve diabetes risk assessment in high-risk relatives." Diabetologia **51**(3): 488-492.

Achenbach, P., M. Hummel, L. Thumer, H. Boerschmann, D. Hofelmann and A. G. Ziegler (2013). "Characteristics of rapid vs slow progression to type 1 diabetes in multiple islet autoantibody-positive children." Diabetologia **56**(7): 1615-1622.

Achenbach, P., K. Koczwara, A. Knopff, H. Naserke, A. G. Ziegler and E. Bonifacio (2004). "Mature high-affinity immune responses to (pro)insulin anticipate the autoimmune cascade that leads to type 1 diabetes." J Clin Invest **114**(4): 589-597.

Achenbach, P., V. Lampasona, U. Landherr, K. Koczwara, S. Krause, H. Grallert, C. Winkler, M. Pfluger, T. Illig, E. Bonifacio and A. G. Ziegler (2009). "Autoantibodies to zinc transporter 8 and SLC30A8 genotype stratify type 1 diabetes risk." Diabetologia **52**(9): 1881-1888.

Achenbach, P., K. Warncke, J. Reiter, H. E. Naserke, A. J. Williams, P. J. Bingley, E. Bonifacio and A. G. Ziegler (2004). "Stratification of type 1 diabetes risk on the basis of islet autoantibody characteristics." Diabetes **53**(2): 384-392.

Ahlqvist, E., P. Storm, A. Karajamaki, M. Martinell, M. Dorkhan, A. Carlsson, P. Vikman, R. B. Prasad, D. M. Aly, P. Almgren, Y. Wessman, N. Shaat, P. Spegel, H. Mulder, E. Lindholm, O. Melander, O. Hansson, U. Malmqvist, A. Lernmark, K. Lahti, T. Forsen, T. Tuomi, A. H. Rosengren and L. Groop (2018). "Novel subgroups of adult-onset diabetes and their association with outcomes: a data-driven cluster analysis of six variables." Lancet Diabetes Endocrinol **6**(5): 361-369.

Ahuja, R., R. Pinyol, N. Reichenbach, L. Custer, J. Klingensmith, M. M. Kessels and B. Qualmann (2007). "Cordon-bleu is an actin nucleation factor and controls neuronal morphology." Cell **131**(2): 337-350.

Aitken, R. J., G. L. Mortimer and K. M. Gillespie (2016). Type 1 Diabetes High-Risk HLA Class II Determination by Polymerase Chain Reaction Sequence-Specific Primers. Type-1 Diabetes: Methods and Protocols. K. M. Gillespie. New York, NY, Springer New York**:** 13-20.

Alkanani, A. K., N. Hara, P. A. Gottlieb, D. Ir, C. E. Robertson, B. D. Wagner, D. N. Frank and D. Zipris (2015). "Alterations in Intestinal Microbiota Correlate With Susceptibility to Type 1 Diabetes." Diabetes **64**(10): 3510-3520.

Allis, C. D. and T. Jenuwein (2016). "The molecular hallmarks of epigenetic control." Nat Rev Genet **17**(8): 487-500.

Almeida, R., I. Ricano-Ponce, V. Kumar, P. Deelen, A. Szperl, G. Trynka, J. Gutierrez-Achury, A. Kanterakis, H. J. Westra, L. Franke, M. A. Swertz, M. Platteel, J. R. Bilbao, D. Barisani, L. Greco, L. Mearin, V. M. Wolters, C. Mulder, M. C. Mazzilli, A. Sood, B. Cukrowska, C. Nunez, R. Pratesi, S. Withoff and C. Wijmenga (2014). "Fine mapping of the celiac disease-associated LPP locus reveals a potential functional variant." Hum Mol Genet **23**(9): 2481-2489.

American Diabetes, A. (2014). "Diagnosis and classification of diabetes mellitus." Diabetes Care **37 Suppl 1**: S81-90.

Arif, S., T. I. Tree, T. P. Astill, J. M. Tremble, A. J. Bishop, C. M. Dayan, B. O. Roep and M. Peakman (2004). "Autoreactive T-cell responses show proinflammatory polarization in diabetes but a regulatory phenotype in health." J Clin Invest **113**(3): 451-463.

Atkinson, M. A., G. S. Eisenbarth and A. W. Michels (2014). "Type 1 diabetes." Lancet **383**(9911): 69-82.

Awata, T., H. Yamashita, S. Kurihara, T. Morita-Ohkubo, Y. Miyashita, S. Katayama, E. Kawasaki, S. Tanaka, H. Ikegami, T. Maruyama, A. Shimada, K. Takahashi, Y. Kawabata, T. Kobayashi, N. Nishida and Y. Mawatari (2013). "A low-frequency GLIS3 variant associated with resistance to Japanese type 1 diabetes." Biochem Biophys Res Commun **437**(4): 521-525.

Bach, J. F. (1994). "Insulin-dependent diabetes mellitus as an autoimmune disease." Endocr Rev **15**(4): 516-542.

Bach, J. F. (2002). "The effect of infections on susceptibility to autoimmune and allergic diseases." N Engl J Med **347**(12): 911-920.

Bach, J. F. (2005). "Infections and autoimmune diseases." J Autoimmun **25 Suppl**: 74-80.

Baekkeskov, S., H. J. Aanstoot, S. Christgau, A. Reetz, M. Solimena, M. Cascalho, F. Folli, H. Richter-Olesen and P. De Camilli (1990). "Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase." Nature **347**(6289): 151-156.

Bain, S. C., G. V. Gill, P. H. Dyer, A. F. Jones, M. Murphy, K. E. Jones, C. Smyth and A. H. Barnett (2003). "Characteristics of Type 1 diabetes of over 50 years duration (the Golden Years Cohort)." Diabet Med **20**(10): 808-811.

Bakay, M., R. Pandey and H. Hakonarson (2013). "Genes involved in type 1 diabetes: an update." Genes (Basel) **4**(3): 499-521.

Balke, E. M., E. V. Balti, B. Van der Auwera, I. Weets, O. Costa, S. Demeester, P. Abrams, K. Casteels, M. Coeckelberghs, S. Tenoutasse, B. Keymeulen, D. G. Pipeleers, F. K. Gorus and R. Belgian Diabetes (2018). "Accelerated Progression to Type 1 Diabetes in the Presence of HLA-A*24 and -B*18 Is Restricted to Multiple Islet Autoantibody-Positive Individuals With Distinct HLA-DQ and Autoantibody Risk Profiles." Diabetes Care **41**(5): 1076-1083.

Barker, J. M., K. J. Barriga, L. Yu, D. Miao, H. A. Erlich, J. M. Norris, G. S. Eisenbarth, M. Rewers and Y. Diabetes Autoimmunity Study in the (2004). "Prediction of autoantibody positivity and progression to type 1 diabetes: Diabetes Autoimmunity Study in the Young (DAISY)." J Clin Endocrinol Metab **89**(8): 3896-3902.

Barker, A., S. J. Sharp, N. J. Timpson, N. Bouatia-Naji, N. M. Warrington, S. Kanoni, L. J. Beilin, S. Brage, P. Deloukas, D. M. Evans, A. Grontved, N. Hassanali, D. A. Lawlor, C. Lecoeur, R. J. Loos, S. J. Lye, M. I. McCarthy, T. A. Mori, N. C. Ndiaye, J. P. Newnham, I. Ntalla, C. E. Pennell, B. St Pourcain, I. Prokopenko, S. M. Ring, N. Sattar, S. Visvikis-Siest, G. V. Dedoussis, L. J. Palmer, P. Froguel, G. D. Smith, U. Ekelund, N. J. Wareham and C. Langenberg (2011). "Association of genetic Loci with glucose levels in childhood and adolescence: a meta-analysis of over 6,000 children." Diabetes **60**(6): 1805-1812.

Barrett, J. C., D. G. Clayton, P. Concannon, B. Akolkar, J. D. Cooper, H. A. Erlich, C. Julier, G. Morahan, J. Nerup, C. Nierras, V. Plagnol, F. Pociot, H. Schuilenburg, D. J. Smyth, H. Stevens, J. A. Todd, N. M. Walker, S. S. Rich and C. Type 1 Diabetes Genetics (2009).

"Genome-wide association study and meta-analysis find that over 40 loci affect risk of type 1 diabetes." Nat Genet **41**(6): 703-707.
Baxter, R. C., D. K. Yue and J. R. Turtle (1976). "Equilibrium binding studies of insulin antibodies in diabetic subjects." Clin Chem **22**(7): 1089-1094.
Bearzatto, M., H. Naserke, S. Piquer, K. Koczwara, V. Lampasona, A. Williams, M. R. Christie, P. J. Bingley, A. G. Ziegler and E. Bonifacio (2002). "Two distinctly HLA-associated contiguous linear epitopes uniquely expressed within the islet antigen 2 molecule are major autoantibody epitopes of the diabetes-specific tyrosine phosphatase-like protein autoantigens." J Immunol **168**(8): 4202-4208.
Begovich, A. B., V. E. Carlton, L. A. Honigberg, S. J. Schrodi, A. P. Chokkalingam, H. C. Alexander, K. G. Ardlie, Q. Huang, A. M. Smith, J. M. Spoerke, M. T. Conn, M. Chang, S. Y. Chang, R. K. Saiki, J. J. Catanese, D. U. Leong, V. E. Garcia, L. B. McAllister, D. A. Jeffery, A. T. Lee, F. Batliwalla, E. Remmers, L. A. Criswell, M. F. Seldin, D. L. Kastner, C. I. Amos, J. J. Sninsky and P. K. Gregersen (2004). "A missense single-nucleotide polymorphism in a gene encoding a protein tyrosine phosphatase (PTPN22) is associated with rheumatoid arthritis." Am J Hum Genet **75**(2): 330-337.
Bell, G. I., S. Horita and J. H. Karam (1984). "A polymorphic locus near the human insulin gene is associated with insulin-dependent diabetes mellitus." Diabetes **33**(2): 176-183.
Bennett, S. T. and J. A. Todd (1996). "Human type 1 diabetes and the insulin gene: principles of mapping polygenes." Annu Rev Genet **30**: 343-370.
Bennett, S. T., A. J. Wilson, F. Cucca, J. Nerup, F. Pociot, P. A. McKinney, A. H. Barnett, S. C. Bain and J. A. Todd (1996). "IDDM2-VNTR-encoded susceptibility to type 1 diabetes: dominant protection and parental transmission of alleles of the insulin gene-linked minisatellite locus." J Autoimmun **9**(3): 415-421.
Bentham, J., D. L. Morris, D. S. C. Graham, C. L. Pinder, P. Tombleson, T. W. Behrens, J. Martin, B. P. Fairfax, J. C. Knight, L. Chen, J. Replogle, A. C. Syvanen, L. Ronnblom, R. R. Graham, J. E. Wither, J. D. Rioux, M. E. Alarcon-Riquelme and T. J. Vyse (2015). "Genetic association analyses implicate aberrant regulation of innate and adaptive immunity genes in the pathogenesis of systemic lupus erythematosus." Nat Genet **47**(12): 1457-1464.
Berger, S. L., T. Kouzarides, R. Shiekhattar and A. Shilatifard (2009). "An operational definition of epigenetics." Genes Dev **23**(7): 781-783.
Bettelli, E., M. Dastrange and M. Oukka (2005). "Foxp3 interacts with nuclear factor of activated T cells and NF-kappa B to repress cytokine gene expression and effector functions of T helper cells." Proc Natl Acad Sci U S A **102**(14): 5138-5143.
Beyerlein, A., E. Donnachie, S. Jergens and A. G. Ziegler (2016). "Infections in Early Life and Development of Type 1 Diabetes." JAMA **315**(17): 1899-1901.
Beyerlein, A., M. Krasmann, E. Thiering, D. Kusian, I. Markevych, O. D'Orlando, K. Warncke, S. Jochner, J. Heinrich and A. G. Ziegler (2015). "Ambient air pollution and early manifestation of type 1 diabetes." Epidemiology **26**(3): e31-32.
Biasucci, G., B. Benenati, L. Morelli, E. Bessi and G. n. Boehm (2008). "Cesarean Delivery May Affect the Early Biodiversity of Intestinal Bacteria." The Journal of Nutrition **138**(9): 1796S-1800S.
Bingley, P. J. (2010). "Clinical applications of diabetes antibody testing." J Clin Endocrinol Metab **95**(1): 25-33.

Bingley, P. J., E. Bonifacio and P. W. Mueller (2003). "Diabetes Antibody Standardization Program: first assay proficiency evaluation." Diabetes **52**(5): 1128-1136.

Bingley, P. J., E. Bonifacio, A. J. Williams, S. Genovese, G. F. Bottazzo and E. A. Gale (1997). "Prediction of IDDM in the general population: strategies based on combinations of autoantibody markers." Diabetes **46**(11): 1701-1710.

Bingley, P. J., E. Bonifacio, A. G. Ziegler, D. A. Schatz, M. A. Atkinson, G. S. Eisenbarth and S. Immunology of Diabetes (2001). "Proposed guidelines on screening for risk of type 1 diabetes." Diabetes Care **24**(2): 398.

Bingley, P. J., D. C. Boulware, J. P. Krischer and G. Type 1 Diabetes TrialNet Study (2016). "The implications of autoantibodies to a single islet antigen in relatives with normal glucose tolerance: development of other autoantibodies and progression to type 1 diabetes." Diabetologia **59**(3): 542-549.

Bingley, P. J., M. R. Christie, E. Bonifacio, R. Bonfanti, M. Shattock, M. T. Fonte, G. F. Bottazzo and E. A. Gale (1994). "Combined analysis of autoantibodies improves prediction of IDDM in isleT-cell antibody-positive relatives." Diabetes **43**(11): 1304-1310.

Bingley, P. J., A. J. Williams and E. A. Gale (1999). "Optimized autoantibody-based risk assessment in family members. Implications for future intervention trials." Diabetes Care **22**(11): 1796-1801.

Bird, A. (2007). "Perceptions of epigenetics." Nature **447**(7143): 396-398.

Bjorksten, B., E. Sepp, K. Julge, T. Voor and M. Mikelsaar (2001). "Allergy development and the intestinal microflora during the first year of life." J Allergy Clin Immunol **108**(4): 516-520.

Bloem, S. J. and B. O. Roep (2017). "The elusive role of B lymphocytes and islet autoantibodies in (human) type 1 diabetes." Diabetologia **60**(7): 1185-1189.

Bonifacio, E., P. J. Bingley, M. Shattock, B. M. Dean, D. Dunger, E. A. Gale and G. F. Bottazzo (1990). "Quantification of islet-cell antibodies and prediction of insulin-dependent diabetes." Lancet **335**(8682): 147-149.

Bonifacio, E., V. Lampasona, L. Bernasconi and A. G. Ziegler (2000). "Maturation of the humoral autoimmune response to epitopes of GAD in preclinical childhood type 1 diabetes." Diabetes **49**(2): 202-208.

Bonifacio, E., K. Warncke, C. Winkler, M. Wallner and A. G. Ziegler (2011). "Cesarean section and interferon-induced helicase gene polymorphisms combine to increase childhood type 1 diabetes risk." Diabetes **60**(12): 3300-3306.

Bosi, E., D. C. Boulware, D. J. Becker, J. H. Buckner, S. Geyer, P. A. Gottlieb, C. Henderson, A. Kinderman, J. M. Sosenko, A. K. Steck, P. J. Bingley and G. Type 1 Diabetes TrialNet Study (2017). "Impact of age and antibody type on progression from single to multiple autoantibodies in type 1 diabetes relatives." J Clin Endocrinol Metab **102**(8): 2881-2886.

Bosi, E., L. Molteni, M. G. Radaelli, L. Folini, I. Fermo, E. Bazzigaluppi, L. Piemonti, M. R. Pastore and R. Paroni (2006). "Increased intestinal permeability precedes clinical onset of type 1 diabetes." Diabetologia **49**(12): 2824-2827.

Bottini, N., L. Musumeci, A. Alonso, S. Rahmouni, K. Nika, M. Rostamkhani, J. MacMurray, G. F. Meloni, P. Lucarelli, M. Pellecchia, G. S. Eisenbarth, D. Comings and T. Mustelin (2004). "A functional variant of lymphoid tyrosine phosphatase is associated with type I diabetes." Nat Genet **36**(4): 337-338.

Brooking, H., R. Ananieva-Jordanova, C. Arnold, M. Amoroso, M. Powell, C. Betterle, R. Zanchetta, J. Furmaniak and B. R. Smith (2003). "A sensitive non-isotopic assay for GAD65 autoantibodies." Clin Chim Acta **331**(1-2): 55-59.

Brooks-Worrell, B., V. H. Gersuk, C. Greenbaum and J. P. Palmer (2001). "Intermolecular antigen spreading occurs during the preclinical period of human type 1 diabetes." J Immunol **166**(8): 5265-5270.

Brooks-Worrell, B. M., D. Nielson and J. P. Palmer (1999). "Insulin autoantibodies and insulin antibodies have similar binding characteristics." Proc Assoc Am Physicians **111**(1): 92-96.

Brorsson, C. A., S. Onengut, W. M. Chen, J. Wenzlau, L. P. Yu, P. Baker, A. J. K. Williams, P. J. Bingley, J. C. Hutton, G. S. Eisenbarth, P. Concannon, S. S. Rich, F. Pociot and T. D. G. Consortium (2015). "Novel Association Between Immune-Mediated Susceptibility Loci and Persistent Autoantibody Positivity in Type 1 Diabetes." Diabetes **64**(8): 3017-3027.

Brown, C. T., A. G. Davis-Richardson, A. Giongo, K. A. Gano, D. B. Crabb, N. Mukherjee, G. Casella, J. C. Drew, J. Ilonen, M. Knip, H. Hyoty, R. Veijola, T. Simell, O. Simell, J. Neu, C. H. Wasserfall, D. Schatz, M. A. Atkinson and E. W. Triplett (2011). "Gut microbiome metagenomics analysis suggests a functional model for the development of autoimmunity for type 1 diabetes." PLoS One **6**(10): e25792.

Brugman, S., F. A. Klatter, J. T. Visser, A. C. Wildeboer-Veloo, H. J. Harmsen, J. Rozing and N. A. Bos (2006). "Antibiotic treatment partially protects against type 1 diabetes in the Bio-Breeding diabetes-prone rat. Is the gut flora involved in the development of type 1 diabetes?" Diabetologia **49**(9): 2105-2108.

Bunce, M., C. M. O'Neill, M. C. Barnardo, P. Krausa, M. J. Browning, P. J. Morris and K. I. Welsh (1995). "Phototyping: comprehensive DNA typing for HLA-A, B, C, DRB1, DRB3, DRB4, DRB5 & DQB1 by PCR with 144 primer mixes utilizing sequence-specific primers (PCR-SSP)." Tissue Antigens **46**(5): 355-367.

Burnet, F. M. (1961). "The new approach to immunology." N Engl J Med **264**: 24-34.

Burrows, S. R., J. Rossjohn and J. McCluskey (2006). "Have we cut ourselves too short in mapping CTL epitopes?" Trends Immunol **27**(1): 11-16.

Butler, A. E., R. Galasso, J. J. Meier, R. Basu, R. A. Rizza and P. C. Butler (2007). "Modestly increased beta cell apoptosis but no increased beta cell replication in recent-onset type 1 diabetic patients who died of diabetic ketoacidosis." Diabetologia **50**(11): 2323-2331.

Caballero-Franco, C. and S. Kissler (2016). "The autoimmunity-associated gene RGS1 affects the frequency of T follicular helper cells." Genes Immun **17**(4): 228-238.

Caillat-Zucman, S., H. J. Garchon, J. Timsit, R. Assan, C. Boitard, I. Djilali-Saiah, P. Bougneres and J. F. Bach (1992). "Age-dependent HLA genetic heterogeneity of type 1 insulin-dependent diabetes mellitus." J Clin Invest **90**(6): 2242-2250.

Caio, G., U. Volta, A. Sapone, D. A. Leffler, R. De Giorgio, C. Catassi and A. Fasano (2019). "Celiac disease: a comprehensive current review." BMC Medicine **17**(1): 142.

Candon, S., A. Perez-Arroyo, C. Marquet, F. Valette, A. P. Foray, B. Pelletier, C. Milani, M. Ventura, J. F. Bach and L. Chatenoud (2015). "Antibiotics in early life alter the gut microbiome and increase disease incidence in a spontaneous mouse model of autoimmune insulin-dependent diabetes." PLoS One **10**(5): e0125448.

Cao, J. (2014). "The functional role of long non-coding RNAs and epigenetics." Biol Proced Online **16**: 11.

Carry, P. M., L. A. Vanderlinden, R. K. Johnson, F. Dong, A. K. Steck, B. I. Frohnert, M. Rewers, I. V. Yang, K. Kechris and J. M. Norris (2020). "DNA methylation near the INS gene is associated with INS genetic variation (rs689) and type 1 diabetes in the Diabetes Autoimmunity Study in the Young." Pediatr Diabetes **21**(4): 597-605.

Castano, L., A. G. Ziegler, R. Ziegler, S. Shoelson and G. S. Eisenbarth (1993). "Characterization of insulin autoantibodies in relatives of patients with type I diabetes." Diabetes **42**(8): 1202-1209.

Cerosaletti, K. and J. H. Buckner (2012). "Protein tyrosine phosphatases and type 1 diabetes: genetic and functional implications of PTPN2 and PTPN22." Rev Diabet Stud **9**(4): 188-200.

Cerosaletti, K., A. Schneider, K. Schwedhelm, I. Frank, M. Tatum, S. Wei, E. Whalen, C. Greenbaum, M. Kita, J. Buckner and S. A. Long (2013). "Multiple autoimmune-associated variants confer decreased IL-2R signaling in CD4+ CD25(hi) T-cells of type 1 diabetic and multiple sclerosis patients." PLoS One **8**(12): e83811.

Chen, Y.-l., Y.-c. Huang, Y.-c. Qiao, W. Ling, Y.-h. Pan, L.-j. Geng, J.-l. Xiao, X.-x. Zhang and H.-l. Zhao (2017). "Climates on incidence of childhood type 1 diabetes mellitus in 72 countries." Scientific Reports **7**(1): 12810.

Chimienti, F., S. Devergnas, F. Pattou, F. Schuit, R. Garcia-Cuenca, B. Vandewalle, J. Kerr-Conte, L. Van Lommel, D. Grunwald, A. Favier and M. Seve (2006). "In vivo expression and functional characterization of the zinc transporter ZnT8 in glucose-induced insulin secretion." J Cell Sci **119**(Pt 20): 4199-4206.

Clarke, T. B., K. M. Davis, E. S. Lysenko, A. Y. Zhou, Y. Yu and J. N. Weiser (2010). "Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity." Nat Med **16**(2): 228-231.

Concannon, P., S. Onengut-Gumuscu, J. A. Todd, D. J. Smyth, F. Pociot, R. Bergholdt, B. Akolkar, H. A. Erlich, J. E. Hilner, C. Julier, G. Morahan, J. Nerup, C. R. Nierras, W. M. Chen, S. S. Rich and C. Type 1 Diabetes Genetics (2008). "A human type 1 diabetes susceptibility locus maps to chromosome 21q22.3." Diabetes **57**(10): 2858-2861.

Congia, M., S. Patel, A. P. Cope, S. De Virgiliis and G. Sonderstrup (1998). "T-cell epitopes of insulin defined in HLA-DR4 transgenic mice are derived from preproinsulin and proinsulin." Proc Natl Acad Sci U S A **95**(7): 3833-3838.

Cooper, J. D., M. J. Simmonds, N. M. Walker, O. Burren, O. J. Brand, H. Guo, C. Wallace, H. Stevens, G. Coleman, C. Wellcome Trust Case Control, J. A. Franklyn, J. A. Todd and S. C. Gough (2012). "Seven newly identified loci for autoimmune thyroid disease." Hum Mol Genet **21**(23): 5202-5208.

Cooper, J. D., D. J. Smyth, A. M. Smiles, V. Plagnol, N. M. Walker, J. E. Allen, K. Downes, J. C. Barrett, B. C. Healy, J. C. Mychaleckyj, J. H. Warram and J. A. Todd (2008). "Meta-analysis of genome-wide association study data identifies additional type 1 diabetes risk loci." Nat Genet **40**(12): 1399-1401.

Coppieters, K. T., A. Wiberg, S. M. Tracy and M. G. von Herrath (2012). "Immunology in the clinic review series: focus on type 1 diabetes and viruses: the role of viruses in type 1 diabetes: a difficult dilemma." Clin Exp Immunol **168**(1): 5-11.

Cotsapas, C., B. F. Voight, E. Rossin, K. Lage, B. M. Neale, C. Wallace, G. R. Abecasis, J. C. Barrett, T. Behrens, J. Cho, P. L. De Jager, J. T. Elder, R. R. Graham, P. Gregersen, L. Klareskog, K.

A. Siminovitch, D. A. van Heel, C. Wijmenga, J. Worthington, J. A. Todd, D. A. Hafler, S. S. Rich, M. J. Daly and F. O. N. o. Consortia (2011). "Pervasive sharing of genetic effects in autoimmune disease." PLoS Genet **7**(8): e1002254.

Couper, J. J., S. Beresford, C. Hirte, P. A. Baghurst, A. Pollard, B. D. Tait, L. C. Harrison and P. G. Colman (2009). "Weight gain in early life predicts risk of islet autoimmunity in children with a first-degree relative with type 1 diabetes." Diabetes Care **32**(1): 94-99.

Couper, J. J., C. Steele, S. Beresford, T. Powell, K. McCaul, A. Pollard, S. Gellert, B. Tait, L. C. Harrison and P. G. Colman (1999). "Lack of association between duration of breast-feeding or introduction of cow's milk and development of islet autoimmunity." Diabetes **48**(11): 2145-2149.

Cudworth, A. G., E. Wolf, A. N. Gorsuch and H. Festenstein (1979). "A new look at HLA genetics with particular reference to type-1 diabetes." Lancet **2**(8139): 389-391.

Dahlquist, G. (2006). "Can we slow the rising incidence of childhood-onset autoimmune diabetes? The overload hypothesis." Diabetologia **49**(1): 20-24.

Dahlquist, G. and L. Mustonen (1994). "Childhood onset diabetes--time trends and climatological factors." Int J Epidemiol **23**(6): 1234-1241.

Dahlquist, G. G., L. G. Blom, L. A. Persson, A. I. Sandstrom and S. G. Wall (1990). "Dietary factors and the risk of developing insulin dependent diabetes in childhood." BMJ **300**(6735): 1302-1306.

Dai, Y. D., G. Carayanniotis and E. Sercarz (2005). "Antigen processing by autoreactive B-cells promotes determinant spreading." Cell Mol Immunol **2**(3): 169-175.

David Male. Immunology: An Illustrated outline. Garland science, Fifth edition-2014.

Decochez, K., I. H. De Leeuw, B. Keymeulen, C. Mathieu, R. Rottiers, I. Weets, E. Vandemeulebroucke, I. Truyen, L. Kaufman, F. C. Schuit, D. G. Pipeleers, F. K. Gorus and R. Belgian Diabetes (2002). "IA-2 autoantibodies predict impending type I diabetes in siblings of patients." Diabetologia **45**(12): 1658-1666.

Delovitch, T. L. and B. Singh (1997). "The nonobese diabetic mouse as a model of autoimmune diabetes: immune dysregulation gets the NOD." Immunity **7**(6): 727-738.

Dendrou, C. A., J. Petersen, J. Rossjohn and L. Fugger (2018). "HLA variation and disease." Nat Rev Immunol **18**(5): 325-339.

Deniro, M. and F. A. Al-Mohanna (2012). "Zinc transporter 8 (ZnT8) expression is reduced by ischemic insults: a potential therapeutic target to prevent ischemic retinopathy." PLoS One **7**(11): e50360.

Devendra, D. and G. S. Eisenbarth (2003). "17. Immunologic endocrine disorders." J Allergy Clin Immunol **111**(2 Suppl): S624-636.

Diabetes Prevention Trial--Type 1 Diabetes Study, G. (2002). "Effects of insulin in relatives of patients with type 1 diabetes mellitus." N Engl J Med **346**(22): 1685-1691.

Diaz-Gallo, L. M., E. Sanchez, N. Ortego-Centeno, J. M. Sabio, F. J. Garcia-Hernandez, E. de Ramon, M. A. Gonzalez-Gay, W. Torsten, H. J. Anders, M. F. Gonzalez-Escribano and J. Martin (2013). "Evidence of new risk genetic factor to systemic lupus erythematosus: the UBASH3A gene." PLoS One **8**(4): e60646.

Diez, J., Y. Park, M. Zeller, D. Brown, D. Garza, C. Ricordi, J. Hutton, G. S. Eisenbarth and A. Pugliese (2001). "Differential splicing of the IA-2 mRNA in pancreas and lymphoid organs as a

permissive genetic mechanism for autoimmunity against the IA-2 type 1 diabetes autoantigen." Diabetes **50**(4): 895-900.

DiLorenzo, T. P. and D. V. Serreze (2005). "The good turned ugly: immunopathogenic basis for diabetogenic CD8+ T-cells in NOD mice." Immunol Rev **204**: 250-263.

DiMeglio, L. A., C. Evans-Molina and R. A. Oram (2018). "Type 1 diabetes." Lancet **391**(10138): 2449-2462.

Dimitri, P., A. M. Habeb, F. Gurbuz, A. Millward, S. Wallis, K. Moussa, T. Akcay, D. Taha, J. Hogue, A. Slavotinek, J. K. Wales, A. Shetty, D. Hawkes, A. T. Hattersley, S. Ellard and E. De Franco (2015). "Expanding the Clinical Spectrum Associated With GLIS3 Mutations." J Clin Endocrinol Metab **100**(10): E1362-1369.

Dominguez-Bello, M. G., E. K. Costello, M. Contreras, M. Magris, G. Hidalgo, N. Fierer and R. Knight (2010). "Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns." Proc Natl Acad Sci U S A **107**(26): 11971-11975.

Dou, Y., H. C. Yim, C. D. Kirkwood, B. R. Williams and A. J. Sadler (2017). "The innate immune receptor MDA5 limits rotavirus infection but promotes cell death and pancreatic inflammation." EMBO J **36**(18): 2742-2757.

Dromey, J. A., S. M. Weenink, G. H. Peters, J. Endl, P. J. Tighe, I. Todd and M. R. Christie (2004). "Mapping of epitopes for autoantibodies to the type 1 diabetes autoantigen IA-2 by peptide phage display and molecular modeling: overlap of antibody and T-cell determinants." J Immunol **172**(7): 4084-4090.

Duarte, G. C. K., T. S. Assmann, C. Dieter, B. M. de Souza and D. Crispim (2017). "GLIS3 rs7020673 and rs10758593 polymorphisms interact in the susceptibility for type 1 diabetes mellitus." Acta Diabetol **54**(9): 813-821.

Dubois-LaForgue, D., J. C. Carel, P. F. Bougneres, J. G. Guillet and C. Boitard (1999). "T-cell response to proinsulin and insulin in type 1 and pretype 1 diabetes." J Clin Immunol **19**(2): 127-134.

Diabetes UK report, 2019 https://www.diabetes.org.uk/resources-s3/2019-02/1362B_Facts%20and%20stats%20Update%20Jan%202019_LOW%20RES_EXTERNAL.pdf

Durinovic-Bello, I., M. Schlosser, M. Riedl, N. Maisel, S. Rosinger, H. Kalbacher, M. Deeg, M. Ziegler, J. Elliott, B. O. Roep, W. Karges and B. O. Boehm (2004). "Pro- and anti-inflammatory cytokine production by autoimmune T-cells against preproinsulin in HLA-DRB1*04, DQ8 Type 1 diabetes." Diabetologia **47**(3): 439-450.

Eisenbarth, G. S. (1986). "Type I diabetes mellitus. A chronic autoimmune disease." N Engl J Med **314**(21): 1360-1368.

Eisenbarth, G. S., R. A. Jackson and A. Pugliese (1992). "Insulin autoimmunity: the rate limiting factor in pre-type I diabetes." J Autoimmun **5 Suppl A**: 241-246.

Ekman, I., T. Vuorinen, M. Knip, R. Veijola, J. Toppari, H. Hyoty, T. Kinnunen, J. Ilonen and J. Lempainen (2019). "Early childhood CMV infection may decelerate the progression to clinical type 1 diabetes." Pediatr Diabetes **20**(1): 73-77.

Ellinghaus, D., L. Jostins, S. L. Spain, A. Cortes, J. Bethune, B. Han, Y. R. Park, S. Raychaudhuri, J. G. Pouget, M. Hubenthal, T. Folseraas, Y. Wang, T. Esko, A. Metspalu, H. J. Westra, L. Franke, T. H. Pers, R. K. Weersma, V. Collij, M. D'Amato, J. Halfvarson, A. B. Jensen, W.

Lieb, F. Degenhardt, A. J. Forstner, A. Hofmann, I. B. D. G. C. International, C. International Genetics of Ankylosing Spondylitis, P. S. C. S. G. International, C. Genetic Analysis of Psoriasis, E. Psoriasis Association Genetics, S. Schreiber, U. Mrowietz, B. D. Juran, K. N. Lazaridis, S. Brunak, A. M. Dale, R. C. Trembath, S. Weidinger, M. Weichenthal, E. Ellinghaus, J. T. Elder, J. N. Barker, O. A. Andreassen, D. P. McGovern, T. H. Karlsen, J. C. Barrett, M. Parkes, M. A. Brown and A. Franke (2016). "Analysis of five chronic inflammatory diseases identifies 27 new associations and highlights disease-specific patterns at shared loci." Nat Genet **48**(5): 510-518.

Emdin, S. O., G. G. Dodson, J. M. Cutfield and S. M. Cutfield (1980). "Role of zinc in insulin biosynthesis. Some possible zinc-insulin interactions in the pancreatic B-cell." Diabetologia **19**(3): 174-182.

Fennessy, M., K. Metcalfe, G. A. Hitman, M. Niven, P. A. Biro, J. Tuomilehto and E. Tuomilehto-Wolf (1994). "A gene in the HLA class I region contributes to susceptibility to IDDM in the Finnish population. Childhood Diabetes in Finland (DiMe) Study Group." Diabetologia **37**(9): 937-944.

Ferrat, L. A., K. Vehik, S. A. Sharp, A. Lernmark, M. J. Rewers, J. X. She, A. G. Ziegler, J. Toppari, B. Akolkar, J. P. Krischer, M. N. Weedon, R. A. Oram, W. A. Hagopian, T. S. Group and Committees (2020). "A combined risk score enhances prediction of type 1 diabetes among susceptible children." Nat Med **26**(8): 1247-1255.

Fichna, M., M. Zurawek, P. Fichna, D. Januszkiewicz and J. Nowak (2012). "Polymorphic variants of the IL2RA gene and susceptibility to type 1 diabetes in the Polish population." Tissue Antigens **79**(3): 198-203.

Floyel, T., C. Brorsson, L. B. Nielsen, M. Miani, C. H. Bang-Berthelsen, M. Friedrichsen, A. J. Overgaard, L. A. Berchtold, A. Wiberg, P. Poulsen, L. Hansen, S. Rosinger, B. O. Boehm, R. Ram, Q. Nguyen, M. Mehta, G. Morahan, P. Concannon, R. Bergholdt, J. H. Nielsen, T. Reinheckel, M. von Herrath, A. Vaag, D. L. Eizirik, H. B. Mortensen, J. Storling and F. Pociot (2014). "CTSH regulates beta-cell function and disease progression in newly diagnosed type 1 diabetes patients." Proc Natl Acad Sci U S A **111**(28): 10305-10310.

Fortune, M. D., H. Guo, O. Burren, E. Schofield, N. M. Walker, M. Ban, S. J. Sawcer, J. Bowes, J. Worthington, A. Barton, S. Eyre, J. A. Todd and C. Wallace (2015). "Statistical colocalization of genetic risk variants for related autoimmune diseases in the context of common controls." Nat Genet **47**(7): 839-846.

Fourlanos, S., M. D. Varney, B. D. Tait, G. Morahan, M. C. Honeyman, P. G. Colman and L. C. Harrison (2008). "Lower risk HLA genotypes account for the rising incidence of type 1 diabetes." Diabetologia **51**: S64-S65.

Fourlanos, S., M. D. Varney, B. D. Tait, G. Morahan, M. C. Honeyman, P. G. Colman and L. C. Harrison (2008). "The rising incidence of type 1 diabetes is accounted for by cases with lower-risk human leukocyte antigen genotypes." Diabetes Care **31**(8): 1546-1549.

Franke, A., T. Balschun, T. H. Karlsen, J. Sventoraityte, S. Nikolaus, G. Mayr, F. S. Domingues, M. Albrecht, M. Nothnagel, D. Ellinghaus, C. Sina, C. M. Onnie, R. K. Weersma, P. C. Stokkers, C. Wijmenga, M. Gazouli, D. Strachan, W. L. McArdle, S. Vermeire, P. Rutgeerts, P. Rosenstiel, M. Krawczak, M. H. Vatn, I. s. group, C. G. Mathew and S. Schreiber (2008). "Sequence variants in IL10, ARPC2 and multiple other loci contribute to ulcerative colitis susceptibility." Nat Genet **40**(11): 1319-1323.

Fugger, L., S. A. Michie, I. Rulifson, C. B. Lock and G. S. McDevitt (1994). "Expression of HLA-DR4 and human CD4 transgenes in mice determines the variable region beta-chain T-cell repertoire and mediates an HLA-DR-restricted immune response." Proc Natl Acad Sci U S A **91**(13): 6151-6155.

Gale, E. A. (2002). "A missing link in the hygiene hypothesis?" Diabetologia **45**(4): 588-594.

Gale, E. A. (2002). "The rise of childhood type 1 diabetes in the 20th century." Diabetes **51**(12): 3353-3361.

Gale, E. A. (2008). "Congenital rubella: citation virus or viral cause of type 1 diabetes?" Diabetologia **51**(9): 1559-1566.

Gale, E. A., P. J. Bingley, C. L. Emmett, T. Collier and G. European Nicotinamide Diabetes Intervention Trial (2004). "European Nicotinamide Diabetes Intervention Trial (ENDIT): a randomised controlled trial of intervention before the onset of type 1 diabetes." Lancet **363**(9413): 925-931.

Gale, E. A. and K. M. Gillespie (2001). "Diabetes and gender." Diabetologia **44**(1): 3-15.

Gardner, S. G., P. J. Bingley, P. A. Sawtell, S. Weeks and E. A. Gale (1997). "Rising incidence of insulin dependent diabetes in children aged under 5 years in the Oxford region: time trend analysis. The Bart's-Oxford Study Group." BMJ **315**(7110): 713-717.

Gardner, S. G., E. A. Gale, A. J. Williams, K. M. Gillespie, K. E. Lawrence, G. F. Bottazzo and P. J. Bingley (1999). "Progression to diabetes in relatives with islet autoantibodies. Is it inevitable?" Diabetes Care **22**(12): 2049-2054.

Garg, G., J. R. Tyler, J. H. Yang, A. J. Cutler, K. Downes, M. Pekalski, G. L. Bell, S. Nutland, M. Peakman, J. A. Todd, L. S. Wicker and T. I. Tree (2012). "Type 1 diabetes-associated IL2RA variation lowers IL-2 signaling and contributes to diminished CD4+CD25+ regulatory T-cell function." J Immunol **188**(9): 4644-4653.

Gartner, A., R. Daniel, D. Farewell, S. Paranjothy, J. Townson and J. W. Gregory (2020). "Demographic and socioeconomic patterns in the risk of alcohol-related hospital admission in children and young adults with childhood onset type-1 diabetes from a record-linked longitudinal population cohort study in Wales." Pediatr Diabetes **21**(7): 1333-1342.

Ge, Y., T. K. Paisie, J. R. B. Newman, L. M. McIntyre and P. Concannon (2017). "UBASH3A Mediates Risk for Type 1 Diabetes Through Inhibition of T-Cell Receptor-Induced NF-kappaB Signaling." Diabetes **66**(7): 2033-2043.

Gerold, K. D., P. Zheng, D. B. Rainbow, A. Zernecke, L. S. Wicker and S. Kissler (2011). "The soluble CTLA-4 splice variant protects from type 1 diabetes and potentiates regulatory T-cell function." Diabetes **60**(7): 1955-1963.

Giannopoulou, E. Z., C. Winkler, R. Chmiel, C. Matzke, M. Scholz, A. Beyerlein, P. Achenbach, E. Bonifacio and A. G. Ziegler (2015). "Islet autoantibody phenotypes and incidence in children at increased risk for type 1 diabetes." Diabetologia **58**(10): 2317-2323.

Gillespie, K. M. (2006). "Type 1 diabetes: pathogenesis and prevention." CMAJ **175**(2): 165-170.

Gillespie, K. M., S. C. Bain, A. H. Barnett, P. J. Bingley, M. R. Christie, G. V. Gill and E. A. Gale (2004). "The rising incidence of childhood type 1 diabetes and reduced contribution of high-risk HLA haplotypes." Lancet **364**(9446): 1699-1700.

Gillespie, K. M., E. A. Gale and P. J. Bingley (2002). "High familial risk and genetic susceptibility in early onset childhood diabetes." Diabetes **51**(1): 210-214.

Gillespie, K. M., S. J. Valovin, J. Saunby, K. M. Hunter, D. A. Savage, D. Middleton, J. A. Todd, P. J. Bingley and E. A. Gale (2000). "HLA class II typing of whole genome amplified mouth swab DNA." Tissue Antigens **56**(6): 530-538.

Gingerich, M. A., V. Sidarala and S. A. Soleimanpour (2020). "Clarifying the function of genes at the chromosome 16p13 locus in type 1 diabetes: CLEC16A and DEXI." Genes & Immunity **21**(2): 79-82.

Ginsberg-Fellner, F., M. E. Witt, B. Fedun, F. Taub, M. J. Dobersen, R. C. McEvoy, L. Z. Cooper, A. L. Notkins and P. Rubinstein (1985). "Diabetes mellitus and autoimmunity in patients with the congenital rubella syndrome." Rev Infect Dis **7 Suppl 1**: S170-176.

Gohlke, H., U. Ferrari, K. Koczwara, E. Bonifacio, T. Illig and A. G. Ziegler (2008). "SLC30A8 (ZnT8) Polymorphism is Associated with Young Age at Type 1 Diabetes Onset." Rev Diabet Stud **5**(1): 25-27.

Gorsuch, A. N., K. M. Spencer, J. Lister, J. M. McNally, B. M. Dean, G. F. Bottazzo and A. G. Cudworth (1981). "Evidence for a long prediabetic period in type I (insulin-dependent) diabetes mellitus." Lancet **2**(8260-61): 1363-1365.

Gotter, J., B. Brors, M. Hergenhahn and B. Kyewski (2004). "Medullary epithelial cells of the human thymus express a highly diverse selection of tissue-specific genes colocalized in chromosomal clusters." J Exp Med **199**(2): 155-166.

Grant, S. F., H. Q. Qu, J. P. Bradfield, L. Marchand, C. E. Kim, J. T. Glessner, R. Grabs, S. P. Taback, E. C. Frackelton, A. W. Eckert, K. Annaiah, M. L. Lawson, F. G. Otieno, E. Santa, J. L. Shaner, R. M. Smith, R. Skraban, M. Imielinski, R. M. Chiavacci, R. W. Grundmeier, C. A. Stanley, S. E. Kirsch, D. Waggott, A. D. Paterson, D. S. Monos, D. E. R. Group, C. Polychronakos and H. Hakonarson (2009). "Follow-up analysis of genome-wide association data identifies novel loci for type 1 diabetes." Diabetes **58**(1): 290-295.

Green, A., C. C. Patterson, E. T. S. G. Europe and Diabetes (2001). "Trends in the incidence of childhood-onset diabetes in Europe 1989-1998." Diabetologia **44 Suppl 3**: B3-8.

Gregersen, P. K., C. I. Amos, A. T. Lee, Y. Lu, E. F. Remmers, D. L. Kastner, M. F. Seldin, L. A. Criswell, R. M. Plenge, V. M. Holers, T. R. Mikuls, T. Sokka, L. W. Moreland, S. L. Bridges, Jr., G. Xie, A. B. Begovich and K. A. Siminovitch (2009). "REL, encoding a member of the NF-kappaB family of transcription factors, is a newly defined risk locus for rheumatoid arthritis." Nat Genet **41**(7): 820-823.

Groom, A., H. R. Elliott, N. D. Embleton and C. L. Relton (2011). "Epigenetics and child health: basic principles." Arch Dis Child **96**(9): 863-869.

Group, T. S. (2008). "The Environmental Determinants of Diabetes in the Young (TEDDY) Study." Ann N Y Acad Sci **1150**: 1-13.

Grunewald, T. G., S. M. Pasedag and E. Butt (2009). "Cell Adhesion and Transcriptional Activity - Defining the Role of the Novel Protooncogene LPP." Transl Oncol **2**(3): 107-116.

Hagglof, B., L. Blom, G. Dahlquist, G. Lonnberg and B. Sahlin (1991). "The Swedish childhood diabetes study: indications of severe psychological stress as a risk factor for type 1 (insulin-dependent) diabetes mellitus in childhood." Diabetologia **34**(8): 579-583.

Hakonarson, H., S. F. Grant, J. P. Bradfield, L. Marchand, C. E. Kim, J. T. Glessner, R. Grabs, T. Casalunovo, S. P. Taback, E. C. Frackelton, M. L. Lawson, L. J. Robinson, R. Skraban, Y. Lu, R. M. Chiavacci, C. A. Stanley, S. E. Kirsch, E. F. Rappaport, J. S. Orange, D. S. Monos, M.

Devoto, H. Q. Qu and C. Polychronakos (2007). "A genome-wide association study identifies KIAA0350 as a type 1 diabetes gene." Nature **448**(7153): 591-594.

Hansen, C. H., L. Krych, D. S. Nielsen, F. K. Vogensen, L. H. Hansen, S. J. Sorensen, K. Buschard and A. K. Hansen (2012). "Early life treatment with vancomycin propagates Akkermansia muciniphila and reduces diabetes incidence in the NOD mouse." Diabetologia **55**(8): 2285-2294.

Hara, N., A. K. Alkanani, D. Ir, C. E. Robertson, B. D. Wagner, D. N. Frank and D. Zipris (2013). "The role of the intestinal microbiota in type 1 diabetes." Clin Immunol **146**(2): 112-119.

Harjutsalo, V., L. Sjoberg and J. Tuomilehto (2008). "Time trends in the incidence of type 1 diabetes in Finnish children: a cohort study." Lancet **371**(9626): 1777-1782.

Harjutsalo, V., R. Sund, M. Knip and P. H. Groop (2013). "Incidence of type 1 diabetes in Finland." JAMA **310**(4): 427-428.

Helgason, T. and M. R. Jonasson (1981). "Evidence for a food additive as a cause of ketosis-prone diabetes." Lancet **2**(8249): 716-720.

Hermann, R., M. Knip, R. Veijola, O. Simell, A. P. Laine, H. K. Akerblom, P. H. Groop, C. Forsblom, K. Pettersson-Fernholm, J. Ilonen and G. FinnDiane Study (2003). "Temporal changes in the frequencies of HLA genotypes in patients with Type 1 diabetes--indication of an increased environmental pressure?" Diabetologia **46**(3): 420-425.

Herold, K. C., B. N. Bundy, S. A. Long, J. A. Bluestone, L. A. DiMeglio, M. J. Dufort, S. E. Gitelman, P. A. Gottlieb, J. P. Krischer, P. S. Linsley, J. B. Marks, W. Moore, A. Moran, H. Rodriguez, W. E. Russell, D. Schatz, J. S. Skyler, E. Tsalikian, D. K. Wherrett, A. G. Ziegler, C. J. Greenbaum and G. Type 1 Diabetes TrialNet Study (2019). "An Anti-CD3 Antibody, Teplizumab, in Relatives at Risk for Type 1 Diabetes." N Engl J Med **381**(7): 603-613.

Herold, K. C., S. E. Gitelman, U. Masharani, W. Hagopian, B. Bisikirska, D. Donaldson, K. Rother, B. Diamond, D. M. Harlan and J. A. Bluestone (2005). "A single course of anti-CD3 monoclonal antibody hOKT3gamma1(Ala-Ala) results in improvement in C-peptide responses and clinical parameters for at least 2 years after onset of type 1 diabetes." Diabetes **54**(6): 1763-1769.

Heuts, F., N. M. Edner and L. S. Walker (2017). "Follicular T Helper Cells: A New Marker of Type 1 Diabetes Risk?" Diabetes **66**(2): 258-260.

Hex, N., C. Bartlett, D. Wright, M. Taylor and D. Varley (2012). "Estimating the current and future costs of Type 1 and Type 2 diabetes in the UK, including direct health costs and indirect societal and productivity costs." Diabet Med **29**(7): 855-862.

Hirschhorn, J. N. (2003). "Genetic epidemiology of type 1 diabetes." Pediatr Diabetes **4**(2): 87-100.

Holman, N., R. J. Young and W. J. Jeffcoate (2012). "Variation in the recorded incidence of amputation of the lower limb in England." Diabetologia **55**(7): 1919-1925.

Holmberg, H., O. Vaarala, V. Sadauskaite-Kuehne, J. Ilonen, Z. Padaiga and J. Ludvigsson (2006). "Higher prevalence of autoantibodies to insulin and GAD65 in Swedish compared to Lithuanian children with type 1 diabetes." Diabetes Res Clin Pract **72**(3): 308-314.

Holopainen, P., M. Arvas, P. Sistonen, K. Mustalahti, P. Collin, M. Maki and J. Partanen (1999). "CD28/CTLA4 gene region on chromosome 2q33 confers genetic susceptibility to celiac disease. A linkage and family-based association study." Tissue Antigens **53**(5): 470-475.

Holz, A., T. Dyrberg, W. Hagopian, D. Homann, M. von Herrath and M. B. Oldstone (2000). "Neither B lymphocytes nor antibodies directed against self antigens of the islets of Langerhans are required for development of virus-induced autoimmune diabetes." J Immunol **165**(10): 5945-5953.
Hon, G., W. Wang and B. Ren (2009). "Discovery and annotation of functional chromatin signatures in the human genome." PLoS Comput Biol **5**(11): e1000566.
Honeyman, M. C., L. C. Harrison, B. Drummond, P. G. Colman and B. D. Tait (1995). "Analysis of families at risk for insulin-dependent diabetes mellitus reveals that HLA antigens influence progression to clinical disease." Mol Med **1**(5): 576-582.
Hong, J. X., G. L. Wilson, C. H. Fox and J. H. Kehrl (1993). "Isolation and characterization of a novel B-cell activation gene." J Immunol **150**(9): 3895-3904.
Hoppu, S., T. Harkonen, M. S. Ronkainen, S. Simell, A. Hekkala, A. Toivonen, J. Ilonen, O. Simell and M. Knip (2006). "IA-2 antibody isotypes and epitope specificity during the prediabetic process in children with HLA-conferred susceptibility to type I diabetes." Clin Exp Immunol **144**(1): 59-66.
Hoppu, S., M. S. Ronkainen, T. Kimpimaki, S. Simell, S. Korhonen, J. Ilonen, O. Simell and M. Knip (2004). "Insulin autoantibody isotypes during the prediabetic process in young children with increased genetic risk of type 1 diabetes." Pediatr Res **55**(2): 236-242.
Howson, J. M., S. Krause, H. Stevens, D. J. Smyth, J. M. Wenzlau, E. Bonifacio, J. Hutton, A. G. Ziegler, J. A. Todd and P. Achenbach (2012). "Genetic association of zinc transporter 8 (ZnT8) autoantibodies in type 1 diabetes cases." Diabetologia **55**(7): 1978-1984.
Howson, J. M., N. M. Walker, D. Clayton, J. A. Todd and C. Type 1 Diabetes Genetics (2009). "Confirmation of HLA class II independent type 1 diabetes associations in the major histocompatibility complex including HLA-B and HLA-A." Diabetes Obes Metab **11 Suppl 1**: 31-45.
Huang, S. Q., N. Zhang, Z. X. Zhou, C. C. Huang, C. L. Zeng, D. Xiao, C. C. Guo, Y. J. Han, X. H. Ye, X. G. Ye, M. L. Ou, B. H. Zhang, Y. Liu, E. Y. Zeng, G. Yang and C. X. Jing (2017). "Association of LPP and TAGAP Polymorphisms with Celiac Disease Risk: A Meta-Analysis." Int J Environ Res Public Health **14**(2): 171.
Hulme, M. A., C. H. Wasserfall, M. A. Atkinson and T. M. Brusko (2012). "Central role for interleukin-2 in type 1 diabetes." Diabetes **61**(1): 14-22.
Hummel, M., E. Bonifacio, H. E. Naserke and A. G. Ziegler (2002). "Elimination of dietary gluten does not reduce titers of type 1 diabetes-associated autoantibodies in high-risk subjects." Diabetes Care **25**(7): 1111-1116.
Hummel, M., E. Bonifacio, S. Schmid, M. Walter, A. Knopff and A. G. Ziegler (2004). "Brief communication: early appearance of islet autoantibodies predicts childhood type 1 diabetes in offspring of diabetic parents." Ann Intern Med **140**(11): 882-886.
Hummel, S. and A. G. Ziegler (2011). "Early determinants of type 1 diabetes: experience from the BABYDIAB and BABYDIET studies." The American Journal of Clinical Nutrition **94**(suppl_6): 1821S-1823S.
Hunt, K. A., A. Zhernakova, G. Turner, G. A. Heap, L. Franke, M. Bruinenberg, J. Romanos, L. C. Dinesen, A. W. Ryan, D. Panesar, R. Gwilliam, F. Takeuchi, W. M. McLaren, G. K. Holmes, P. D. Howdle, J. R. Walters, D. S. Sanders, R. J. Playford, G. Trynka, C. J. Mulder, M. L. Mearin, W. H. Verbeek, V. Trimble, F. M. Stevens, C. O'Morain, N. P. Kennedy, D. Kelleher,

D. J. Pennington, D. P. Strachan, W. L. McArdle, C. A. Mein, M. C. Wapenaar, P. Deloukas, R. McGinnis, R. McManus, C. Wijmenga and D. A. van Heel (2008). "Newly identified genetic risk variants for celiac disease related to the immune response." Nat Genet **40**(4): 395-402.

Hussen, H. I., M. Persson and T. Moradi (2015). "Maternal overweight and obesity are associated with increased risk of type 1 diabetes in offspring of parents without diabetes regardless of ethnicity." Diabetologia **58**(7): 1464-1473.

Hyoty, H. (2002). "Enterovirus infections and type 1 diabetes." Ann Med **34**(3): 138-147.

Hyttinen, V., J. Kaprio, L. Kinnunen, M. Koskenvuo and J. Tuomilehto (2003). "Genetic liability of type 1 diabetes and the onset age among 22,650 young Finnish twin pairs: a nationwide follow-up study." Diabetes **52**(4): 1052-1055.

Ichikawa, S., N. Fukuhara, H. Katsushima, T. Takahashi, J. Yamamoto, H. Yokoyama, O. Sasaki, O. Fukuhara, J. Nomura, K. Ishizawa, R. Ichinohasama, A. Muto, K. Igarashi and H. Harigae (2014). "Association between BACH2 expression and clinical prognosis in diffuse large B-cell lymphoma." Cancer Sci **105**(4): 437-444.

Insel, R. and J. L. Dunne (2016). "JDRF's vision and strategy for prevention of type 1 diabetes." Pediatr Diabetes **17 Suppl 22**: 87-92.

International Multiple Sclerosis Genetics, C., D. A. Hafler, A. Compston, S. Sawcer, E. S. Lander, M. J. Daly, P. L. De Jager, P. I. de Bakker, S. B. Gabriel, D. B. Mirel, A. J. Ivinson, M. A. Pericak-Vance, S. G. Gregory, J. D. Rioux, J. L. McCauley, J. L. Haines, L. F. Barcellos, B. Cree, J. R. Oksenberg and S. L. Hauser (2007). "Risk alleles for multiple sclerosis identified by a genomewide study." N Engl J Med **357**(9): 851-862.

Islam, S. T., S. Srinivasan and M. E. Craig (2014). "Environmental determinants of type 1 diabetes: a role for overweight and insulin resistance." J Paediatr Child Health **50**(11): 874-879.

Izzo, V., M. Pinelli, N. Tinto, M. V. Esposito, A. Cola, M. P. Sperandeo, F. Tucci, S. Cocozza, L. Greco and L. Sacchetti (2011). "Improving the estimation of celiac disease sibling risk by non-HLA genes." PLoS One **6**(11): e26920.

Jacobsen, L. M., L. Bocchino, C. Evans-Molina, L. DiMeglio, R. Goland, D. M. Wilson, M. A. Atkinson, T. Aye, W. E. Russell, J. M. Wentworth, D. Boulware, S. Geyer and J. M. Sosenko (2020). "The risk of progression to type 1 diabetes is highly variable in individuals with multiple autoantibodies following screening." Diabetologia **63**(3): 588-596.

Jansen, M. A., D. van den Heuvel, K. V. van der Zwet, V. W. Jaddoe, A. Hofman, J. C. Escher, P. L. Fraaij, H. Hooijkaas, M. C. van Zelm and H. A. Moll (2016). "Herpesvirus Infections and Transglutaminase Type 2 Antibody Positivity in Childhood: The Generation R Study." J Pediatr Gastroenterol Nutr **63**(4): 423-430.

Jeong, E. G., Y. H. Soung, J. W. Lee, S. H. Lee, S. W. Nam, J. Y. Lee, N. J. Yoo and S. H. Lee (2006). "ERBB3 kinase domain mutations are rare in lung, breast and colon carcinomas." Int J Cancer **119**(12): 2986-2987.

Jermendy, A., I. Szatmari, A. Korner, A. J. Szabo, P. Toth-Heyn and R. Hermann (2018). "Association between interferon-induced helicase (IFIH1) rs1990760 polymorphism and seasonal variation in the onset of type 1 diabetes mellitus." Pediatr Diabetes **19**(2): 300-304.

Jerram, S. T., M. N. Dang and R. D. Leslie (2017). "The Role of Epigenetics in Type 1 Diabetes." Curr Diab Rep **17**(10): 89.

Jevnikar, Z., M. Rojnik, P. Jamnik, B. Doljak, U. P. Fonovic and J. Kos (2013). "Cathepsin H mediates the processing of talin and regulates migration of prostate cancer cells." J Biol Chem **288**(4): 2201-2209.

Jin, G.-X., Y.-Y. Zhou, L. Yu and Y.-X. Bi (2015). "Correlation between single nucleotide polymorphism of FCRL-3 gene and Graves' disease in Han population of northern Anhui province, China." International Journal of Clinical and Experimental Medicine **8**(8): 12624-12630.

Jin, Y., S. A. Birlea, P. R. Fain, K. Gowan, S. L. Riccardi, P. J. Holland, C. M. Mailloux, A. J. Sufit, S. M. Hutton, A. Amadi-Myers, D. C. Bennett, M. R. Wallace, W. T. McCormack, E. H. Kemp, D. J. Gawkrodger, A. P. Weetman, M. Picardo, G. Leone, A. Taieb, T. Jouary, K. Ezzedine, N. van Geel, J. Lambert, A. Overbeck and R. A. Spritz (2010). "Variant of TYR and autoimmunity susceptibility loci in generalized vitiligo." N Engl J Med **362**(18): 1686-1697.

Johnson, R. K., L. A. Vanderlinden, F. Dong, P. M. Carry, J. Seifert, K. Waugh, H. Shorrosh, T. Fingerlin, B. I. Frohnert, I. V. Yang, K. Kechris, M. Rewers and J. M. Norris (2020). "Longitudinal DNA methylation differences precede type 1 diabetes." Sci Rep **10**(1): 3721.

Johnson, K., R. Wong, K. J. Barriga, G. Klingensmith, A. G. Ziegler, M. J. Rewers and A. K. Steck (2012). "rs11203203 is associated with type 1 diabetes risk in population pre-screened for high-risk HLA-DR,DQ genotypes." Pediatr Diabetes **13**(8): 611-615.

Jostins, L., S. Ripke, R. K. Weersma, R. H. Duerr, D. P. McGovern, K. Y. Hui, J. C. Lee, L. P. Schumm, Y. Sharma, C. A. Anderson, J. Essers, M. Mitrovic, K. Ning, I. Cleynen, E. Theatre, S. L. Spain, S. Raychaudhuri, P. Goyette, Z. Wei, C. Abraham, J. P. Achkar, T. Ahmad, L. Amininejad, A. N. Ananthakrishnan, V. Andersen, J. M. Andrews, L. Baidoo, T. Balschun, P. A. Bampton, A. Bitton, G. Boucher, S. Brand, C. Buning, A. Cohain, S. Cichon, M. D'Amato, D. De Jong, K. L. Devaney, M. Dubinsky, C. Edwards, D. Ellinghaus, L. R. Ferguson, D. Franchimont, K. Fransen, R. Gearry, M. Georges, C. Gieger, J. Glas, T. Haritunians, A. Hart, C. Hawkey, M. Hedl, X. Hu, T. H. Karlsen, L. Kupcinskas, S. Kugathasan, A. Latiano, D. Laukens, I. C. Lawrance, C. W. Lees, E. Louis, G. Mahy, J. Mansfield, A. R. Morgan, C. Mowat, W. Newman, O. Palmieri, C. Y. Ponsioen, U. Potocnik, N. J. Prescott, M. Regueiro, J. I. Rotter, R. K. Russell, J. D. Sanderson, M. Sans, J. Satsangi, S. Schreiber, L. A. Simms, J. Sventoraityte, S. R. Targan, K. D. Taylor, M. Tremelling, H. W. Verspaget, M. De Vos, C. Wijmenga, D. C. Wilson, J. Winkelmann, R. J. Xavier, S. Zeissig, B. Zhang, C. K. Zhang, H. Zhao, I. B. D. G. C. International, M. S. Silverberg, V. Annese, H. Hakonarson, S. R. Brant, G. Radford-Smith, C. G. Mathew, J. D. Rioux, E. E. Schadt, M. J. Daly, A. Franke, M. Parkes, S. Vermeire, J. C. Barrett and J. H. Cho (2012). "Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease." Nature **491**(7422): 119-124.

Kahn, H. S., T. M. Morgan, L. D. Case, D. Dabelea, E. J. Mayer-Davis, J. M. Lawrence, S. M. Marcovina, G. Imperatore and S. f. D. i. Y. S. Group (2009). "Association of type 1 diabetes with month of birth among U.S. youth: The SEARCH for Diabetes in Youth Study." Diabetes Care **32**(11): 2010-2015.

Kass, I., D. E. Hoke, M. G. S. Costa, C. F. Reboul, B. T. Porebski, N. P. Cowieson, H. Leh, E. Pennacchietti, J. McCoey, O. Kleifeld, C. Borri Voltattorni, D. Langley, B. Roome, I. R. Mackay, D. Christ, D. Perahia, M. Buckle, A. Paiardini, D. De Biase and A. M. Buckle (2014). "Cofactor-dependent conformational heterogeneity of GAD65 and its role in

autoimmunity and neurotransmitter homeostasis." Proceedings of the National Academy of Sciences **111**: E2524-E2529.

Katsarou, A., S. Gudbjornsdottir, A. Rawshani, D. Dabelea, E. Bonifacio, B. J. Anderson, L. M. Jacobsen, D. A. Schatz and A. Lernmark (2017). "Type 1 diabetes mellitus." Nat Rev Dis Primers **3**: 17016.

Kaur, S., A. H. Mirza, C. A. Brorsson, T. Floyel, J. Storling, H. B. Mortensen, F. Pociot and G. Hvidoere International Study (2016). "The genetic and regulatory architecture of ERBB3-type 1 diabetes susceptibility locus." Mol Cell Endocrinol **419**: 83-91.

Kawasaki, E. and K. Eguchi (2004). "Is Type 1 diabetes in the Japanese population the same as among Caucasians?" Ann N Y Acad Sci **1037**: 96-103.

Kawasaki, E., M. Uga, K. Nakamura, G. Kuriya, T. Satoh, K. Fujishima, M. Ozaki, N. Abiru, H. Yamasaki, J. M. Wenzlau, H. W. Davidson, J. C. Hutton and K. Eguchi (2008). "Association between anti-ZnT8 autoantibody specificities and SLC30A8 Arg325Trp variant in Japanese patients with type 1 diabetes." Diabetologia **51**(12): 2299-2302.

Kemppainen, K. M., A. N. Ardissone, A. G. Davis-Richardson, J. R. Fagen, K. A. Gano, L. G. Leon-Novelo, K. Vehik, G. Casella, O. Simell, A. G. Ziegler, M. J. Rewers, A. Lernmark, W. Hagopian, J. X. She, J. P. Krischer, B. Akolkar, D. A. Schatz, M. A. Atkinson, E. W. Triplett and T. S. Group (2015). "Early childhood gut microbiomes show strong geographic differences among subjects at high risk for type 1 diabetes." Diabetes Care **38**(2): 329-332.

Kennedy, G. C., M. S. German and W. J. Rutter (1995). "The minisatellite in the diabetes susceptibility locus IDDM2 regulates insulin transcription." Nat Genet **9**(3): 293-298.

Kharroubi, A. T. and H. M. Darwish (2015). "Diabetes mellitus: The epidemic of the century." World J Diabetes **6**(6): 850-867.

Khong, J. J., K. P. Burdon, Y. Lu, K. Laurie, L. Leonardos, P. N. Baird, S. Sahebjada, J. P. Walsh, A. Gajdatsy, P. R. Ebeling, P. S. Hamblin, R. Wong, S. P. Forehan, S. Fourlanos, A. P. Roberts, M. Doogue, D. Selva, G. W. Montgomery, S. Macgregor and J. E. Craig (2016). "Pooled genome wide association detects association upstream of FCRL3 with Graves' disease." BMC Genomics **17**(1): 939.

Kikutani, H. and S. Makino (1992). "The murine autoimmune diabetes model: NOD and related strains." Adv Immunol **51**: 285-322.

Kim, S. J., D. G. Jeong, S. K. Jeong, T. S. Yoon and S. E. Ryu (2007). "Crystal structure of the major diabetes autoantigen insulinoma-associated protein 2 reveals distinctive immune epitopes." Diabetes **56**(1): 41-48.

Kimpimäki, T., A. Kupila, A. M. Hämäläinen, M. Kukko, P. Kulmala, K. Savola, T. Simell, P. Keskinen, J. Ilonen, O. Simell and M. Knip (2001). "The First Signs of β-Cell Autoimmunity Appear in Infancy in Genetically Susceptible Children from the General Population: The Finnish Type 1 Diabetes Prediction and Prevention Study." The Journal of Clinical Endocrinology & Metabolism **86**(10): 4782-4788.

Kirchhoff, K., F. Machicao, A. Haupt, S. A. Schafer, O. Tschritter, H. Staiger, N. Stefan, H. U. Haring and A. Fritsche (2008). "Polymorphisms in the TCF7L2, CDKAL1 and SLC30A8 genes are associated with impaired proinsulin conversion." Diabetologia **51**(4): 597-601.

Knip, M. (2002). "Can we predict type 1 diabetes in the general population?" Diabetes Care **25**(3): 623-625.

Knip, M., M. Kukko, P. Kulmala, R. Veijola, O. Simell, H. K. Akerblom and J. Ilonen (2002). "Humoral beta-cell autoimmunity in relation to HLA-defined disease susceptibility in preclinical and clinical type 1 diabetes." Am J Med Genet **115**(1): 48-54.

Knip, M., H. Siljander, J. Ilonen, O. Simell and R. Veijola (2016). "Role of humoral beta-cell autoimmunity in type 1 diabetes." Pediatr Diabetes **17 Suppl 22**: 17-24.

Knip, M., R. Veijola, S. M. Virtanen, H. Hyoty, O. Vaarala and H. K. Akerblom (2005). "Environmental triggers and determinants of type 1 diabetes." Diabetes **54 Suppl 2**: S125-136.

Kochi, Y., K. Myouzen, R. Yamada, A. Suzuki, T. Kurosaki, Y. Nakamura and K. Yamamoto (2009). "FCRL3, an autoimmune susceptibility gene, has inhibitory potential on B-cell receptor-mediated signaling." J Immunol **183**(9): 5502-5510.

Kochi, Y., R. Yamada, A. Suzuki, J. B. Harley, S. Shirasawa, T. Sawada, S. C. Bae, S. Tokuhiro, X. Chang, A. Sekine, A. Takahashi, T. Tsunoda, Y. Ohnishi, K. M. Kaufman, C. P. Kang, C. Kang, S. Otsubo, W. Yumura, A. Mimori, T. Koike, Y. Nakamura, T. Sasazuki and K. Yamamoto (2005). "A functional variant in FCRL3, encoding Fc receptor-like 3, is associated with rheumatoid arthritis and several autoimmunities." Nat Genet **37**(5): 478-485.

Kohner, E., J. Allwinkle, J. Andrews, R. Baker, F. Brown, H. Cheng, M. Gray, S. Grindey, I. Koppel, B. Martin, J. Reckless, D. Rothman, M. Sculpher, F. Talbot, N. Vaughan and J. Wilkinson (1996). "Report of the Visual Handicap Group." Diabet Med **13**(9 Suppl 4): S13-26.

Kometani, K., R. Nakagawa, R. Shinnakasu, T. Kaji, A. Rybouchkin, S. Moriyama, K. Furukawa, H. Koseki, T. Takemori and T. Kurosaki (2013). "Repression of the transcription factor Bach2 contributes to predisposition of IgG1 memory B cells toward plasma cell differentiation." Immunity **39**(1): 136-147.

Kontiainen, S., T. Scheinin, A. Schlenzka, J. Maenpaa, L. Groop and S. Koskimies (1988). "Differences in HLA types in children with insulin-dependent diabetes diagnosed in 1960s, 1970s, and 1980s." Lancet **2**(8604): 219.

Krischer, J. P., X. Liu, K. Vehik, B. Akolkar, W. A. Hagopian, M. J. Rewers, J. X. She, J. Toppari, A. G. Ziegler, A. Lernmark and T. S. Group (2019). "Predicting IsleT-cell Autoimmunity and Type 1 Diabetes: An 8-Year TEDDY Study Progress Report." Diabetes Care **42**(6): 1051-1060.

Krischer, J. P., K. F. Lynch, A. Lernmark, W. A. Hagopian, M. J. Rewers, J. X. She, J. Toppari, A. G. Ziegler, B. Akolkar and T. S. Group (2017). "Genetic and Environmental Interactions Modify the Risk of Diabetes-Related Autoimmunity by 6 Years of Age: The TEDDY Study." Diabetes Care **40**(9): 1194-1202.

Krischer, J. P., K. F. Lynch, D. A. Schatz, J. Ilonen, A. Lernmark, W. A. Hagopian, M. J. Rewers, J. X. She, O. G. Simell, J. Toppari, A. G. Ziegler, B. Akolkar, E. Bonifacio and T. S. Group (2015). "The 6 year incidence of diabetes-associated autoantibodies in genetically at-risk children: the TEDDY study." Diabetologia **58**(5): 980-987.

Korsgren, O. Skog and K. Dahl-Jorgensen (2016). "Insulitis and characterisation of infiltrating T-cells in surgical pancreatic tail resections from patients at onset of type 1 diabetes." Diabetologia **59**(3): 492-501.

Krogvold, L., B. Edwin, T. Buanes, G. Frisk, O. Skog, M. Anagandula, O. Korsgren, D. Undlien, M. C. Eike, S. J. Richardson, P. Leete, N. G. Morgan, S. Oikarinen, M. Oikarinen, J. E. Laiho, H. Hyoty, J. Ludvigsson, K. F. Hanssen and K. Dahl-Jorgensen (2015). "Detection of a low-

grade enteroviral infection in the islets of langerhans of living patients newly diagnosed with type 1 diabetes." Diabetes **64**(5): 1682-1687.
Kumar, H., T. Kawai and S. Akira (2009). "Pathogen recognition in the innate immune response." Biochem J **420**(1): 1-16.
Kutlu, B., D. Burdick, D. Baxter, J. Rasschaert, D. Flamez, D. L. Eizirik, N. Welsh, N. Goodman and L. Hood (2009). "Detailed transcriptome atlas of the pancreatic beta cell." BMC Med Genomics **2**: 3.
Kyogoku, C., C. D. Langefeld, W. A. Ortmann, A. Lee, S. Selby, V. E. Carlton, M. Chang, P. Ramos, E. C. Baechler, F. M. Batliwalla, J. Novitzke, A. H. Williams, C. Gillett, P. Rodine, R. R. Graham, K. G. Ardlie, P. M. Gaffney, K. L. Moser, M. Petri, A. B. Begovich, P. K. Gregersen and T. W. Behrens (2004). "Genetic association of the R620W polymorphism of protein tyrosine phosphatase PTPN22 with human SLE." Am J Hum Genet **75**(3): 504-507.
Kyvik, K. O., A. Green and H. Beck-Nielsen (1995). "Concordance rates of insulin dependent diabetes mellitus: a population based study of young Danish twins." BMJ **311**(7010): 913-917.
Lamb, M. M., X. Yin, G. O. Zerbe, G. J. Klingensmith, D. Dabelea, T. E. Fingerlin, M. Rewers and J. M. Norris (2009). "Height growth velocity, islet autoimmunity and type 1 diabetes development: the Diabetes Autoimmunity Study in the Young." Diabetologia **52**(10): 2064-2071.
Lambert, A. P., K. M. Gillespie, G. Thomson, H. J. Cordell, J. A. Todd, E. A. Gale and P. J. Bingley (2004). "Absolute risk of childhood-onset type 1 diabetes defined by human leukocyte antigen class II genotype: a population-based study in the United Kingdom." J Clin Endocrinol Metab **89**(8): 4037-4043.
Lampasona, V., M. Bearzatto, S. Genovese, E. Bosi, M. Ferrari and E. Bonifacio (1996). "Autoantibodies in insulin-dependent diabetes recognize distinct cytoplasmic domains of the protein tyrosine phosphatase-like IA-2 autoantigen." J Immunol **157**(6): 2707-2711.
Lampasona, V. and D. J. C. D. R. Liberati (2016). "Islet Autoantibodies." **16**(6): 53.
Lampasona, V., M. Schlosser, P. W. Mueller, A. J. Williams, J. M. Wenzlau, J. C. Hutton and P. Achenbach (2011). "Diabetes antibody standardization program: first proficiency evaluation of assays for autoantibodies to zinc transporter 8." Clin Chem **57**(12): 1693-1702.
Larsen, C. E. and C. A. Alper (2004). "The genetics of HLA-associated disease." Curr Opin Immunol **16**(5): 660-667.
Lavrikova, E. Y., A. G. Nikitin, T. L. Kuraeva, V. A. Peterkova, N. M. Tsitlidze, D. A. Chistiakov and V. V. Nosikov (2011). "The carriage of the type 1 diabetes-associated R262W variant of human LNK correlates with increased proliferation of peripheral blood monocytes in diabetic patients." Pediatr Diabetes **12**(2): 127-132.
Ledford, H. (2008). "Language: Disputed definitions." Nature **455**(7216): 1023-1028.
Leete, P., A. Willcox, L. Krogvold, K. Dahl-Jorgensen, A. K. Foulis, S. J. Richardson and N. G. Morgan (2016). "Differential Insulitic Profiles Determine the Extent of beta-Cell Destruction and the Age at Onset of Type 1 Diabetes." Diabetes **65**(5): 1362-1369.
Lemaire, K., F. Chimienti and F. Schuit (2012). "Zinc transporters and their role in the pancreatic beta-cell." J Diabetes Investig **3**(3): 202-211.

Lettre, G. and J. D. Rioux (2008). "Autoimmune diseases: insights from genome-wide association studies." Hum Mol Genet **17**(R2): R116-121.

Li, E. (2002). "Chromatin modification and epigenetic reprogramming in mammalian development." Nat Rev Genet **3**(9): 662-673.

Liberati, D., R. C. Wyatt, C. Brigatti, I. Marzinotto, M. Ferrari, E. Bazzigaluppi, E. Bosi, B. T. Gillard, K. M. Gillespie, F. Gorus, I. Weets, E. Balti, L. Piemonti, P. Achenbach, A. J. K. Williams and V. Lampasona (2018). "A novel LIPS assay for insulin autoantibodies." Acta Diabetol **55**(3): 263-270.

Libman, I. M., E. Barinas-Mitchell, S. Marcovina, F. Bacha, T. Hannon, H. Tfayli, S. J. Lee, S. Bansal, R. Robertson and S. Arslanian (2011). "β-Cell autoimmunity in overweight non-diabetic youth: any implications?" Pediatric diabetes **12**(3 Pt 2): 207-211.

Lichti-Kaiser, K., G. ZeRuth and A. M. Jetten (2014). "Transcription Factor Gli-Similar 3 (Glis3): Implications for the Development of Congenital Hypothyroidism." J Endocrinol Diabetes Obes **2**(2): 1024.

Lin, X., Y. Zhang and Q. Chen (2016). "FCRL3 gene polymorphisms as risk factors for rheumatoid arthritis." Hum Immunol **77**(2): 223-229.

Lipman, T. H., Y. Chang and K. M. Murphy (2002). "The epidemiology of type 1 diabetes in children in Philadelphia 1990-1994: evidence of an epidemic." Diabetes Care **25**(11): 1969-1975.

Lis, J., A. Jarzab and D. Witkowska (2012). "[Molecular mimicry in the etiology of autoimmune diseases]." Postepy Hig Med Dosw (Online) **66**: 475-491.

Liu, J. Z., S. van Sommeren, H. Huang, S. C. Ng, R. Alberts, A. Takahashi, S. Ripke, J. C. Lee, L. Jostins, T. Shah, S. Abedian, J. H. Cheon, J. Cho, N. E. Dayani, L. Franke, Y. Fuyuno, A. Hart, R. C. Juyal, G. Juyal, W. H. Kim, A. P. Morris, H. Poustchi, W. G. Newman, V. Midha, T. R. Orchard, H. Vahedi, A. Sood, J. Y. Sung, R. Malekzadeh, H. J. Westra, K. Yamazaki, S. K. Yang, C. International Multiple Sclerosis Genetics, I. B. D. G. C. International, J. C. Barrett, B. Z. Alizadeh, M. Parkes, T. Bk, M. J. Daly, M. Kubo, C. A. Anderson and R. K. Weersma (2015). "Association analyses identify 38 susceptibility loci for inflammatory bowel disease and highlight shared genetic risk across populations." Nat Genet **47**(9): 979-986.

Liu, R., W. A. Paxton, S. Choe, D. Ceradini, S. R. Martin, R. Horuk, M. E. MacDonald, H. Stuhlmann, R. A. Koup and N. R. Landau (1996). "Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection." Cell **86**(3): 367-377.

Long, A. E., K. M. Gillespie, R. J. Aitken, J. C. Goode, P. J. Bingley and A. J. Williams (2013). "Humoral responses to islet antigen-2 and zinc transporter 8 are attenuated in patients carrying HLA-A*24 alleles at the onset of type 1 diabetes." Diabetes **62**(6): 2067-2071.

Long, A. E., K. M. Gillespie, S. Rokni, P. J. Bingley and A. J. Williams (2012). "Rising incidence of type 1 diabetes is associated with altered immunophenotype at diagnosis." Diabetes **61**(3): 683-686.

Long, A. E., A. T. Gooneratne, S. Rokni, A. J. Williams and P. J. Bingley (2012). "The role of autoantibodies to zinc transporter 8 in prediction of type 1 diabetes in relatives: lessons from the European Nicotinamide Diabetes Intervention Trial (ENDIT) cohort." J Clin Endocrinol Metab **97**(2): 632-637.

Long, A. E., I. V. Wilson, D. J. Becker, I. M. Libman, V. C. Arena, F. S. Wong, A. K. Steck, M. J. Rewers, L. Yu, P. Achenbach, R. Casas, J. Ludvigsson, A. J. K. Williams and K. M. Gillespie (2018). "Characteristics of slow progression to diabetes in multiple islet autoantibody-positive

individuals from five longitudinal cohorts: the SNAIL study." Diabetologia **61**(6): 1484-1490.

Ludvigsson, J., U. Gustafsson-Stolt, P. E. Liss, T. Svensson and A. S. Group (2002). "Mothers of children in ABIS, a population-based screening for prediabetes, experience few ethical conflicts and have a positive attitude." Ann N Y Acad Sci **958**: 376-381.

Luster, A. D. (1998). "Chemokines--chemotactic cytokines that mediate inflammation." N Engl J Med **338**(7): 436-445.

Lynch, B. L., J. Dean, D. Brady and C. De Gascun (2018). "Adenovirus Type 4 Respiratory Infections among Civilian Adults, Northeastern United States, 2011-2015." Emerg Infect Dis **24**(7): 1392-1393.

Maahs, D. M., N. A. West, J. M. Lawrence and E. J. Mayer-Davis (2010). "Epidemiology of type 1 diabetes." Endocrinol Metab Clin North Am **39**(3): 481-497.

Maffeis, C., A. Martina, M. Corradi, S. Quarella, N. Nori, S. Torriani, M. Plebani, G. Contreas and G. E. Felis (2016). "Association between intestinal permeability and faecal microbiota composition in Italian children with beta cell autoimmunity at risk for type 1 diabetes." Diabetes Metab Res Rev **32**(7): 700-709.

Malek, T. R. and I. Castro (2010). "Interleukin-2 receptor signaling: at the interface between tolerance and immunity." Immunity **33**(2): 153-165.

Mally, M. I., V. Cirulli, T. Otonkoski, G. Soto and A. Hayek (1996). "Ontogeny and tissue distribution of human GAD expression." Diabetes **45**(4): 496-501.

Marroqui, L., I. Santin, R. S. Dos Santos, L. Marselli, P. Marchetti and D. L. Eizirik (2014). "BACH2, a candidate risk gene for type 1 diabetes, regulates apoptosis in pancreatic beta-cells via JNK1 modulation and crosstalk with the candidate gene PTPN2." Diabetes **63**(7): 2516-2527.

Martin, S., D. Wolf-Eichbaum, G. Duinkerken, W. A. Scherbaum, H. Kolb, J. G. Noordzij and B. O. Roep (2001). "Development of type 1 diabetes despite severe hereditary B-cell deficiency." N Engl J Med **345**(14): 1036-1040.

Martino, G., L. M. Grimaldi, E. Bazzigaluppi, N. Passini, F. Sinigaglia and L. Rogge (1996). "The insulin-dependent diabetes mellitus-associated ICA 105 autoantigen in stiff-man syndrome patients." J Neuroimmunol **69**(1-2): 129-134.

McKeon, A., M. T. Robinson, K. M. McEvoy, J. Y. Matsumoto, V. A. Lennon, J. E. Ahlskog and S. J. Pittock (2012). "Stiff-man syndrome and variants: clinical course, treatments, and outcomes." Arch Neurol **69**(2): 230-238.

Mbunwe, E., B. J. Van der Auwera, I. Vermeulen, S. Demeester, A. Van Dalem, E. V. Balti, S. Van Aken, L. Derdelinckx, H. Dorchy, J. De Schepper, C. van Schravendijk, J. M. Wenzlau, J. C. Hutton, D. Pipeleers, I. Weets, F. K. Gorus and R. Belgian Diabetes (2013). "HLA-A*24 is an independent predictor of 5-year progression to diabetes in autoantibody-positive first-degree relatives of type 1 diabetic patients." Diabetes **62**(4): 1345-1350.

McKinney, P. A., R. Parslow, K. Gurney, G. Law, H. J. Bodansky and D. R. Williams (1997). "Antenatal risk factors for childhood diabetes mellitus; a case-control study of medical record data in Yorkshire, UK." Diabetologia **40**(8): 933-939.

McLaughlin, K. A., C. C. Richardson, A. Ravishankar, C. Brigatti, D. Liberati, V. Lampasona, L. Piemonti, D. Morgan, R. G. Feltbower and M. R. Christie (2016). "Identification of

Tetraspanin-7 as a Target of Autoantibodies in Type 1 Diabetes." Diabetes **65**(6): 1690-1698.
McLaughlin, K. A., C. C. Richardson, S. Williams, E. Bonifacio, D. Morgan, R. G. Feltbower, M. Powell, B. Rees Smith, J. Furmaniak and M. R. Christie (2015). "Relationships between major epitopes of the IA-2 autoantigen in Type 1 diabetes: Implications for determinant spreading." Clin Immunol **160**(2): 226-236.
Mehers, K. L. and K. M. Gillespie (2008). "The genetic basis for type 1 diabetes." Br Med Bull **88**(1): 115-129.
Miao, D., K. M. Guyer, F. Dong, L. Jiang, A. K. Steck, M. Rewers, G. S. Eisenbarth and L. Yu (2013). "GAD65 autoantibodies detected by electrochemiluminescence assay identify high risk for type 1 diabetes." Diabetes **62**(12): 4174-4178.
Mikk, M. L., T. Heikkinen, M. I. El-Amir, M. Kiviniemi, A. P. Laine, T. Harkonen, R. Veijola, J. Toppari, M. Knip, J. Ilonen and R. Finnish Paediatric Diabetes (2017). "The association of the HLA-A*24:02, B*39:01 and B*39:06 alleles with type 1 diabetes is restricted to specific HLA-DR/DQ haplotypes in Finns." HLA **89**(4): 215-224.
Mikkelsen, K. H., F. K. Knop, T. Vilsboll, M. Frost, J. Hallas and A. Pottegard (2017). "Use of antibiotics in childhood and risk of Type 1 diabetes: a population-based case-control study." Diabet Med **34**(2): 272-277.
Mlynarski, W. M., G. P. Placha, P. P. Wolkow, J. P. Bochenski, J. H. Warram and A. S. Krolewski (2005). "Risk of diabetic nephropathy in type 1 diabetes is associated with functional polymorphisms in RANTES receptor gene (CCR5): a sex-specific effect." Diabetes **54**(11): 3331-3335.
Mohan, J. F. and E. R. Unanue (2012). "Unconventional recognition of peptides by T-cells and the implications for autoimmunity." Nat Rev Immunol **12**(10): 721-728.
Moore, F., M. L. Colli, M. Cnop, M. I. Esteve, A. K. Cardozo, D. A. Cunha, M. Bugliani, P. Marchetti and D. L. Eizirik (2009). "PTPN2, a candidate gene for type 1 diabetes, modulates interferon-gamma-induced pancreatic beta-cell apoptosis." Diabetes **58**(6): 1283-1291.
Morgan, N. G. and S. J. Richardson (2014). "Enteroviruses as causative agents in type 1 diabetes: loose ends or lost cause?" Trends Endocrinol Metab **25**(12): 611-619.
Morris, A. P., B. F. Voight, T. M. Teslovich, T. Ferreira, A. V. Segre, V. Steinthorsdottir, R. J. Strawbridge, H. Khan, H. Grallert, A. Mahajan, I. Prokopenko, H. M. Kang, C. Dina, T. Esko, R. M. Fraser, S. Kanoni, A. Kumar, V. Lagou, C. Langenberg, J. Luan, C. M. Lindgren, M. Muller-Nurasyid, S. Pechlivanis, N. W. Rayner, L. J. Scott, S. Wiltshire, L. Yengo, L. Kinnunen, E. J. Rossin, S. Raychaudhuri, A. D. Johnson, A. S. Dimas, R. J. Loos, S. Vedantam, H. Chen, J. C. Florez, C. Fox, C. T. Liu, D. Rybin, D. J. Couper, W. H. Kao, M. Li, M. C. Cornelis, P. Kraft, Q. Sun, R. M. van Dam, H. M. Stringham, P. S. Chines, K. Fischer, P. Fontanillas, O. L. Holmen, S. E. Hunt, A. U. Jackson, A. Kong, R. Lawrence, J. Meyer, J. R. Perry, C. G. Platou, S. Potter, E. Rehnberg, N. Robertson, S. Sivapalaratnam, A. Stancakova, K. Stirrups, G. Thorleifsson, E. Tikkanen, A. R. Wood, P. Almgren, M. Atalay, R. Benediktsson, L. L. Bonnycastle, N. Burtt, J. Carey, G. Charpentier, A. T. Crenshaw, A. S. Doney, M. Dorkhan, S. Edkins, V. Emilsson, E. Eury, T. Forsen, K. Gertow, B. Gigante, G. B. Grant, C. J. Groves, C. Guiducci, C. Herder, A. B. Hreidarsson, J. Hui, A. James, A. Jonsson, W. Rathmann, N. Klopp, J. Kravic, K. Krjutskov, C. Langford, K. Leander, E. Lindholm, S. Lobbens, S. Mannisto, G. Mirza, T. W. Muhleisen, B. Musk, M. Parkin, L. Rallidis, J. Saramies, B. Sennblad, S. Shah,

G. Sigurethsson, A. Silveira, G. Steinbach, B. Thorand, J. Trakalo, F. Veglia, R. Wennauer, W. Winckler, D. Zabaneh, H. Campbell, C. van Duijn, A. G. Uitterlinden, A. Hofman, E. Sijbrands, G. R. Abecasis, K. R. Owen, E. Zeggini, M. D. Trip, N. G. Forouhi, A. C. Syvanen, J. G. Eriksson, L. Peltonen, M. M. Nothen, B. Balkau, C. N. Palmer, V. Lyssenko, T. Tuomi, B. Isomaa, D. J. Hunter, L. Qi, C. Wellcome Trust Case Control, G. Meta-Analyses of, I. Insulin-related traits Consortium, A. T. C. Genetic Investigation of, C. Asian Genetic Epidemiology Network-Type 2 Diabetes, C. South Asian Type 2 Diabetes, A. R. Shuldiner, M. Roden, I. Barroso, T. Wilsgaard, J. Beilby, K. Hovingh, J. F. Price, J. F. Wilson, R. Rauramaa, T. A. Lakka, L. Lind, G. Dedoussis, I. Njolstad, N. L. Pedersen, K. T. Khaw, N. J. Wareham, S. M. Keinanen-Kiukaanniemi, T. E. Saaristo, E. Korpi-Hyovalti, J. Saltevo, M. Laakso, J. Kuusisto, A. Metspalu, F. S. Collins, K. L. Mohlke, R. N. Bergman, J. Tuomilehto, B. O. Boehm, C. Gieger, K. Hveem, S. Cauchi, P. Froguel, D. Baldassarre, E. Tremoli, S. E. Humphries, D. Saleheen, J. Danesh, E. Ingelsson, S. Ripatti, V. Salomaa, R. Erbel, K. H. Jockel, S. Moebus, A. Peters, T. Illig, U. de Faire, A. Hamsten, A. D. Morris, P. J. Donnelly, T. M. Frayling, A. T. Hattersley, E. Boerwinkle, O. Melander, S. Kathiresan, P. M. Nilsson, P. Deloukas, U. Thorsteinsdottir, L. C. Groop, K. Stefansson, F. Hu, J. S. Pankow, J. Dupuis, J. B. Meigs, D. Altshuler, M. Boehnke, M. I. McCarthy, D. I. G. Replication and C. Meta-analysis (2012). "Large-scale association analysis provides insights into the genetic architecture and pathophysiology of type 2 diabetes." Nat Genet **44**(9): 981-990.

Mujoo, K., B. K. Choi, Z. Huang, N. Zhang and Z. An (2014). "Regulation of ERBB3/HER3 signaling in cancer." Oncotarget **5**(21): 10222-10236.

Myhr, C. B., M. A. Hulme, C. H. Wasserfall, P. J. Hong, P. S. Lakshmi, D. A. Schatz, M. J. Haller, T. M. Brusko and M. A. Atkinson (2013). "The autoimmune disease-associated SNP rs917997 of IL18RAP controls IFNgamma production by PBMC." J Autoimmun **44**: 8-12.

Mziaut, H., S. Kersting, K.-P. Knoch, W.-H. Fan, M. Trajkovski, K. Erdmann, H. Bergert, F. Ehehalt, H.-D. Saeger and M. Solimena (2008). "ICA512 signaling enhances pancreatic β-cell proliferation by regulating cyclins D through STATs." **105**(2): 674-679.

Nagata, S., T. Ise and I. Pastan (2009). "Fc receptor-like 3 protein expressed on IL-2 nonresponsive subset of human regulatory T-cells." J Immunol **182**(12): 7518-7526.

Nakanishi, K., T. Kobayashi, T. Murase, T. Nakatsuji, H. Inoko, K. Tsuji and K. Kosaka (1993). "Association of HLA-A24 With Complete β-Cell Destruction in IDDM." Diabetes **42**(7): 1086-1093.

Nakanishi, K., T. Kobayashi, T. Murase, T. Naruse, Y. Nose and H. Inoko (1999). "Human leukocyte antigen-A24 and -DQA1*0301 in Japanese insulin-dependent diabetes mellitus: independent contributions to susceptibility to the disease and additive contributions to acceleration of beta-cell destruction." J Clin Endocrinol Metab **84**(10): 3721-3725.

Nakayama, M., N. Abiru, H. Moriyama, N. Babaya, E. Liu, D. Miao, L. Yu, D. R. Wegmann, J. C. Hutton, J. F. Elliott and G. S. Eisenbarth (2005). "Prime role for an insulin epitope in the development of type 1 diabetes in NOD mice." Nature **435**(7039): 220-223.

Naserke, H. E., A. G. Ziegler, V. Lampasona and E. Bonifacio (1998). "Early development and spreading of autoantibodies to epitopes of IA-2 and their association with progression to type 1 diabetes." J Immunol **161**(12): 6963-6969.

Nejentsev, S., J. D. Cooper, L. Godfrey, J. M. Howson, H. Rance, S. Nutland, N. M. Walker, C. Guja, C. Ionescu-Tirgoviste, D. A. Savage, D. E. Undlien, K. S. Ronningen, E. Tuomilehto-Wolf, J.

Tuomilehto, K. M. Gillespie, S. M. Ring, D. P. Strachan, B. Widmer, D. Dunger and J. A. Todd (2004). "Analysis of the vitamin D receptor gene sequence variants in type 1 diabetes." Diabetes **53**(10): 2709-2712.

Nejentsev, S., J. M. Howson, N. M. Walker, J. Szeszko, S. F. Field, H. E. Stevens, P. Reynolds, M. Hardy, E. King, J. Masters, J. Hulme, L. M. Maier, D. Smyth, R. Bailey, J. D. Cooper, G. Ribas, R. D. Campbell, D. G. Clayton, J. A. Todd and C. Wellcome Trust Case Control (2007). "Localization of type 1 diabetes susceptibility to the MHC class I genes HLA-B and HLA-A." Nature **450**(7171): 887-892.

Nejentsev, S., N. Walker, D. Riches, M. Egholm and J. A. Todd (2009). "Rare variants of IFIH1, a gene implicated in antiviral responses, protect against type 1 diabetes." Science **324**(5925): 387-389.

Nerup, J., P. Platz, O. O. Andersen, M. Christy, J. Lyngsoe, J. E. Poulsen, L. P. Ryder, L. S. Nielsen, M. Thomsen and A. Svejgaard (1974). "HL-A antigens and diabetes mellitus." Lancet **2**(7885): 864-866.

Nistico, L., R. Buzzetti, L. E. Pritchard, B. Van der Auwera, C. Giovannini, E. Bosi, M. T. Larrad, M. S. Rios, C. C. Chow, C. S. Cockram, K. Jacobs, C. Mijovic, S. C. Bain, A. H. Barnett, C. L. Vandewalle, F. Schuit, F. K. Gorus, R. Tosi, P. Pozzilli and J. A. Todd (1996). "The CTLA-4 gene region of chromosome 2q33 is linked to, and associated with, type 1 diabetes. Belgian Diabetes Registry." Hum Mol Genet **5**(7): 1075-1080.

Noble, J. A., A. M. Valdes, T. L. Bugawan, R. J. Apple, G. Thomson and H. A. Erlich (2002). "The HLA class I A locus affects susceptibility to type 1 diabetes." Hum Immunol **63**(8): 657-664.

Noble, J. A., A. M. Valdes, M. Cook, W. Klitz, G. Thomson and H. A. Erlich (1996). "The role of HLA class II genes in insulin-dependent diabetes mellitus: molecular analysis of 180 Caucasian, multiplex families." Am J Hum Genet **59**(5): 1134-1148.

Nogueira, T. C., F. M. Paula, O. Villate, M. L. Colli, R. F. Moura, D. A. Cunha, L. Marselli, P. Marchetti, M. Cnop, C. Julier and D. L. Eizirik (2013). "GLIS3, a susceptibility gene for type 1 and type 2 diabetes, modulates pancreatic beta cell apoptosis via regulation of a splice variant of the BH3-only protein Bim." PLoS Genet **9**(5): e1003532.

Nokoff, N. J., M. Rewers and M. Cree Green (2012). "The interplay of autoimmunity and insulin resistance in type 1 diabetes." Discov Med **13**(69): 115-122.

Noorchashm, H., Y. K. Lieu, N. Noorchashm, S. Y. Rostami, S. A. Greeley, A. Schlachterman, H. K. Song, L. E. Noto, A. M. Jevnikar, C. F. Barker and A. Naji (1999). "I-Ag7-mediated antigen presentation by B lymphocytes is critical in overcoming a checkpoint in T-cell tolerance to islet beta cells of nonobese diabetic mice." J Immunol **163**(2): 743-750.

Norris, J. M., K. Barriga, G. Klingensmith, M. Hoffman, G. S. Eisenbarth, H. A. Erlich and M. Rewers (2003). "Timing of initial cereal exposure in infancy and risk of islet autoimmunity." JAMA **290**(13): 1713-1720.

Olmos, P., R. A'Hern, D. A. Heaton, B. A. Millward, D. Risley, D. A. Pyke and R. D. Leslie (1988). "The significance of the concordance rate for type 1 (insulin-dependent) diabetes in identical twins." Diabetologia **31**(10): 747-750.

Olsson, A. H., P. Volkov, K. Bacos, T. Dayeh, E. Hall, E. A. Nilsson, C. Ladenvall, T. Ronn and C. Ling (2014). "Genome-wide associations between genetic and epigenetic variation influence

mRNA expression and insulin secretion in human pancreatic islets." PLoS Genet **10**(11): e1004735.

Onkamo, P., S. Vaananen, M. Karvonen and J. Tuomilehto (1999). "Worldwide increase in incidence of Type I diabetes--the analysis of the data on published incidence trends." Diabetologia **42**(12): 1395-1403.

Ono, A., K. Kono, D. Ikebe, A. Muto, J. Sun, M. Kobayashi, K. Ueda, J. V. Melo, K. Igarashi and S. Tashiro (2007). "Nuclear positioning of the BACH2 gene in BCR-ABL positive leukemic cells." Genes Chromosomes Cancer **46**(1): 67-74.

Onuma, H., R. Kawamura, Y. Tabara, M. Yamashita, J. Ohashi, E. Kawasaki, A. Imagawa, Y. Yamada, D. Chujo, K. Takahashi, T. Suehiro, Y. Takata, H. Osawa and H. Makino (2019). "Variants in the BACH2 and CLEC16A gene might be associated with susceptibility to insulin-triggered type 1 diabetes." J Diabetes Investig **10**(6): 1447-1453.

Orban, T., B. Bundy, D. J. Becker, L. A. DiMeglio, S. E. Gitelman, R. Goland, P. A. Gottlieb, C. J. Greenbaum, J. B. Marks, R. Monzavi, A. Moran, P. Raskin, H. Rodriguez, W. E. Russell, D. Schatz, D. Wherrett, D. M. Wilson, J. P. Krischer, J. S. Skyler and G. Type 1 Diabetes TrialNet Abatacept Study (2011). "Co-stimulation modulation with abatacept in patients with recent-onset type 1 diabetes: a randomised, double-blind, placebo-controlled trial." Lancet **378**(9789): 412-419.

Ostman, J., G. Lonnberg, H. J. Arnqvist, G. Blohme, J. Bolinder, A. Ekbom Schnell, J. W. Eriksson, S. Gudbjornsdottir, G. Sundkvist and L. Nystrom (2008). "Gender differences and temporal variation in the incidence of type 1 diabetes: results of 8012 cases in the nationwide Diabetes Incidence Study in Sweden 1983-2002." J Intern Med **263**(4): 386-394.

Palmer, J. P., C. M. Asplin, P. Clemons, K. Lyen, O. Tatpati, P. K. Raghu and T. L. Paquette (1983). "Insulin antibodies in insulin-dependent diabetics before insulin treatment." Science **222**(4630): 1337-1339.

Pani, M. A., M. Knapp, H. Donner, J. Braun, M. P. Baur, K. H. Usadel and K. Badenhoop (2000). "Vitamin D receptor allele combinations influence genetic susceptibility to type 1 diabetes in Germans." Diabetes **49**(3): 504-507.

Parikka, V., K. Nanto-Salonen, M. Saarinen, T. Simell, J. Ilonen, H. Hyoty, R. Veijola, M. Knip and O. Simell (2012). "Early seroconversion and rapidly increasing autoantibody concentrations predict prepubertal manifestation of type 1 diabetes in children at genetic risk." Diabetologia **55**(7): 1926-1936.

Parkkola, A., A. P. Laine, M. Karhunen, T. Harkonen, S. J. Ryhanen, J. Ilonen, M. Knip and R. Finnish Pediatric Diabetes (2017). "HLA and non-HLA genes and familial predisposition to autoimmune diseases in families with a child affected by type 1 diabetes." PLoS One **12**(11): e0188402.

Pasi, K. J., C. A. Sabin, P. V. Jenkins, H. L. Devereux, C. Ononye and C. A. Lee (2000). "The effects of the 32-bp CCR-5 deletion on HIV transmission and HIV disease progression in individuals with haemophilia." Br J Haematol **111**(1): 136-142.

Pastore, M. R., E. Bazzigaluppi, C. Belloni, C. Arcovio, E. Bonifacio and E. Bosi (2003). "Six months of gluten-free diet do not influence autoantibody titers, but improve insulin secretion in subjects at high risk for type 1 diabetes." J Clin Endocrinol Metab **88**(1): 162-165.

Patricia M. Heavey, I. R. R. (1999). "The Gut Microflora of the Developing Infant: Microbiology and Metabolism." Microbial Ecology in Health and Disease **11**(2): 75-83.

Patterson, C. C., G. G. Dahlquist, E. Gyurus, A. Green, G. Soltesz and E. S. Group (2009). "Incidence trends for childhood type 1 diabetes in Europe during 1989-2003 and predicted new cases 2005-20: a multicentre prospective registration study." Lancet **373**(9680): 2027-2033.

Patterson, C. C., V. Harjutsalo, J. Rosenbauer, A. Neu, O. Cinek, T. Skrivarhaug, B. Rami-Merhar, G. Soltesz, J. Svensson, R. C. Parslow, C. Castell, E. J. Schoenle, P. J. Bingley, G. Dahlquist, P. K. Jarosz-Chobot, D. Marciulionyte, E. F. Roche, U. Rothe, N. Bratina, C. Ionescu-Tirgoviste, I. Weets, M. Kocova, V. Cherubini, N. Rojnic Putarek, C. E. deBeaufort, M. Samardzic and A. Green (2019). "Trends and cyclical variation in the incidence of childhood type 1 diabetes in 26 European centres in the 25 year period 1989-2013: a multicentre prospective registration study." Diabetologia **62**(3): 408-417.

Pawlowicz, M., R. Filipow, G. Krzykowski, A. Stanislawska-Sachadyn, L. Morzuch, J. Kulczycka, A. Balcerska and J. Limon (2016). "Coincidence of PTPN22 c.1858CC and FCRL3 -169CC genotypes as a biomarker of preserved residual beta-cell function in children with type 1 diabetes." Pediatr Diabetes **18**(8): 696-705.

Payton, M. A., C. J. Hawkes and M. R. Christie (1995). "Relationship of the 37,000- and 40,000-M(r) tryptic fragments of islet antigens in insulin-dependent diabetes to the protein tyrosine phosphatase-like molecule IA-2 (ICA512)." The Journal of Clinical Investigation **96**(3): 1506-1511.

Pearson, G., B. Chai, T. Vozheiko, X. Liu, M. Kandarpa, R. C. Piper and S. A. Soleimanpour (2018). "Clec16a, Nrdp1, and USP8 Form a Ubiquitin-Dependent Tripartite Complex That Regulates beta-Cell Mitophagy." Diabetes **67**(2): 265-277.

Pescovitz, M. D., C. J. Greenbaum, B. Bundy, D. J. Becker, S. E. Gitelman, R. Goland, P. A. Gottlieb, J. B. Marks, A. Moran, P. Raskin, H. Rodriguez, D. A. Schatz, D. K. Wherrett, D. M. Wilson, J. P. Krischer, J. S. Skyler and C. D. S. G. Type 1 Diabetes TrialNet Anti (2014). "B-lymphocyte depletion with rituximab and beta-cell function: two-year results." Diabetes Care **37**(2): 453-459.

Petersen, J. S., K. O. Kyvik, P. J. Bingley, E. A. Gale, A. Green, T. Dyrberg and H. Beck-Nielsen (1997). "Population based study of prevalence of isleT-cell autoantibodies in monozygotic and dizygotic Danish twin pairs with insulin dependent diabetes mellitus." BMJ **314**(7094): 1575-1579.

Petit, M. M., R. Mols, E. F. Schoenmakers, N. Mandahl and W. J. Van de Ven (1996). "LPP, the preferred fusion partner gene of HMGIC in lipomas, is a novel member of the LIM protein gene family." Genomics **36**(1): 118-129.

Petronis, A. (2010). "Epigenetics as a unifying principle in the aetiology of complex traits and diseases." Nature **465**(7299): 721-727.

Pihoker, C., L. K. Gilliam, C. S. Hampe and A. Lernmark (2005). "Autoantibodies in diabetes." Diabetes **54 Suppl 2**: S52-61.

Piquer, S., C. Belloni, V. Lampasona, E. Bazzigaluppi, M. Vianello, B. Giometto, E. Bosi, G. F. Bottazzo and E. Bonifacio (2005). "Humoral autoimmune responses to glutamic acid decarboxylase have similar target epitopes and subclass that show titer-dependent disease association." Clin Immunol **117**(1): 31-35.

Plagnol, V., J. M. Howson, D. J. Smyth, N. Walker, J. P. Hafler, C. Wallace, H. Stevens, L. Jackson, M. J. Simmonds, C. Type 1 Diabetes Genetics, P. J. Bingley, S. C. Gough and J. A. Todd

(2011). "Genome-wide association analysis of autoantibody positivity in type 1 diabetes cases." PLoS Genet **7**(8): e1002216.

Plot, L., H. Amital, O. Barzilai, M. Ram, N. Bizzaro and Y. Shoenfeld (2009). "Infections may have a protective role in the etiopathogenesis of celiac disease." Ann N Y Acad Sci **1173**: 670-674.

Pociot, F., B. Akolkar, P. Concannon, H. A. Erlich, C. Julier, G. Morahan, C. R. Nierras, J. A. Todd, S. S. Rich and J. Nerup (2010). "Genetics of type 1 diabetes: what's next?" Diabetes **59**(7): 1561-1571.

Pugliese, A. (1997). "Parental gender effects on the insulin-gene-associated susceptibility to insulin-dependent diabetes mellitus." Eur J Clin Invest **27**(5): 359.

Pugliese, A. (1998). "Insulin expression in the thymus, tolerance, and type 1 diabetes." Diabetes Metab Rev **14**(4): 325-327.

Pugliese, A., M. Zeller, A. Fernandez, Jr., L. J. Zalcberg, R. J. Bartlett, C. Ricordi, M. Pietropaolo, G. S. Eisenbarth, S. T. Bennett and D. D. Patel (1997). "The insulin gene is transcribed in the human thymus and transcription levels correlated with allelic variation at the INS VNTR-IDDM2 susceptibility locus for type 1 diabetes." Nat Genet **15**(3): 293-297.

Pundziute-Lycka, A., G. Dahlquist, L. Nystrom, H. Arnqvist, E. Bjork, G. Blohme, J. Bolinder, J. W. Eriksson, G. Sundkvist, J. Ostman and G. Swedish Childhood Diabetes Study (2002). "The incidence of Type I diabetes has not increased but shifted to a younger age at diagnosis in the 0-34 years group in Sweden 1983-1998." Diabetologia **45**(6): 783-791.

Qu, H. Q., A. Montpetit, B. Ge, T. J. Hudson and C. Polychronakos (2007). "Toward further mapping of the association between the IL2RA locus and type 1 diabetes." Diabetes **56**(4): 1174-1176.

Rakyan, V. K., H. Beyan, T. A. Down, M. I. Hawa, S. Maslau, D. Aden, A. Daunay, F. Busato, C. A. Mein, B. Manfras, K. R. Dias, C. G. Bell, J. Tost, B. O. Boehm, S. Beck and R. D. Leslie (2011). "Identification of type 1 diabetes-associated DNA methylation variable positions that precede disease diagnosis." PLoS Genet **7**(9): e1002300.

Ramos-Lopez, E., B. Lange, H. Kahles, H. S. Willenberg, G. Meyer, M. Penna-Martinez, N. Reisch, S. Hahner, J. Seissler and K. Badenhoop (2008). "Insulin gene polymorphisms in type 1 diabetes, Addison's disease and the polyglandular autoimmune syndrome type II." BMC Med Genet **9**: 65.

Rasmussen, T., L. C. Stene, S. O. Samuelsen, O. Cinek, T. Wetlesen, P. A. Torjesen and K. S. Ronningen (2009). "Maternal BMI before pregnancy, maternal weight gain during pregnancy, and risk of persistent positivity for multiple diabetes-associated autoantibodies in children with the high-risk HLA genotype: the MIDIA study." Diabetes Care **32**(10): 1904-1906.

Redondo, M. J., J. Jeffrey, P. R. Fain, G. S. Eisenbarth and T. Orban (2008). "Concordance for islet autoimmunity among monozygotic twins." N Engl J Med **359**(26): 2849-2850.

Reetz, A., M. Solimena, M. Matteoli, F. Folli, K. Takei and P. De Camilli (1991). "GABA and pancreatic beta-cells: colocalization of glutamic acid decarboxylase (GAD) and GABA with synaptic-like microvesicles suggests their role in GABA storage and secretion." EMBO J **10**(5): 1275-1284.

Regnell, S. E. and Å. Lernmark (2017). "Early prediction of autoimmune (type 1) diabetes." Diabetologia **60**(8): 1370-1381.

Renault, L., B. Bugyi and M. F. Carlier (2008). "Spire and Cordon-bleu: multifunctional regulators of actin dynamics." Trends Cell Biol **18**(10): 494-504.

Rewers, M., H. Hyoty, A. Lernmark, W. Hagopian, J. X. She, D. Schatz, A. G. Ziegler, J. Toppari, B. Akolkar, J. Krischer and T. S. Group (2018). "The Environmental Determinants of Diabetes in the Young (TEDDY) Study: 2018 Update." Curr Diab Rep **18**(12): 136.

Rewers, M., T. L. Bugawan, J. M. Norris, A. Blair, B. Beaty, M. Hoffman, R. S. McDuffie, Jr., R. F. Hamman, G. Klingensmith, G. S. Eisenbarth and H. A. Erlich (1996). "Newborn screening for HLA markers associated with IDDM: diabetes autoimmunity study in the young (DAISY)." Diabetologia **39**(7): 807-812.

Rich, S. S. and P. Concannon (2015). "Summary of the Type 1 Diabetes Genetics Consortium Autoantibody Workshop." Diabetes Care **38 Suppl 2**: S45-48.

Richard-Miceli, C. and L. A. Criswell (2012). "Emerging patterns of genetic overlap across autoimmune disorders." Genome Med **4**(1): 6.

Richardson, C. C., J. A. Dromey, K. A. McLaughlin, D. Morgan, H. J. Bodansky, R. G. Feltbower, A. H. Barnett, G. V. Gill, S. C. Bain and M. R. Christie (2013). "High frequency of autoantibodies in patients with long duration type 1 diabetes." Diabetologia **56**(11): 2538-2540.

Risch, N. (1987). "Assessing the role of HLA-linked and unlinked determinants of disease." Am J Hum Genet **40**(1): 1-14.

Robles, D. T., G. S. Eisenbarth, T. Wang, H. A. Erlich, T. L. Bugawan, S. R. Babu, K. Barriga, J. M. Norris, M. Hoffman, G. Klingensmith, L. Yu, M. Rewers and Y. Diabetes Autoimmunity Study in the (2002). "Millennium award recipient contribution. Identification of children with early onset and high incidence of anti-islet autoantibodies." Clin Immunol **102**(3): 217-224.

Robertson, C. C., J. R. J. Inshaw, S. Onengut-Gumuscu, W. M. Chen, D. F. Santa Cruz, H. Yang, A. J. Cutler, D. J. M. Crouch, E. Farber, S. L. Bridges, Jr., J. C. Edberg, R. P. Kimberly, J. H. Buckner, P. Deloukas, J. Divers, D. Dabelea, J. M. Lawrence, S. Marcovina, A. S. Shah, C. J. Greenbaum, M. A. Atkinson, P. K. Gregersen, J. R. Oksenberg, F. Pociot, M. J. Rewers, A. K. Steck, D. B. Dunger, C. Type 1 Diabetes Genetics, L. S. Wicker, P. Concannon, J. A. Todd and S. S. Rich (2021). "Fine-mapping, trans-ancestral and genomic analyses identify causal variants, cells, genes and drug targets for type 1 diabetes." Nat Genet **53**(7): 962-971.

Rodriguez-Calvo, T., S. Sabouri, F. Anquetil and M. G. von Herrath (2016). "The viral paradigm in type 1 diabetes: Who are the main suspects?" Autoimmun Rev **15**(10): 964-969.

Ronkainen, M. S., S. Hoppu, S. Korhonen, S. Simell, R. Veijola, J. Ilonen, O. Simell and M. Knip (2006). "Early epitope- and isotype-specific humoral immune responses to GAD65 in young children with genetic susceptibility to type 1 diabetes." Eur J Endocrinol **155**(4): 633-642.

Rosenzwajg, M., G. Churlaud, A. Hartemann and D. Klatzmann (2014). "Interleukin 2 in the pathogenesis and therapy of type 1 diabetes." Curr Diab Rep **14**(12): 553.

Roychoudhuri, R., K. Hirahara, K. Mousavi, D. Clever, C. A. Klebanoff, M. Bonelli, G. Sciumè, H. Zare, G. Vahedi, B. Dema, Z. Yu, H. Liu, H. Takahashi, M. Rao, P. Muranski, J. G. Crompton, G. Punkosdy, D. Bedognetti, E. Wang, V. Hoffmann, J. Rivera, F. M. Marincola, A. Nakamura, V. Sartorelli, Y. Kanno, L. Gattinoni, A. Muto, K. Igarashi, J. J. O'Shea and N. P.

Restifo (2013). "BACH2 represses effector programs to stabilize T(reg)-mediated immune homeostasis." Nature **498**(7455): 506-510.

Rudy, G., N. Stone, L. C. Harrison, P. G. Colman, P. McNair, V. Brusic, M. B. French, M. C. Honeyman, B. Tait and A. M. Lew (1995). "Similar peptides from two beta cell autoantigens, proinsulin and glutamic acid decarboxylase, stimulate T-cells of individuals at risk for insulin-dependent diabetes." Mol Med **1**(6): 625-633.

Rui, J., S. Deng, J. Lebastchi, P. L. Clark, S. Usmani-Brown and K. C. Herold (2016). "Methylation of insulin DNA in response to proinflammatory cytokines during the progression of autoimmune diabetes in NOD mice." Diabetologia **59**(5): 1021-1029.

Saiz, A., Y. Blanco, L. Sabater, F. Gonzalez, L. Bataller, R. Casamitjana, L. Ramio-Torrenta and F. Graus (2008). "Spectrum of neurological syndromes associated with glutamic acid decarboxylase antibodies: diagnostic clues for this association." Brain **131**(Pt 10): 2553-2563.

Santosh, B., A. Varshney and P. K. Yadava (2015). "Non-coding RNAs: biological functions and applications." Cell Biochem Funct **33**(1): 14-22.

Schmidli, R. S., P. G. Colman, E. Bonifacio, G. F. Bottazzo and L. C. Harrison (1994). "High level of concordance between assays for glutamic acid decarboxylase antibodies. The First International Glutamic Acid Decarboxylase Antibody Workshop." Diabetes **43**(8): 1005-1009.

Senee, V., C. Chelala, S. Duchatelet, D. Feng, H. Blanc, J. C. Cossec, C. Charon, M. Nicolino, P. Boileau, D. R. Cavener, P. Bougneres, D. Taha and C. Julier (2006). "Mutations in GLIS3 are responsible for a rare syndrome with neonatal diabetes mellitus and congenital hypothyroidism." Nat Genet **38**(6): 682-687.

Shan, Z., W. Bao, Y. Zhang, Y. Rong, X. Wang, Y. Jin, Y. Song, P. Yao, C. Sun, F. B. Hu and L. Liu (2014). "Interactions between zinc transporter-8 gene (SLC30A8) and plasma zinc concentrations for impaired glucose regulation and type 2 diabetes." Diabetes **63**(5): 1796-1803.

Shapiro, M. R., P. Thirawatananond, L. Peters, R. C. Sharp, S. Ogundare, A. L. Posgai, D. J. Perry and T. M. Brusko (2021). "De-coding genetic risk variants in type 1 diabetes." Immunol Cell Biol **99**(5): 496-508.

Sheehan, A., A. Freni Sterrantino, D. Fecht, P. Elliott and S. Hodgson (2020). "Childhood type 1 diabetes: an environment-wide association study across England." Diabetologia **63**(5): 964-976.

Sims, E. K., S. Geyer, S. B. Johnson, I. Libman, L. M. Jacobsen, D. Boulware, L. E. Rafkin, D. Matheson, M. A. Atkinson, H. Rodriguez, M. Spall, H. E. Larsson, D. K. Wherrett, C. J. Greenbaum, J. Krischer, L. A. DiMeglio and G. Type 1 Diabetes TrialNet Study (2020). "Erratum. Who Is Enrolling? The Path to Monitoring in Type 1 Diabetes TrialNet's Pathway to Prevention. Diabetes Care 2019;42:2228-2236." Diabetes Care **43**(4): 934-934.

Skrivarhaug, T., L. C. Stene, A. K. Drivvoll, H. Strom, G. Joner and G. Norwegian Childhood Diabetes Study (2014). "Incidence of type 1 diabetes in Norway among children aged 0-14 years between 1989 and 2012: has the incidence stopped rising? Results from the Norwegian Childhood Diabetes Registry." Diabetologia **57**(1): 57-62.

Skyler, J. S. (2013). "The compelling case for anti-CD3 in type 1 diabetes." Diabetes **62**(11): 3656-3657.

Smyth, D. J., J. D. Cooper, R. Bailey, S. Field, O. Burren, L. J. Smink, C. Guja, C. Ionescu-Tirgoviste, B. Widmer, D. B. Dunger, D. A. Savage, N. M. Walker, D. G. Clayton and J. A. Todd (2006). "A genome-wide association study of nonsynonymous SNPs identifies a type 1 diabetes locus in the interferon-induced helicase (IFIH1) region." Nat Genet **38**(6): 617-619.

Smyth, D., J. D. Cooper, J. E. Collins, J. M. Heward, J. A. Franklyn, J. M. Howson, A. Vella, S. Nutland, H. E. Rance, L. Maier, B. J. Barratt, C. Guja, C. Ionescu-Tirgoviste, D. A. Savage, D. B. Dunger, B. Widmer, D. P. Strachan, S. M. Ring, N. Walker, D. G. Clayton, R. C. Twells, S. C. Gough and J. A. Todd (2004). "Replication of an association between the lymphoid tyrosine phosphatase locus (LYP/PTPN22) with type 1 diabetes, and evidence for its role as a general autoimmunity locus." Diabetes **53**(11): 3020-3023.

Smyth, D. J., V. Plagnol, N. M. Walker, J. D. Cooper, K. Downes, J. H. Yang, J. M. Howson, H. Stevens, R. McManus, C. Wijmenga, G. A. Heap, P. C. Dubois, D. G. Clayton, K. A. Hunt, D. A. van Heel and J. A. Todd (2008). "Shared and distinct genetic variants in type 1 diabetes and celiac disease." N Engl J Med **359**(26): 2767-2777.

So, M., C. O'Rourke, H. T. Bahnson, C. J. Greenbaum and C. Speake (2020). "Autoantibody Reversion: Changing Risk Categories in Multiple-Autoantibody-Positive Individuals." Diabetes Care **43**(4): 913-917.

Soderbergh, A., A. G. Myhre, O. Ekwall, G. Gebre-Medhin, H. Hedstrand, E. Landgren, A. Miettinen, P. Eskelin, M. Halonen, T. Tuomi, J. Gustafsson, E. S. Husebye, J. Perheentupa, M. Gylling, M. P. Manns, F. Rorsman, O. Kampe and T. Nilsson (2004). "Prevalence and clinical associations of 10 defined autoantibodies in autoimmune polyendocrine syndrome type I." J Clin Endocrinol Metab **89**(2): 557-562.

Solimena, M., R. Dirkx, Jr., J. M. Hermel, S. Pleasic-Williams, J. A. Shapiro, L. Caron and D. U. Rabin (1996). "ICA 512, an autoantigen of type I diabetes, is an intrinsic membrane protein of neurosecretory granules." EMBO J **15**(9): 2102-2114.

Soltesz, G., C. C. Patterson, G. Dahlquist and E. S. Group (2007). "Worldwide childhood type 1 diabetes incidence--what can we learn from epidemiology?" Pediatr Diabetes **8 Suppl 6**: 6-14.

Spalinger, M. R., J. Zeitz, L. Biedermann, J. B. Rossel, M. C. Sulz, P. Frei, S. Scharl, S. R. Vavricka, M. Fried, G. Rogler, M. Scharl and I. B. D. C. S. G. Swiss (2016). "Genotype-Phenotype Associations of the CD-Associated Single Nucleotide Polymorphism within the Gene Locus Encoding Protein Tyrosine Phosphatase Non-Receptor Type 22 in Patients of the Swiss IBD Cohort." PLoS One **11**(7): e0160215.

Stanley, H. M., J. M. Norris, K. Barriga, M. Hoffman, L. Yu, D. Miao, H. A. Erlich, G. S. Eisenbarth, M. Rewers and Y. Diabetes Autoimmunity Study in the (2004). "Is presence of islet autoantibodies at birth associated with development of persistent islet autoimmunity? The Diabetes Autoimmunity Study in the Young (DAISY)." Diabetes Care **27**(2): 497-502.

Steck, A. K., T. K. Armstrong, S. R. Babu, G. S. Eisenbarth and C. Type 1 Diabetes Genetics (2011). "Stepwise or linear decrease in penetrance of type 1 diabetes with lower-risk HLA genotypes over the past 40 years." Diabetes **60**(3): 1045-1049.

Steck, A. K., F. Dong, R. Wong, A. Fouts, E. Liu, J. Romanos, C. Wijmenga, J. M. Norris and M. J. Rewers (2014). "Improving prediction of type 1 diabetes by testing non-HLA genetic variants in addition to HLA markers." Pediatr Diabetes **15**(5): 355-362.

Steck, A. K., W. Zhang, T. L. Bugawan, K. J. Barriga, A. Blair, H. A. Erlich, G. S. Eisenbarth, J. M. Norris and M. J. Rewers (2009). "Do non-HLA genes influence development of persistent islet autoimmunity and type 1 diabetes in children with high-risk HLA-DR,DQ genotypes?" Diabetes **58**(4): 1028-1033.

Stene, L. C. and M. Rewers (2012). "Immunology in the clinic review series; focus on type 1 diabetes and viruses: the enterovirus link to type 1 diabetes: critical review of human studies." Clin Exp Immunol **168**(1): 12-23.

Stewart, C. J., N. J. Ajami, J. L. O'Brien, D. S. Hutchinson, D. P. Smith, M. C. Wong, M. C. Ross, R. E. Lloyd, H. Doddapaneni, G. A. Metcalf, D. Muzny, R. A. Gibbs, T. Vatanen, C. Huttenhower, R. J. Xavier, M. Rewers, W. Hagopian, J. Toppari, A. G. Ziegler, J. X. She, B. Akolkar, A. Lernmark, H. Hyoty, K. Vehik, J. P. Krischer and J. F. Petrosino (2018). "Temporal development of the gut microbiome in early childhood from the TEDDY study." Nature **562**(7728): 583-588.

Swainson, L. A., J. E. Mold, U. D. Bajpai and J. M. McCune (2010). "Expression of the autoimmune susceptibility gene FcRL3 on human regulatory T-cells is associated with dysfunction and high levels of programmed cell death-1." J Immunol **184**(7): 3639-3647.

Tait, B. D., P. G. Colman, G. Morahan, L. Marchinovska, E. Dore, S. Gellert, M. C. Honeyman, K. Stephen and A. Loth (2003). "HLA genes associated with autoimmunity and progression to disease in type 1 diabetes." Tissue Antigens **61**(2): 146-153.

Takeyama, N., Y. Ano, G. Wu, N. Kubota, K. Saeki, A. Sakudo, E. Momotani, K. Sugiura, M. Yukawa and T. Onodera (2009). "Localization of insulinoma associated protein 2, IA-2 in mouse neuroendocrine tissues using two novel monoclonal antibodies." Life Sci **84**(19-20): 678-687.

Tang, Q., J. Y. Adams, C. Penaranda, K. Melli, E. Piaggio, E. Sgouroudis, C. A. Piccirillo, B. L. Salomon and J. A. Bluestone (2008). "Central role of defective interleukin-2 production in the triggering of islet autoimmune destruction." Immunity **28**(5): 687-697.

Thauvin-Robinet, C., L. Duplomb-Jego, F. Limoge, D. Picot, A. Masurel, B. Terriat, C. Champilou, D. Minot, J. St-Onge, P. Kuentz, Y. Duffourd, J. Thevenon, J. B. Riviere and L. Faivre (2016). "Homozygous FIBP nonsense variant responsible of syndromic overgrowth, with overgrowth, macrocephaly, retinal coloboma and learning disabilities." Clin Genet **89**(5): e1-4.

Thomas, N. J., J. M. Dennis, S. A. Sharp, A. Kaur, S. Misra, H. C. Walkey, D. G. Johnston, N. S. Oliver, W. A. Hagopian, M. N. Weedon, K. A. Patel and R. A. Oram (2021). "DR15-DQ6 remains dominantly protective against type 1 diabetes throughout the first five decades of life." Diabetologia. 10.1007/s00125-021-05513-4.

Todd, J. A. (2010). "Etiology of type 1 diabetes." Immunity **32**(4): 457-467.Todd, J. A. (2018). "Evidence that UBASH3 is a causal gene for type 1 diabetes." Eur J Hum Genet **26**(7): 925-927.

Todd, J. A., N. M. Walker, J. D. Cooper, D. J. Smyth, K. Downes, V. Plagnol, R. Bailey, S. Nejentsev, S. F. Field, F. Payne, C. E. Lowe, J. S. Szeszko, J. P. Hafler, L. Zeitels, J. H. Yang, A. Vella, S. Nutland, H. E. Stevens, H. Schuilenburg, G. Coleman, M. Maisuria, W. Meadows, L. J. Smink, B. Healy, O. S. Burren, A. A. Lam, N. R. Ovington, J. Allen, E. Adlem, H. T. Leung, C. Wallace, J. M. Howson, C. Guja, C. Ionescu-Tirgoviste, F. Genetics of Type 1 Diabetes in, M. J. Simmonds, J. M. Heward, S. C. Gough, C. Wellcome Trust Case Control, D. B. Dunger,

L. S. Wicker and D. G. Clayton (2007). "Robust associations of four new chromosome regions from genome-wide analyses of type 1 diabetes." Nat Genet **39**(7): 857-864.

Trajkovski, M., H. Mziaut, A. Altkruger, J. Ouwendijk, K. P. Knoch, S. Muller and M. Solimena (2004). "Nuclear translocation of an ICA512 cytosolic fragment couples granule exocytosis and insulin expression in {beta}-cells." J Cell Biol **167**(6): 1063-1074.

Trajkovski, M., H. Mziaut, S. Schubert, Y. Kalaidzidis, A. Altkruger and M. Solimena (2008). "Regulation of insulin granule turnover in pancreatic beta-cells by cleaved ICA512." J Biol Chem **283**(48): 33719-33729.

Tuomilehto-Wolf, E. and J. Tuomilehto (1991). "HLA antigens in insulin-dependent diabetes mellitus." Ann Med **23**(5): 481-488.

Ueda, H., J. M. Howson, L. Esposito, J. Heward, H. Snook, G. Chamberlain, D. B. Rainbow, K. M. Hunter, A. N. Smith, G. Di Genova, M. H. Herr, I. Dahlman, F. Payne, D. Smyth, C. Lowe, R. C. Twells, S. Howlett, B. Healy, S. Nutland, H. E. Rance, V. Everett, L. J. Smink, A. C. Lam, H. J. Cordell, N. M. Walker, C. Bordin, J. Hulme, C. Motzo, F. Cucca, J. F. Hess, M. L. Metzker, J. Rogers, S. Gregory, A. Allahabadia, R. Nithiyananthan, E. Tuomilehto-Wolf, J. Tuomilehto, P. Bingley, K. M. Gillespie, D. E. Undlien, K. S. Ronningen, C. Guja, C. Ionescu-Tirgoviste, D. A. Savage, A. P. Maxwell, D. J. Carson, C. C. Patterson, J. A. Franklyn, D. G. Clayton, L. B. Peterson, L. S. Wicker, J. A. Todd and S. C. Gough (2003). "Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease." Nature **423**(6939): 506-511.

Umemura, T., M. Ota, H. Hamano, Y. Katsuyama, K. Kiyosawa and S. Kawa (2006). "Genetic association of Fc receptor-like 3 polymorphisms with autoimmune pancreatitis in Japanese patients." Gut **55**(9): 1367-1368.

Vafiadis, P., S. T. Bennett, J. A. Todd, J. Nadeau, R. Grabs, C. G. Goodyer, S. Wickramasinghe, E. Colle and C. Polychronakos (1997). "Insulin expression in human thymus is modulated by INS VNTR alleles at the IDDM2 locus." Nat Genet **15**(3): 289-292.

Vafiadis, P., H. Ounissi-Benkalha, M. Palumbo, R. Grabs, M. Rousseau, C. G. Goodyer and C. Polychronakos (2001). "Class III alleles of the variable number of tandem repeat insulin polymorphism associated with silencing of thymic insulin predispose to type 1 diabetes." J Clin Endocrinol Metab **86**(8): 3705-3710.

Vahasalo, P. (1992). "Autoantibodies to insulin have similar affinity to that of antibodies to exogenous insulin but lower binding capacity." Eur J Clin Invest **22**(12): 772-776.

Valdes, A. M., H. A. Erlich and J. A. Noble (2005). "Human leukocyte antigen class I B and C loci contribute to Type 1 Diabetes (T1D) susceptibility and age at T1D onset." Hum Immunol **66**(3): 301-313.

van Belle, T. L., K. T. Coppieters and M. G. von Herrath (2011). "Type 1 diabetes: etiology, immunology, and therapeutic strategies." Physiol Rev **91**(1): 79-118.

Vang, T., M. Congia, M. D. Macis, L. Musumeci, V. Orru, P. Zavattari, K. Nika, L. Tautz, K. Tasken, F. Cucca, T. Mustelin and N. Bottini (2005). "Autoimmune-associated lymphoid tyrosine phosphatase is a gain-of-function variant." Nat Genet **37**(12): 1317-1319.

Vardi, P., A. G. Ziegler, J. H. Mathews, S. Dib, R. J. Keller, A. T. Ricker, J. I. Wolfsdorf, R. D. Herskowitz, A. Rabizadeh, G. S. Eisenbarth and *et al*. (1988). "Concentration of insulin autoantibodies at onset of type I diabetes. Inverse log-linear correlation with age." Diabetes Care **11**(9): 736-739.

Vatanen, T., E. A. Franzosa, R. Schwager, S. Tripathi, T. D. Arthur, K. Vehik, Å. Lernmark, W. A. Hagopian, M. J. Rewers, J.-X. She, J. Toppari, A.-G. Ziegler, B. Akolkar, J. P. Krischer, C. J. Stewart, N. J. Ajami, J. F. Petrosino, D. Gevers, H. Lähdesmäki, H. Vlamakis, C. Huttenhower and R. J. Xavier (2018). "The human gut microbiome in early-onset type 1 diabetes from the TEDDY study." Nature **562**(7728): 589-594.

Vehik, K., R. F. Hamman, D. Lezotte, J. M. Norris, G. J. Klingensmith, M. Rewers and D. Dabelea (2008). "Trends in high-risk HLA susceptibility genes among Colorado youth with type 1 diabetes." Diabetes Care **31**(7): 1392-1396.

Veijola, R., H. Reijonen, P. Vahasalo, E. Sabbah, P. Kulmala, J. Ilonen, H. K. Akerblom and M. Knip (1996). "HLA-DQB1-defined genetic susceptibility, beta cell autoimmunity, and metabolic characteristics in familial and nonfamilial insulin-dependent diabetes mellitus. Childhood Diabetes in Finland (DiMe) Study Group." J Clin Invest **98**(11): 2489-2495.

Verge, C. F., D. Stenger, E. Bonifacio, P. G. Colman, C. Pilcher, P. J. Bingley and G. S. Eisenbarth (1998). "Combined use of autoantibodies (IA-2 autoantibody, GAD autoantibody, insulin autoantibody, cytoplasmic isleT-cell antibodies) in type 1 diabetes: Combinatorial Islet Autoantibody Workshop." Diabetes **47**(12): 1857-1866.

Virtanen, S. M., E. Hypponen, E. Laara, P. Vahasalo, P. Kulmala, K. Savola, L. Rasanen, A. Aro, M. Knip and H. K. Akerblom (1998). "Cow's milk consumption, disease-associated autoantibodies and type 1 diabetes mellitus: a follow-up study in siblings of diabetic children. Childhood Diabetes in Finland Study Group." Diabet Med **15**(9): 730-738.

Virtanen, S. M., E. Laara, E. Hypponen, H. Reijonen, L. Rasanen, A. Aro, M. Knip, J. Ilonen and H. K. Akerblom (2000). "Cow's milk consumption, HLA-DQB1 genotype, and type 1 diabetes: a nested case-control study of siblings of children with diabetes. Childhood diabetes in Finland study group." Diabetes **49**(6): 912-917.

Virtanen, S. M., H. M. Takkinen, J. Nevalainen, C. Kronberg-Kippila, M. Salmenhaara, L. Uusitalo, M. G. Kenward, M. Erkkola, R. Veijola, O. Simell, J. Ilonen and M. Knip (2011). "Early introduction of root vegetables in infancy associated with advanced ss-cell autoimmunity in young children with human leukocyte antigen-conferred susceptibility to Type 1 diabetes." Diabet Med **28**(8): 965-971.

Vlajinac, H., S. Sipetic, J. Marinkovic, M. Bjekic, N. Kocev and S. Sajic (2006). "The Belgrade childhood diabetes study - comparison of children with type 1 diabetes with their siblings." Paediatr Perinat Epidemiol **20**(3): 238-243.

Volta, U., A. Fabbri, C. Parisi, M. Piscaglia, G. Caio, F. Tovoli and E. Fiorini (2010). "Old and new serological tests for celiac disease screening." Expert Review of Gastroenterology & Hepatology **4**(1): 31-35.

Volta, U., A. Granito, E. Fiorini, C. Parisi, M. Piscaglia, G. Pappas, P. Muratori and F. B. Bianchi (2008). "Usefulness of antibodies to deamidated gliadin peptides in celiac disease diagnosis and follow-up." Dig Dis Sci **53**(6): 1582-1588.

von Herrath, M. (2009). "Diabetes: A virus-gene collaboration." Nature **459**(7246): 518-519.

Wabl, M., M. Cascalho and C. Steinberg (1999). "Hypermutation in antibody affinity maturation." Curr Opin Immunol **11**(2): 186-189.

Waddington, C. H. (1968). "Towards a theoretical biology." Nature **218**(5141): 525-527.

Waddington, C. H. (2012). "The epigenotype. 1942." Int J Epidemiol **41**(1): 10-13.

Wahlberg, J., O. Vaarala, J. Ludvigsson and A. B.-s. group (2006). "Dietary risk factors for the emergence of type 1 diabetes-related autoantibodies in 21/2 year-old Swedish children." Br J Nutr **95**(3): 603-608.

Walunas, T. L., C. Y. Bakker and J. A. Bluestone (1996). "CTLA-4 ligation blocks CD28-dependent T-cell activation." J Exp Med **183**(6): 2541-2550.

Wang, H., Y. Jin, M. V. P. Linga Reddy, R. Podolsky, S. Liu, P. Yang, B. Bode, J. Chip Reed, R. D. Steed, S. W. Anderson, L. Steed, D. Hopkins, Y. Huang and J.-X. She (2010). "Genetically Dependent ERBB3 Expression Modulates Antigen Presenting Cell Function and Type 1 Diabetes Risk." PLoS ONE **5**(7): e11789.

Warram, J. H., A. S. Krolewski and C. R. Kahn (1988). "Determinants of IDDM and perinatal mortality in children of diabetic mothers." Diabetes **37**(10): 1328-1334.

Wasserfall, C., K. Nead, C. Mathews and M. A. Atkinson (2011). "The threshold hypothesis: solving the equation of nurture vs nature in type 1 diabetes." Diabetologia **54**(9): 2232.

Wellcome Trust Case Control, C. (2007). "Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls." Nature **447**(7145): 661-678.

Wen, X. and Y. Yang (2017). "Emerging roles of GLIS3 in neonatal diabetes, type 1 and type 2 diabetes." J Mol Endocrinol **58**(2): R73-R85.

Wenzlau, J. M., K. Juhl, L. Yu, O. Moua, S. A. Sarkar, P. Gottlieb, M. Rewers, G. S. Eisenbarth, J. Jensen, H. W. Davidson and J. C. Hutton (2007). "The cation efflux transporter ZnT8 (Slc30A8) is a major autoantigen in human type 1 diabetes." Proc Natl Acad Sci U S A **104**(43): 17040-17045.

Wenzlau, J. M., Y. Liu, L. Yu, O. Moua, K. T. Fowler, S. Rangasamy, J. Walters, G. S. Eisenbarth, H. W. Davidson and J. C. Hutton (2008). "A common nonsynonymous single nucleotide polymorphism in the SLC30A8 gene determines ZnT8 autoantibody specificity in type 1 diabetes." Diabetes **57**(10): 2693-2697.

Wild, S., G. Roglic, A. Green, R. Sicree and H. King (2004). "Global prevalence of diabetes: estimates for the year 2000 and projections for 2030." Diabetes Care **27**(5): 1047-1053.

Wilkin, T. J. (2001). "The accelerator hypothesis: weight gain as the missing link between Type I and Type II diabetes." Diabetologia **44**(7): 914-922.

Wilkin, T. J. (2008). "Diabetes: 1 and 2, or one and the same? Progress with the accelerator hypothesis." Pediatr Diabetes **9**(3 Pt 2): 23-32.

Williams, A. J., R. J. Aitken, M. A. Chandler, K. M. Gillespie, V. Lampasona and P. J. Bingley (2008). "Autoantibodies to islet antigen-2 are associated with HLA-DRB1*07 and DRB1*09 haplotypes as well as DRB1*04 at onset of type 1 diabetes: the possible role of HLA-DQA in autoimmunity to IA-2." Diabetologia **51**(8): 1444-1448.

Williams, A. J. K., V. Lampasona, M. Schlosser, P. W. Mueller, D. L. Pittman, W. E. Winter, B. Akolkar, R. Wyatt, C. Brigatti, S. Krause and P. Achenbach (2015). "Detection of Antibodies Directed to the N-Terminal Region of GAD Is Dependent on Assay Format and Contributes to Differences in the Specificity of GAD Autoantibody Assays for Type 1 Diabetes." **64**(9): 3239-3246.

Williams, A. J. K., V. Lampasona, R. Wyatt, C. Brigatti, K. M. Gillespie, P. J. Bingley and P. Achenbach (2015). "Reactivity to N-Terminally Truncated GAD65(96–585) Identifies GAD Autoantibodies That Are More Closely Associated With Diabetes Progression in Relatives of Patients With Type 1 Diabetes." **64**(9): 3247-3252.

Williams, G. M., A. E. Long, I. V. Wilson, R. J. Aitken, R. C. Wyatt, T. J. McDonald, F. S. Wong, A. T. Hattersley, A. J. Williams, P. J. Bingley and K. M. Gillespie (2016). "Beta cell function and ongoing autoimmunity in long-standing, childhood onset type 1 diabetes." Diabetologia **59**(12): 2722-2726.

Winkler, C., J. Krumsiek, F. Buettner, C. Angermuller, E. Z. Giannopoulou, F. J. Theis, A. G. Ziegler and E. Bonifacio (2014). "Feature ranking of type 1 diabetes susceptibility genes improves prediction of type 1 diabetes." Diabetologia **57**(12): 2521-2529.

Xu, A., W. Zhu, T. Li, X. Li, J. Cheng, C. Li, P. Yi and L. Liu (2015). "Interleukin-10 gene transfer into insulin-producing beta cells protects against diabetes in non-obese diabetic mice." Mol Med Rep **12**(3): 3881-3889.

Xu, K., M. Zha, X. Wu, Z. Yu, R. Yu, X. Xu, H. Chen and T. Yang (2011). "Association between rs13266634 C/T polymorphisms of solute carrier family 30 member 8 (SLC30A8) and type 2 diabetes, impaired glucose tolerance, type 1 diabetes--a meta-analysis." Diabetes Res Clin Pract **91**(2): 195-202.

Yamanouchi, J., D. Rainbow, P. Serra, S. Howlett, K. Hunter, V. E. Garner, A. Gonzalez-Munoz, J. Clark, R. Veijola, R. Cubbon, S. L. Chen, R. Rosa, A. M. Cumiskey, D. V. Serreze, S. Gregory, J. Rogers, P. A. Lyons, B. Healy, L. J. Smink, J. A. Todd, L. B. Peterson, L. S. Wicker and P. Santamaria (2007). "Interleukin-2 gene variation impairs regulatory T-cell function and causes autoimmunity." Nat Genet **39**(3): 329-337.

Yang, B. T., T. A. Dayeh, C. L. Kirkpatrick, J. Taneera, R. Kumar, L. Groop, C. B. Wollheim, M. D. Nitert and C. Ling (2011). "Insulin promoter DNA methylation correlates negatively with insulin gene expression and positively with HbA(1c) levels in human pancreatic islets." Diabetologia **54**(2): 360-367.

Yang, J., R. N. Tamura, U. M. Uusitalo, C. A. Aronsson, K. Silvis, A. Riikonen, N. Frank, G. Joslowski, C. Winkler, J. M. Norris and S. M. Virtanen (2017). "Vitamin D and probiotics supplement use in young children with genetic risk for type 1 diabetes." Eur J Clin Nutr **71**(12): 1449-1454.

Yang, J. H., A. J. Cutler, R. C. Ferreira, J. L. Reading, N. J. Cooper, C. Wallace, P. Clarke, D. J. Smyth, C. S. Boyce, G. J. Gao, J. A. Todd, L. S. Wicker and T. I. Tree (2015). "Natural Variation in Interleukin-2 Sensitivity Influences Regulatory T-Cell Frequency and Function in Individuals With Long-standing Type 1 Diabetes." Diabetes **64**(11): 3891-3902.

Yang, Y. and L. Chan (2016). "Monogenic Diabetes: What It Teaches Us on the Common Forms of Type 1 and Type 2 Diabetes." Endocr Rev **37**(3): 190-222.

Yang, Y., X. Su, K. Zhang and R. Zhou (2013). "The Fc receptor-like 3 gene polymorphisms and susceptibility to autoimmune diseases: an updated meta-analysis." Autoimmunity **46**(8): 547-558.

Yassouridis, C., F. Leisch, C. Winkler, A. G. Ziegler and A. Beyerlein (2017). "Associations of growth patterns and islet autoimmunity in children with increased risk for type 1 diabetes: a functional analysis approach." Pediatr Diabetes **18**(2): 103-110.

Ye, J., A. E. Long, J. A. Pearson, H. Taylor, P. J. Bingley, A. J. Williams and K. M. Gillespie (2015). "Attenuated humoral responses in HLA-A*24-positive individuals at risk of type 1 diabetes." Diabetologia **58**(10): 2284-2287.

Ye, J., M. Vives-Pi and K. M. Gillespie (2014). "Maternal microchimerism: increased in the insulin positive compartment of type 1 diabetes pancreas but not in infiltrating immune cells or replicating islet cells." PLoS One **9**(1): e86985.

Ye, J., T. G. Richardson, W. L. McArdle, C. L. Relton, K. M. Gillespie, M. Suderman and G. Hemani (2018). "Identification of loci where DNA methylation potentially mediates genetic risk of type 1 diabetes." J Autoimmun **93**: 66-75.

Yoon, J. W., H. S. Jun and P. Santamaria (1998). "Cellular and molecular mechanisms for the initiation and progression of beta cell destruction resulting from the collaboration between macrophages and T-cells." Autoimmunity **27**(2): 109-122.

Yu, L., M. Rewers, R. Gianani, E. Kawasaki, Y. Zhang, C. Verge, P. Chase, G. Klingensmith, H. Erlich, J. Norris and G. S. Eisenbarth (1996). "Antiislet autoantibodies usually develop sequentially rather than simultaneously." The Journal of Clinical Endocrinology & Metabolism **81**(12): 4264-4267.

Zheng, P., Z. Li and Z. Zhou (2018). "Gut microbiome in type 1 diabetes: A comprehensive review." Diabetes Metab Res Rev **34**(7): e3043.

Ziegler, A. G., E. Bonifacio and B.-B. S. Group (2012). "Age-related islet autoantibody incidence in offspring of patients with type 1 diabetes." Diabetologia **55**(7): 1937-1943.

Ziegler, A. G., M. Hummel, M. Schenker and E. Bonifacio (1999). "Autoantibody appearance and risk for development of childhood diabetes in offspring of parents with type 1 diabetes: the 2-year analysis of the German BABYDIAB Study." Diabetes **48**(3): 460-468.

Ziegler, A. G. and G. T. Nepom (2010). "Prediction and pathogenesis in type 1 diabetes." Immunity **32**(4): 468-478.

Ziegler, A. G., M. Rewers, O. Simell, T. Simell, J. Lempainen, A. Steck, C. Winkler, J. Ilonen, R. Veijola, M. Knip, E. Bonifacio and G. S. Eisenbarth (2013). "Seroconversion to multiple islet autoantibodies and risk of progression to diabetes in children." JAMA **309**(23): 2473-2479.

Ziegler, A. G., S. Schmid, D. Huber, M. Hummel and E. Bonifacio (2003). "Early infant feeding and risk of developing type 1 diabetes-associated autoantibodies." JAMA **290**(13): 1721-1728.

Zoledziewska, M., G. Costa, M. Pitzalis, E. Cocco, C. Melis, L. Moi, P. Zavattari, R. Murru, R. Lampis, L. Morelli, F. Poddie, P. Frongia, P. Pusceddu, M. Bajorek, A. Marras, A. M. Satta, A. Chessa, M. Pugliatti, S. Sotgiu, M. B. Whalen, G. Rosati, F. Cucca and M. G. Marrosu (2009). "Variation within the CLEC16A gene shows consistent disease association with both multiple sclerosis and type 1 diabetes in Sardinia." Genes Immun **10**(1): 15-17.

Appendix

A. Non-*HLA* SNPs in T1D patients diagnosed under 21 years old

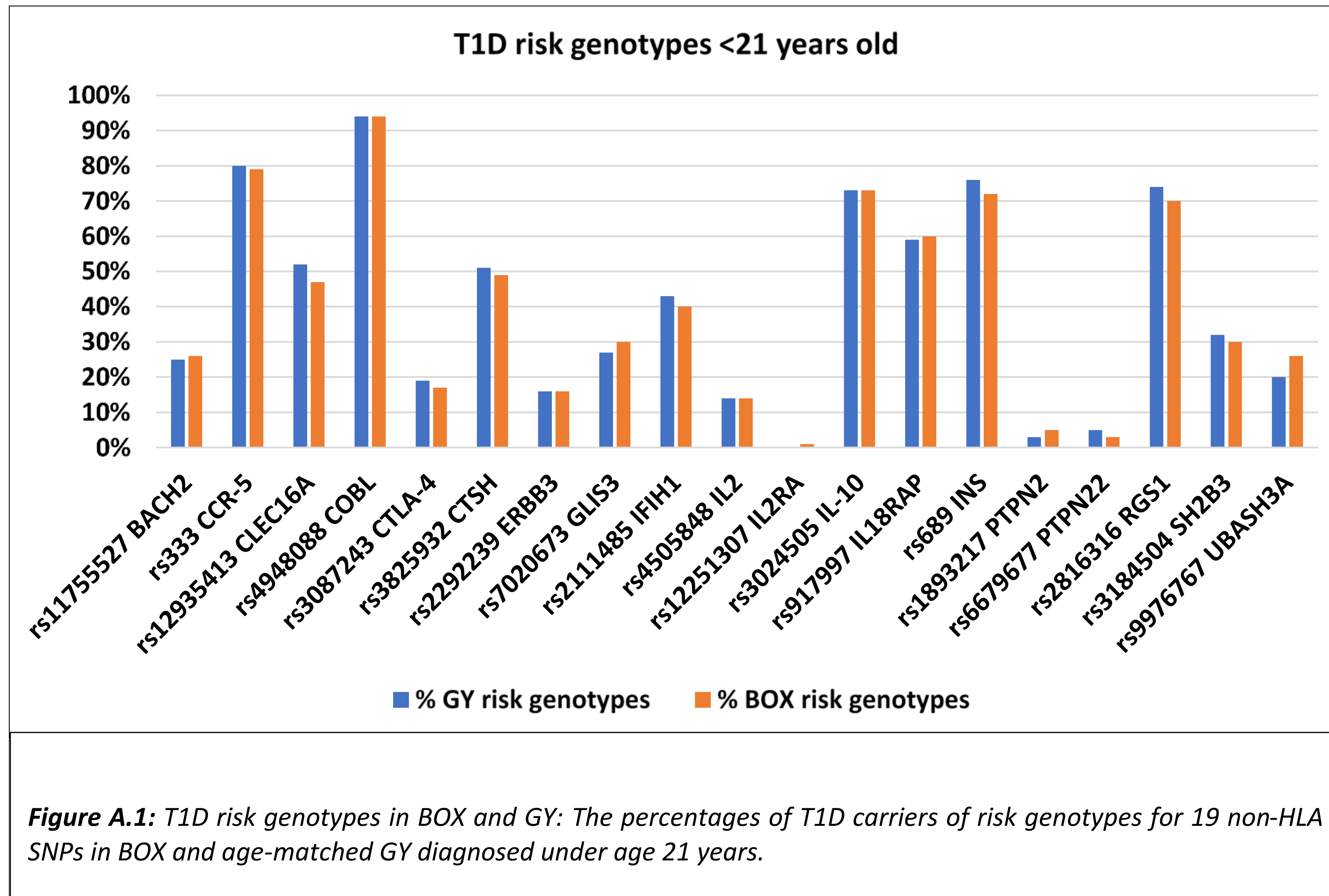

Figure A.1: *T1D risk genotypes in BOX and GY: The percentages of T1D carriers of risk genotypes for 19 non-HLA SNPs in BOX and age-matched GY diagnosed under age 21 years.*

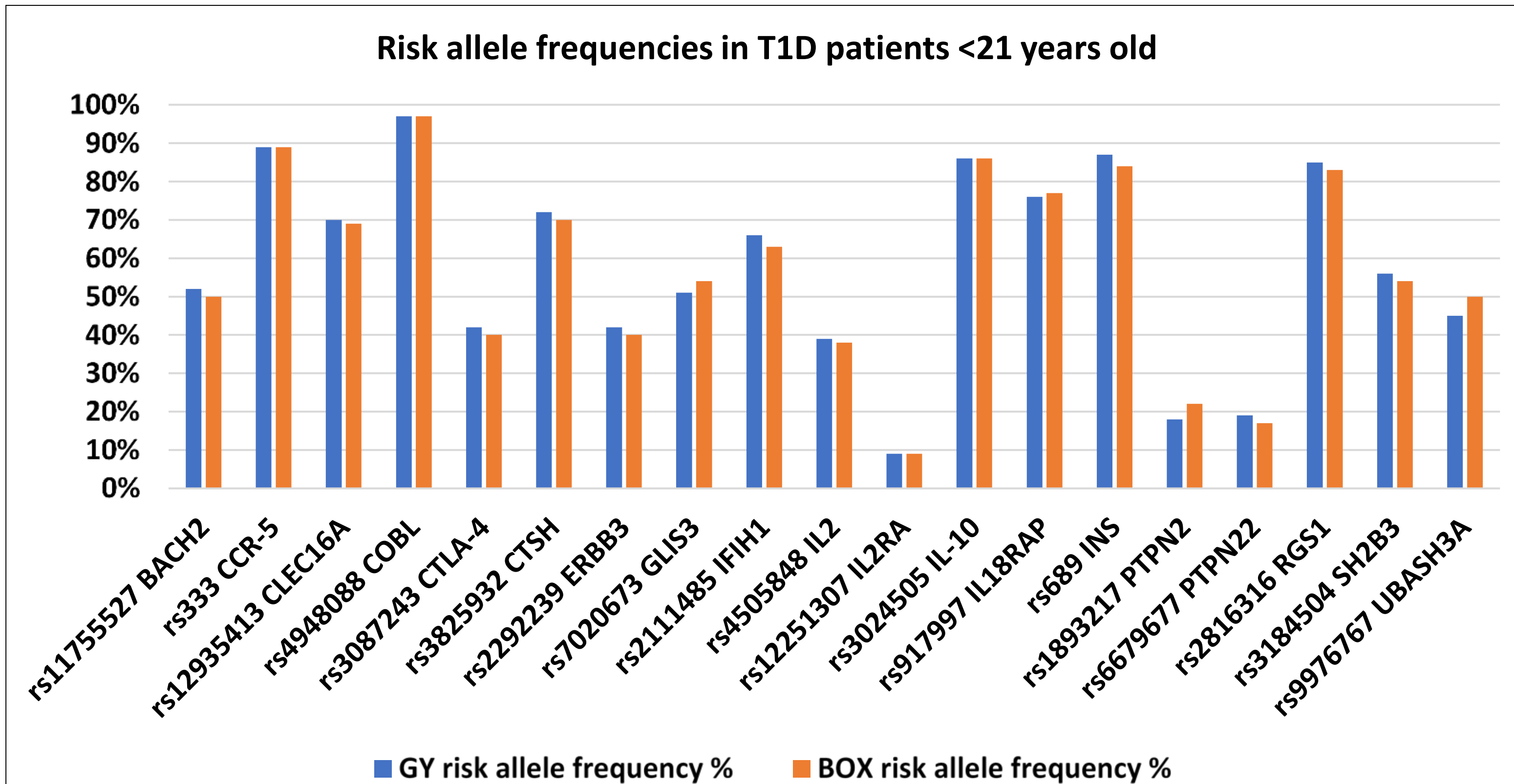

Figure A.2: *Risk allele frequencies in BOX and GY: The percentages of T1D risk allele frequencies for 19 non-HLA SNPs in BOX and age-matched GY diagnosed under age 21 years. The changes were modest overall. The biggest change was a 5% increase for the UBASH3A risk allele between the GY and BOX cohorts.*

Taqman Probe	Allele 1/Allele 2	Genotype	% BOX males	% GY males	% BOX females	% GY females
rs11755527 *BACH2*	C/G	C/C	26%	22%	26%	20%
		C/G	50%	54%	47%	54%
		G/G	24%	25%	27%	26%
rs333 *CCR-5*	WT/Δ32	WT/WT	80%	80%	77%	81%
		WT/Δ32	19%	18%	22%	19%
		Δ32/Δ32	1%	2%	1%	1%
rs12935413 *CLEC16A/KIAA0350*	A/G	A/A	9%	12%	10%	12%
		A/G	43%	40%	43%	34%
		G/G	48%	48%	47%	55%
rs4948088 *COBL*	A/C	A/A	0%	0%	0%	0%
		A/C	5%	7%	6%	5%
		C/C	95%	93%	94%	95%
rs3087243 *CTLA-4*	A/G	A/A	17%	18%	17%	20%
		A/G	47%	51%	46%	43%
		G/G	36%	31%	37%	37%
rs3825932 *CTSH*	C/T	C/C	47%	49%	51%	53%
		C/T	44%	44%	41%	41%
		T/T	9%	7%	8%	6%
rs2292239 *ERBB3*	G/T = A/G	G/G	39%	30%	34%	34%
		G/T	43%	53%	51%	51%
		T/T	18%	17%	15%	15%
rs7020673 *GLIS3*	C/G	C/C	20%	21%	22%	28%
		C/G	49%	54%	50%	45%
		G/G	31%	25%	28%	27%

Table A.1a: *Genotypes by gender in BOX and GY-Part A: The percentages of males and females carrying the risk genotypes (highlighted in pink) in BOX and age-matched GY under the age of 21 years.*

Taqman Probe	Allele 1/Allele 2	Genotype	% BOX males	% GY males	% BOX females	% GY females
rs2111485 *IFIH1*	C/T = A/G	C/C	15%	9%	13%	12%
		C/T	45%	43%	48%	49%
		T/T	40%	48%	39%	39%
rs4505848 *IL2*	A/G	A/A	37%	37%	39%	35%
		A/G	50%	45%	45%	54%
		G/G	13%	18%	16%	11%
rs12251307 *IL2RA*	C/T	C/C	81%	84%	83%	80%
		C/T	18%	16%	16%	20%
		T/T	1%	0%	1%	0%
rs3024505 *IL-10*	A/G	A/A	2%	2%	3%	2%
		A/G	24%	24%	26%	25%
		G/G	75%	74%	71%	73%
rs917997 *IL18RAP*	A/G = C/T	C/C	62%	60%	58%	57%
		C/T	32%	35%	38%	35%
		T/T	7%	5%	5%	8%
rs689 *INS*	A/T	A/A	3%	3%	5%	2%
		A/T	23%	21%	26%	23%
		T/T	73%	76%	69%	75%
rs1893217 *PTPN2*	A/G	A/A	62%	67%	63%	67%
		A/G	34%	29%	33%	30%
		G/G	4%	4%	5%	2%
rs6679677 *PTPN22*	A/C	A/A	3%	2%	3%	8%
		A/C	29%	30%	26%	26%
		C/C	68%	68%	71%	66%
rs2816316 *RGS1*	A/G	A/A	69%	75%	71%	72%
		A/G	28%	22%	27%	25%
		G/G	3%	2%	3%	3%
rs3184504 *SH2B3*	C/T = A/G	C/C	21%	23%	21%	16%
		C/T	48%	48%	50%	49%
		T/T	31%	28%	29%	35%
rs9976767 *UBASH3A*	A/G	A/A	27%	29%	25%	29%
		A/G	48%	50%	49%	52%
		G/G	25%	21%	26%	19%

Table A.1b: *Genotypes by gender in BOX and GY-Part A: The percentages of males and females carrying the risk genotypes (highlighted in pink) in BOX and age-matched GY under the age of 21 years.*

<u>B. Non-*HLA* SNPs in under 5 and 10 years old children</u>

GY < 10 years						
Non-*HLA* SNP	genotypes by number and percentage					
	11		12		22	
UBASH3A	29	25%	63	55%	23	20%
INS	1	1%	27	24%	85	75%
COBL	0	0%	7	6%	107	94%
PTPN22	9	8%	26	23%	78	69%
CCR5	92	84%	18	16%	0	0%
PTPN2	66	62%	35	33%	6	6%
CLEC16A	18	16%	38	34%	55	50%
IL18RAP	62	55%	44	39%	6	5%
SH2B3	21	19%	50	46%	38	35%
BACH2	23	21%	66	59%	23	21%
CTSH	56	51%	45	41%	8	7%
IL2RA	95	86%	16	14%	0	0%
IL2	28	26%	58	55%	20	19%
GLIS3	25	22%	54	48%	33	29%
ERBB3	42	38%	55	50%	14	13%
IL10	3	3%	27	24%	81	73%
RGS1	88	79%	23	21%	1	1%
CTLA4	25	22%	57	50%	32	28%
IFIH1	10	9%	56	48%	50	43%

Table A.2: *GY children diagnosed under the age of 10 years (n=115): The numbers and percentages of T1D non-HLA SNPs genotypes.*

BOX < 10 years (1985-1995)						
Non-*HLA* SNP	genotypes by number and percentage					
	11		12		22	
UBASH3A	77	28%	127	46%	74	27%
INS	13	5%	66	23%	208	72%
COBL	0	0%	11	4%	278	96%
PTPN22	7	2%	92	32%	190	66%
CCR5	232	81%	54	19%	1	0%
PTPN2	182	63%	94	32%	15	5%
CLEC16A	28	10%	121	42%	140	48%
IL18RAP	160	55%	109	38%	20	7%
SH2B3	52	18%	144	50%	93	32%
BACH2	72	25%	144	50%	73	25%
CTSH	145	50%	117	41%	26	9%
IL2RA	237	82%	52	18%	0	0%
IL2	109	38%	125	43%	54	19%
GLIS3	55	19%	149	52%	85	29%
ERBB3	108	38%	123	43%	57	20%
IL10	5	2%	70	24%	212	74%
RGS1	207	72%	70	24%	10	3%
CTLA4	63	22%	132	46%	95	33%
IFIH1	45	16%	115	40%	129	45%

Table A.3: *BOX children diagnosed between 1985-1995 with T1D under the age of 10 years: The numbers and percentages of T1D non-HLA SNPs genotypes. The total number of BOX children under 10 years old was 291, of whom 55% are males.*

BOX < 10 years (1995-2005)						
Non-*HLA* SNP	genotypes by number and percentage					
	11		12		22	
UBASH3A	80	20%	142	54%	64	26%
INS	14	3%	64	21%	217	76%
COBL	0	0%	21	2%	274	98%
PTPN22	7	4%	84	35%	204	61%
CCR5	232	79%	58	21%	3	0%
PTPN2	180	58%	111	39%	4	3%
CLEC16A	25	9%	136	43%	134	47%
IL18RAP	176	61%	96	32%	23	7%
SH2B3	67	17%	139	49%	89	34%
BACH2	75	23%	144	55%	75	22%
CTSH	156	50%	121	41%	18	9%
IL2RA	249	84%	43	16%	3	0%
IL2	102	36%	149	48%	44	15%
GLIS3	58	23%	149	50%	88	27%
ERBB3	105	36%	144	47%	46	17%
IL10	6	2%	71	28%	218	70%
RGS1	198	74%	90	23%	7	3%
CTLA4	51	21%	133	52%	111	27%
IFIH1	46	15%	141	43%	108	42%

Table A.4: *BOX children (1995-2005) diagnosed with T1D under the age of 10 years: The numbers and percentages of T1D non-HLA SNPs genotypes. The total number of BOX children under 10 years old was 295, of whom 49% are males.*

GY < 5 years						
Non-*HLA* SNP	genotypes by number and percentage					
	11		12		22	
UBASH3A	8	24%	19	58%	6	18%
INS	0	0%	11	33%	22	67%
COBL	0	0%	4	12%	29	88%
PTPN22	2	6%	6	18%	25	76%
CCR5	25	81%	6	19%	0	0%
PTPN2	2	6%	6	19%	23	74%
CLEC16A	5	16%	14	45%	12	39%
IL18RAP	20	65%	10	32%	1	3%
SH2B3	4	13%	11	37%	15	50%
BACH2	7	23%	16	52%	8	26%
CTSH	14	45%	17	55%	0	0%
IL2RA	26	84%	5	16%	0	0%
IL2	5	17%	19	63%	6	20%
GLIS3	3	10%	14	45%	14	45%
ERBB3	7	23%	18	58%	6	19%
IL10	2	6%	8	26%	21	68%
RGS1	24	77%	7	23%	0	0%
CTLA4	7	21%	16	48%	10	30%
IFIH1	3	9%	16	47%	15	44%

Table A.5: *GY children diagnosed with T1D under the age of 5 years: The numbers and percentages of T1D non-HLA SNPs genotypes. The total number of BOX children diagnosed under 5 years was 37, of whom 46% are males.*

BOX < 5 years (1985-1995)						
Non-*HLA* SNP	genotypes by number and percentage					
	11		12		22	
UBASH3A	26	28%	72	50%	35	22%
INS	4	5%	29	22%	103	74%
COBL	0	0%	3	7%	134	93%
PTPN22	5	2%	48	28%	84	69%
CCR5	108	79%	28	20%	0	1%
PTPN2	79	61%	54	38%	4	1%
CLEC16A	13	8%	59	46%	65	45%
IL18RAP	84	60%	44	33%	9	8%
SH2B3	23	23%	67	47%	47	30%
BACH2	31	26%	76	49%	30	26%
CTSH	68	53%	56	41%	13	6%
IL2RA	115	84%	22	15%	0	1%
IL2	50	35%	66	51%	21	15%
GLIS3	31	20%	69	51%	37	30%
ERBB3	49	36%	64	49%	23	16%
IL10	3	2%	38	24%	95	74%
RGS1	100	67%	31	31%	4	2%
CTLA4	29	17%	71	45%	37	38%
IFIH1	21	16%	59	48%	57	37%

Table A.6: *BOX children diagnosed with T1D between 1985-1995 under the age of 5 years: The numbers and percentages of T1D non-HLA SNPs genotypes. The total number of BOX children diagnosed under 5 years was 137, of whom 55% are males.*

BOX < 5 years (1995-2005)						
Non-*HLA* SNP	genotypes by number and percentage					
	11		12		22	
UBASH3A	28	28%	55	55%	17	17%
INS	5	5%	23	22%	77	73%
COBL	0	0%	9	9%	96	91%
PTPN22	4	4%	25	24%	76	72%
CCR5	85	83%	18	17%	0	0%
PTPN2	62	59%	40	38%	3	3%
CLEC16A	9	9%	50	48%	46	44%
IL18RAP	65	62%	34	32%	6	6%
SH2B3	28	27%	48	46%	29	28%
BACH2	29	28%	57	54%	19	18%
CTSH	52	50%	44	42%	9	9%
IL2RA	90	86%	13	12%	2	2%
IL2	34	32%	53	50%	18	17%
GLIS3	19	18%	57	54%	29	28%
ERBB3	36	34%	46	44%	23	22%
IL10	2	2%	22	21%	81	77%
RGS1	68	65%	34	32%	3	3%
CTLA4	20	19%	49	47%	36	34%
IFIH1	12	11%	48	46%	45	43%

Table A.7: *BOX children diagnosed with T1D between 1995-2005 under the age of 5 years: The numbers and percentages of T1D non-HLA SNPs genotypes. The total number of BOX children diagnosed under 5 years was 105, of whom 50% are males.*

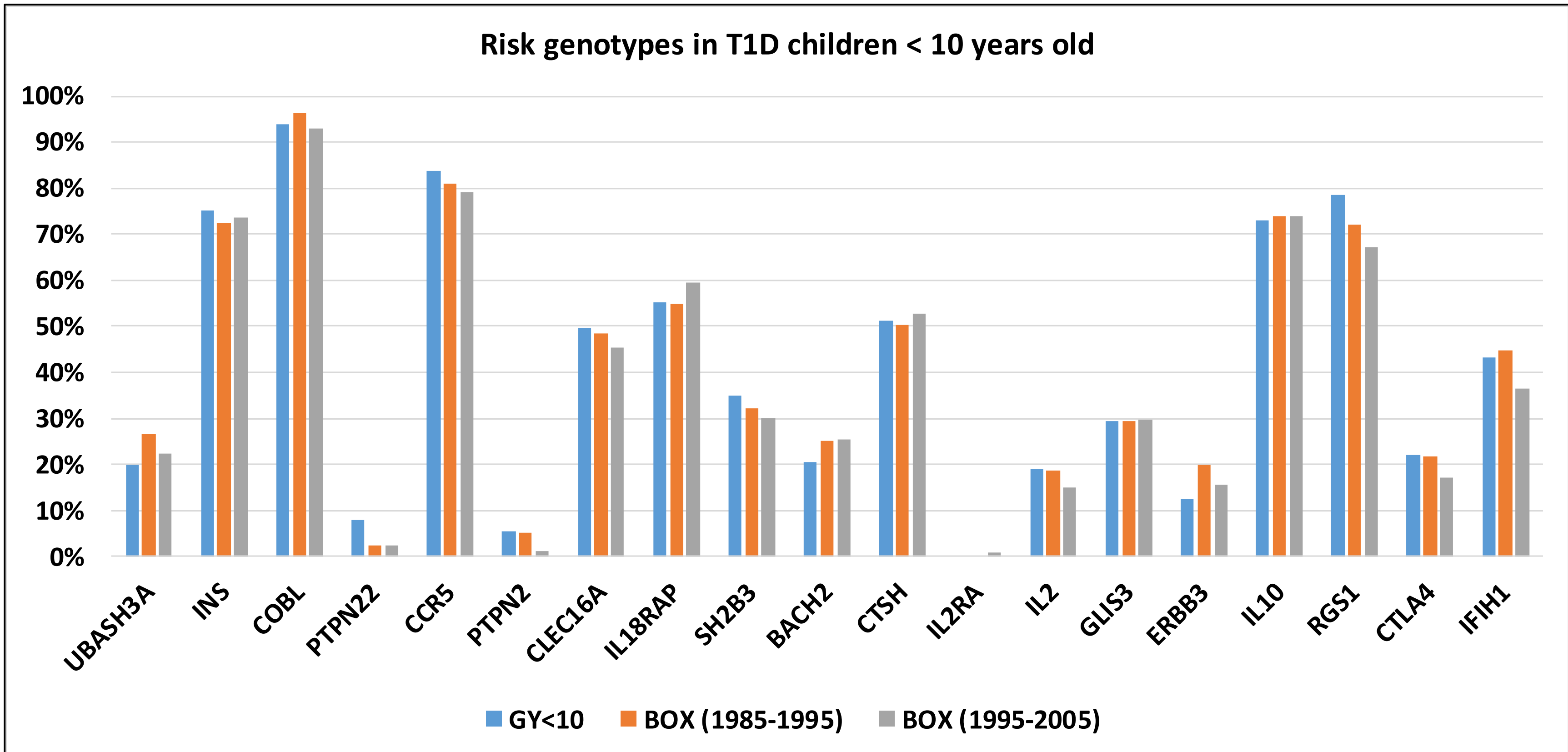

Figure A.3: *Non-HLA risk genotypes in T1D GY and BOX children under the age of 10 years by decade of diagnosis: GY are blue bars and BOX (1995-1985) are orange bars while BOX (1995-2005) are grey bars.*

C. Non-*HLA* SNPs and islet autoantibodies associations at diagnosis

FCRL3 genotypes	IA-2A positive	IA-2A negative
11	151 (89%)	19 (11%)
12	202 (75%)	68 (25%)
22	82 (71%)	33 (29%)

Table A.8: *FCRL3 genotypes and IA-2A: The number and percentage of FCRL3 genotypes by their association with IA-2A at onset in newly-diagnosed BOX participants. The CC (11) genotype had the highest association with IA-2A positivity. P<0.001 chi-square test.*

FCRL3 genotypes	IA-2βA positive	IA-2βA negative
11	135 (79%)	35 (21%)
12	173 (65%)	95 (35%)
22	66 (58%)	48 (42%)

Table A.9: *FCRL3 genotypes and IA-2βA: The number and percentage of FCRL3 genotypes by their association with IA-2βA at onset in newly-diagnosed BOX participants. The CC (11) genotype had the highest association with IA-2βA positivity. P<0.001 chi-square test.*

FCRL3 genotypes	ZnT8A positive	ZnT8A negative
11	140 (82%)	30 (18%)
12	172 (64%)	98 (36%)
22	74 (64%)	41 (36%)

Table A.10: *FCRL3 genotypes and ZnT8A: The number and percentage of FCRL3 genotypes by their association with ZnT8A at onset in newly-diagnosed BOX participants. The CC (11) genotype had the highest association with positivity (p<0.001) chi-square test.*

RELA genotypes	GADA positive	GADA negative
11	278 (80%)	71 (20%)
12	138 (77%)	41 (23%)
22	20 (100%)	0 (0%)

Table A.11: *RELA genotypes and GADA: The number and percentage of each of the RELA genotypes by their association with GADA at onset in newly-diagnosed BOX participants. The TT (22) genotype had the highest association with GADA positivity (p=0.055) chi-square test.*

LPP genotypes	GADA positive	GADA negative
11	114 (88%)	15 (12%)
12	216 (79%)	57 (21%)
22	112 (75%)	37 (25%)

Table A.12: *LPP genotypes and GADA: The number and percentage of LPP genotypes by their association with GADA at onset in newly-diagnosed BOX participants. The GG (11) genotype had the highest association with GADA positivity (p=0.018) chi-square test.*

LPP genotypes	IA-2βA positive	IA-2βA negative
11	95 (74%)	33 (26%)
12	188 (70%)	82 (30%)
22	88 (60%)	58 (40%)

Table A.13: *LPP genotypes and IA-2βA: The number and percentage of LPP genotypes by their association with IA-2βA at onset in newly diagnosed BOX participants. The GG (11) genotype had the strongest association with IA-2βA positivity (p=0.036) chi-square test.*

BACH2 genotypes	GADA positive	GADA negative
11	102 (72%)	40 (28%)
12	249 (82%)	55 (18%)
22	124 (83%)	26 (17%)

Table A.14: *BACH2 genotypes and GADA: The number and percentage of BACH2 genotypes by their association with GADA at onset in newly-diagnosed BOX participants. The GG (22) T1D risk genotype had the strongest association with GADA positivity (puncorr=0.028). Chi-square test without Bonferroni correction.*

CCR-5 genotypes	GADA positive	GADA negative
11	380 (82%)	86 (18%)
12	88 (72%)	35 (28%)
22	3 (60%)	2 (40%)

Table A.15: *CCR-5 genotypes and GADA: The number and percentage of CCR-5 genotypes by their association with GADA at onset in newly-diagnosed BOX participants. The WTWT (11) T1D risk genotype had the strongest association with GADA positivity (puncorr=0.029). Chi-square test without Bonferroni correction.*

UBASH3A genotypes	GADA positive	GADA negative
11	114 (74%)	41 (26%)
12	206 (82%)	44 (1%)
22	127 (83%)	26 (17)

Table A.16: *UBASH3A genotypes and GADA: The number and percentage of UBASH3A genotypes by their association with GADA at onset in newly-diagnosed BOX individuals. The GG (22) T1D risk genotype had a borderline association with GADA positivity (puncorr=0.055). Chi-square test without Bonferroni correction.*

CTLA-4 genotypes	IA-2βA positive	IA-2βA negative
11	73 (71%)	30 (29%)
12	207 (71%)	83 (29%)
22	122 (60%)	81 (40%)

Table A.17: *CTLA-4 genotypes and IA-2βA: The number and percentage of CTLA-4 genotypes by their association with IA-2βA at onset in newly-diagnosed BOX individuals. The AA (11) T1D risk genotype had the strongest association with IA-2βA positivity (puncorr=0.023). Chi- square test without Bonferroni correction.*

CTLA-4 genotypes	IAA positive	IAA negative
11	44 (60%)	30 (40%)
12	142 (74%)	49 (26%)
22	107 (70%)	47 (30%)

Table A.18: *CTLA-4 genotypes and IAA: The number and percentage of CTLA-4 genotypes by their association with IAA at onset in newly-diagnosed BOX participants. The GG (22) T1D risk genotype had a borderline association with IAA positivity (puncorr=0.059). Chi-square test without Bonferroni correction.*

IL2 genotypes	IAA positive	IAA negative
11	100 (63%)	60 (37%)
12	147 (74%)	53 (26%)
22	43 (78%)	12 (22%)

Table A.19: *IL2 genotypes and IAA: The number and percentage of IL2 genotypes by their association with IAA at onset in newly-diagnosed BOX participants. The GG (22) T1D risk genotype had a borderline association with IAA positivity (puncorr=0.028). Chi-square test without Bonferroni correction.*

D. Golden Years dataset

Sample number	GY number	Sex	Age at diagnosis	Duration of diabetes	DR	DQ	DRB1	DQA1	DQB1	DRB1	DQA1	DQB1
2	WDDP002	M	16	54	3/4	2/8	*0304	*05011-02	*02	*0401	*03011/12	*0302
3	WDAD003	M	10	62	4/4	7/8	*0405	*03011/12	0301/4	*0405	*03011/12	*0302
6	WDRB006	F	11	52	3/3	2/2	*0304	*05011-02	*02	*0304	*05011-02	*02
8	WDSG008	M	7	58	1/4	5/8	*0103	0101/4	*05	*0401	*03011/12	*0302
9	WDSC009	M	20	55	4/4	7/8	*04	*03011/12	0301/4	*04	*03011/12	*0302
12	WDRH012	M	16	61	3/4	2/8	*0304	*05011-02	*02	*0401	*03011/12,	*0302
13	WDGD013	M	14	54	3/4	2/8	*0304	*05011-02	*02	*0404	*03011/12,	*0302
15	WDWL015	M	8	53	4/8	1.x/4	*0401	*03011/12	*0604-9	*0801-11	*0401	*04
16	WDDR016	M	19	63	4/13	8/1.18	*0401	*03011/12	*0302	*13	*0102/3	0603
18	WDJF018	M	13	51	3/3	2/2	*0304	*05011-02	*02	*0304	*05011-02	*02
24	WDMO024	F	10	56	3/4	2/8	*0304	*05011-02	*02	*0401	03011/12	*0302
27	WDED027	F	20	53	3/4	2/8	*0304	*05011-02	*02	*04	*03011/12	*0302
31	WDJD031	M	18	56	7/7	2/2	*0701	*0201	*02	*0701	*0201	*02
32	WDJV032	F	11	65	4/4	8/8	*0401	*03011/12	*0302	*0401	*03011/12	*0302
33	WDJS033	M	13	55	1/4	5/7	*01	0101/4	*05	*0401	*03011/12	*0301/4
34	WDJC034	M	13	56	3/4	2/8	*0304	*05011-02	*02	*0401	*03011/12	*0302
36	WDBP036	F	17	53	4/9	8/9	*0401	*03011/12	*0302	*0901	*03011/12	*03032
38	WDPW038	F	11	54	3/3	2/2	*0301	*05011-02	*02	*0301	*05011-02	*02
40	WDAJ040	F	15	61	3/7	2/2	*0304	*05011-02	*02	*0701	*0201	*02
41	WDJM041	F	13	52	3/4	2/8	*0304	*05011-02	*02	*04	*03011/12	*0302
42	WDPB042		12	61	1/4	5/8	*01	*0101/4	*05	*0404	*03011/12	*0302
46	WDCS046	M	9	52	1/4	5/8	*01	*0101/4	*05	*04	*03011/12	*0302
50	WDMM050	F	9	58	4/13	7/1.x	*0401	*03011/12	*0301/4	*13	*0102-3	*0604-9
52	WDMA052	F	19	54	1/4	5/8	*01	*0101/4	*05	*04	*03011/12	*0302
53	WDRM053	F	15	61	4/4	7/8	*04	*03011/12	*0301/4	*04	*03011/12	*0302
55	WDAM055	M	20	52	3/13	2/1.x	*0304	*05011-02	*02	*13	*0102/3	*0604-9
57	WDDA057	F	11	52	4/8	8/4	*04	*03011/12	*0302	*0801-11	*0401	*04

Table A.20: *A representation of the Golden Years dataset.*

E. BOX Study dataset

IDF	IDS	DM	DR Allele 1	DR Allele 2	DQB1 Allele 1	DQB1 Allele 2	Date of diagnosis	Sex	Age of diagnosis
251	4	1	04	04	0301	0302	06/03/1985	2	11
252	3	1	04	06	0302	0603	10/04/1985	1	15
253	5	1	03	04	0201	0302	09/08/1985	2	16
254	3	1	04	04	0302	0302	08/05/1985	2	18
255	5	1	03	04	02	0302	17/07/1985	2	12
256	4	1	01	04	0302	0501	10/04/1985	1	19
258	5	1	01	04	0302	0501	15/09/1985	1	11
259	3	1	04	09	0302	0303	24/06/1985	1	14
261	3	1	03	04	02	0302	15/06/1985	2	5
262	3	1	04	04	0302	0302	13/02/1985	1	10
265	4	1	03	04	02	0302	26/08/1985	2	13
267	3	1	01	04	0302	0501	14/10/1985	2	15
268	4	1	03	03	02	0201	15/04/1985	1	16
270	5	1	03	04	02	0302	20/01/1985	2	14
272	6	1	03	04	0201	0302	05/11/1985	1	13
274	4	1	03	07	02	02	04/11/1985	1	10
275	3	1	04	07	02	0302	15/10/1985	1	17
276	3	1	01	04	0302	0501	16/12/1985	1	8
277	7	1	03	04	02	0302	15/04/1985	2	12
278	4	1	04	11	0301	0302	15/07/1985	1	17
279	6	1	04	09	0302	0303	14/11/1985	2	9
280	4	1	02	03	02	NON	22/10/1985	1	18
281	3	1	01	04	0301	0501	12/08/1985	1	14
282	3	1	03	03	0201	0201	09/07/1985	1	15
283	3	1	01	04	0302	0501	16/01/1986	1	9
285	4	1	04	04	0302	0302	15/08/1985	1	10
286	3	1	03	04	02	0302	15/11/1985	2	20
287	6	1	04	04	0301	0302	18/01/1985	1	17

Table A.21: *A representation of the BOX study dataset.*