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# Characterising The Clinical Heterogeneity Of Type 1 Diabetes

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in fulfilment of the requirements  
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## Declaration

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The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I have not previously submitted this material, either in part or in full, for a degree at this or any other institution.



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Myra Sui Yen Poon


24 October 2015

## Ethics approval

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All studies in this thesis were approved by the Ethics Committee of the Sydney Children's Hospitals Network.

All participants or guardians gave written informed consent for analysis of their results.



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Myra Sui Yen Poon

24 October 2015

## **Dedication**

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This thesis is dedicated to my parents, Christopher and Mary Poon, for their love, encouragement and support.

## Acknowledgements

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I wish to acknowledge the following people who have supported me throughout my PhD. To my supervisors, Professor Kim Donaghue and Professor Maria Craig - thank you for your encouragement, guidance and generously giving your time to provide invaluable feedback. Kim, you have been, and continue to be, a wonderful mentor and advisor.

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Finally to my husband, Cliff and son, Kal without whose love, patience and belief, this journey would not have been completed.



## **Abstracts presented**

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Results from the research in this thesis were presented at both national and international meetings as outlined below:

**1. International Society for Paediatrics and Adolescent Diabetes Annual Meeting, September 2014, Toronto, Canada**

Poster Presentation: *Do markers of subclinical autonomic neuropathy predict mortality in young people with T1D?*

**2. Joint meeting of the European Society for Paediatric Endocrinology and the Lawson Wilkins Pediatric Endocrine Society, September 2013, Milan, Italy**

Poster Presentation: *Are environmental and genetic factors at type 1 diabetes diagnosis associated with the development of microvascular complications?*

**3. Australasian Paediatric Endocrine Group Annual Scientific Meeting, July 2013, Sydney, Australia**

Oral Presentation: *Viruses and new onset type 1 diabetes*

**4. International Society for Paediatrics and Adolescent Diabetes Annual Meeting, October 2012, Istanbul, Turkey**

Oral Presentation: *The association between vitamin D deficiency and retinopathy in type 1 diabetes is not explained by changes in retinal vascular calibre*

**5. Australasian Paediatric Endocrine Group Annual Scientific Meeting, July 2012,  
Queenstown, New Zealand**

Oral Presentation: *The association between vitamin D deficiency and retinopathy in type 1 diabetes is not explained by changes in retinal vascular calibre*

**6. Australasian Paediatric Endocrine Group Annual Scientific Meeting, August  
2011, Perth, Australia**

Oral Presentation: *The role of environmental factors in type 1 diabetes*

**7. European Society for Paediatric Endocrinology Annual Scientific Meeting, June  
2011, Hangzhou, China**

Poster Presentation: *Vitamin D deficiency is associated with EV infection in new onset type 1 diabetes*

## **Grants and Scholarships**

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**National Health and Medical Research Council Postgraduate Medical Research Scholarship 2010 – 2013** – *“Environmental risk factors for type 1 diabetes and long term complications”*

**Diabetes Australia Research Trust Grant 2012** - *“The role of environmental factors in the development of microvascular and macrovascular complications in type 1 diabetes – a 14 year follow up of an incident cohort”*

**Australasian Paediatric Endocrine Group and Novo Nordisk research grant 2011-2012** – *“The role of environmental factors in the development of microvascular and macrovascular complications in type 1 diabetes – a 14 year follow up of an incident cohort”*

**Novo Nordisk Regional Diabetes Support Scheme grant 2011** – *“The role of environmental factors in diabetes complications – a 12 year follow up of an incident cohort”*

**Juvenile Diabetes Research Foundation and Royal Australasian College of Physicians Fellowship 2010** – *“Environmental risk factors for type 1 diabetes and complications”*

## Awards

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The following prizes were awarded for research presentations related to this thesis.

### **Sapphire Bioscience Prize for Excellence**

2012 Postgraduate Student Conference, Discipline of Paediatrics and Child Health, The Children's Hospital at Westmead at Western Clinical School

### **Pathtech Prize for Excellence,**

2013 Postgraduate Student Conference, Discipline of Paediatrics and Child Health, The Children's Hospital at Westmead at Western Clinical School

## Publications related to thesis

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1. **Poon M**, Rawlinson WD, Craig ME. Diabetes and Viruses in Australia and the Asia Pacific Region. In *Diabetes and Viruses*, Ed: Hyoty H, Toniolo A. Springer, 207-18, 2013.
2. **Poon M**, Craig ME, Kaur H, Cusumano J, Sasongko MB, Wong TY, Donaghue KC. Vitamin D Deficiency Is Not Associated with Changes in Retinal Geometric Parameters in Young People with Type 1 Diabetes. *Journal of Diabetes Research*, Volume 2013 (2013), Article ID 280691, 5 pages.

## List of Abbreviations

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1,25(OH) <sub>2</sub> D <sub>3</sub>	1,25 dihydroxyvitamin D <sub>3</sub>
25OHD	25-hydroxyvitamin D
ACR	Albumin-creatinine Ratio
AdDit	Adolescent Type 1 Diabetes Cardio-Renal Interventional trial
AER	Albumin excretion rate
AGE	Advanced glycation End-products
AIRE	Autoimmune regulator gene
BMI	Body mass index
C pneumoniae	Chlamydia pneumoniae
CB4	Coxsackie B4
CIMT	Carotid intima media thickness
CMV	Cytomegalovirus
CSII	Continuous subcutaneous insulin infusion
DAISY	Diabetes Autoimmunity Study in the Young
DCCT	Diabetes Control and Complications Trial
DIAMOND	Multinational Project for Childhood Diabetes
DiME	Childhood Diabetes in Finland
DiPP	Diabetes Prediction and Prevention Study
DMO	Diabetic macular oedema
DPT-1	Diabetes Prevention Trial - Type 1
DR	Diabetic retinopathy
EDC	Epidemiology of Diabetes Complications
EDIC	Epidemiology of Diabetes Interventions and Complications
eGDR	Estimated glucose disposal rate
EGFR	Estimated glomerular filtration rate
ENDIT	European Nicotinamide Diabetes Intervention Trial
ETDRS	Early Treatment Diabetes Retinopathy
EURODIAB	European Diabetes Study Group
EV	EV
FoxP3	Foxhead box P3
FPIR	First phase insulin response
GAD	Glutamic acid decarboxylase autoantibody
GDR	Glucose disposal rate
GFR	Glomerular filtration rate
GBM	Glomerular basement membrane
H pylori	Helicobacter pylori
HbA1c	Glycated haemoglobin
HBV	Hepatitis B virus
HLA	Human leukocyte antigen
HLA-DQ8	DRB1*04-DQA1*0301-DQB1*0302
HLA-DQ2	DRB1*0301-DQA1*0501-DQB1*0201
HLA-DR6	DRB1*1505-DQA1*0102-DQB1*0602
HOMA-IR	Homeostatic model assessment - insulin resistance
IA	Islet autoimmunity
IAA	Insulin autoantibody
IA2	Tyrosine-phosphatase-like insulinoma antigen

ICA	Islet cell autoantibodies
MDI	Multiple daily Injections
MHC	Major histocompatibility complex
NGF	Nerve growth factor
NHANES	National Health and Nutrition Examination Survey
PCR	Polymerase chain reaction
PDR	Proliferative diabetic retinopathy
ROS	Reactive oxygen species
SMR	Standardised mortality ratio
T1D	Type 1 diabetes
T2D	Type 2 diabetes
Treg	Regulatory T cells
UV	Ultraviolet
VDR	Vitamin D receptor
VEGF	Vascular endothelial growth factor
WESDR	Winconsin Epidemiologic Study of Diabetes Retinopathy
ZnT8A	Zinc transporter 8A

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## Abstract

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There is growing evidence that type 1 diabetes is not a single disease with a predictable course, but rather comprises a number of disease phenotypes characterised by distinct immunologic, genetic and metabolic features. Indeed, a recent shift towards onset in patients at lower genetic risk highlights the increasing importance of environmental and metabolic factors in disease pathogenesis. These factors may contribute to development of microvascular complications and mortality risk. This thesis aims to characterise the heterogeneity of type 1 diabetes and its complications by describing four cohorts diagnosed between 1973 and 2014.

There was a significant reduction in the frequency of high-risk genotypes between two incident cohorts presenting to our hospital over 15 years (Cohort A: diagnosed 1997-1999 and Cohort B: diagnosed 2010-14). The proportion of children of Australian/European ethnicity also declined. The difference in proportion of patients with the high-risk genotype was independent of ethnicity. Vitamin D levels were lower in Cohort B and there was a higher rate of vitamin D deficiency. Differences in vitamin D levels were independent of ethnicity. Bicarbonate levels have been shown to influence vitamin D levels, however the differences in vitamin D between groups was independent of bicarbonate levels. Further analysis of Cohort B revealed that those aged less than five years were taller, heavier, had lower vitamin D levels and more islet autoantibodies, which may be suggestive of a greater autoimmune response, compared with those aged over five years.

To investigate whether factors at type 1 diabetes diagnosis influence the development of microvascular complications, Cohort A was followed longitudinally for fifteen years. The presence of HLA-DRB1\*04-DQB1\*0302 was associated with reduced risk of autonomic neuropathy and DRB1\*04 or \*03 associated with increased time to onset of microalbuminuria. EV infection at diagnosis was associated with reduced risk of retinopathy suggesting that genetics and response to inflammation may modify the other known risk factors.

The fourth study explored the previously reported association between vitamin D deficiency and retinopathy in Cohort C. In this cohort, vitamin D deficiency was associated with a two-fold increased risk of retinopathy but was not associated with changes in retinal vascular geometry parameters.

The role of microvascular disease in mortality was explored in Cohort D (diagnosed 1973-1993). In a longitudinal study after median follow up 23 years, standardised mortality was increased in patients before 45 years of age, particularly in females. Elevation in albumin excretion rate and autonomic neuropathy predicted mortality, highlighting the importance of screening for early signs of microvascular disease and markers of subclinical autonomic neuropathy.

Findings from this thesis contribute to understanding the heterogeneity of T1D and its disease course, identification of novel risk factors for microvascular complications and early risk factors for increased mortality. These findings may allow appropriate risk stratification and targeted intervention to prevent type 1 diabetes onset, development of microvascular complications and to reduce mortality.

## Chapter 1 Hypotheses and aims

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## **Overview**

The overarching theme of this thesis is the heterogeneity of type 1 diabetes throughout the disease course. This thesis proposes that genetic and environmental factors contribute to this heterogeneity. The environmental factors of particular interest are adiposity, viruses and vitamin D. Moreover, the relative importance of genetic and environmental factors may have evolved over time. This will be demonstrated by comparing features of two incident cohorts diagnosed fifteen years apart. An historical cohort will be reassessed as young adults and to investigate the role of factors at diagnosis in the development of microvascular complications. The role of vitamin D in the development of retinal vascular changes will be studied. Finally the impact of adolescent complication screening assessment on mortality will be evaluated.

## **Hypotheses**

1. Environmental risk factors and adiposity are associated with the increased proportion of children with type 1 diabetes who carry low risk HLA genotypes
2. Features at diagnosis (adiposity, viral trigger, vitamin D status, HLA genotype) influence the development of complications in young people with type 1 diabetes
3. Vitamin D deficiency mediates changes in retinal vascular geometry

4. Adolescent autonomic dysfunction, microvascular complications and glycaemic control in adolescence predict mortality in type 1 diabetes patients 23 years later

## **Aims**

1. To characterise a contemporary incident cohort of young people with type 1 diabetes and determine if:

a) Those with low risk HLA genotypes differ in adiposity, viruses or vitamin D deficiency from those with high risk HLA genotypes

b) Children with type 1 diabetes diagnosis <5 years of age have different characteristics to those diagnosed >5 years of age

2. To determine if adiposity, viruses or vitamin D deficiency, are more prevalent at the diagnosis of type 1 diabetes in children diagnosed currently compared with those diagnosed 15 years earlier

3. To determine if features at the diagnosis of type 1 diabetes (adiposity, viral trigger, vitamin D deficiency, HLA genotype) are associated with increased risk of microvascular complications in an incident cohort after 15 years diabetes duration

4. To determine if vitamin D deficiency is associated with adverse retinal vascular geometry parameters

5. To determine if adolescent complications, including subclinical autonomic neuropathy, predict mortality in young adults with type 1 diabetes 23 years later

## Chapter 2 Literature Review

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## **2.1 Epidemiology of Type 1 diabetes**

Type 1 diabetes (T1D) is an autoimmune condition affecting children, adolescents and young adults. It is generally thought that disease is precipitated by immune associated destruction of insulin-producing pancreatic  $\beta$  cells which results from the influence of environmental factors on genetically susceptible individuals. The environmental triggers have yet to be fully elucidated.

There is a wide variation in T1D incidence around the world. Incidence rates in children aged 14 years or under were reported by the International Diabetes Federation in 2013 and ranged from 0.1 per 100 000 in South East Asia to 57.6 per 100 000 in Finland. Asian countries tended to have very low incidence. The highest incidence rates were seen in Scandinavian countries, Saudi Arabia and the United Kingdom, however even within these continents there was wide variation in incidence (Patterson, Guariguata, et al. 2014a).

Incidence rates of T1D have been increasing over time and the average annual global increase in incidence was 2.8% (95% CI 2.4-3.2%) between 1990 and 1999. A slightly higher rate of increase of 3.4% (95% CI 2.7-4.3%) was seen in the later half of the study period. The greatest increase in rate has been in the youngest age group - 4.0% in the 0-4 year age group compared with 2.1% in the 10-14 year age group (The DIAMOND Project Group 2006).

The incidence of T1D increased in Australia from 19.8 per 100 000 in 2000 to 23.4 per 100 000 in 2006, representing an average increase of 2.8% (95% CI 1.5, 4.1) per year (Catanzariti et al. 2009). During this period, incidence increased with age, ranging from

14.8 per 100,000 in children aged 0-4 years to 29.0 per 100,000 in those aged 10-14 years. Incidence was higher in boys in the 0-4 year and 10-14 year age groups. In a subsequent study from New South Wales, the incidence of T1D in 10-18 year old children increased by 3.8% per year between 2001 and 2008 (Tran et al. 2014). In Finland and Sweden the incidence has plateaued from 2005 (Berhan et al. 2011; Harjutsalo et al. 2013) with perhaps a shift to a younger age at diagnosis (Karvonen et al. 1999; S. G. Gardner et al. 1997).

Seasonal variation in incidence accompanies the geographic variability, with more cases diagnosed in autumn and winter than summer (Patterson, Gyürüs, et al. 2014b). Some of the geographic differences in incidence may be due to genetic variability of different ethnic groups. Alternatively, differences in environmental exposures in different regions may account for variability in incidence.

## **2.2 Pathophysiology and natural history of type 1 diabetes**

T1D results from the progressive loss of pancreatic beta cell function by an immune-mediated process. Studies in animal models of T1D and affected individuals demonstrate that disease results from dysregulation of self-tolerance processes with production of pathogenic auto-reactive CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes and autoantibodies targeting pancreatic islet cell antigens. The resulting pancreatic beta cell destruction leads to reduction in insulin production and hyperglycaemia. The onset and tempo of this process is variable, influenced by a number of factors, and may occur over years. Clinical T1D occurs when 70-80% of the beta cell mass has been destroyed (Muir et al. 2014).

### **Triggers for autoimmunity**

The trigger for initiation of the autoimmune process has not been clearly elucidated, however there is evidence that auto reactive T cells play an early role in the process. In the Non-Obese Diabetic (NOD) mouse model of T1D, CD11+ dendritic cells and ER-MP23+ macrophages are seen at 3 weeks of age. Shortly after, auto reactive T cells are found in the pancreatic draining lymph nodes. The transfer of T cells from a NOD mouse is sufficient to induce diabetes in young irradiated recipients (Miller et al. 1988; Peterson & Haskins 1996; Christianson et al. 1993). In humans, the MHC class II locus, associated with antigen presentation to T cells, confers genetic susceptibility (Erlich et al, 2008).

Neonatal beta cell apoptosis, a physiological process that is part of organ remodelling, may be the mechanism by which self-antigens are presented to the immune system. The initial islet cell damage, when it occurs at this time, may release further antigens with subsequent epitope spreading, leading to amplification of the immune response. The islet auto-antigens insulin, glutamine acid decarboxylase and zinc transporter 8, as well as islet-specific glucose-6-phosphatase catalytic subunit-related protein and chromogranin A, are specific T cell targets (Bluestone et al. 2010).

### **The role of insulin in autoimmunity**

Insulin is further implicated in two additional pathogenic mechanisms. Firstly, there may be defective negative selection of thymic T-cells that are reactive to insulin. Evidence for this has been derived from individuals with spontaneous mutations of the autoimmune regulator gene (AIRE). AIRE controls the ectopic expression of insulin by thymic medullary epithelial cells, which is important for T cell development and

education to self-antigens. Approximately 20% of individuals with AIRE mutations develop T1D, presumably due to inability to select against islet antigen reactivity (J. M. Gardner et al. 2009). Secondly, there is an association between T1D and polymorphisms of the insulin promoter gene, which also controls expression of insulin in the thymus. This gene has a variable number of tandem repeats and shorter forms are associated with T1D susceptibility (Anderson & Su 2011). The resultant alteration in thymic expression of insulin may influence the autoimmune repertoire. Finally, T cells that recognise insulin as a target have been isolated from pancreatic samples of early insulinitis.

### **Histological studies**

Direct examination of pancreatic tissue informs our understanding of the pathophysiological processes involved in T1D. Many studies have reported the type and sequence of inflammatory cell infiltrates during insulinitis shortly after diagnosis. In a Japanese study of pancreatic biopsies from recently diagnosed T1D patients, insulinitis was seen in 50% of specimens. Of these, the infiltrating cells were identified as CD8+ and CD4+ T lymphocytes, B lymphocytes and macrophages, with CD8+ T lymphocytes predominating. In addition, in half of the specimens there was evidence of MHC class I and II antigen hyperexpression in islet and endothelial cells, compared with normal pancreata. In a small number of patients, intercellular adhesion molecule-1 and lymphocyte function-associated antigen-3 were increased (Itoh et al. 1993). The second study included postmortem pancreatic specimens obtained within 18 months of T1D diagnosis. The predominant inflammatory cell types found were CD8+ T cells and macrophages in both early and late stages of insulinitis. CD4+ T cells were also seen but were fewer in number. Numbers of CD20+ cells increased with duration of insulinitis

suggesting recruitment towards the later stages of the process. Almost one-quarter of the islets contained insulin. There was no correlation between age and duration of diabetes and percentage of insulin-containing cells or degree of insulinitis (Willcox et al. 2009).

A new initiative, The Juvenile Diabetes Research Foundation Network for Pancreatic Organ Donors with Diabetes (nPOD) has provided fascinating insights into the process of insulinitis. Samples from pancreatic donor recipients who have diabetes recurrence despite immunosuppression, show evidence of insulinitis that is identical to that seen in patients with spontaneous diabetes in native pancreata. Insulinitis and diabetes recurrence post-transplantation correlates with the reappearance of diabetes autoantibodies and is associated with selective reduction of C-peptide secretion. Autoreactive T cells are seen in the circulation and correlate with disease severity and progression. These antigen-specific autoreactive T cells can also mediate beta cell destruction in immunodeficient mice. Samples from native pancreata of T1D patients confirm the important role of islet-autoantibody-specific CD8<sup>+</sup> T lymphocytes in insulinitis. Insulinitis is also seen in donors with multiple islet autoantibodies who do not have clinical disease (Pugliese et al, 2014).

The presence of insulin-producing beta cells have been described well after diagnosis. In patients with childhood-onset T1D after a mean duration of 14 years, a small study of predominantly cadaveric specimens found insulin-producing beta cells were absent in the majority of patients. However a small number had some remaining beta cells with corresponding detectable serum C-peptide levels. Of these, two patterns of beta cell survival were seen; either a lobular pattern of abnormal beta cells or a reduced number

of beta cells with normal appearance (Gianani et al. 2010). More recent nPOD studies have also demonstrated the patchy nature of insulinitis and the presence of insulin-producing cells many years after the onset of clinical disease{Pugliese:2014jka}.

### **The role of T and B Lymphocytes**

Regulatory T cells (Treg) suppress auto-reactive T cells thereby inducing immune tolerance and their role in T1D pathogenesis has been investigated. Treg cells are characterised by the presence of Foxhead box P3 (FoxP3) which acts as a master regulator of Treg development and function (Fontenot et al. 2003; Hori et al. 2003). The premise underlying this research has been that alterations in the number or function of Treg cells predisposes to autoimmunity. Loss of function mutations of the FoxP3 lead to conditions associated with immune dysregulation, polyendocrinopathy and enteropathy (Verbsky & Chatila 2013). However studies of Treg cells in peripheral blood have shown widely discrepant results with findings of increased, decreased or normal Treg frequency and function. Reasons for the lack of consensus may include the definition used to identify Treg cells, variation in characteristics of patients included or assay type. In addition, peripheral Treg numbers or function are not likely to be reflective of pathogenic pancreatic Treg activity (Tan et al. 2014). Some studies have suggested that T effector cells in T1D patients are resistant to suppression by Treg, demonstrating that defective Treg action is not the predominant pathogenic process (Lawson et al. 2008; Schneider et al. 2008).

As well as T lymphocytes, more recently B lymphocytes have also been implicated in pathogenesis, in addition to their role in the production of islet autoantibodies. B

lymphocytes act as antigen-presenting cells and express high levels of HLA class II antigens. In humans with recent onset T1D, a randomised controlled trial of rituximab, a selective CD20 monoclonal antibody, demonstrated reduction in the decline of C peptide production between 3 and 12 months. In addition, stimulated C peptide levels were higher in the treatment group compared with controls. HbA1c and insulin requirement were also reduced (Pescovitz et al. 2009).

### **The role of islet cell autoantibodies**

In the majority of affected individuals there is evidence of autoimmunity at diagnosis, but prospective studies indicate that the development of autoantibodies precedes the onset of clinical disease by months to years, and rarely, more than a decade. Autoantibodies to islet antigens glutamic acid decarboxylase (GAD), insulin (IAA) and/or tyrosine-phosphatase-like insulinoma antigen (IA2) are present in at least 90% of patients. Additional autoantibodies, such as Zinc Transporter 8 (ZnT8A) have been identified more recently and can influence presentation at T1D onset (Salonen et al. 2013). The presence of autoantibodies to thyroid-related antigens and tissue transglutaminase reflects a wider autoimmune process.

Prospective studies of children genetically at-risk of T1D have also provided information on the sequence of autoantibody development. Islet autoantibodies may develop in the first year of life (A. G. Ziegler et al. 1993). IAA is usually the first autoantibody to appear and is predominantly found in young children (A. G. Ziegler et al. 1999). One study has demonstrated that this autoantibody was present in 90% of patients who developed diabetes at less than 5 years of age, whereas it is present in less than 50% of those diagnosed at over 15 years of age (Vardi et al. 1988).

However the precise role of autoantibodies in the pathogenesis of T1D in humans has not been clarified. T1D has been reported in a patient with severe hereditary B lymphocyte and resultant immunoglobulin deficiency (S. Martin et al. 2001). In addition, there is evidence of transplacental passage of GAD and IA-2 autoantibodies from T1D mothers to their children, however the majority of these children do not develop the disease (A. G. Ziegler et al. 1993; Hamalainen et al. 2000). High islet autoantibody levels are birth are usually transient (Naserke et al. 2001). Indeed the presence of islet autoantibodies at birth reduces the risk of the development of islet immunity and T1D compared with children without positive autoantibodies (5 year risk of seroconversion 1.3% vs 5.3%,  $p=0.008$ ; 8 year risk of diabetes 1.1% vs 3%,  $p=0.04$ ) (Koczwara et al. 2003). Finally a small proportion of patients with clinical T1D are persistently autoantibody negative and are clinically indistinguishable from antibody positive patients (Hameed et al. 2010). Collectively, these studies argue against pathogenicity of islet autoantibodies.

The clinical utility of autoantibodies has to date been in identification of subjects who are at increased risk of T1D development. In children of parents with T1D, early seroconversion and the presence of multiple islet autoantibodies strongly predict the development of disease (Yu et al. 2000; Naserke et al. 2001; Parikka et al. 2012). Recent pooled analysis of three prospective birth cohort studies demonstrated progression to T1D was almost 70% in children with multiple islet-autoantibodies and only 0.4% in those who had no islet antibodies. Children who seroconverted to islet autoimmunity at younger than three years of age had the fastest rate of progression to T1D, as did those with the high-risk HLA genotype DR3/DR4-DQ8 (A.-G. Ziegler et al. 2013). Therefore the



pattern of autoantibody development may predict younger age of onset, suggesting a more aggressive phenotype.

Specific autoantibodies are associated with different HLA types. HLA-DR4-DQ8 is associated with IA-2 autoantibodies and HLA-DR3-DQ2 is associated with GAD autoantibodies in European populations (Craig et al. 2014). These differences in T1D pathogenesis, autoantibody profile and islet cell preservation support the possibility of different phenotypes within T1D.

### **2.3 The changing genetic profile of T1D**

More than sixty genes are now thought to influence the development of T1D, many of which are involved in immune regulation (Barrett et al. 2009). Four genes or genetic regions in particular have been implicated due to odds ratio > 1.1 - Human Leukocyte Antigen (HLA) class II genes on chromosome 6, PTPN22, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and the insulin gene.

#### **Human Leucocyte Antigen Susceptibility Alleles**

The Human Leucocyte Antigen (HLA) class I and II regions on chromosome 6 demonstrate the most consistent association with T1D risk in Caucasian populations (Skrodenienė et al. 2009), accounting for 30-60% of genetic risk (Sheehy et al. 1989; Noble et al. 1996). Genes within this region code for the highly polymorphic molecules which are expressed on multiple immune cells including dendritic cells, B cells, macrophages and thymic epithelial cells. These molecules are known as the Major

Histocompatibility Complex (MHC) II and constitute part of the binding groove for antigen presentation to CD4+ and CD8+ T lymphocytes. Thus they play a vital in selecting targets for immune attack. Specific alleles are implicated in susceptibility to or protection from multiple autoimmune conditions (Noble & Erlich 2012; Erlich et al. 2008).

The majority of studies investigate specific risk alleles on HLA class II regions. The HLA DRB1-DQB1-DQA1 regions are most commonly associated with T1D and both susceptibility and protective alleles have been identified in large studies of affected individuals (Sheehy, M.J. et al. 1989; Noble, J.A. et al., 1996; Erlich et al. 2008; Lambert et al. 2004). These studies compare the frequency distributions of DR-DQ haplotypes in probands with those in population or family-based controls to determine the haplotypes most commonly associated with affected individuals (Table 2.1). The highest risk genotype is well-established as DRB1\*0301-DQA1\*0501-DQB1\*0201/DRB1\*0401-DQA1\*0301-DQB1\*0302 (Erlich et al. 2008; Lambert et al. 2004). More detailed analyses have identified several HLA epitopes, with associated alleles, which influence susceptibility or resistance to T1D (Roark et al. 2013). Susceptibility can be modified by HLA class I alleles such as HLA-B\*39 (Mikk et al. 2014).

Unique protective and susceptibility HLA-DRB1, DQB1 and DQA1 genotypes are found in different ethnic populations, accounting for some of the differences in T1D incidence and risk. For example, specific HLA DRB1-DQA1-DQB1 haplotypes were found to confer susceptibility and protection in African American T1D, but not European, patients (Noble et al. 2013). A study of T1D patients in Israel found that the distribution of specific susceptibility alleles differed between Ashkenazi, non-Ashkenazi and other

Arabic patients. In addition, different HLA-DRB1\*04 susceptibility alleles were found in these groups compared with studies in other populations. DRB1\*0402/05 was more common compared with \*0401 in other populations (O. J. Kwon et al. 2001). In the East Asian subset of the Type 1 Diabetes Genetics Consortium study (T1DGC), the DRB1\*0901-DQA1\*0301-DQB1\*0303 haplotype was associated with T1D (OR 2.23 (0.67-7.78) (Erlich et al. 2008). Delli et al demonstrated that the high-risk DQ8 haplotype and DQ8/DQ2 genotypes were more common in patients of Swedish origin compared with those of non-Swedish origin (43% and 30% vs 27% and 21%, p<0.02). Conversely the DQ2 haplotype was more common in patients of non-Swedish origin. Autoantibody profiles also differed between the two groups(Delli et al. 2010).

Table 2.1 Diabetes-associated HLA risk alleles (adapted from Erlich et al, 2008)

DRB1	DQA1	DQB1	OR (95% CI)
<b>Susceptible</b>			
0405	0301	0302	11.37 (2.71-47.68)
0401	0301	0302	8.39 (6.97-11.80)
0301	0501	0201	3.64 (2.89-4.58)
0402	0301	0302	3.63 (1.76-7.49)
0404	0301	0302	1.59 (1.01-2.49)
<b>Protective</b>			
0701	0201	0303	0.02 (0.00-0.13)
1401	0101	0503	0.02 (0.00 – 0.32)
1501	0102	0602	0.03 (0.01-0.07)
1104	0501	0301	0.07 (0.02-0.3)
1303	0501	0301	0.1 (0.08-0.64)

### Reduction in proportion of patients with high-risk HLA class II genotypes

In recent decades, a change in the genetic profile of newly diagnosed patients has been demonstrated (Table 2.2). In Finland, the frequency of the HLA-DR3 haplotype was higher in patients diagnosed in the 1960s compared with those diagnosed one to two decades later (Kontiainen et al. 1988). Subsequently, Hermann et al found that the

combined frequency of four high-risk haplotypes was higher in patients diagnosed between 1939 and 1965 compared with those diagnosed 1990-2001. The high risk genotype HLA-(DR3)-DQA1\*05-DQB1\*02/(DR4)-DQB1\*0302 was more common in the earlier compared with the later time period particularly in patients diagnosed before 1960 compared with those diagnosed after 1990 (28.8% vs 18.2%). In addition, the proportion of patients carrying protective genotypes was lower in patients diagnosed before 1965 compared with those diagnosed after 1990 (Hermann et al. 2003).

Similarly, in British patients diagnosed between 1922 and 1946, the proportion carrying the highest risk HLA genotype was higher compared with age (at diagnosis) and sex-matched controls diagnosed between 1985 and 2002. The difference in frequencies of these genotypes was even greater in patients diagnosed when younger than 5 years old. However this study did not demonstrate an increased proportion of protective haplotypes in the later cohort (Gillespie et al. 2004).

Fourlanos et al examined the HLA genotypes of Australian patients diagnosed between 1950 and 2005 and also found that a higher proportion of patients diagnosed in earlier decades had the high risk genotype DR3/4 compared with those diagnosed more recently. The proportion of patients with intermediate-risk genotypes increased (Fourlanos et al. 2008). In these four studies, the ethnic compositions of the groups compared were similar, discounting the effect of immigration.

Table 2.2 Studies examining differences in HLA genotypes between earlier and later cohorts

Study population and country	Time period of diagnosis	Outcome
<b><i>Long-surviving cohorts</i></b>		
Konttinen, 1988 Finland White Finnish, stable population	1960s vs 1980s	HLA-DR3 54% vs 39%
Hermann, 2003 Finland White Finnish, <2% immigration rate	1939-1965 vs 1990-2001	(DR3)-DQA1*05-DQB1*02/(DR4)-DQB1*0302 25.3% vs 18.2%  Combined frequency of 4 high risk haplotypes 68.5% vs 62.3%  Protective haplotypes 6.0% vs 13.2%
Gillespie, 2004 United Kingdom 95% White	1922-1946 vs 1985-2002 Age and sex matched cohorts	HLA-DR3-DQ2/DR4-DQ8 (highest risk) 47% vs 35%  Subset <5y at diagnosis, 63% vs 42%
Fourlanos, 2008 Australia White Caucasian	1950-1969 vs 2000-2005	HLA-DR3/4 (highest risk) 79% vs 28%  D4,X and DR3,X (lower risk) 20% vs 48%
<b><i>Recent cohorts</i></b>		
Resic Lindehammer, 2008 Sweden White	1986-1987 vs 2003-2005	HLA-DQA1*0501-B1*0201/*0301-*0302 36% vs 19%
Vehik, 2008 Colorado, United States Non-Hispanic white and Hispanic	1978-1988 vs 2002-2004	HLA-DRB1*03-DQB1*02/DRB1*04-DQB1*03 39% vs 28%
Steck et al, 2011 Colorado, United States Majority Caucasian	Pre-1985 vs 1985-2006	High-risk HLA-DR3/4-DQB1*0302 <5y group: 50% vs 39%, p=0.004; 6-10y group: 58%, vs 35%, p=0.002

However studies comparing HLA genotypes between long-surviving cohorts and contemporary cohorts cannot exclude a possible interaction between HLA genotype and decreased mortality. In addition, there may be a confounding effect of decreased mortality in contemporary populations. Studies investigating more recently diagnosed cohorts can overcome these issues. Similar reductions in the highest risk genotypes have been demonstrated in Sweden and Colorado, US when patients diagnosed in the 1960s-1980s were compared with those diagnosed after 2000 (Resic-Lindehammer et al. 2008; Vehik et al. 2008; Steck et al. 2011). In the large multicentre study of participants in the T1DGC and patients at the Barbara Davies Diabetes Centre in Colorado, there was an increase in the proportion of patients with other genotypes in the T1DGC.

The consistent changes in HLA risk genotypes from different cohorts over the past ninety years support the hypothesis that the environment has become more permissive for the development of T1D in patients, particularly in those at lower genetic risk, and may be associated with a trend to onset of disease at a younger age (Karvonen et al. 1999).

### **Ethnic diversity of Australia**

The previous studies have predominantly investigated populations of European descent. Therefore it would be important to determine the trends in HLA risk genotypes in more ethnically diverse groups. The proportion of the Australian population born overseas increased from 22% in the 1990s to 28% in 2013 (Figure 2.1) (Australian Bureau of Statistics 2013). Almost 30% of migrants were born in the United Kingdom or New Zealand. Numbers of migrants from regions with low T1D incidence, such as China,

India and South East Asia, are increasing. This thesis examines changes in the genetic risk patterns in our clinic population over fifteen years and explores the role of environmental triggers for T1D onset.

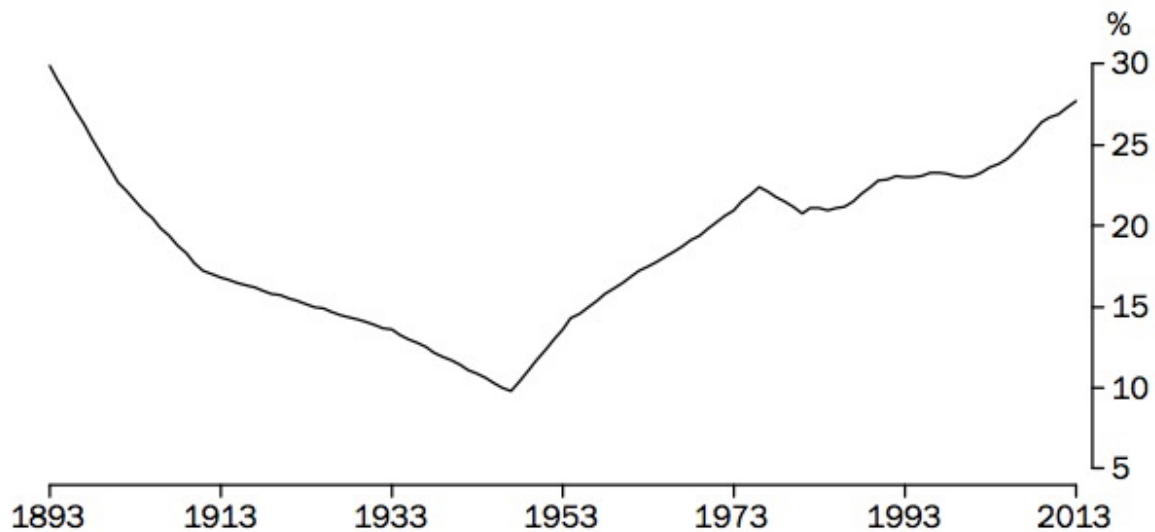


Figure 2.1 Rates of migration to Australia (from ABS, 2013, Migration, Australia, 3412.0)

## 2.4 Environmental factors in type 1 diabetes onset

### Genes, environment and gene-environment interaction

In addition to the changing genetic profile of recently diagnosed patients, a number of epidemiological studies support the role of environmental factors in T1D incidence and suggest an interaction between genetic risk and the environment in T1D pathogenesis. Cow's milk protein, vitamin D and viruses are amongst several environmental factors that have been investigated.

The wide variation in geographic and seasonal incidence of T1D, (Patterson et al. 2014b) and a decreasing gradient of T1D incidence from northern to southern countries

suggest environmental factors may be implicated. However countries such as Sardinia (high incidence in Southern Europe) and Iceland (low incidence in Northern Europe), are exceptions to this pattern (Karvonen et al. 1993). The north-south gradient in incidence is also seen within larger continents (Karvonen et al. 1993; Yang et al. 1998). Some studies have demonstrated variation in season of birth, suggesting the influence of early life exposures (Kahn et al. 2009; Jongbloet et al. 1998; Rothwell et al. 1996), but this finding has not been confirmed in other studies (McKinney et al. 2001).

Concordance rates in monozygotic twins are only between 40% and 65% (Nisticò et al. 2012; Redondo et al. 2008; Hyttinen et al. 2003), highlighting the role of environmental factors, and there is a shift to younger age of T1D onset (The DIAMOND Project Group 2006; Karvonen et al. 1999; S. G. Gardner et al. 1997).

Migration studies provide insight into the relative roles of genetic predisposition and environmental exposures, however the results of these studies are variable. Several have examined T1D incidence of children of Sardinian heritage (a region with high T1D incidence) whose parents have migrated to other Italian regions or other countries. In Italian regions with low T1D incidence, children with two Sardinian parents had the highest incidence compared with children for whom one or neither parent was Sardinian (Calori et al. 1998; Muntoni et al. 1997; Bruno et al. 2000). Children originating from Sardinia and living in Germany, had a higher T1D prevalence than German children (2.3% vs 0.11%) (Ehehalt et al. 2009).

Additional studies support the importance of inherent genetic risk. In Germany, incidence rates of non-German children were lower than those of German children and



closer to rates in their country of origin (Neu et al. 2001). In a Swedish population study, offspring of parents born in Sweden or Finland had the highest incidence, the lowest incidence was in children whose parents originated from countries of very low or low T1D incidence (OR 0.21 (95% CI 0.11-0.41) and 0.37 (95% CI 0.29-0.48), and those with parents of different background had intermediate incidence (Hjern & Söderström 2007). Children born in Italy to parents who had migrated from developing countries had a younger age of onset of T1D compared with those born overseas to immigrant parents and also Italian children, suggesting the influence of environmental factors in utero or the first year of life (Cadario et al. 2004).

However additional studies of migrant children suggest that the environment modifies genetic risk. T1D incidence rates in South Asian children living in the UK were similar to rates in white/other ethnic group children and higher than that in their country of origin. There was also a more rapid rise in incidence in migrant children (Feltbower et al. 2002, Raymond et al. 2001). The prevalence of T1D in children of Sardinian origin living in Germany was higher than that of children living in Sardinia (Ehehalt et al. 2009). Among T1D patients of Jewish Ethiopian heritage who had migrated to Israel, incidence was reported as high, however comparative data of incidence in the country of origin was not available (Zung et al. 2004). In patients with two susceptible haplotypes, there was a negative correlation between age of T1D onset and time between migration and birth of the affected child, suggesting that the changed environment resulted in earlier onset of disease in those at highest genetic risk. Finally, a recent study of Somali children with T1D in Finland demonstrated incidence rates comparable with that of the background population (40/10000 in Somali children vs 37/10000 in Finnish children).

The majority of the Somali children were born in Finland and just over half had the high risk haplotype DR3-DQ2(Oilinki et al. 2011).

### **Potential Environmental Factors**

A number of potential dietary environmental triggers have been examined. The introduction of cow's milk protein in early life has been implicated in epidemiological studies(Elliott et al. 1999; Virtanen et al. 2000; Verge et al. 1994; Saukkonen et al. 1998; Lamb et al. 2014). In contrast, the observational Australian Baby Diab and Finnish MIDIA studies demonstrated that duration of exclusive breastfeeding, introduction of mixed-feeding or solids were not associated with the development of islet autoimmunity {Couper:1999ud, LundBlix:2015wn}. Similarly a randomised, double-blind controlled trial of hydrolysed cows' milk protein formula in infancy did not reduce the risk of autoantibody development in genetically-susceptible infants (Knip et al. 2014). In the Finnish MIDIA study, breastfeeding for at least 12 months reduced the risk of T1D in children with high-risk HLA genotypes (HR 0.37, 95% CI 0.15-0.93) and risk of progression from islet autoimmunity to T1D (HR 0.35, 95% CI 0.13-0.94) (Lund-Blix et al. 2015). The DAISY group demonstrated that early (<4 months) and late (>6 months) introduction of solids was associated with increased risk of T1D, as was early introduction of fruit. There was a protective effect of breastfeeding at the time of introduction of cereals (Frederiksen et al. 2013). Maternal intake of fatty acids during pregnancy was not associated with the development of clinical T1D in offspring (Niinistö et al. 2013).

Recent studies have suggested a link between dietary and environmental vitamin D and T1D.

### **2.4.1 Vitamin D and type 1 diabetes**

#### **The role of vitamin D in health and immunity**

Vitamin D<sub>3</sub> (cholecalciferol) is a steroid hormone that is largely synthesised in the skin by the action of ultraviolet (UV) radiation on 7-dehydrocholesterol. Small amounts of vitamin D, in the form of vitamin D<sub>2</sub> (ergocalciferol), can be obtained from the diet. Both vitamin D<sub>2</sub> and D<sub>3</sub> undergo two successive hydroxylation steps - first in the liver converting it to 25-(OH)-vitamin D, and subsequently in the kidney, by 1 $\alpha$ -hydroxylase, to its active form, 1 $\alpha$ 25(OH)<sub>2</sub> vitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>), also known as calcitriol. Calcitriol regulates gene transcription via the vitamin D receptor in the nuclei of target cells (Ross et al. 2011). This process is controlled by parathyroid hormone and serum phosphate.

Vitamin D deficiency is emerging as a significant issue in Australia with rates ranging from 10-78%. Groups at highest risk of deficiency include those of Pacific Island or Maori descent, African refugees, those with dark skin or infants of mothers wearing covering clothing (Paxton et al. 2013). A case series of rickets from our centre demonstrated that prevalence increased between 1993 and 2003. Over three quarters of those affected were born in Australia and cases were almost exclusively recent migrant children or first generation offspring of immigrant parents (Robinson 2005).

Vitamin D has a well-established role in calcium metabolism and bone turnover. However the ubiquitous nature of the vitamin D receptor suggests a wider action. The vitamin D response elements, which are a marker of vitamin D action, are found in a

large number of human genes, including those involved in cell proliferation, cell differentiation and apoptosis. Vitamin D also has a significant role in immune regulation, with particular relevance to autoimmune disease aetiology (Casteels et al. 1995).  $1\alpha$ -hydroxylase is present in cells of the immune system including dendritic cells (Hewison et al. 2003) and macrophages (Overbergh et al. 2000), which act as antigen presenting cells. Active  $1,25(\text{OH})_2\text{D}_3$  is produced in these cells and influences their function. For example,  $1,25(\text{OH})_2\text{D}_3$  inhibits interleukin-12 secretion, an important cytokine involved in the differentiation and activation of T-helper 1 (Th1) cells which are one of the main effector cells in autoimmune disease.  $1,25(\text{OH})_2\text{D}_3$  also has direct suppressive effects on Th1 cell cytokine production with resultant suppression of autoimmune disease (Lemire 1995). The effect of  $1,25(\text{OH})_2\text{D}_3$  on dendritic cells is to promote the development of cells with greater self-tolerance and the induction of regulatory rather than effector T cells (Penna et al. 2007). Thus, conversely, vitamin D deficiency may result in greater Th1 action and the development of dendritic cells with less self-tolerance and the induction of effector T cells.

### **T1D incidence varies with latitude**

As ultraviolet (UV) radiation is involved in the activation of 25OHD, it is not surprising that 25OHD levels vary with latitude. Lower 25OHD levels are associated with increasing latitude where UV radiance is lower. A relationship between T1D and latitude has also been observed. In Australia, the prevalence of T1D was positively correlated with latitude (Pearson  $r=0.77$ ,  $p=0.026$ ) and that the prevalence increased almost three-fold over the north-south latitude gradient. T1D prevalence was inversely correlated with regional, average UV radiance (Pearson  $r=-0.80$ ,  $p=0.018$ ) (Staples et al. 2002). Similar results have been demonstrated in North America (Karvonen et al. 1993)

and China (Yang et al. 1998). In a worldwide study there was a higher incidence of T1D at higher latitudes in both hemispheres ( $R^2$  for latitude = 0.25,  $p < 0.0001$ ) (Mohr et al. 2008). A prospective Swedish study demonstrated that incidence rates were inversely related to aggregate mean monthly temperature and sunlight hours (Dahlquist & Mustonen 1994). The influence of UV radiation on autoimmune diseases such as T1D may be mediated via 25OHD and its effect on immune function.

### **The association between vitamin D and T1D - animal studies**

Animal studies have highlighted the association between 25OHD and T1D using the NOD mouse, which develops diabetes spontaneously. When 25OHD deficiency was induced in NOD mice in utero and in early life, a higher percentage developed diabetes at 250 days (35% male and 66% female 25OHD deficient mice vs 15% and 45% control mice,  $p = 0.05$ ). Treatment with  $1,25(\text{OH})_2\text{D}_3$  from day 21 of life in NOD mice was associated with a lower incidence and severity of insulinitis than in controls at day 100 (42% vs 75%,  $p < 0.025$ ) (Mathieu et al. 1992). Treatment reduced the cumulative incidence of T1D at 200 days (56% in controls vs 8% in cases,  $p < 0.001$ ) and restored suppressor cell function – a known defect in NOD mice (Mathieu et al. 1994).

### **The association between vitamin D and T1D - epidemiological studies**

The European Diabetes Study Group (EURODIAB) study reported a reduced risk of T1D in patients who had received vitamin D supplementation in early infancy compared with controls after adjustment for possible confounders (duration of breastfeeding <3 months, maternal age >35 years, birth weight <2500g, study centre) (The EURODIAB Study Group 1999). A large Finnish prospective case-control study also found a lower rate of T1D in children who had received 25OHD supplementation in the first year of

life, regardless of whether the dose was given regularly or irregularly (rate ratio 0.12, 95% CI 0.03-0.51 for regular dose; 0.16, 95% CI 0.04-0.74 for irregular dose). Children with suspected rickets had a 3-fold rate of developing T1DM (rate ratio 3.0, 95% CI 1.0-9.0) (Hypponen et al. 2001). The use of cod liver oil, but not other 25OHD supplements in the first year of life was associated with a significantly lower risk of T1D development in a cross-sectional case-control study conducted in Norway (adjusted OR 0.74, 95% CI 0.56-0.99,  $p=0.04$ ) (Stene et al. 2003). However the results seen in this study may be due long-chain n-3 fatty acids which can reduce production of interleukin-1 and tumour necrosis factor (Endres et al. 1989) as well as reducing HLA class II production on activated monocytes, thereby inhibiting their antigen-presenting function (D. A. Hughes & Pinder 2000). Both of these processes are implicated in the pathogenesis of T1D.

Two Italian studies have reported conflicting results on the association of vitamin D supplementation and T1D. A case-control study involving patients in Rome and its province did not find an association between vitamin D supplementation and T1D. However this is a region with a predominantly sunny climate where vitamin D levels in the whole population may be high enough to have a protective effect against T1D (Visalli et al. 2003). Conversely, a case-control study in the Pavia province (North Italy) did demonstrate a reduced risk of T1D in patients who had 25OHD supplementation during lactation (OR 0.33, 95% CI 0.14-0.81,  $p=0.015$ ) (Tenconi et al. 2007).

A meta-analysis including some of these studies concluded that patients who had received vitamin D supplementation in infancy had a 29% reduced risk of developing T1D (Dong et al. 2013). In an earlier analysis, there was evidence of a dose-response relationship and reduced risk when supplementation was under 6 months of age

(Zipitis & Akobeng 2008). However none of the studies included in the meta-analyses incorporated biochemical assessment of vitamin D status and relied on questionnaires to obtain information regarding supplementation dose and frequency, leading to the possible introduction of recall bias. Quantification of the total 25OHD intake from diet or sun exposure was not performed. An attempt to control for potential confounding factors, such as duration of breastfeeding and maternal age was made in individual studies. However it is possible that some unrecognised, but significant confounding factors could have influenced the result.

More recently, analysis of the longitudinal DAISY cohort demonstrated no association between vitamin D intake or 25OHD levels and of islet autoimmunity or progression to T1D (Simpson et al. 2011).

Studies of antenatal vitamin D intake suggest protection against T1D development. In a Norwegian study, there was a reduced risk of T1D in children whose mothers had used cod liver oil during pregnancy (OR 0.36, 0.14-0.9,  $p=0.03$ ) (Stene et al. 2000). Antenatal intake of 25OHD from food or supplements was protective against seroconversion to T1D autoimmunity in a Swedish study and the DAISY cohort (Brekke & Ludvigsson 2007; Fronczak et al. 2003). All of these studies relied on dietary history and not direct measurement of biomarkers of vitamin D and therefore results may be influenced by recall bias.

### **Vitamin D levels at T1D diagnosis**

A number of studies have investigated vitamin D levels in patients at diagnosis of T1D with varying results. An older study in Danish adults demonstrated normal 25OHD

levels in a small number of newly diagnosed patients (Storm et al. 1983). In contrast, in German patients aged 11-46 years, 1,25(OH)<sub>2</sub>D<sub>3</sub> levels were significantly lower in those newly diagnosed with T1D compared with controls (39±2 pg/mL vs 55±4 pg/mL, p<0.01). In addition, there was loss of seasonal variation of vitamin D levels in patients compared with controls (Baumgartl et al. 1991). Italian adolescents with mean age 14.6 years demonstrated significantly lower median levels of 25OHD and 1,25(OH)<sub>2</sub>D<sub>3</sub> compared with controls who were matched for age, sex and geographic location (p<0.01 and p<0.03). There was no correlation between season of diagnosis and vitamin D levels, with low levels found even when diagnosis was during the summer months. This study highlights the presence of low vitamin D levels in newly diagnosed patients living in a country with high levels of sunshine. This suggests that patients who are genetically pre-disposed to T1D may also be at higher risk of vitamin D deficiency (Pozzilli et al. 2005). Alternatively, metabolic derangement at the time of diagnosis may influence 25OHD levels.

In young Swedish adults with T1D with samples taken close to the time of diagnosis, 25OHD levels were significantly lower compared with controls (82.5nmol/L + 1.3 vs 96.7nmol/L +2, p<0.001) and males compared with females (77.9+1.4 vs 90.1+2.4 nmol/L, p<0.0001). In this study, vitamin D levels < 80nmol/L were considered to represent deficiency and 54% of patients fell into this category. There was a similar seasonal variation in levels in patients and controls (Littorin et al. 2006). Of note, 25OHD levels were measured up to 17 years after diagnosis on samples stored at -20°C, however stability of vitamin D samples over time has been reported (Agborsangaya et al. 2009).



There has been one Australian study of 25OHD levels at diagnosis. This demonstrated low 25OHD levels (<50nmol/L) in 14 out of 64 patients. Low 25OHD levels were associated with acidosis, and bicarbonate levels explained 20% of the variation in 25OHD levels. In multiple logistic regression analysis, ethnicity was an independent explanatory variable for 25OHD levels. Follow up 25OHD measurements taken up to 300 days later had normalised in the majority of patients. The authors postulated that either acidosis interfered with 25OHD metabolism or that low 25OHD may predispose patients to acidosis, due to its effect on insulin secretion and sensitivity (Huynh et al. 2009).

Similarly, Indian children with new onset T1D had lower vitamin D levels compared with controls. However there was no difference in vitamin D levels when stratified according to presence of DKA (Borkar et al. 2009).

### **Vitamin D levels and T1D**

Low vitamin D levels have also been reported at varying stages after diagnosis. Australian children and adolescents with T1D were more than three times more likely to have 25OHD deficiency than historical controls. Mean 25OHD levels were significantly lower in T1D patients compared with controls (54.7nmol/L vs 64.6nmol/L,  $p=0.0005$ ) (Greer et al. 2007). 25OHD insufficiency or deficiency was demonstrated in 76% of T1D patients. Patients with 25OHD deficiency were older, had longer diabetes duration and lower HbA1c levels. In multivariate analysis, ethnicity was significantly associated with 25OHD levels, with lower 25OHD levels in non-white patients (Svoren et al. 2009).

Previous studies have examined cross-sectional data in individual cohorts. There have been no studies of change in rates of vitamin D deficiency over time which may be a factor explaining the increasing incidence of T1D.

### **The vitamin D receptor and T1D**

Vitamin D exerts its effect via the nuclear vitamin D receptor (VDR), resulting in promotion of gene transcription. Rates of single nucleotide polymorphisms (SNPs) of the VDR (including ApaI, FokI, TaqI and BsmI) differ in T1D compared with controls, and either confer susceptibility or protection. However results have not been consistent across several populations with some studies finding a positive association (Shimada et al. 2008; De Azevêdo Silva et al. 2013) and others demonstrating a negative association (Ramos-Lopez et al. 2007; García et al. 2007; Nejentsev et al. 2004). A meta-analysis demonstrated no association between VDR SNPs and T1D (Guo 2006). As many of these SNPs (excluding FokI) are in non-coding regions and therefore not of functional significance, they may in fact be in linkage disequilibrium with other true causal variants. Alternatively, environmental exposure to vitamin D, through diet, supplements or UVB irradiation may alter the risk associated with particular SNPs. Ponsonby et al analysed several studies in relation to ambient winter UV radiation levels and found changes in the association between SNPs and T1D which varied with UV radiation levels. There was an increase in the log odds ratio of the F and B alleles of FokI and BsmI with increase in UV radiation and decrease in the association between TaqI T allele and UV radiation (Ponsonby et al. 2008).

#### **2.4.2 Viruses and type 1 diabetes**

Circumstantial evidence of the role of infection is provided by studies examining T1D incidence in relation to household crowding (Patterson et al. 1996) as well as seasonality and temporal relationships between infectious epidemics and T1D incidence. A number of viruses have been linked with the pathogenesis of T1D including rubella, rotavirus and Enterovirus (EV) (Honeyman et al. 1998; Honeyman et al. 2000; Coulson et al. 2002; Honeyman et al. 2010), however the strongest evidence exists for EV (van der Werf et al. 2007; Craig et al. 2013).

EVs are members of the Picornaviridae family and include coxsackievirus A and B, echoviruses and polioviruses. The earliest evidence was provided by Yoon et al who demonstrated that an EV strain isolated from the pancreas of a child who died following acute onset of T1D was able to induce diabetes in mice (Yoon et al. 1979). Coxsackieviruses (CV) displays tropism for pancreatic tissue in vivo (Ylipaasto et al. 2004; Laitinen et al. 2014) and in vitro (Roivainen et al. 2000). This is supported by the discovery of the Coxsackie Adenovirus receptor and the antibody specific for EV VP1 capsid protein in pancreatic islets in post mortem studies (M. Oikarinen et al. 2008; Richardson et al. 2012).

The rate of background EV varies, with lower rates of infection in countries with a high T1D incidence (Viskari et al. 2004). This suggests that lower rates of infection in early life predisposes to more severe or complicated infections in later childhood. A recent retrospective cohort study from Taiwan has demonstrate higher rates of T1D in patients with prior EV infection (incidence rate ratio 1.48 (95% CI 1.19-1.83) (Lin et al.

2014). Subsequently, a number of lines of evidence have emerged, supporting the role of EV as an environmental trigger of T1D.

### **EV and T1D onset – serological evidence**

Serological evidence exists for a link between EV and the onset of T1D. A number of studies have demonstrated higher rates of CV and echovirus IgM seropositivity in newly diagnosed T1D patients compared with controls (Friman et al. 1985; Frisk et al. 1985; Frisk et al. 1992; Frisk & Diderholm 1997; King et al. 1983; Banatvala et al. 1985; Helfand et al. 1995). Helfand et al also demonstrated that case children were more likely to be positive for 2 or more serotypes than control children. A recent cross-sectional study conducted in several European countries found that neutralising antibodies against CVB1 were more common in newly diagnosed T1D patients compared with matched controls. There was no difference in antibodies against other CV strains. The authors suggested that testing of antibodies against multiple EV strains simultaneously may mask the presence of increases in individual serotypes (S. Oikarinen et al. 2014).

### **EV and T1D onset – molecular evidence**

Direct molecular evidence of coxsackievirus B4 (CVB4) infection at the time of diabetes diagnosis has been demonstrated in a number of studies. EV RNA with sequence homology to CVB3 and CVB4 was detected in 64% of T1D children compared with 4% of controls (Clements et al. 1995). In a study from our centre, EV RNA was detected in 30% of newly diagnosed T1D patients compared with 4% of controls (odds ratio 11.1, 95% CI 4.7-25.7,  $p < 0.001$ ). EV 71 was the predominant subtype and detected in 25% of patients (Craig, Howard, et al. 2003a). In an adult study, CVB4 RNA was detected in 42% of newly diagnosed T1D patients but not in controls or those with T2D (Andréoletti et

al. 1997). EV has been detected in gut mucosa of adults with T1D at higher rates than controls using in situ hybridisation, immunohistochemistry and PCR (M. Oikarinen et al. 2012). A meta-analysis has demonstrated an almost ten-fold risk of EV detected by molecular methods and T1D (Yeung et al. 2011).

### **In utero EV and T1D**

A number of studies have examined a potential link between in utero EV infection and development of T1D. Higher rates of CVB IgM antibodies have been found in mothers whose offspring subsequently developed T1D under 3 years of age compared with control mothers (26% vs 3%,  $p < 0.005$ ) (Hyöty et al. 1995). Dahlquist et al demonstrated that mothers of children who subsequently developed T1D had EV and CVB3 IgM detected at higher rates than controls during early pregnancy and at birth (Dahlquist, Frisk, et al. 1995a; Dahlquist, Ivarsson, et al. 1995b; Dahlquist et al. 1999). A later study demonstrated that although the rate of in utero infection was higher in mothers whose children subsequently develop T1D, the difference was only small (Viskari et al. 2012).

### **Prospective studies of EV and genetically at-risk children**

A number of prospective studies in children genetically at risk of T1D have investigated rates of EV infection prior to seroconversion to islet autoimmunity (Table 2.3). Several have demonstrated higher rates of EV infection in those who develop islet autoantibodies compared with those who do not (Hyöty et al. 1995; Salminen et al. 2002; Sadeharju, Hamalainen, et al. 2003a; Salminen et al. 2004; Sadeharju et al. 2001). Lonnrot et al demonstrated an association between EV and elevation in ICA and GAD autoantibody titres, but not IAA or IA-2A (Lonnrot, M. et al., 2000). The risk of EV

infection is often highest in the 6 months immediately preceding seroconversion with autoantibodies (OR 7.7, 95% CI 1.9-31.5,  $p < 0.004$ ) (Oikarinen et al. 2010). In contrast, two studies did not demonstrate higher rates of EV RNA in stool of autoantibody positive patients (Simonen-Tikka et al. 2011; Tapia et al. 2010). The differences in these studies may relate to geographic variations in circulating viruses and/or methodological differences in virus detection methods.

Three studies investigating EV infection and progression to T1D found higher rates in those who developed T1D compared with those who did not (Table 2.4) (Hyöty et al. 1995; S. Oikarinen et al. 2011; S. Oikarinen et al. 2014; Stene et al. 2010).

Table 2.3 Prospective studies investigating association between EV and development of islet autoimmunity

Study and country	Study group	Outcome
<b>Hyoty, 1995 Finland</b>	DiME Siblings of T1D probands who progressed to T1D (cases n=22) vs those who did not (controls n=110)	19% of infections in cases associated with increased ICA antibody vs 3% in controls, p<0.001
<b>Lonnrot, 2000 Finland</b>	DiME	EV RNA in 12% of serum samples from cases vs 2% of controls (OR 7.1, p<0.01)  All positive serum samples from 6/11 pre-diabetic patients; 5/11 RNA negative  EV RNA associated with increases in ICA and GADA, but not IAA or IA-2A
<b>Salminen, 2000 Finland</b>	DIPP Birth cohort with HLA risk genotypes for diabetes  Patients who developed autoimmunity (n=20) vs those who did not (n=104)	EV infection preceded first appearance of autoantibodies in 22% of cases compared with 14% of controls, particularly in the six months immediately prior to seroconversion
<b>Saderhaju, 2001 Finland</b>	DIPP Patients who developed autoimmunity (n=21) vs those who did not (n=104)	Higher CVB4-IgG, echovirus IgA and IgG and peptide IgG in cases vs controls, males vs females, HLA-DQB1*0302/x vs others
<b>Salminen, 2003 Finland</b>	DIPP Patients who developed autoimmunity (n=41) vs those who did not (n=196)	EV infection ( $\geq$ two-fold increase in IgG and IgA or detection of EV RNA) in 22% sample intervals in cases vs 14% of controls (p=0.004) prior to islet autoimmunity
<b>Saderhaju, 2003</b>	TRIGR Patients who developed islet autoantibodies (n=19) vs those who did not (n=84)	EV infections more common in islet autoantibody positive children vs controls (0.83 vs 0.29 infections/child, p=0.01)  Higher echovirus levels in cases vs controls (p=0.0009)

Table 2.3 Prospective studies investigating association between EV and development of islet autoimmunity (cont'd)

Study and country	Study group	Outcome
<b>Salminen, 2004 Finland</b>	DIPP Patients who developed autoimmunity (n=12) vs those who did not (n=53)	42% case children repeatedly EV RNA positive (stool) vs 11% controls, p=0.02  EV infection (stool, EV antibody or RNA) in 83% case children prior to islet cell autoimmunity vs 42% controls
<b>Tapia, 2010 Norway</b>	MIDIA Patients with highest risk HLA genotype persistently positive for $\geq 2$ islet autoantibodies (n=27) vs autoantibody negative (n=54)	No increased rate of EV RNA positivity in stool prior to seroconversion to islet autoimmunity compared with controls (12.7% vs 13.6%, p=0.97)
<b>Simonen-Tikka, 2011 Germany</b>	BabyDiet Offspring or siblings of T1D patients with high-risk HLA genotypes Patients who developed islet autoimmunity (n=22) vs those who did not (n=82)	No difference in EV RNA detection (stool) between cases and controls (18% vs 24%, p=0.5) in first year of life

Table 2.4 Prospective studies investigating association between EV and progression to T1D

Study and country	Study group	Outcome
<b>Oikarinen, 2010 Finland</b>	DIPP Patients who developed T1D (n=38) vs those who did not (n=140)	EV RNA detected from 5.1% cases vs 1.9% controls (p<0.01)  Risk of EV infection highest in the 6 months immediately preceding autoantibody seroconversion (OR 7.7, 95% CI 1.9-31.5, p<0.004), particularly in boys
<b>Stene, 2010 Denver, US</b>	DAISY 50 patients who progressed from islet autoantibody positivity to T1D	Patients more likely to be diagnosed with T1D following the detection of EV RNA in serum than after negative EV RNA test (OR 7.02, 95% CI 1.95-25.3)
<b>Oikarinen, 2014 European countries</b>	Patients who developed T1D (n=249) vs those who did not (n=249)	Antibodies against CVB1 higher in case vs control children (OR 1.7)



## **Proposed mechanisms of the role of EV**

The mechanism by which EVs trigger T1D onset has not been fully elucidated, but there is evidence for both direct infection of islet cells, bystander activation and molecular mimicry. Islet cell tropism by EVs has been shown in a number of studies. Yin et al have demonstrated persistent infection of islet cells by CVB4 without associated morphological changes and a resultant reduction in glucose-stimulated insulin secretion (Yin et al. 2002). Ylipaasto et al studied autopsy pancreata specimens of infants who died of fulminant CV infection as well as adolescents and adults with T1D. Seven out of twelve infant autopsy specimens and four out of sixty-five adult T1D patient specimens had evidence of EV RNA in pancreatic islets but not in exocrine tissues. Insulinitis was found in six of the seven infant specimens with EV RNA. There was no evidence of EV RNA in forty control specimens taken from patients without T1D (Ylipaasto et al. 2004). Richardson et al found EV capsid protein in multiple islets of 61% of recent-onset T1D patients compared with only 7.7% of neonatal and paediatric non-diabetic controls ( $\chi^2=29.71$ ,  $p<0.001$ ) (Richardson et al. 2009). In infants who had died of CV myocarditis, EV capsid protein was found in the islet endocrine cells in seven of twenty specimens (Foulis et al. 1990). Recently, histological examination of fresh pancreatic tissue soon after T1D diagnosis has demonstrated higher rates of EV capsid protein compared with controls and associated hyperexpression of class I HLA molecules (Krogvold et al. 2014). In addition, CVB infection of islet cells results in reduction of insulin gene expression and differential expression of genes involved in cytokine synthesis such as interferon  $\beta$  (IFN $\beta$ ), interferon induced with helicase C domain 1 (IF1H1) (Anagandula et al. 2013), thus providing insight into the pathogenetic mechanism.

Infection of islet cells by EV could promote islet cell destruction and release of sequestered antigens, polyclonal activation of lymphocytes by a superantigen effect, release of inflammatory cytokines promoting inflammation and stimulation of the production of MHC and co-stimulatory molecules. All these processes enhance the production of self-reacting lymphocytes and the process of autoimmunity (Afonso & Mallone 2013). A genome-wide association study of T1D patients has identified a mutation in the IFH11 gene, which promotes the apoptosis of virus-infected cells, thus providing a molecular link between virus infection and T1D development (Smyth et al. 2006).

Molecular mimicry is another putative mechanism by which EV infections induce or promote T1D development. Sequence homology exists between the beta cell protein GAD65 and cox ssp, an enzyme involved in CV replication. Tian et al demonstrated that cross-reactive T-cell responses are induced by both of these proteins in the NOD mouse, which has a specific MHC allele. They concluded that these T cell responses may be restricted to individuals carrying risk MHC alleles for T1D (Tian et al. 1994). Peripheral blood mononuclear cell proliferation was induced by the specific peptide sequence from GAD65 which has sequence homology with the P2-C protein of CVB in patients newly diagnosed with T1D or islet cell autoimmunity but not in healthy controls (Atkinson et al. 1994). Antibodies produced to CVB4 also cross-react with epitopes contained by tyrosine phosphatases (IA-2, IAR) which are major target autoantigens in T1D (Harkönen et al. 2002).

### **Other viruses and islet cell autoimmunity**

In parallel with studies of EV, Honeyman et al have investigated the association between rotavirus (RV) and T1D, which reinforce proposed mechanisms for virus-triggered autoimmunity. They have demonstrated sequence homology between VP7, an immunogenic protein of RV, and IA2 and GAD65 epitope peptides (Honeyman et al. 1998). In addition, they demonstrated a temporal association between RV infection and the development of islet autoimmunity in children at genetic risk of developing T1D (Honeyman et al. 2000). They have also shown that RV displays islet cell tropism (Coulson et al. 2002). More recently, they reported similar binding affinities between RV, IA2 and GAD65 peptides and HLA-DRB1\*04 molecules and comparable T cell proliferative responses between the three peptides. Finally, when T cells stimulated to proliferate by an IA2 epitope were re-exposed to RV, they expressed IFN-gamma providing evidence of cross-reactivity between the self antigen and rotavirus (Honeyman et al. 2010).

### **HLA genotype and EV**

Host factors related to HLA genotype may influence susceptibility to EV serotypes and induce different immune responses. In a Swedish study, all newly diagnosed T1D patients who were seropositive for CBV IgM at diagnosis had HLA-DR4 or HLA-DQ 3/4 genotypes. Patients positive for CBV2, 3 and 5 had HLA-DR4 and DQ4 patterns. This was significantly different from those who were positive for CVB4 who had HLA DR 3 or DQ 3 patterns (Fohlman et al. 1987). HLA-DR3/4 risk alleles were associated with a greater immune response to CVB4 and poliovirus immunisation than HLA- DR2 protective alleles (Sadeharju, Knip, et al. 2003b). Additionally, a study from our centre demonstrated that patients in whom EV RNA was detected were less likely to have HLA-

DRB1\*03-DQB\*02 (OR 0.46, 0.24-0.87,  $p=0.02$ ) (Craig, Howard, et al. 2003a). The conflicting findings may be due to different methods for detection of viral infection (serology vs molecular testing) and the possibility that different strains of CBV were tested, with only some strains involved in T1D pathogenesis (Frisk & Diderholm 1997).

### **2.4.3 Adiposity and type 1 diabetes**

#### **The accelerator hypothesis**

The 'accelerator hypothesis' proposes that T1D and T2D are conditions along a continuum, set against different genetic backgrounds. Both result in insulin dependence, however the tempo of progression to this point differs depending on the type and number of accelerators present. Increasing body weight, resulting in insulin resistance, is identified as the common environmental link accelerating beta cell destruction. This theory suggests that the worldwide trend of increasing adiposity in children and adults is also responsible for the increase in T1D incidence (Wilkin 2009; Kibirige et al. 2003).

A component of the accelerator hypothesis suggests that the children who are more overweight will develop T1D earlier, with no increase in lifetime risk, and this is supported by a number of studies reporting an inverse relationship between body mass index (BMI)-SDS and age of onset (Evertsen et al. 2009; Betts et al. 2005; Kordonouri & Hartmann 2005; Clarke et al. 2006; Kibirige et al. 2003; Islam et al. 2014). Betts et al also demonstrated that waist circumference SDS, as a measure of adiposity, was substantially greater in children with T1D than the population average (Betts et al. 2005). In the US SEARCH for Diabetes in Youth study, the association between BMI-SDS and younger age of onset was only seen in the subset with C-peptide levels below the

median range suggesting that adiposity as an accelerator acts late in the course of diabetes pathogenesis (Dabelea et al. 2006). In contrast to the earlier reports, a study from Melbourne, Australia found no association between BMI-SDS and younger age of onset (O Connell et al. 2007).

Change in growth parameters in early life may also influence T1D development. Kibirige et al demonstrated that the change in weight SDS from birth was significantly correlated with age at diagnosis (Kibirige et al. 2003). A retrospective Swedish study demonstrated that children who developed diabetes were heavier and taller in the first year of life than controls. Case children also had greater weight and height gain (measured by SDS change from birth) in the first 7 years of life compared with controls (Ljungkrantz et al. 2008).

### **Change in BMI SDS over time**

The accelerator hypothesis also predicts that there would be an increase in BMI SDS over time, coincident with the increase in T1D incidence. This prediction is supported by a number of studies. In Finland, there was a positive correlation between height, weight and BMI z scores and incidence of T1D between 1979 and 1993 (Knip et al. 2008). In the UK, small but significant correlations were found between weight and BMI SDS and year of onset so that more recently diagnosed patients tended to be heavier ( $r=0.26$ ,  $P=0.001$  for weight SDS and  $r=0.27$ ,  $p<0.001$  for BMI SDS (Betts et al. 2005). Two US studies reported a significant increase in the proportion of overweight and obese children diagnosed between 1979 and 2004 (Evertsen et al. 2009; Libman et al. 2003). There was a discrepancy between the two studies; in the earlier study, the greatest increase was in children aged over 11 years. In the more recent study, the

greatest increase was in those less than 9 years at diagnosis. In contrast, one Australian study found that age at diagnosis increased, despite a small but significant increase in BMI-SDS in T1D patients between 1992 and 2003, and there was no association between BMI-SDS and age at presentation (O'Connell et al. 2007).

Two studies from our centre suggest that BMI-SDS has stabilised over a decade. Clarke et al found no change in BMI-SDS at diagnosis over the period 1976-2004 (Clarke et al. 2006). In follow up of this study, Islam et al examined the change in BMI-SDS from 1990-2009 (Islam et al. 2014). Although BMI-SDS increased between 1990 and 1994, there was subsequently no significant change in BMI-SDS from 1995 to 2009. Over the same time period, there was no change in proportion of overweight and obese children, ethnicity, and diabetic ketoacidosis. Therefore it appears that adiposity may play a diminishing role in diabetes pathogenesis.

### **Adiposity and autoimmunity**

Prospective studies of at risk populations provide additional insight into factors initiating autoimmunity and progression of disease. Some support the role of adiposity earlier in the course of disease. Weight and BMI z scores and change in z scores between birth and age two years were predictors of risk for seroconversion to islet cell autoimmunity in the AusDiab study of first-degree relatives of T1D probands followed from birth. This effect persisted after controlling for HLA-type. (Couper & Donaghue 2009). Conversely this association with weight and BMI was not demonstrated in the DAISY cohort (Lamb et al. 2009), nor the German BABY-DIAB study (Winkler et al. 2009). In addition, the BABY-DIAB study did not demonstrate an association between

homeostatic model assessment – insulin resistance (HOMA-IR) and the development of islet autoimmunity.

### **Adiposity and T1D risk**

Weight and obesity are also associated with T1D in the general population. A recent meta-analysis comprising over 2600 cases from 9 European studies reported a significant association between childhood obesity, BMI and weight for height and T1D. Childhood obesity doubled the odds of subsequent T1D (pooled odds ratio 2.03 (95% CI 1.46-2.80)). There was also a continuous association between childhood BMI and subsequent T1D, supporting a dose-response relationship between weight and risk (Verbeeten et al. 2010).

Insulin resistance, as a consequence of excess weight, also contributes to disease progression. A prospective study of first degree relatives of T1D patients found that insulin resistance relative to insulin secretion was higher at baseline and follow up in patients who progressed to T1D than those who did not (Furlanos et al. 2004). A study in identical twins of T1D patients found similar results, suggesting that the changes in insulin secretion that precede diabetes onset are not genetically determined (Hawa et al. 2005). In a subset of autoantibody-positive siblings of patients with T1D studied in the Finnish DiME cohort, first phase insulin response (FPIR) and decreased insulin sensitivity in relation to FPIR predicted the onset of clinical T1D (Mrena et al. 2006). In the Diabetes Prevention Trial - Type 1 (DPT-1), first or second-degree relatives were assessed as being of moderate or high risk for progression to T1D based on first-phase insulin response to intravenous glucose tolerance test (IVGTT). In both groups, patients with higher insulin resistance (as measured by HOMA-IR) was associated with greater

risk of developing T1D (moderate risk, HR 2.7 (95% CI 1.45-5.06; high risk HR 1.83 (95% CI 1.19-2.82)) (Xu et al. 2007). Subgroup analysis of participants in the European Nicotinamide Diabetes Intervention Trial (ENDIT) confirmed that HOMA-IR was associated with an increased risk of T1D development in only the subset of patients with reduced first-phase insulin response (Bingley et al. 2008).

A recent Swedish paper proposed that the effect of weight on diabetes development may be restricted by HLA-related genetic risk. In new onset T1D patients aged less than 18 years, high-risk HLA genotypes were negatively associated with BMI. In addition, the proportion of patients with the low risk genotype DQA1\*0501-DQB1\*0201/\*0501-DQB1\*0201 increased with increasing BMI. The odds ratio of obesity was 1.8 (95% CI 1.21-2.61) in patients with this genotype (Carlsson et al. 2011).

In support of the accelerator hypothesis, adiposity appears to play a role in the development of autoimmunity and T1D with younger age of onset in those with greater genetic risk and concurrent increases in T1D incidence and BMI over recent times. In our centre, there has been a plateau in BMI-SDS of newly diagnosed patients since 1995 suggesting a role for additional factors in pathogenesis. However the effect of weight on disease pathogenesis may be modified by genetic risk and this observation warrants more detailed investigation.



## **2.5 Vascular Complications of Type 1 Diabetes**

### **2.5.1 Diabetic Retinopathy**

Diabetic retinopathy (DR) is a significant cause of blindness globally. The cumulative incidence of DR is 90% after 30 years diabetes duration (Joergensen et al. 2011; Skrivarhaug, Fosmark, et al. 2006b; M. S. Roy et al. 2004). However early retinopathy is already present in up to 24% of childhood-onset patients with minimum diabetes duration of 5 years (Donaghue, Craig, et al. 2005a; Mayer-Davis et al. 2012). Vision-threatening DR was estimated to be present in 30% of T1D patients in a cross-sectional US (M. S. Roy et al. 2004). More recent reports have demonstrated a decline in prevalence (Downie et al. 2011; LeCaire et al. 2013).

Retinopathy may worsen significantly in adolescence (B. E. K. Klein et al. 1990), possibly due to deterioration in glycaemic control and/or the increase in pubertal hormones, resulting in increased insulin resistance. Therefore the identification of modifiable risk factors and determining characteristics of high-risk patients is crucial to the prevention of significant later morbidity.

#### **Risk Factors**

The major modifiable risk factors associated with DR are hyperglycaemia, hypertension and elevated cholesterol (Mayer-Davis et al. 2012). The landmark Diabetes Control and Complications Trial (DCCT) demonstrated that intensive insulin therapy, with the goal of achieving blood glucose concentrations close to the normal range, reduced the adjusted mean risk of incident DR by 76% compared with conventional therapy. In patients with retinopathy at baseline, intensive therapy slowed the progression of

retinopathy by 54% and reduced the development of proliferative or severe non-proliferative retinopathy by 47% (The Diabetes Control and Complications Trial Group 1993). Following the completion of the DCCT, all patients were recommended intensive insulin therapy and the majority were subsequently followed in the Epidemiology of Diabetes Interventions and Complications (EDIC) study. After four years, patients who had been in the intensive insulin therapy group had a lower risk of worsening retinopathy (odds reduction 72% vs 87%,  $p > 0.001$ ) despite convergence of HbA1c values of the two DCCT treatment groups (The Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) Research Group 2000a), suggesting a role for metabolic memory (L. Zhang et al. 2012b). The Wisconsin Epidemiologic Study of Diabetes Retinopathy (WESDR) confirmed that higher HbA1c or an increase in HbA1c after 4 years was associated with progression of retinopathy and increased risk of proliferative retinopathy (R. Klein et al. 1998; R. Klein et al. 2008). Thirty year follow up of the DCCT has demonstrated that intensive therapy reduces the risk of DR progression and severe retinal outcomes by at least 50% (Aiello for the DCCT/EDIC Research Group 2013).

Hypertension is another significant risk factor for DR. Follow up of patients in the WESDR after 25 years demonstrated that higher diastolic blood pressure at baseline was associated with retinopathy progression and an increased risk of proliferative retinopathy (R. Klein et al. 2008). In a study from our centre, elevated systolic and diastolic blood pressure in adolescents with T1D was associated with an increased risk of DR development, independent of microalbuminuria, HbA1c, duration and age. The cumulative incidence of DR was greater in those with systolic and diastolic blood

pressures on or above the 90<sup>th</sup> centile compared with those below the 90<sup>th</sup> centile (58% vs 35%,  $p=0.03$  for systolic BP, 57% vs 33%,  $p=0.005$ ) (Gallego et al. 2008).

Duration of diabetes also influences DR development with most studies demonstrating an increasing cumulative incidence with time (R. Klein et al. 1998; R. Klein et al. 2008). However the risk varies according to the timing of diabetes onset with respect to age and puberty. Whilst diabetes during the pre-pubertal years does contribute to the risk of DR, duration of diabetes in the post-pubertal years may increase the risk more than the pre-pubertal years (B. E. K. Klein et al. 1990). In a study from our centre, pre-pubertal years of diabetes increased the risk of DR by 28% compared with 36% for post-pubertal years (Donaghue et al. 2003). Similarly, a Danish study found that the risk for pubertal diabetes duration was twice that of pre-pubertal duration (Olsen et al. 2004).

Age and pubertal stage are also risk factors for the presence of retinopathy. In an incident cohort assessed 6 years after diagnosis, retinopathy was present in 12% of pre-pubertal compared with 29% of post-pubertal children and 8% and 25% of children aged less or more than 11 years, respectively (Donaghue, Craig, et al. 2005a). In contrast, retrospective analysis of the FinnDiane cohort demonstrated that diabetes diagnosis at less than 15 years of age was associated with a significantly higher risk of proliferative retinopathy compared with those diagnosed at over 15 years of age (Hietala et al. 2010).

Sex-related differences in DR are also apparent; male sex is a risk factor for DR (R. Klein et al. 2008; R. Klein et al. 1998). T1D onset in the pubertal years doubled the risk of DR

in males compared with females. In addition, sex-related differences in DR prevalence were noted after 40 years diabetes duration, when the overall risk of DR was 39% higher in males versus females (Harjutsalo et al. 2011).

Despite these known risk factors, analysis of the DCCT data demonstrated that approximately 10% of patients with good control ( $\text{HbA1c} \leq 6.87\%$ ) developed retinopathy and over 40% of those with poor control ( $\text{HbA1c} \geq 9.49\%$ ) did not develop retinopathy (L. Zhang et al. 2001). This highlights the role of additional factors, such as genetic risk or environmental exposures, which may influence the development of retinopathy. For example, the association between HLA risk genotype and retinopathy is incompletely understood. Cross-sectional analysis of WESDR data and other cohorts demonstrate an association between high risk HLA-DR alleles and prevalence or DR (Cruickshanks et al. 1992; D. Agardh et al. 1996). However this was not supported in a subsequent study of fourteen year incidence and progression of DR in the WESDR cohort (Wong et al. 2002).

### **Pathophysiology**

A number of intracellular processes underlie the development of DR. These include activation of the polyol pathway, protein kinase C, accumulation of advanced glycation end-products (AGE), formation of reactive oxygen species (ROS), upregulation of inflammatory processes and production of angiogenic growth factors (Sultan et al. 2012; Tarr et al. 2013). Vascular endothelial growth factor (VEGF), which regulates both physiological and pathological angiogenesis, is produced by ischaemic retinal cells in the presence of hypoxia. The production of VEGF results in the influx of inflammatory cells, breakdown of the blood-retinal barrier and vascular leakage (Ishida et al. 2003).

Other features of DR include endothelial cell proliferation and death, basement membrane thickening and loss of pericytes (Sultan et al. 2012).

There are 2 recognised stages of diabetic retinopathy.

### *1. Non-proliferative (background) retinopathy*

This is characterised by microaneurysms, retinal haemorrhages, hard exudates, cotton wool spots and intraretinal microvascular abnormalities. The latter include beading, constriction or dilatation and vessel tortuosity (R. Klein et al. 2004; R. Klein et al. 2012) (N. Cheung et al. 2009). Grading of non-proliferative retinopathy ranges from mild to severe. Mild to moderate grades do not threaten vision and do not always progress to proliferative retinopathy. Severe non-proliferative retinopathy is associated with increasing vessel obstruction resulting in ischaemia and infarctions of retinal nerve fibres and production of cotton wool spots. Intraretinal microvascular abnormalities also progress.

### *2. Proliferative diabetic retinopathy (PDR)*

Neovascularisation in the retinal or posterior vitreal surface is the hallmark of proliferative retinopathy. These vessels may rupture and cause vision-threatening bleeding. More severe PDR may be associated with fibrosis and adhesions, which can result in haemorrhage or retinal detachment. The degree of visual loss is determined by the location and extent of the neovascularisation and signs of vitreous or pre-retinal haemorrhages (Donaghue et al. 2014). Proliferative retinopathy is associated with a 4 year increased risk of visual loss, cardiovascular disease, diabetic nephropathy and mortality (R. Klein et al. 1992).

## **Diabetic Macular Oedema (DMO)**

Diabetic macular oedema occurs rarely in childhood or adolescence but is the leading cause of blindness in adults with diabetes. It is characterised by retinal thickening and hard exudates in the macula region or within one disc diameter of the centre of the macula. It may progress independently and is assessed separately from the other stages of retinopathy and may cause significant visual loss (N. Cheung et al. 2010).

## **Treatment**

The mainstay of treatment for vision-threatening DR is laser photocoagulation. This may be pan-retinal for proliferative retinopathy or macular (focal) for macular oedema. In the former, laser burns are directed throughout the retina, sparing the macular region, to inhibit and promote regression of neovascularisation (N. Cheung et al. 2010). Two large studies have demonstrated reduction in risk of severe vision loss (in more advanced retinopathy) and reduction in risk of progression to severe proliferative retinopathy from severe non-proliferative retinopathy by up to 50% (Early Treatment Diabetic Retinopathy Study Group 1991; The Diabetic Retinopathy Study Research Group 1981). Macular laser reduces the risk of visual loss due to macular oedema by 50%. Laser photocoagulation is not indicated for mild or moderate non-proliferative retinopathy.

Surgical vitrectomy is used for treatment of persistent vitreal haemorrhage and traction retinal detachment. Whilst it may reduce the risk of retinal neovascularisation and macular oedema, the risk of iris neovascularisation and cataracts is increased. Novel therapies include intraocular anti-VEGF and corticosteroid injections. However their use must be weighed against the risk of systemic absorption which may impact on

cardiovascular risk, as well as risk of localised complications such as cataract formation, retinal detachment, vitreous haemorrhage, infection, and potential loss of neural retinal cells (N. Cheung et al. 2010).

### **2.5.2 Retinal Vascular Geometry**

Microvascular complications are responsible for significant morbidity in T1D patients. Retinopathy affects 90% of patients after 30 years diabetes duration and nephropathy occurs in up to 40% after 25 years. Biennial screening for early signs of retinopathy is recommended to target patients for intensive glycaemic control and modification of risk factors. However despite these measures, many patients will progress to vision-threatening retinopathy and require intervention in an attempt to limit further progression. Thus novel, non-invasive techniques to identify patients at increased risk prior to the development of features of retinopathy are vital.

Changes in retinal vasculature have been found in association with hypertension, ageing and other disease states (Stanton et al. 1995; Witt et al. 2006; C. Y. Cheung et al. 2011), highlighting the relationship between microvascular and microvascular disease. Measurement of specific retinal vascular parameters provides a valuable tool for assessment of diabetic microvascular complications and risk stratification. Adverse changes in retinal vascular geometric parameters are associated with risk factors for microvascular complications. In a cross-sectional study of young people with T1D in our centre, increasing diabetes duration was associated with increased arteriolar branching angle and optimality deviation; elevated HbA1c was associated with increased arteriolar tortuosity; increasing systolic blood pressure was associated with decreasing

arteriolar length-diameter ratio. Increasing total cholesterol was associated with increasing arteriolar length-diameter ratio (Sasongko et al. 2010).

In addition to their association with risk factors, retinal vascular measures also predict subsequent development of complications. Early reports from the Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR) demonstrated that increased venular calibre was associated with the four year incidence of proliferative retinopathy and both increased arteriolar and venular calibres were associated with the four year progression of retinopathy (R. Klein et al. 2004; Wong et al. 2004). In longitudinal studies of young people with T1D aged 12-20 years from our centre, larger arteriolar calibre (Alibrahim et al. 2006), increasing arteriolar tortuosity (Sasongko et al. 2012) and lower arteriolar length-diameter ratio predicted incident retinopathy (Benitez-Aguirre et al. 2011). In a cross-sectional study of young people with T1D, changes in fractal dimension were associated with retinopathy (N. Cheung et al. 2009), however no association was found when longitudinal data from the same cohort was analysed (Lim et al. 2009), suggesting that these changes were consequential to the retinopathy.

Examination of the retinal vasculature can also provide information on renal disease, therefore contributing to the wider understanding of the pathophysiological processes underlying microvascular disease. In the WESDR, increased venular diameters were predictive of gross proteinuria and renal insufficiency independent of risk factors for renal disease (Wong et al. 2004). In a cross sectional study of young people with T1D, increasing arteriolar tortuosity was associated with early kidney dysfunction (Sasongko et al. 2012) and in subsequent longitudinal follow up, venular length-diameter ratio and tortuosity predicted incident renal dysfunction (Benitez-Aguirre et al. 2012).



The mechanisms underlying these changes in retinal vascular geometry are incompletely understood. In particular, whether the environmental factors associated with microvascular disease are also associated with retinal vascular geometry changes. We have previously found that vitamin D deficiency is associated with retinopathy. In chapter 7, we investigated whether vitamin D deficiency was associated with adverse changes in retinal vascular parameters.

### **2.5.3 Diabetic Nephropathy**

Diabetic nephropathy is a leading cause of end stage renal failure in Western countries. The incidence of nephropathy in T1D ranges from 22%-41% after 20-25 years diabetes duration (Romero-Aroca et al. 2012; Tryggvason et al. 2005; Andersen et al. 1983; Mollsten et al. 2010). More recently, lower rates of nephropathy and microalbuminuria have been reported after 24 years of follow up (7.8% and 14.9%, respectively) (Skriverhaug, Bangstad, et al. 2006a). The incidence of end stage renal disease (ESRD) ranges from 1-7.8% after 25-30 years diabetes duration (Mollsten et al. 2010; Finne et al. 2005; Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) Research Group et al. 2009). Worldwide surveys reported that rates of ESRD due to T1D declined at a rate of 6.4-7.8% per year between 1998-2002 (ESRD Incidence Study Group et al. 2006; Stewart et al. 2007). However recent reports from the Joslin centre demonstrate 15 year cumulative risks of ESRD of 52% and pre-ESRD death of 11% in patients with macroalbuminuria at baseline, despite improvements in cholesterol level, blood pressure and increased use of antihypertensive medication and lipid treatment over this period (Rosolowsky et al. 2011). The FinnDiane group also reported a cumulative incidence of ESRD of 36% in patients with macroalbuminuria after 9.9 years of follow up (Forsblom et al. 2011).

Similarly, no change in microalbuminuria rates over time were reported after 10 years follow up in the Oxford Regional Prospective Study groups diagnosed 1986-1996, despite improvements in glycaemic control (Amin et al. 2008). Therefore it appears that the introduction of antihypertensive and lipid-lowering treatments, as well as stricter glycaemic targets may only delay the onset of ESRD (Rosolowsky et al. 2011; Marshall 2012).

Epidemiological studies from the 1980s reported a 49% mortality rate by seven years after the appearance of proteinuria, and an overall mortality rate of 83%. The majority of deaths were due to uraemia, ischaemic heart disease or stroke. A Danish study reported similar findings (Borch-Johnsen et al. 1985).

### **Stages of diabetic nephropathy**

Traditionally, five distinct phases of nephropathy are described. It was thought that patients progressed sequentially from hyperfiltration to increasing albumin excretion with eventual development of ESRD. Recently it has been shown that proteinuria did not precede, but occurred concurrently with the onset of chronic renal disease. In this cohort, a proportion of patients had microalbuminuria or improved to normoalbuminuria prior to the onset of chronic kidney disease suggesting that progression may not be stepwise (Perkins et al. 2009).

### ***Stages of diabetic nephropathy***

1. Normal glomerular morphology with increased glomerular filtration rate/hyperfiltration
2. Early histological changes with elevation of albumin excretion

3. Microalbumuria
4. Overt proteinuria
5. End stage renal disease.

#### *1. Hyperfiltration with normal glomerular morphology*

Renal hypertrophy and increased glomerular filtration rate (GFR) may be seen in up to 50% of patients soon after the onset of diabetes. Early studies demonstrated that hyperfiltration (GFR>125mL/min/1.74m<sup>2</sup>) at initial assessment was a significant predictor of nephropathy, independent of glycaemic control (Mogensen 1986; Rudberg et al. 2007). Elevated eGFR is accompanied by an increase in kidney volume in patients predisposed to microalbuminuria and is associated with subsequent faster decline in GFR (Zerbini et al. 2006). In the early stages of nephropathy, a faster rate of early eGFR decline is a better predictor of future ESRD than HbA1c, Albumin-Creatinine ratio (ACR) and systolic blood pressure (Skupien et al. 2012). Blood pressure is usually normal and microalbuminuria is reversed with improved glycaemic control. There are no glomerular or vascular structural changes on histology (Bogdanović 2008).

More recently the occurrence of hyperfiltration as the first step in the development of microalbuminuria and diabetic nephropathy has been challenged. A report from the FinnDiane group found that patients with hyperfiltration (using eGFR levels above the 90<sup>th</sup> percentile of the normal population as the threshold) were not more likely to develop microalbuminuria than those with normal eGFR. The distribution of eGFR also was not significantly different from the general Finnish population (Thomas et al. 2012). This confirmed an earlier report from the Joslin First Kidney Study which demonstrated that hyperfiltration resolved in a number of patients and the presence of

hyperfiltration did not increase the risk of development of microalbuminuria (Ficociello et al. 2009).

### *2. Early structural changes and changes in albumin excretion rate*

Thickening of the glomerular basement membrane (GBM) and mesangial matrix expansion are the first measurable histological changes of diabetic nephropathy. Progression leads to a reduction in the filtration surface of the glomerulus. Accompanying changes can be seen in the arterioles, tubules and interstitium (Fioretto et al. 2008). GBM width may be a predictor of nephropathy progression. In a longitudinal study of normoalbuminuric patients with renal biopsy at baseline, those who progressed to proteinuria or ESRD had greater glomerular basement membrane width than non-progressors ( $567.36 \pm 104.1$  vs  $459.56 \pm 85.7$ ) (Caramori et al. 2013).

Urinary albumin excretion rate (AER) begins to rise and is the earliest sign of diabetic nephropathy (Parving et al. 1982). This can be seen as early as 6 years after diagnosis (Donaghue, Craig, et al. 2005a). Early elevation of AER and the rate of rise of ACR, even within the normal range, are predictors of microalbuminuria (Hovind 2004). ACR in the upper tertile for age at 11 – 15 years, together with elevated HbA1c is highly predictive of the later development of microalbuminuria (Dunger et al. 2007).

### *3. Incipient – microalbuminuria*

Microalbuminuria is defined as an elevated AER  $>20$  micrograms/min and  $<200$  micrograms/min or ACR 2.5-25mg/mmol/L or 30-300mg/L males and 3.5-25mg/mmol/L in females (Donaghue et al. 2014). The prevalence of microalbuminuria ranges from 9-34% after 10 years of diabetes (Chaturvedi et al. 2001; Schultz et al.

1999) and 34-50% after 18-19 years of diabetes (Amin et al. 2008). Microalbuminuria has been shown to progress to overt proteinuria at a rate of 2-13 per 100 person-years (Rossing et al. 2005; Viberti 1994). Patients who develop micro- or macroalbuminuria have higher albumin excretion rates within the normal range at baseline (AER 2.9 vs 11.5 mg/day) and albumin excretion rate is predictive of future macroalbuminuria (Kern et al. 2010).

Microalbuminuria may be intermittent or transient. In a small prospective cohort of young adults followed for 7 years, 56% of patients with MA at baseline reverted to normoalbuminuria, 33% remained microalbuminuric and 6% progressed to diabetic nephropathy. Risk factors for progression were higher systolic blood pressure, HbA1c and triglycerides. In logistic regression, duration 10-14 years and HbA1c >10% were associated with increased risk of progression (Tabaei et al. 2001). These findings were confirmed in the EURODIAB cohort; in patients with microalbuminuria at baseline, 50.6% regressed to normoalbuminuria, 35.5% remained microalbuminuric and 13.9% progressed to macroalbuminuria. This confirmed HbA1c as well as higher albumin excretion rate and higher body mass index as risk factors for progression. Patients with lower AER were more likely to revert to normoalbuminuria (Giorgino et al. 2004). Conversely, in a prospective study of patients who had microalbuminuria at baseline, only 16% regressed after 10 years, and this was explained by antihypertensive medication in more than half of these (Rossing et al. 2005). Differences in rate of regression may be due to variations in glycaemic control.

#### *4. Overt proteinuria*

This stage is associated with the development of clinical proteinuria (>0.5g/24h). The increasing albumin excretion rate is accompanied by a rise in blood pressure of 3mmHg per year and declining GFR (10mL/min per year). Macroalbuminuria is associated with development of end stage renal disease and significantly increased coronary mortality (Borch-Johnsen et al. 1985; Krolewski et al. 1985; Krolewski et al. 1991).

#### *5. End stage renal disease*

End stage renal disease is defined as  $GFR < 15 \text{ mL/min/1.73m}^2$  and usually occurs within 5-10 years of the onset of proteinuria (Bogdanović 2007). Patients at this stage require renal replacement therapy or renal transplantation.

### **Risk Factors**

The Diabetes Control and Complications study demonstrated that intensive insulin therapy reduced the cumulative risk of development of microalbuminuria by 34% in patients with normal albumin excretion rate at baseline. In patients with early microvascular complications (secondary prevention cohort) the cumulative risk reduction of microalbuminuria was 43% and risk of clinical albuminuria was also reduced by 56% (Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) Research Group 1995). Similar findings were demonstrated in a subset of adolescents in the secondary prevention cohort (Diabetes Control and Complications Trial Research Group 1994). In the subsequent EDIC study, the risk of incident microalbuminuria was reduced by 86% in the former intensive therapy group (Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) Research Group 2000a).

Intensive therapy reduced the risk of impaired glomerular filtration rate in the DCCT and EDIC combined; however this finding was not independent of HbA1c or AER. During the course of the two studies, the rate of decline in GFR was slower in the intensive therapy group (Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) Research Group 2011).

Poor glycaemic control, associated with chronic hyperglycaemia, is known to be the major risk factor for the development of nephropathy (Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) Research Group 2011). Other risk factors include elevated cholesterol levels, systolic blood pressure and CRP, which is a marker of inflammation (Skriverhaug, Bangstad, et al. 2006a; Salgado et al. 2010). In earlier cohorts, male sex was associated with greater risk of nephropathy development. Some recent studies have found that male preponderance is no longer a feature (Costacou et al. 2011; Mollsten et al. 2010; Svensson et al. 2006). In other contemporary studies, a sex-related difference in risk was still present, although this was dependent on the age of onset of diabetes, with the difference apparent in patients with post-pubertal age of onset (Harjutsalo et al. 2011). Older age at diabetes onset, particularly in the post-pubertal years, is also associated with greater nephropathy risk (Svensson et al. 2006) (Harjutsalo et al. 2011). Proliferative retinopathy is an independent predictor of nephropathy after 25 years (Karlberg et al. 2011).

Other markers of future nephropathy have also been investigated. These include urinary *N*-acetyl-D-glucosaminidase (NAG), which predicts microalbuminuria and macroalbuminuria, and pentosidine and advanced glycation end product (AGE)

fluorescence, which predict macroalbuminuria and microalbuminuria, respectively (Kern et al. 2010). Neutrophil-gelatinase- associated lipocalin (NGAL) also correlated with AER in a small study group of children without evidence of nephropathy (Zachwieja et al. 2010). In animal studies, absence of vascular endothelial growth factor A production induced glomerular scarring, apoptosis and proteinuria in a diabetic mouse model (Sivaskandarajah et al. 2012).

## **Treatment**

Blockade of the renin-angiotensive-aldosterone system is an established treatment of diabetic nephropathy. A recent meta-analysis found that these agents reduced albumin excretion by 60-67% in patients with microalbuminuria. Treatment with ACEI resulted in fewer patients progressing to macroalbuminuria (RR=0.39, 95% CI 0.23-0.64, p=0005) and also increased the rate of regression to normoalbuminuria (RR=5.81 (CI 2.05–16.43, P=0.001) (Hirst et al. 2011).

Pancreatic transplantation can reverse interstitial and tubular changes of diabetic nephropathy after 10 years. In a small study of 13 patients post-islet cell transplant, glomerular and tubular basement widths and mesangial matrix were reduced and even normalised in 8 patients who underwent repeat biopsy after 10 years (Mauer & Fioretto 2013).

### **2.5.4 Peripheral Neuropathy**

Peripheral neuropathy in T1D can be classified as focal/multifocal or generalised. Focal neuropathy includes mononeuropathy (e.g. carpal tunnel syndrome, peroneal nerve



palsy, cranial nerve palsy); multifocal neuropathy may involve spinal radiculoplexuses at any level. Generalised neuropathy may be either typical or atypical. In the typical form, a distal, symmetrical, sensorimotor polyneuropathy is described in a 'glove and stocking' distribution. This form of neuropathy is characterised by decreased sensation and neuropathic sensory symptoms that include numbness, stabbing, burning or aching pain. This may also be accompanied by hyperalgesia (Blankenburg et al. 2012). Clinically, symmetrical reduction of distal sensation or decreased or absent ankle reflexes may be elicited. Sensory neuropathy is followed by loss of motor function later in the disease course. Atypical peripheral neuropathies may have an acute, subacute or chronic onset with either a monophasic or fluctuating course and are associated with pain and symptoms of autonomic dysfunction (Donaghue et al. 2014; Tesfaye et al. 2010).

In young people the prevalence of diabetic neuropathy ranges from less than 10% to 27% (Eppens et al. 2006; Mohsin et al. 2005; Cho et al. 2011; Downie et al. 2011; Jaiswal et al. 2013; Moser et al. 2013). Prevalence rates up to 35% have been reported in adults after 25 years diabetes duration (Pambianco et al. 2006; Tesfaye et al. 1996).

### **Pathophysiology**

Risk factors for typical generalised peripheral neuropathy include hyperglycaemia, diabetes duration and cardiovascular risk factors such as hypertension and lipid abnormalities (Jaiswal et al. 2013). It is associated with the presence of retinopathy and microalbuminuria (Tefaye et al. 1996; Dyck et al. 1999; Jaiswal et al. 2013). This suggests a shared pathophysiological basis involving the accumulation of advanced glycation end products, increased oxidative stress and increased polyol flux, as well as

neural hypoxia and ischaemia (Cameron et al. 2001). Small nerve fibre dysfunction precedes large fibre dysfunction; progression of small fibre dysfunction is associated with worsening peripheral neuropathy (Breiner et al. 2014). High resolution magnetic resonance neurography has suggested a role for proximal, multifocal fascicular injury in pathogenesis (Pham et al. 2011).

### **Assessment**

A history of sensory disturbance or neuropathic pain may be elicited and physical examination includes examination of ankle reflexes, vibration perception and light touch sensation. Nerve conduction studies of the peroneal, median and sural nerves may be performed and changes may precede features of clinical neuropathy (S.-S. Lee et al. 2010; Hyllienmark et al. 2013) and correlate with tactile hypoesthesia (Blankenburg et al. 2012). More recently, other simpler testing modalities have been employed including vibration perception and thermal threshold testing (C. L. Martin et al. 2010a).

### **Treatment**

Short-term improvement in HbA1c and triglycerides may result in improvement in sensory nerve conduction velocity (Perkins et al. 2010). First line therapies for symptomatic treatment include tricyclic antidepressants, anticonvulsants and the serotonin and norepinephrine reuptake inhibitor duloxetine, as well as opiate analgesia (Chong & Hester 2006). Topical local anaesthetic treatment may be beneficial. Intravenous antioxidant treatment with alpha-lipoic acid has proven efficacy in randomised controlled trials and meta-analysis (D. Ziegler et al. 2004).

### **2.5.5 Autonomic Neuropathy**

Diabetic autonomic neuropathy is associated with significant morbidity and mortality in T1D. Symptoms are diverse and include postural hypotension, gastrointestinal disturbance such as gastric fullness, vomiting and diarrhoea, sweating abnormalities, bladder paresis, impotence and retrograde ejaculation. Subclinical signs of autonomic dysfunction may be seen in adolescence with changes in cardiovascular reflexes and abnormalities in pupil size and reflexes (Pena et al. 1995; Maguire et al. 2007). Studies of cardiovascular reflexes demonstrate a shift in autonomic balance with reduced vagal regulation and increased sympathetic activity after five to ten years diabetes duration (Lucini et al. 2009; Jaiswal et al. 2012).

#### **Risk Factors**

Abnormalities in autonomic function are associated with other microvascular complications. In young people with T1D from our centre, small pupil size was associated with the development of retinopathy and microalbuminuria after twelve years of follow up (Maguire et al. 2007). In the SEARCH Cardiovascular Disease Study, early cardiac autonomic neuropathy was associated with microalbuminuria (Jaiswal et al. 2012). A recent report from the Adolescent Type 1 Diabetes Cardio-Renal Interventional Trial demonstrated that patients with ACR in the upper tertile had faster heart rate and reduced heart rate variability compared with those in the lower tertiles, independent of age and HbA1c (Cho et al. 2015).

Similar to other microvascular complications, risk factors include longer diabetes duration, poor glycaemic control, female sex and lipid abnormalities (Pena et al. 1995; Schwingshandl et al. 1993; Jaiswal et al. 2012).

## **Genetic Factors**

Genetic factors also confer susceptibility to autonomic neuropathy. The HLA-DR3/4 genotype was associated with six times the odds of cardiovascular autonomic neuropathy compared with other HLA-DR genotypes (Barzilay et al. 1992). We have previously shown that pupillary abnormalities were strongly associated with the Z-2/Z-2 genotype of the aldose reductase gene. This variant conferred three times the risk compared with those with protective or non-susceptible genotypes (Donaghue, Margan, et al. 2005b). Glutathione S-transferase gene polymorphisms have also been associated with cardiovascular autonomic neuropathy (Vojtková et al. 2013).

## **Testing**

Traditionally, measures of cardiovascular reflexes are used to assess for autonomic neuropathy. These include heart rate response to deep breathing, postural changes and Valsalva manoeuvre, postural blood pressure changes and changes in QT interval. Measurement of heart rate variability at rest has been enhanced by advances in technology including high frequency spectral analysis. Changes in specific parameters act as markers of parasympathetic/sympathetic balance.

Resting pupil measurements and pupillary responses to light (change in pupil size, maximum constriction velocity, reflex amplitude), collectively termed pupillometry, have been used as sensitive measures of autonomic dysfunction and reference ranges based on normal control populations have been established. Abnormalities are seen before changes in cardiovascular reflexes (Pena et al. 1995).

## **2.6 Environmental and Genetic Factors in Vascular Complications of Type 1 Diabetes**

Novel risk factors for microvascular complications have been investigated in an effort to understand and reduce their incidence.

### **2.6.1 Vitamin D and Microvascular Complications**

#### **Vitamin D and diabetic retinopathy**

An association between vitamin D deficiency and DR has been demonstrated in T2D patients. In a small Turkish study there was an inverse relationship between 1,25(OH)<sub>2</sub>D<sub>3</sub> levels and severity of diabetic retinopathy (Aksoy et al. 2000). In the Third National Health and Nutrition Examination Survey (NHANES) the prevalence of vitamin D deficiency increased with increasing severity of diabetic retinopathy, ranging from 27.9% in those with no retinopathy to 64.6% in those with proliferative retinopathy. However this study did not distinguish between participants with type 1 and type 2 diabetes (Patrick et al. 2012). In a smaller cross-sectional study of T2D patients, there was a significant difference in 25OHD levels between groups stratified according to the presence or absence of diabetes and severity of retinopathy. 25OHD levels were lower with increasing severity of retinopathy and were lowest in those with proliferative retinopathy (Payne et al. 2012). However a more recent prospective study of predominantly male adults with T2D found no association between vitamin D status and the incidence of retinopathy or renal disease (Alele et al. 2013).

The association between 25OHD deficiency and retinopathy in T1D has been studied with varying results. In a cross-sectional study of adolescents and young adults with

T1D, we recently found that low vitamin D (<50nmol/L) was associated with twice the risk of retinopathy, independent of diabetes duration and HbA1c (H. Kaur et al. 2011). In contrast, in a prospective study of Danish T1D patients diagnosed between 1979 and 1984, there was no relationship between severe vitamin D deficiency (defined as 25OHD <15nmol/L, <10<sup>th</sup> percentile) and the development of background or proliferative retinopathy after 26 years (Joergensen et al. 2011). However baseline vitamin D levels were measured 3 years after diagnosis.

VDR polymorphisms may also influence the risk of diabetic retinopathy in T1D. In a French cohort, the prevalence of Taq1 wild type TT genotype was lower in patients with severe diabetic retinopathy compared with controls without retinopathy, particularly in patients with diabetes duration greater than 25 years (Taverna et al. 2002). In contrast, the FF genotype of the Fok1 polymorphism was associated with a lower risk of severe diabetic retinopathy (OR 0.54, 95% CI 0.32-0.90, corrected P=0.025) (Taverna 2005).

### **Vitamin D and diabetic nephropathy**

The role of 25OHD in diabetic nephropathy has been studied in patients with T1D and T2D. In a Japanese study, serum levels of 25OHD were significantly decreased in one hundred patients with T2D compared with controls. In patients with early diabetic nephropathy, higher albumin excretion rate was associated with decreased 25OHD levels (Inukai et al. 2000). This was confirmed in a study of Chinese T2D patients (Huang et al. 2012). In an Italian study of T1D patients with microalbuminuria, 25OHD and 1,25(OH)<sub>2</sub>D<sub>3</sub> levels were lower compared with those with normoalbuminuria and a control group (Verrotti et al. 1999). However in a Danish prospective study of T1D patients, severe vitamin D deficiency (10<sup>th</sup> percentile of the study group, 25OHD

<15nmol/L) did not predict the development of micro- or macroalbuminuria (Joergensen et al. 2011). In patients with T2D, polymorphisms of the VDR gene may either protect (R. J. L. Martin et al. 2010b) or increase the risk (Nosratabadi et al. 2010; H. Zhang et al. 2012a; Vedralová et al. 2012) of nephropathy.

Animal studies provide insight into the possible mechanisms underlying the association between vitamin D deficiency and albuminuria. Mouse models of T2D without nephropathy demonstrate up regulation of genes involved in calcium and vitamin D metabolism (Wang et al. 2006; Tourigny et al. 2012). VDR knockout mice develop more severe nephropathy than diabetic wild-type mice (Z. Zhang et al. 2007). VDR activators, calcitriol and paricalcitol, reduce glomerular expression of inflammatory cytokine mRNA as well as IL-6 and MCP-1 protein, even at doses that did not prevent proteinuria (Sanchez-Nino et al. 2012). Transgenic mice with human VDR expressed in the podocytes had less albuminuria compared with wild-type mice. Treatment with a vitamin D analogue further reduced albuminuria, podocyte loss and fibrosis suggesting that VDR signalling in podocytes is the mechanism for the renoprotection induced by vitamin D (Wang et al. 2012). Increased urinary megalin, 25OHD and vitamin D binding protein as well as up regulation of  $1\alpha$  hydroxylase genes and genetic targets of  $1,25$  hydroxylase were found in a mouse model of T1D (Fowlkes et al. 2011). One study of T1D patients with nephropathy or retinopathy demonstrated a negative correlation between 25OHD levels and inflammatory markers (high-sensitivity C-reactive protein, NGkB activity and Toll-like receptor 4 expression) highlighting the role of vitamin D and inflammation in the development of microvascular complications (Devaraj et al. 2011).

## **Vitamin D and neuropathy**

Several animal studies have demonstrated the neurotropic effects of vitamin D mediated by nerve growth factor (NGF). NGF is required for development and maintenance of neurons in the peripheral and central nervous system. Treatment of mouse fibroblasts, rat astrocytes and rat brain with 1,25(OH)<sub>2</sub>D<sub>3</sub> results in an increase in NGF mRNA and extracellular protein (Wion et al. 1991; Carlson & Kenny 2007). Upregulation of VDR and vitamin D analogues result in a further increase in NGF (Musiol & Feldman 1996). Rats treated with streptozotocin to induce diabetes demonstrate depletion of NGF in muscle and skin. A novel agent CB1093 was shown to increase NGF production in non-diabetic rats and NGF depletion was prevented with CB1093 in diabetic rats (Riaz et al. 1999). Increased VDR expression has been reported in dorsal root ganglia neurons of diabetic rats, indicating a potential role for vitamin D in treatment of diabetic neuropathy (Filipović et al. 2013).

In humans, NGF is produced by epidermal keratinocytes. NGF levels are significantly reduced in skin in diabetics compared with controls. These reduced levels may be associated with small sensory nerve fibre dysfunction in early diabetic peripheral neuropathy. Therefore NGF may play a role in the pathogenesis of diabetic neuropathy (Anand et al. 1996). Treatment of human keratinocytes with Tacalcitol, a vitamin D<sub>3</sub> analogue, results in a dose-dependant increase in expression of NGF mRNA (Fukuoka et al. 2001) suggesting that vitamin D analogues may have a role as potential therapeutic agents in diabetic neuropathy.

The association between vitamin D and peripheral neuropathy has been investigated in a number of studies in T2D. A small study in T2D patients found a significant



improvement in subjective pain scores with oral treatment in those with vitamin D insufficiency (P. Lee & Chen 2008). In a cross-sectional study of 210 T2D patients, 81% of patients with diabetic neuropathy had vitamin D deficiency compared with only 60.4% of patients without. In this study, diabetic neuropathy was defined using a neuropathy symptom score, neuropathy disability score and nerve conduction studies. Mean 25OHD levels were lower in the group with diabetic neuropathy (36.9nmol/L vs 58.3nmol/L). In binary logistic regression analysis, diabetic peripheral neuropathy was significantly associated with vitamin D deficiency after inclusion of potential confounders such as duration of diabetes, HbA1c and LDL-cholesterol (Shehab et al. 2011). In the 2001-2004 NHANES conducted in the US, vitamin D insufficiency (defined as <30ng/mL) was associated with self-reported numbness and composite paraesthesia, after adjustment for cardiovascular risk factors (Soderstrom et al. 2011).

Topical compounds containing vitamin D3 have been used for treatment of diabetic neuropathy. In a small randomised controlled trial of patients with T1D and T2D, topical QR-333, a compound containing quercetin, ascorbyl palmitate and vitamin D3, reduced severity of numbness, jolting pain and irritation compared with baseline levels. However the effect of vitamin D3 alone could not be assessed (Valensi et al. 2005).

Although there are numerous studies documenting an association between vitamin D levels and microvascular complications, there is a paucity of studies including patients with T1D. Given the potential role of 25OHD in T1D pathogenesis (Mathieu et al. 2005), and the multiple reports of low vitamin D levels in association with T1D, it would be beneficial to explore the effect of 25OHD levels on the development of complications.

### **2.6.2 Infection and vascular complications**

Inflammation plays a key role in the development of microvascular complications. To further understand the triggers and causal pathways, a number of studies have investigated the concurrent role of infection, with associated inflammation, and microvascular disease. The effect of infection on complications may be mediated locally, for example intraocular infection can promote progression of DR (Dev et al., 1999). Alternatively, generalised infection or the inflammation associated with distant infection may influence changes in microvascular and microvascular structures.

Several studies have linked *Chlamydia pneumoniae* (*C pneumoniae*) infection with atherosclerosis. In a Japanese study of non-diabetic patients *Chlamydia* TWAR strain IgG and IgM antibody levels were elevated with acute myocardial infarction (AMI) and chronic coronary heart disease (CCHD) compared with controls (Saikku et al. 1988). Other studies have linked increased cardiovascular risk with *C pneumoniae* serology (Pesonen et al. 2007) and found bacterial components in atherosclerotic plaques (O'Connor et al. 2001; Shi & Tokunaga 2002). Previous infection with *C pneumoniae* is a predictor for increased carotid intima media thickness (CIMT) and carotid artery stenosis (Espinola-Klein et al. 2000). In a study of T2D patients, anti-*C pneumoniae* IgG titres were higher in patients with retinopathy compared with those without documenting an association between previous infection and risk of microvascular disease (Banaee et al. 2015).

Patients with T2D are at increased risk of *Helicobacter pylori* (*H pylori*) infection. *H pylori* has been found at higher rates in patients with autonomic and peripheral neuropathy, retinopathy and nephropathy and increases the odds of thrombotic

cerebral ischaemia (Hamed et al. 2008). A study of Japanese patients with T2D demonstrated increased arterial stiffness (as measured by pulse wave velocity) and seropositivity to H pylori (Ohnishi et al. 2008). H pylori infection is associated with production of inflammatory cytokines interleukin-6 and tumour necrosis factor- $\alpha$ , which mediate the effect on microvascular structures (Hamed et al. 2008).

Viral infections have also been implicated in the pathogenesis of vascular disease. Chinese T2D patients with chronic hepatitis B viral (HBV) infection had a higher frequency of retinopathy than non-HBV-infected patients at baseline and infection increased the risk of end-stage renal disease (Cheng et al. 2006). There is serological evidence for higher infection rates in patients with coronary events compared with controls (Reunanen et al. 2002; Roivainen et al. 1998), and increased risk of CHD with higher HSV titres (Pesonen et al. 2007) In addition, EV, cytomegalovirus (CMV) and have been identified in atherosclerotic plaques (T. W. Kwon et al. 2004; Schanen et al. 2007), (Chiu 1999; Shi & Tokunaga 2001; Shi & Tokunaga 2002). Previous CMV infection is a predictor for increased CIMT and carotid artery stenosis (Espinola-Klein et al. 2000).

The process of atherosclerosis begins in childhood, with demonstration of the presence of aortic plaques even during infancy. It is therefore possible that childhood infections contribute to its pathogenesis. Children with acute infection had a greater increase in CIMT three months after recovery compared with controls (Liuba 2003). Increased aortic intima media thickness was demonstrated in children with persistent seropositivity to C pneumoniae or treatment of Chlamydial infections compared with controls (Volanen et al. 2008; Volanen et al. 2006). Increased carotid artery stiffness

was shown in patients with T1D with an increased number of reported respiratory infections (Odermarsky et al. 2008).

There is a paucity of studies investigating the role of infection in vascular complications in T1D. As infection is considered to play a role in the aetiology of T1D (Craig et al. 2013), its influence on the development of diabetes complications warrants further study.

### **2.6.3 Adiposity and Vascular Complications**

T1D patients are at significantly increased risk of cardiovascular disease with hazard ratios ranging from three to seven times the risk of the general population. Women with T1D appear to be at a much greater risk compared with men (Soedamah-Muthu et al. 2006), which highlights the loss of protection against cardiovascular disease with female sex found in females without T1D.

Since the DCCT, intensive glycaemic control has been a treatment goal of T1D management to prevent microvascular and macrovascular complications. Poor glycaemic control predisposes directly to cardiovascular disease. Some studies demonstrate a J-shaped curve of the association between glycaemic control and cardiovascular disease, whilst in others the risk increases linearly with increasing HbA1c (Eeg-Olofsson et al. 2010). However later analysis of the DCCT cohort found that intensive treatment was associated with the prevalence of the metabolic syndrome and the greatest weight gain over the nine year study period (Kilpatrick et al. 2007). When the group was stratified according to quartiles of weight gain, those in the fourth quartile (with greatest weight gain) had significantly higher blood pressure, triglyceride

and total cholesterol levels, insulin requirements and waist-hip ratios compared with the other three quartiles (Purnell et al. 1998). After follow up for six years in the EDIC study, the fourth quartile maintained greater waist circumference, BMI and insulin requirements and had higher carotid intima thickness (Purnell et al. 2013). Therefore optimising glycaemic control with intensive insulin therapy is a double-edged sword.

Adiposity is a risk factor for macrovascular disease in adults and is an element of the metabolic syndrome. The metabolic syndrome and its components are significant predictors of mortality, coronary artery disease and renal failure in T1D (Orchard et al. 2003; Pambianco et al. 2007). In adult patients with T1D, the presence of coronary artery calcium is associated with visceral and subcutaneous adiposity and BMI in men and all three adiposity measures plus waist circumference in women (Conway et al. 2007).

Decreased insulin sensitivity, as a consequence of adiposity, is associated with vascular complications. Low glucose disposal rate (GDR), measured directly by euglycemic clamp, was associated with microalbuminuria in a small group of T1D patients (Yip et al. 1993). An indirect measure of insulin resistance, the estimated glucose disposal rate (eGDR), derived from HbA1c, waist-hip ratio and hypertension correlates with the more invasive euglycaemic clamp (Williams et al. 2015). Low eGDR correlates with high insulin resistance and is associated with microalbuminuria, overt nephropathy, retinopathy, neuropathy and coronary artery disease (Orchard et al. 2002; Chillarón et al. 2009; Kilpatrick et al. 2007; Olson et al. 2002; Orchard et al. 2003). Moreover, it is an independent predictor of macrovascular disease, lower extremity arterial disease events and mortality (Olson et al. 2002; Orchard et al. 2003; Kilpatrick et al. 2007).

Adiposity is an increasing finding in young people and adults with T1D and is also a risk factor for microvascular disease (van Vliet et al. 2010; Conway et al. 2010). In longitudinal studies of young people from our centre, obesity at baseline increased the risk of microalbuminuria by three times after a median of 6 years follow up (Stone et al. 2006) and BMI >95<sup>th</sup> percentile was significantly associated with risk of retinopathy (Gallego et al. 2008). With steady but high rates of adiposity in T1D, new patients may be at greater risk of vascular disease. In this thesis, we will investigate the role of adiposity, quantified by BMI-SDS and the development of microvascular complications after fifteen years follow up.

#### **2.6.4 Genetic Factors in Microvascular Complications**

Familial clustering of microvascular complications has been shown in a number of studies suggesting that genetic factors play an important role in incidence. In families with several members with T1D, higher rates of complications are found in relatives of T1D probands who have retinopathy and nephropathy compared with relatives of probands without complications (Seaquist et al. 1989; Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) Research Group 1997; Harjutsalo et al. 2004; Monti et al. 2007). Concordance in severity of complications has also been demonstrated. In the DCCT/EDIC study, severe retinopathy was three times more likely in a relative if the proband had severe retinopathy (Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) Research Group 1997). In the study by Harjutsalo et al, the risk of nephropathy in the sibling increased with severity of renal disease in the proband (Harjutsalo et al. 2004). A

diagnosis of T2D in a parent can also increase the risk of nephropathy (Monti et al. 2007).

### **HLA-risk alleles and microvascular complications**

The influence of HLA-class I and II risk alleles on the development of microvascular complications have been investigated with conflicting results, with some demonstrating a positive association (Cruickshanks et al. 1992; D. Agardh et al. 1996; Mimura et al. 2003; Lipner et al. 2013) but not others (Wong et al. 2002; E. Agardh et al. 2004; Falck et al. 1997; Jensen et al. 2011). Lipner et al found that the HLA-DRB1\*03:01 allele and HLA-DQA1\*05:01-DQB1\*02:01 haplotype (which are in linkage disequilibrium) were protective against one or more microvascular complications, and retinopathy after adjustment for age at diabetes onset, diabetes duration and sex (Lipner et al. 2013). In contrast, Agardh et al found that the HLA-DQB1\*0201/0302 genotype was associated with an increased risk of proliferative retinopathy in one study (D. Agardh et al. 1996), but this finding was not replicated in a later study (E. Agardh et al. 2004). In a cross-sectional study of WESDR patients, HLA-DR4, but not -DR3, was associated with five times increased risk of retinopathy (Cruickshanks et al. 1992). However in a subsequent longitudinal study, neither allele was associated with incident or progression of retinopathy (Wong et al. 2002). The WESDR included patients with both T1D and T2D, although diagnosis at younger than 30 years of age and the use of insulin were used as proxy selection criteria to identify T1D patients. Three additional studies have not shown an association between -DR3 or -DR4 and retinopathy (Falck et al. 1997; Jensen et al. 2011; Lipner et al. 2013). Two studies have demonstrated an association between various HLA class I alleles and retinopathy (Mimura et al. 2003; Lipner et al. 2013).

Fewer studies have investigated the association between HLA alleles and diabetic nephropathy, again with varying results. In adults with ESRD, the frequency of HLA-B antigens was higher and B7 and B12 were lower (Barbosa 1981). In contrast, two studies have found no difference in the frequency of HLA-A,B,C, DR, DQA1 and DQB1 alleles in patients with and without nephropathy (Chowdhury et al. 1999; Walton et al. 1984). In the Genetics of Kidneys in Diabetes (GoKinD) study, patients with HLA-DRB1\*04 were 50% less likely to have nephropathy compared with those negative for – DRB1\*04 (Cordovado et al. 2007). Distribution of HLA alleles differs between ethnic groups and may account for variations in incidence of end stage renal disease (Freedman et al. 1993).

### **Susceptibility Genes**

In examination of genes involved in the pathogenesis of microvascular complications, several susceptibility (Wessman et al. 2011; Pezzolesi et al. 2009; Fagerholm et al. 2012) and protective (Syreeni et al. 2011) loci have been identified in Caucasian populations. Enzymes encoded by some of these regions are important in epigenetic modifications, which promote the inflammatory process of microvascular complications. Single nucleotide polymorphisms (SNP) in the angiotensin II type 1 receptor (Möllsten et al. 2011) and ACE insertion/deletion genes (Yu et al. 2012) are associated with diabetic nephropathy, however the findings varied according to sex and ethnicity. SNPs in the matrix metalloproteinase genes, which are important in extracellular matrix remodelling are also associated with nephropathy risk (Kure et al. 2011). Susceptibility loci on chromosome 22q11, 3q21-125 and 19q13 have been identified for diabetic nephropathy (Wessman et al. 2011). Allelic variations in the superoxide dismutase gene was associated with reduced eGFR, prevalence of diabetic



nephropathy and incidence of microalbuminuria in European Caucasian T1D patients (Mohammedi et al. 2011).

The association between HLA risk alleles and microvascular complications remains unclear. It is likely that the discrepant results are related to study design and methods. Several previous studies have used age at diabetes onset and use of insulin as proxy measures to identify patients with T1D (Wong et al. 2002). In other studies, the presence or absence of complications has been ascertained by questionnaire or qualitative measures (Chowdhury et al. 1999; Jensen et al. 2011; Lipner et al. 2013). In this thesis, we aim to study the longitudinal association between HLA class II risk alleles and microvascular complications assessed using standardised quantitative techniques.

## **2.7 Therapeutics**

The discovery of insulin in the early twentieth century and the use of bovine insulin in patients in 1922 revolutionised the treatment of T1D and significantly improved the life expectancy of patients (Rosenfeld 2002). The addition of protamine and zinc resulted in slow-release insulins with treatment effect of 24-36 hours. The production of synthetic insulin increased in scale from 1963 with the use of recombinant DNA techniques and has made treatment widely accessible. Since then, manipulation of the insulin molecule by amino acid substitutions has produced insulin analogues with variable onset and duration of action therefore allowing the evolution of insulin treatment regimens which more closely mimic the physiological production of insulin throughout the day and maintain near-normoglycemia without increasing the risk of hypoglycaemia (Aathira 2014d). Insulin delivery has also progressed from intermittent subcutaneous injection

to production of pumps to provide continuous subcutaneous insulin infusion (Malik & Taplin 2014}.

Injection of short and long acting insulin analogues twice daily was the standard treatment regimen prior to the publication of the DCCT in 1993. Patients randomised to intensive insulin therapy had significant reductions in incident retinopathy, albuminuria and clinical neuropathy compared with the conventional treatment group (one or two daily injections). Patients with mild retinopathy at baseline had slowed progression of retinopathy with intensive insulin therapy (Diabetes Control and Complications Study Group 1993}. Following this study, multiple daily injection (MDI) regimens have become the standard of therapy with the goal of reducing HbA1c. At the end of the DCCT study, all patients were recommended intensive therapy and have undergone periodic follow up in the EDIC study. Over time, there has been convergence of HbA1c levels between treatment groups (Genuth et al. 2013). Long term follow up after thirty years of the DCCT/EDIC and The Pittsburgh Epidemiology of Diabetes Complications (EDC) cohorts demonstrated retinopathy, nephropathy and cardiovascular disease rates of 47-50%, 17-25% and 14%, respectively in the conventional and EDC groups and less than half these rates in the intensively treated groups. This highlights the hypothesised role of “metabolic memory” in the pathophysiology of microvascular complications (Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) Research Group et al. 2009).

The use of continuous subcutaneous insulin infusion (CSII) via insulin pump has become more common in the last decade. This allows a more physiologic delivery of

insulin with adjustable basal insulin and prandial bolus insulin. Early indications for use included frequent and/or severe hypoglycaemia, poor glycaemic control, dawn phenomenon and the desire for a more flexible lifestyle (Danne et al. 2014). However there is evidence of improved glycaemic control with CSII compared with MDI (MD et al. 2010). The use of continuous subcutaneous glucose monitoring (CGM) and CSII in combination is progressing towards “closed-loop” insulin delivery systems.

## **2.8 Mortality and Type 1 Diabetes**

Mortality rates are increased in patients with T1D compared with the background population in several studies. Collectively, these demonstrate standardised mortality rates ranging from 2.0-7.55 (Joergensen et al. 2011; Patterson et al. 2007; Skrivarhaug et al. 2005; Dahlquist & Källén 2005; Podar et al. 2000; Edge et al. 1999; Laing et al. 2003; O'Grady et al. 2012; Lind et al. 2014). In the younger age groups, deaths are often attributed to acute complications of diabetes, such as diabetic ketoacidosis and hypoglycaemia (Patterson et al. 2007; O'Grady et al. 2012; Edge et al. 1999). In older patients, cardiovascular disease and chronic renal disease account for a greater proportion of deaths.

In young with T1D people, standardised mortality is two to three times the age-matched population (Edge et al. 1999; Patterson et al. 2007; O'Grady et al. 2012), even after relatively short follow up times of less than ten years. In some studies, acute complications of diabetes account for the majority of deaths (Edge et al. 1999; O'Grady et al. 2012), however in others only approximately one-third can be attributed to diabetes (Dahlquist & Källén 2005; Patterson et al. 2007). Up to 50% of deaths in young people are unrelated to diabetes (Dahlquist & Källén 2005; Patterson et al. 2007).

Highest rates of death occur in those aged under four years and over ten years, particularly in males (Edge et al. 1999; O'Grady et al. 2012).

T1D may be associated with increased rates of deaths due to accidents and suicides. A recent study of the Swedish National Diabetes Register demonstrated that the risk of any unnatural death was over twice that of the control population, with three times the relative risk of suicide and double the risk of accidental death. These risks were particularly increased when a subgroup of patients registered at age <40 years was examined as a proxy for those with T1D. A significant number of suicides were due to deliberate or accidental self-poisoning and transportation accidents. The proportions of diabetes patients with cardiovascular disease, stroke and any mental illness were greater than controls (Webb et al. 2014). These findings were in contrast with an earlier Swedish study which did not find an excess risk of deaths due to accidents or suicide compared with controls (Dahlquist & Källén 2005). In the 2007 EURODIAB report there was a non-significant excess of accidental or violent deaths. The rate of suicides was only slightly increased (Patterson et al. 2007). A recent meta-analysis examining risk of suicide reported that T1D patients were 3-4 times more likely to attempt suicide than the general population. Patients also had a 61% higher risk of experiencing suicidal thoughts than individuals without DM-1 (Pompili et al. 2014).

Longer-term cohort studies demonstrate a high rate of deaths due to microvascular complications, particularly renal disease. Ewing et al recognised that diabetic autonomic neuropathy increased mortality to 50% in five years compared with only 15% in those without neuropathy. Rates of retinopathy, nephropathy and peripheral neuropathy were also higher in those with autonomic neuropathy suggesting a common

pathophysiological process (Ewing et al. 1980). In this study, the majority of deaths were due to renal failure and Orchard et al subsequently proposed that the excess mortality in the presence of autonomic neuropathy could be explained by associations with other microvascular complications and cardiovascular risk (Orchard et al. 1996). In this later study, patients were assessed by only one test of autonomic function (expiration/inspiration ratio).

Cardiovascular disease also accounts for a significant proportion of deaths, although some studies have demonstrated a decline in cardiovascular deaths in recent cohorts (Gregg et al. 2012; Allemann et al. 2009). Initial analysis of Australian data from 1997 to 2010 found that crude death rates due to cardiovascular disease fell over time. However the authors noted that deaths due to cardiovascular disease were under-reported and when cause of death was re-classified, the rates of cardiovascular deaths increased in both males and females over this time (Harding et al. 2014).

Risk factors for mortality reflect known risks for microvascular and macrovascular disease. The EURODIAB Prospective Cohort Study found that age at baseline, HbA1C, waist-to-hip ratio, pulse pressure and non-HDL cholesterol were all risk factors for mortality after seven years follow up. The most important microvascular complications for predicting mortality were macroalbuminuria, peripheral and autonomic neuropathy (Soedamah-Muthu et al. 2008). The Early Treatment Diabetes Retinopathy Study (ETDRS) assessed differences in characteristics and risk factors for mortality in 3711 patients with T1D. Acute coronary events were the most common cause, accounting for 55% of deaths. Patients who died were more likely to be older, non-white, use diuretic or antihypertensive medications and have higher systolic blood pressure, total

cholesterol, triglycerides and fibrinogen. After adjustment for age, sex, race, duration, HbA1c and all statistically significant covariates for mortality including microvascular disease, markers of renal disease and diminished vibratory sense, amputation and poor visual acuity were significantly associated with mortality (Cusick et al. 2005).

Higher standardised mortality rates are also seen in females compared with males in both young (Patterson et al. 2007; Edge et al. 1999) and older cohorts (Allemann et al. 2009; Secrest et al. 2010). A recent meta-analysis has reported a pooled women-to-men ratio of SMR for all-cause mortality of 1.37. Similar increased ratios were noted for fatal renal and cardiovascular diseases and incident stroke (Huxley et al. 2015). While it is difficult to explain the higher female death rates in the younger patients, the findings in older cohorts suggest that the established protection from cardiovascular disease associated with female gender is diminished in patients with T1D. Indeed in the meta-analysis by Huxley et al, the pooled women-to-men ratio of SMR for incident coronary heart disease was 2.54 (Huxley et al. 2015). Results from a Scottish study confirmed a greater reduction in life expectancy for women than men (Livingstone et al. 2015).

Encouragingly, a decline in mortality rates has been reported in long-term cohorts. Thirty-year mortality rates in patients diagnosed between 1965 to 1979 in Allegheny County, Pennsylvania declined across time periods (SMR 9.3 between 1965-69 to 5.6 between 1975-79) (Secrest et al. 2010). An Australian study found that SMR decreased from 4.2 to 3.08 in males between 1997 and 2010, and from 3.92 to 3.46 in females over the same period ( $P_{\text{trend}} < 0.01$ ) (Harding et al. 2014). This study confirms findings from Canada, Switzerland, the United Kingdom and the United States, although some of these studies do not distinguish between T1D and T2D (Allemann et al. 2009; Lind et al. 2013;

Gregg et al. 2012). Two studies have reported that mortality rates were not increased in T1D in the absence of nephropathy (Orchard et al. 2010; Groop et al. 2009).

Reduction in mortality rates may occur with improvements in glycaemic control. Follow up of participants from the DCCT has shown that intensive therapy reduces all-cause mortality risk (HR 0.67, 95% CI 0.46-0.99) (Orchard et al. 2015). These studies support efforts to improve glycaemic control and consequent microvascular complications in order to reduce mortality in T1D to rates comparable with the general population. Identification of additional early risk factors will assist in risk stratification. This thesis reports the association of early markers of microvascular disease and subclinical autonomic neuropathy and mortality in a cohort of young people with T1D followed for twenty-three years.

## Chapter 3 Study Design, Subjects and Methods

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### **3.1 Overview**

This chapter describes the study populations and designs. Methods specific to each study are described below.

### **3.2 Study Populations**

Cohort A: an historical incident cohort of 206 children and young people (median age 8.1 years, male 39%) diagnosed with T1D at The Children's Hospital at Westmead between April 1997 and September 1999

Cohort B: a contemporary incident cohort of 213 children and young people (median age 8.8 years, 50% male) diagnosed with T1D at The Children's Hospital at Westmead and St George Hospital between August 2010 and January 2014

Cohort C: 481 young people with T1D (median age 15.4 years, male 52%) mean diabetes duration 7.2+3.5 years, examined for complications at the Children's Hospital at Westmead between 2009 and 2010

Cohort D: 413 young people diagnosed with T1D between 1973 and 1993 who attended for their first diabetes complications assessment between 1990 and 1995 (median age 14.4 years, male 48%)

### **3.3 Study Designs**

Study 1 was a cross-sectional study of Cohort B to examine the association between HLA genotype (low vs high risk) and age at diagnosis (<5 years vs >5 years) and adiposity, viruses or vitamin D deficiency at diagnosis of T1D.

Study 2 examined the difference in rates of adiposity, viruses or vitamin D deficiency at T1D diagnosis between Cohort A and Cohort B, diagnosed fifteen years later.

Study 3 was a prospective longitudinal study of 156 young people from Cohort A who were examined repeatedly for complications. The aim was to determine if features at diagnosis of T1D (adiposity, viral trigger, vitamin D deficiency, HLA genotype) predict risk of microvascular complications after 15 years

Study 4 was a cross-sectional study of Cohort C to determine if vitamin D deficiency is associated with adverse retinal vascular geometry parameters

Study 5 was a longitudinal cohort study of young people diagnosed with T1D between 1973 and 1993, who attended their first diabetes complications assessment between 1990 and 1995 to determine whether adolescent complications, including subclinical autonomic neuropathy predict mortality 23 years later

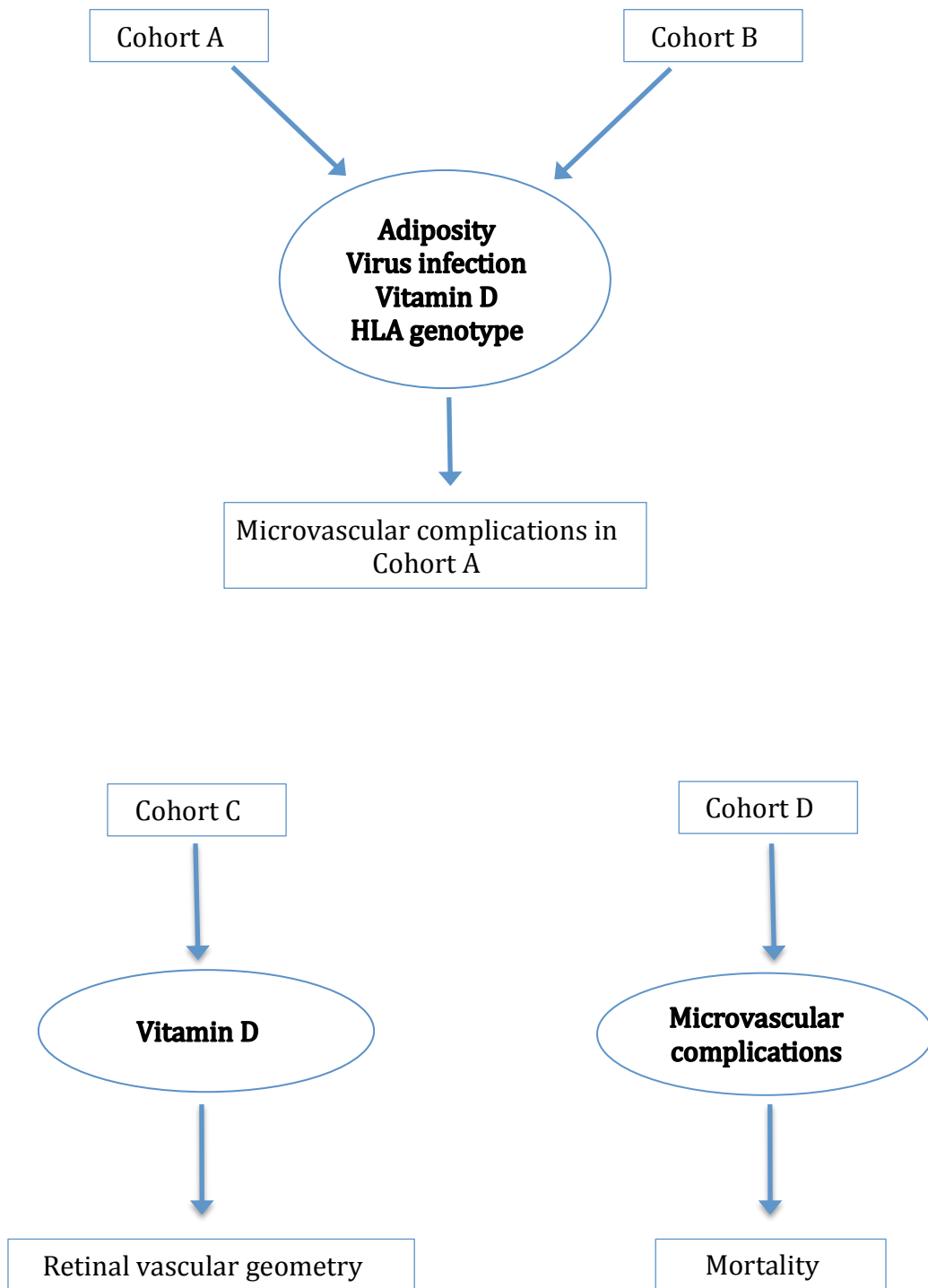


Figure 3.1 Overview of Cohorts and studies

### **3.4 Clinical Assessment**

Height was measured using a Harpenden stadiometer and weight measured using digital scales. Body mass index (BMI) was calculated according to the formula  $\text{weight}/\text{height}^2$ . Measurements were taken at the first clinic visit at least one month after diagnosis to allow for correction of dehydration and metabolic derangement, and at all subsequent complications assessments visits. Anthropometric measures were converted to standard deviation scores according to Centre for Disease Control reference (Kuczmarski et al. 2002). Patients were classified as overweight or obese according to age-related reference standards corresponding to BMI  $25\text{kg}/\text{m}^2$  and  $30\text{kg}/\text{m}^2$ , respectively (Cole et al. 2000).

### **3.5 Ethnicity**

The country of birth of the patient, parents and grandparents were ascertained using a standard questionnaire or by interview. Ethnic groups were defined according to the Australian Bureau of Statistics standardised codes (Australian Bureau of Statistics 2005) as shown in Table 3.1. Ethnicity of the child was ascribed according to the combined birthplaces of parents and grandparents. Patients were stratified as European/Australian (group 1) or non-European/Australian (groups 2-7). Patients with more than one ethnic origin were classified as group 8.

Table 3.1 Ethnic groups

Ethnic group
<b>1. Australian/New Zealander/European (Eastern or Western)/ North American</b>
<b>2. Oceanian Indigenous</b>
<b>3. Southern European</b>
<b>4. Middle Eastern/North African</b>
<b>5. Asian</b>
<b>6. Latino/Hispanic</b>
<b>7. African</b>
<b>8. Mixed</b>

### **3.6 Samples and testing methods**

Whole blood, serum, throat swabs or stool were collected within two weeks of diagnosis and samples were stored at 4°C until processing. Plasma was separated from blood collected in EDTA.

#### ***Acid-Base status***

Acid-base status was measured at diagnosis by venous blood gas. Diabetic ketoacidosis was defined as pH<7.3 or bicarbonate <15nmol/L, hyperglycaemia and ketosis (Wolfsdorf et al. 2014).

#### ***Diabetes-associated autoantibodies***

Between 1997 and 1999, autoantibodies to insulin (IAA), glutamic acid decarboxylase (GAD) and islet-antigen-2 (IA2) were measured using an in-house competitive radioimmunoassay. From 2010, GAD and IA2 autoantibodies were changed to a commercially available radioimmunoassay (RSR Limited, UK).

### ***C peptide***

C peptide was determined using a chemiluminescent enzyme assay (IMMULITE 1000(r) analyser, Siemens Healthcare, UK).

### ***Vitamin D radioimmunoassay***

Whole blood was centrifuged at 3000 rpm for 10 minutes and serum extracted and stored at -80°C until testing. 25 hydroxycholecalciferol (25OHD) levels were measured using radioimmunoassay (DiaSorin, Stillwater, MN).

### ***HLA genotyping – Cohort A***

In Cohort B, HLA genotyping of HLA-DRB1 and DQB1 alleles was performed on whole blood at the Red Cross Blood Bank, Sydney as previously described (Craig, Howard, et al. 2003a). Briefly, exon 2 polymorphisms were determined from PCR-amplified DNA using sequence-specific oligonucleotides and alleles assigned on the basis of hybridization patterns.

HLA genotypes were classified into the following groups:

- HLA DRB1\*03-DQB1\*02/DRB1\*04-DQB1\*0302 (having both high risk alleles)
- HLA-DRB1\*03-DQB1\*02/x or DRB1\*04-DQB1\*0302/y (either heterozygous or homozygous for risk alleles)
- z/z (patients without either risk allele).

### ***HLA genotyping – Cohort B***

Saliva samples for HLA genotyping were collected using Oragene DNA saliva kits (DNA Genotek Inc, Kanata, Ontario) at least 30 minutes after consumption of any food or drink. Samples were stored at room temperature until testing, which was performed in the immunogenetics laboratory of the Royal Perth Hospital, Western Australia. For DRB1, exon 2 was amplified by PCR and sequencing performed using the 3730 DNA analyser (Applied Biosystems, Life Technologies, Carlsbad, Ca). DQA1 and DQB1 were tested using a DQA/DQB SSO genotyping kit (One Lambda, Canoga Park, Ca). If further typing was required for DQB1, PCR was used to amplify Exon 2 and Exon 3, and sequencing performed using the 3730 DNA analyser.

HLA genotypes were classified into the following groups (Erlich et al. 2008):

-HLA-DRB1\*04-DQA1\*0301-DQB1\*0302/DRB1\*0301-DQA1\*0501-DQB1\*0201 (DR4-DQ8/DR3-DR2)

-HLA-DRB1\*04-DQA1\*0301-DQB1\*0302/x (DR4-DQ8/x)

-HLA-DRB1\*0301-DQA1\*0501-DQB1\*0201/y (DR3-DQ2/y)

-z/z

### ***Virus studies – Cohort B***

EDTA and whole blood samples collected from Cohort B were centrifuged at 3000 rpm for 10 minutes as soon as possible after collection and plasma or serum extracted into aliquots with minimum volume 250 microlitre. Extracted plasma or serum samples were frozen at -80°C and transported at -20°C to the laboratory for testing. Between August 2010 and August 2011, throat swabs were placed in viral transport media and

stored at 4°C until testing. After August 2011, the throat swabs were placed in MEM viral culture medium and frozen at -80°C until testing. Stool samples were divided and frozen at -80°C.

#### *Multiplex virus polymerase chain reaction (PCR) (VDL 10)*

Plasma, throat swabs and stool were tested for EV, cytomegalovirus (CMV), Herpes Simplex virus (HSV), Epstein Barr virus (EBV) and Varicella zoster virus (VZV) with multiplex polymerase chain reaction (mPCR) as previously described (McIver et al. 2005). Plasma or serum were also tested using an EV nested RT-PCR. In brief, nucleic acid was extracted using the MagNa Pure LC (Roche JE379, Basel, Switzerland). Extracted DNA was amplified in the Bio-Rad MyCycler Thermal Cycler using two rounds of PCR with stock primers specific for each virus. Routine testing for virus contamination was performed. Virus was detected using two methods: 1) agarose gel electrophoresis and viewed under ultraviolet light, 2) enzyme-linked immunosorbent assay (Dig Detection Kit).

#### ***Virus Studies – Cohort A***

Plasma and stool were tested for EV using a monoplex RT-PCR as previously described (Craig, Howard, et al. 2003a).

### **3.7 Diabetes Complications Assessment**

Participants attended the Diabetes Complications Assessment Service at The Children's Hospital at Westmead for assessment for complications.



### ***Clinical Assessment***

Participants were seen by a physician and a structured medical history was obtained including documentation of episodes of ketosis, significant hypoglycaemic episodes and current insulin treatment. Height was measured to the nearest 0.1cm using a standard stadiometer and weight was measured to the nearest 0.1kg using a digital scale. BMI was calculated and expressed as age and sex adjusted standard deviation scores.

### ***Blood Pressure***

Manual blood pressure was taken using a conventional sphygmomanometer using an appropriately-sized cuff with the participant seated after five minutes rest. Blood pressure standard deviation scores based on age, height and gender, were used in the analysis (Jackson et al. 2007).

### ***Retinopathy***

Retinopathy was assessed by 7 field fundal photography using a Topcon Fundus camera (TRC 50-VT, Tokyo Optical Co., Tokyo) after dilatation of the pupils with cyclopentolate 1% and phenylephrine 2.5%. Non-simultaneous photographic pairs were taken of seven standardised fields in each eye and then viewed with a Donaldson Stereoviewer, which provided a three-dimensional representation of the fundus and enabled microaneurysms to be more easily distinguished from haemorrhages and artefacts. The IMAGEnet 2000 Lite system was used to digitise images from September 2004. Photographs were screened for the presence of microaneurysms by a trained orthoptist (AP). Images were graded by the same ophthalmologist (SH) according to the Early Treatment Diabetic Retinopathy Study (ETDRS) adaptation of the modified Airlie House

classification of diabetic retinopathy (Klein, Klein 1989). The grading system is as follows:

Level 10: no retinopathy

Level 21: microaneurysms only or haemorrhages only

Level 31: microaneurysms plus one or more of the following: definite haemorrhages, hard exudates or venous loops; questionable soft exudates, intraretinal microvascular abnormalities or venous beading

Level 41-51: moderately severe non-proliferative retinopathy

Level 61 and 65: proliferative retinopathy

Level 71: proliferative retinopathy with high-risk characteristics

Level 80: eye unable to be graded because of vitreous haemorrhage obscuring the retina

Retinopathy was defined as the presence of at least one microaneurysm or one haemorrhage (grade 21) in one eye.

### ***Nephropathy***

AER was assessed from three consecutive timed overnight urine specimens. If these were not available, ACR was calculated from an early morning urine specimen provided on the day of assessment. Albumin was measured using polyclonal radioimmunoassay (Pharmacia RIA, Beckman Coulter, Australia) prior to 2000, nephelometric assay using an IMAGE analyser ( $IMAGE = (0.8734 \times \text{radio-immunoassay value}) - 0.501$ ;  $r = 0.99$ ) from 2000 to 2003 and competitive chemiluminescence immunoassay using the IMMULITE analyser (Diagnostic Products, Los Angeles, CA) thereafter. Creatinine was measured by Jaffe reaction, Dimension ARX (Dade Behring, Newark, DE).

Early elevation of AER was defined as AER  $>7.5\mu\text{g}/\text{min}$  or mean ACR  $>1.0\text{mg}/\text{mmol}$  (males) and  $1.4\text{mg}/\text{mmol}$  (females) in two out of three consecutive timed overnight samples to allow for wide intra-individual variation. This cutoff was selected because the 95<sup>th</sup> percentile was  $7.2\ \mu\text{g}/\text{min}$  in 690 non-diabetic Australian children aged  $11.5\pm 3.38$  years (Couper et al. 1994) and  $7.6\ \mu\text{g}/\text{min}$  in 41 non-diabetic American adolescents (Garg et al. 1990).

Microalbuminuria was defined as AER  $>20 - <200\mu\text{g}/\text{min}$  or ACR  $2.3\text{-}25\text{mg}/\text{mmol}/\text{L}$  (males) or  $3.5\text{-}25\text{mg}/\text{mmol}/\text{L}$  (females) or albumin concentration  $30\text{-}300\text{mg}/\text{L}$ . Proteinuria was defined as AER  $>200\mu\text{g}/\text{min}$ .

### ***Peripheral neuropathy***

Peripheral nerve function was assessed by thermal threshold testing for hot and cold at the left foot using the TSA-II Neurosensory Analyzer (Medoc Ltd). Vibration threshold at the left medial malleolus and left great toe was measured with the VAS-3000 Vibratory Sensory Analyzer (Medoc Ltd). Peripheral nerve abnormalities were defined as  $< 95\%$  of the normal range in a nondiabetic adolescent control group.

### ***Pupillometry***

Pupillary autonomic function was assessed using an infrared pupillometer (Pupilsan, Fairvill Medical Optics) as previously described (Schwingshandl et al. 1993). Parameters included were pupil diameter before and three seconds after a light stimulus was delivered, reflex pupillary amplitude and maximum constriction velocity. Reference ranges were derived from 122 non-diabetic control subjects (Schwingshandl et al. 1993). Abnormal cardiovascular and pupillary tests were defined as less than the

fifth percentile of the reference range (Donaghue et al. 1993). Small resting pupil diameter, defined as <5.3mm, was also used as an outcome because we have previously shown this cut off predicts subsequent development of retinopathy and microalbuminuria (Maguire et al. 2007).

### **Retinal Vascular Geometry**

Retinal vascular geometric measures were performed on digitised left eye retinal images using a semi-automated computer programme (Singapore Vessel I Assessment, SIVA) (Sasongko et al. 2010).

1. Vessel calibre
2. Vessel tortuosity
3. Branching angle
4. Optimality deviation
5. Length-diameter ratio

Vessel calibre is described as central retinal arteriolar (CRAE) and venular (CRVE) equivalents. These were calculated from the calibres of the largest six arterioles and venules of the left eye.

Vessel tortuosity is a measure of the curvature of the vessels and is calculated as the integral of the total squared curvature along the vessel length divided by the arc length. Normal vessels are usually straight and smooth and changes in vessel tortuosity are associated with blood pressure (Taarnhoj et al. 2008).

Branching angle measures the angle between the first two daughter vessels. Changes in branching angle result in alterations in blood flow and 75 degrees has been proposed as the optimal branching angle to maximise blood flow efficiency and reduce diffusion distance. An increased branching angle results in reduced blood flow. A decreased branching angle is associated with ageing and hypertension (Stanton et al. 1995).

Optimality deviation quantifies the degree to which the vasculature deviates from an optimal state of microvascular networks and is a measure of the diameter of the daughter vessels relative to the parent vessels. Increased arteriolar optimality deviation is associated with BMI, independent of smoking in healthy adults (A. D. Hughes et al. 2009).

Length-diameter ratio measures the length between the midpoints of the first and second vessel branches divided by the diameter of the parent vessel at the first branch. Increased arteriolar length-diameter ratio is associated with older age and hypertension in healthy adults (A. D. Hughes et al. 2009).

## Chapter 4

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**The interaction between environmental and genetic factors at type 1 diabetes presentation in a contemporary incident cohort**

**Aims:** To characterise a contemporary incident cohort of young people with type 1 diabetes and determine if those with low risk HLA genotypes differ in adiposity, viruses or vitamin D deficiency from those with high risk HLA genotypes

To determine if children with diagnosis <5 years of age have different characteristics to those diagnosed >5 years of age

## **Introduction**

In recent years, the incidence of T1D has increased worldwide at a rate of 3% per year. This rate of rise is faster than can be attributed to an increase in genetic susceptibility. Indeed there appears to be a trend towards disease in patients at lower genetic risk. Studies from the United Kingdom, Finland, Sweden, Colorado and Australia have demonstrated an increase in the proportion of patients with moderate or low risk HLA genotypes and a corresponding decrease in patients with high risk genotypes in recent cohorts compared with those diagnosed several decades earlier (Furlanos et al. 2008; Gillespie et al. 2004; Hermann et al. 2003; Carlsson et al. 2011; Vehik et al. 2008). In addition, incidence rates have increased most rapidly in the youngest age groups reflecting a shift to an earlier age of diabetes onset.

As T1D results from an interaction between environmental triggers and genetic risk, this provides evidence for a greater influence of environmental or other factors on the pathogenesis of the disease. Cow's milk protein (TRIGR Study Group 2007; Knip et al. 2014), infectious triggers (Craig et al. 2013), ultraviolet exposure (Mohr et al. 2008) and vitamin D (Dong et al. 2013; Hypponen et al. 2001; Stene et al. 2003; Zipitis & Akobeng 2008; Littorin et al. 2006) have been proposed as potential environmental factors influencing disease pathogenesis. Furthermore, rising levels of obesity have been linked to the increasing incidence of T1D by the accelerator hypothesis which suggests that the ensuing insulin resistance promotes beta cell destruction and more rapid disease onset (Wilkin 2009; Wilkin 2011). This study focuses on the role of viruses, vitamin D and adiposity in T1D pathogenesis.

We hypothesised that environmental risk factors and adiposity are associated with the increased proportion of children with T1D who carry low risk HLA genotypes. The aim of the study was to characterise a contemporary incident cohort of young people with T1D and determine if there are differences in adiposity, viruses or vitamin D deficiency between those with low risk HLA genotypes those with high-risk HLA genotypes and younger and older children.

## **Method**

### **Study population**

An incident cohort of children and young people newly diagnosed with T1D aged less than 16 years was recruited between August 2010 and January 2014 at the Children's Hospital at Westmead (CHW) and St George Hospital, Kogarah (SGH) New South Wales. This group formed Cohort B. Informed consent was obtained from all parents/guardians. The study was approved by the Research Ethics Committee of the Sydney Children's Hospitals Network and South Eastern Sydney Local Health District.

### **Biochemical and clinical measurements**

Height and weight were measured as described in Chapter 3 – Methods.

Clinical care was transferred to hospitals geographically closer to home for 22% of recruited patients. Therefore measurements for height and weight at first outpatient clinic were not available for n=43 patients and also contributed to incomplete sample collection at CHW.



## **Samples and testing methods**

Whole blood, serum, throat swabs or stool were collected within two weeks of diagnosis and samples were stored at 4°C until processing. Islet cell autoantibodies were tested by radioimmunoassay including Glutamic Acid Decarboxylase (GAD), insulin (IAA) and islet-antigen-2 (IA2) autoantibodies as described in Chapter 3 - Methods. Positive levels were defined as GAD  $\geq 0.6$ u/mL, IAA  $\geq 53$ u/mL and IA2  $\geq 0.8$ u/mL. Thyroid peroxidase and anti-thyroglobulin autoantibodies as well as thyroid function were measured.

Acid-base status was measured at diagnosis by venous blood gas. Diabetic ketoacidosis was defined as pH < 7.3 or bicarbonate < 15nmol/L, hyperglycaemia and ketosis (Wolfsdorf et al. 2014).

25-hydroxyvitamin D was measured using radioimmunoassay (DiaSorin, Stillwater, MN). Vitamin D deficiency was defined as levels less than 50nmol/L (Holick et al. 2011; Paxton et al. 2013).

C peptide levels were measured by chemiluminescent enzyme assay (IMMULITE 1000(r), Siemens, UK). Normal range was defined as 0.2-0.65nmol/L, limit of detection 0.05nmol/L.

Patients were defined as having T1D on clinical grounds and if at least one islet cell autoantibody was positive and/or required treatment with insulin (Craig et al. 2014)

## HLA typing

Saliva samples for HLA typing were collected using Oragene DNA saliva kits (DNA Genotek Inc, Kanata, Ontario) as described in methods (chapter 3). In brief, for DRB1, exon 2 was amplified by PCR and sequencing performed using the 3730 DNA analyser (Applied Biosystems, Life Technologies, Carlsbad, Ca). DQA1 and DQB1 were tested using a DQA/DQB SSO genotyping kit (One Lambda, Canoga Park, Ca). If further typing was required for DQB1, Exons 2 and 3 were amplified by PCR and sequencing performed using the 3730 DNA analyser.

HLA risk genotypes were defined as follows:

-HLA-DRB1\*04-DQA1\*0301-DQB1\*0302/DRB1\*0301-DQA1\*0501-DQB1\*0201 (DR4-DQ8/DR3-DQ2)

-HLA-DRB1\*04-DQA1\*0301-DQB1\*0302/x (DR4-DQ8/x)

-HLA-DRB1\*0301-DQA1\*0501-DQB1\*0201/y (DR3-DQ2/y)

-z/z

## Virus testing

Plasma was separated from blood collected in EDTA and serum from whole blood. The majority of blood samples were processed immediately; however there was a delay until processing in 9%. Maximum time to processing was 60 hours. Processed samples were stored at  $-80^{\circ}\text{C}$  until testing. From August 2010 until August 2011, throat swabs were stored in viral transport medium prior to testing. After August 2011, throat swab samples were stored in MEM viral culture medium and frozen at  $-80^{\circ}\text{C}$  prior to processing. Stool samples were stored at  $4^{\circ}\text{C}$  for a maximum of 48 hours prior to storage at  $-80^{\circ}\text{C}$ .

Plasma, throat swabs, stool and stool were tested by multiplex PCR for EBV, CMV, HSV, VZV and EV. A subset of plasma and serum samples were tested by EV nested RT-PCR as described in Chapter 3 – Methods.

### **Ethnicity**

Details of country of birth and ethnicity of patients, parents and grandparents were collected by standardised questionnaire or interview. Ethnicity was defined using Australian Bureau of Statistics standardised codes as described in methods (Chapter 3) and grouped as shown in table 3.1.

### **Statistical Analysis**

Descriptive statistics are presented as mean ( $\pm$  SD) for normally distributed continuous variables and median [interquartile range] for skewed variables. Vitamin D was analysed as a categorical variable (deficient vs not deficient) and as a continuous variable. Differences in frequency of categorical variables between patients stratified by HLA risk groups and age (<5 years vs >5 years) were assessed using chi-square tests. The Student t test or analysis of variance were used to assess differences in means between groups.

## **Results**

### **Patient recruitment**

Between August 2010 and January 2014, two hundred and seventy-five patients were diagnosed with T1D at CHW; of these, two hundred and eighteen patients (79%) were recruited to the study. Three further patients were recruited at SGH.

There was no difference in age or sex between recruited and non-recruited patients (n=57)(median age recruited 8.8y [5.5-11.8] vs non-recruited 8.7y [6.1-12.2], p=0.8; recruited 77.5% female vs non-recruited 82.2% female).

The diagnosis was subsequently changed from T1D to T2D (n=5), monogenic diabetes (n = 2) or stress-induced hyperglycaemia (n=1) and these eight patients were excluded from further analysis. Of the remaining 213 patients, 106 (50%) were male with median age 8.8 years.

### **Ethnicity (n=163/213)**

The breakdown of ethnic groups is shown in Figure 4.1. The largest ethnic groups were Australian/New Zealand/European (37%) followed by Asian (15%) and Middle Eastern/North African (10%). A proportion of patients were of mixed ethnicity (23%), most commonly Australian/Southern European (10% of the whole group).

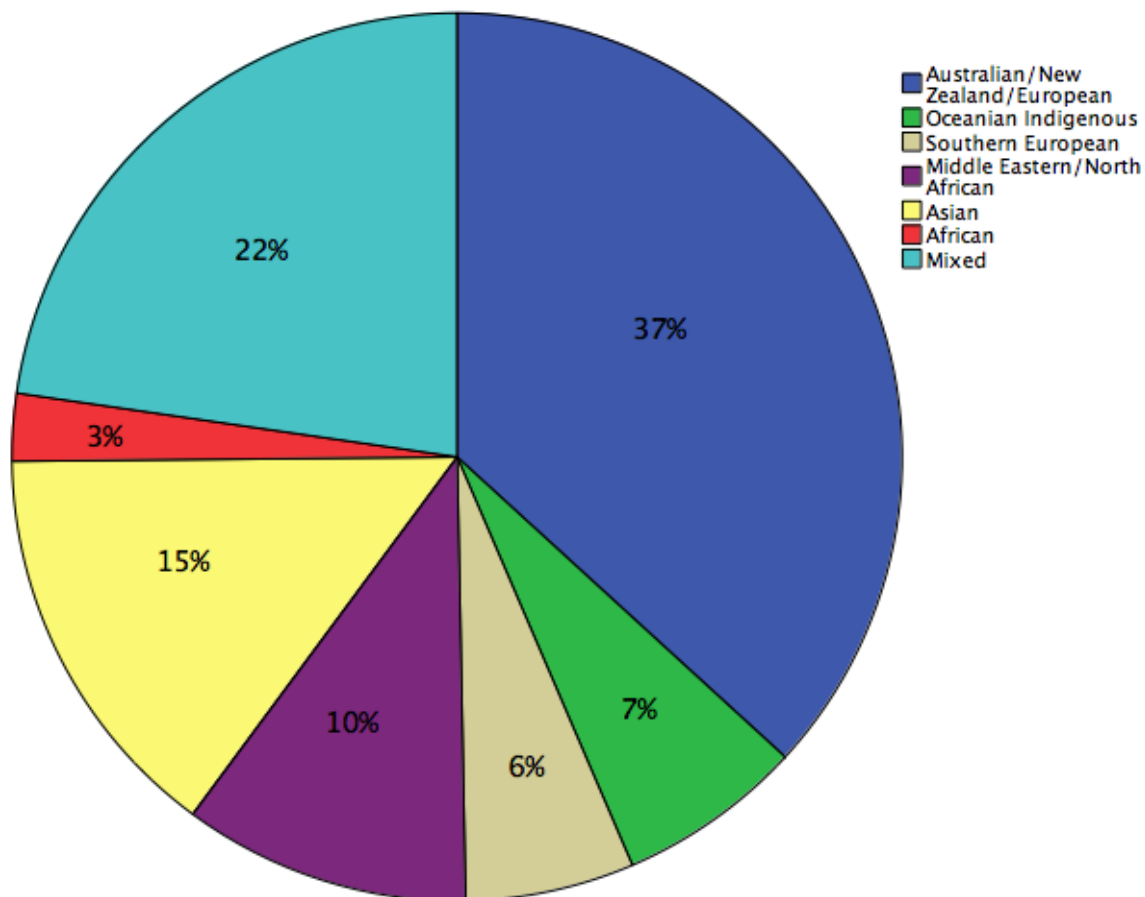


Figure 4.1. Proportion of patients according to ethnic groups

### Diabetes-associated autoantibodies

Autoantibody testing was not performed in 9/213 (4.2%) patients due to insufficient sample at diagnosis. These patients had clinical features of T1D and were commenced on insulin within one day of diagnosis. Of the samples tested, IAA was positive in 137/183 (75%), GAD positive in 199/200 (99.5%) and IA2 positive in 195/196 (99.5%). At least one autoantibody was positive in 204/204 (100%), two autoantibodies positive in 198/204 (97%) and all three autoantibodies positive in 129/203 (64%). Anti-thyroglobulin antibodies were positive in 26/197 (13%) and anti-thyroid peroxidase antibodies positive in 10/193 (5%); together 14% had evidence of

thyroid autoimmunity. Anti-gliadin or anti-transglutaminase antibodies were positive in 12/195 patients (6.2%).

### **HLA typing (n=180/213 tested)**

The highest risk HLA genotype (DRB1\*04-DQA1\*0301-DQB1\*302/DRB1\*0301-DQA1\*0501-DQB1\*0201) was present in 23.3%, DRB1\*04-DQA1\*0301-DQB1\*0302/x in 25.6%, DRB1\*0301-DQA1\*0501-DQB1\*0201/y in 32.2% and z/z genotype in 18.9%. There were no patients with protective HLA alleles.

### **Vitamin D (n=158/213 tested)**

Median vitamin D level was 58.9nmol/L [IQR 38.1-72.9] and 64/158 (40.5%) were deficient (<50nmol/L).

### **Virus testing**

At least one sample for virus testing (plasma/serum, throat swab or stool) was collected from 206/213 (97%) of patients. EV was detected in 14/189 plasma or serum samples, 6/147 throat swabs and 4/27 stool samples. In total, EV was detected in 22/206 (10.7%) of patients. EV was detected in both plasma and throat swab in two patients. Other viruses detected were EBV (1/167 plasma samples, 15/147 throat swabs; total 7.7%), HSV (1/167 plasma, 4/147 throat swabs; total 2.4%), HCMV (1/147 throat swabs; total 0.4%) and VZV (1/147 throat swabs; total 0.4%). EV was the only virus detected in stool. In total, evidence of viral infection was detected in 45/206 patients (22%). One patient had more than one virus isolated (HSV in plasma and EBV from throat swab).

### Comparison between groups at different genetic risk

Characteristics in the group stratified according to HLA risk genotype are shown in Table 4.1. Mean age was lower in patients with the high risk HLA-DR3/4 genotype compared with the z/z genotype (mean age 7.7 years (95% CI 6.5-9.1) vs 9.9 years (95% CI 8.8-11.0), p=0.02). There was no difference in sex, BMI z score, proportion of Australian/European patients or rates of DKA, vitamin D deficiency or overweight/obesity between groups.

Table 4.1 Comparison of characteristics stratified by HLA risk genotype

	<b>DR4-DQ8/ DR3-DQ2 (n=42)</b>	<b>DR4-DQ8/x (n=46)</b>	<b>DR3-DQ2/y (n=58)</b>	<b>z/z (n=34)</b>	<b>p</b>
<b>Male (%)</b>	47.6	54.3	51.7	41.2	0.7
<b>Age (years) *</b>	7.7 (6.5-9.1)	9.1 (7.9-10.2)	8.6 (7.7-9.5)	9.9 (8.8-11.0)	0.02
<b>Australian/ European (%)</b>	37.5	48.6	28.3	25.9	0.2
<b>DKA (%)</b>	38.9	45.5	37.9	25.8	0.4
<b>Vitamin D deficiency (%)</b>	35.7	38.2	39.6	40.0	0.99
<b>BMI z score</b>	0.6 (0.3-0.9)	0.7 (0.5-1.0)	0.8(0.5-1.0)	0.6 (0.3-0.9)	1.0
<b>Overweight or obese (%)</b>	23.3	35.3	40.0	22.6	0.3
<b>Virus infection (%)</b>	26	21	20	21	0.9

\*mean (95% CI)

### Characteristics of younger patients

Characteristics for the group stratified into age <5 years vs >5 years are shown in Table 4.2. There were a greater proportion of males patients aged less than 5 years at diagnosis. Younger patients were also more likely to have 3 positive islet

autoantibodies. A greater proportion carried the high-risk HLA genotype DR3-DQ2/DR4-DQ8, although the latter result did not reach statistical significance. Median vitamin D level was lower, however there was no difference in the proportion with vitamin D deficiency or DKA.

Median height and weight z scores were significantly higher and vitamin D levels lower in the younger patients compared with older patients. However there was no difference in BMI z score or proportion of overweight or obese patients.

Table 4.2 Comparison of patient characteristics stratified by age group

	<b>Total group (n=213)</b>	<b>Age under 5 years (n=45)</b>	<b>Age over 5 years (n=168)</b>	<b>p*</b>
<b>Male (%)</b>	50	64	46	0.03
<b>3 positive antibodies (%)</b>	64	82	59	0.004
<b>DR3-DQ2/DR4-DQ8 (%)</b>	26	41	23	0.05
<b>European/Australian (%)</b>	37	27	40	0.2
<b>Height z score</b>	0.4±1.1	1.4 [-0.2-2.0]	0.3 [-0.3-1.0]	<0.0001
<b>Weight z score</b>	0.7±0.8	1.0 [0.3-2.4]	0.7 [0.2-1.1]	0.002
<b>BMI z score</b>	0.7±0.9	0.9 [0.2-1.6]	0.7 [0.2-1.4]	0.38
<b>Overweight (%)</b>	31.3	33.3	30.8	0.82
<b>Obese (%)</b>	5.1	7.4	4.5	0.62
<b>Vitamin D (nmol/L)</b>	58.9 [38.1-72.9]	34.5 [27.0-60.1]	57.5 [39.3-73.0]	0.04
<b>Vitamin D deficiency (%)</b>	40.5	51.9	38.2	0.20
<b>EV infection (%)</b>	10.7	16.3	8.8	0.16
<b>Other viruses (%)</b>	11.2	4.5	13.0	0.18

\*P values for comparison age under 5 years vs over 5 years

## Discussion

Young people with T1D demonstrate heterogeneous characteristics at presentation, which vary with age at diagnosis and genetic risk. Children aged less than 5 years at



diagnosis were more likely to be male, have a more marked autoimmune response (with 3 positive autoantibodies), greater height and weight z scores and lower vitamin D levels compared with those older than 5 years.

This highlights the important role of environmental triggers at or close to the time of the onset of clinical disease as demonstrated in a number of previous studies (Stene et al. 2010; S. Oikarinen et al. 2011; Yeung et al. 2011; Raab et al. 2014; Franchi et al. 2013; Svoren et al. 2009; The et al. 2013; Pozzilli et al. 2005). Some epidemiological studies have reported a link between vitamin D intake in early life and T1D development (Dong et al. 2013; Zipitis & Akobeng 2008; Hypponen et al. 2001). Exposure to environmental triggers may be responsible for earlier disease onset in genetically susceptible patients and the shift to younger age of onset (Karvonen et al. 1999).

Several earlier studies have demonstrated an inverse relationship between BMI-SDS and age of T1D onset (Clarke et al. 2006; Evertsen et al. 2009; Betts et al. 2005; Dabelea et al. 2006; Kibirige et al. 2003; Islam et al. 2014). We have previously shown that children with T1D are heavier than the general population (Islam 2014; Clarke 2005). These studies are consistent with the accelerator hypothesis, which suggests that excess weight, and the resultant insulin resistance, acts as an accelerator for clinical presentation of T1D. In keeping with our previous reports, we found that younger patients were heavier and taller than their peers compared with older patients. It is surprising that there was no difference in BMI z score or rates of overweight and obesity, however this may reflect our small sample size compared with previous study groups.

In our study, children with high-risk HLA genotypes were younger compared with those with the z/z genotype. However we did not find a difference in hypothesised aetiological agents, such as virus infection, vitamin D deficiency or adiposity when stratified according to HLA risk genotype.

Children with the highest risk genotype present at an earlier age and prospective studies report faster progression once two or more autoantibodies are present (A.-G. Ziegler et al. 2013; Tait et al. 1995; Caillat-Zucman et al. 1992). Previous studies included only Australian/European patients. Our study confirms and extends these previous findings to a more ethnically diverse group in which over 65% of patients were of either mixed or non-Australian/European ethnicity.

An interaction between obesity and HLA risk genotypes was observed in a Swedish study of young people with T1D. Higher rates of obesity were found in patients with lower risk HLA genotypes, but no association between BMI and high-risk HLA genotypes was reported. They proposed that obesity increased the risk of T1D particularly in patients with DQA1\*0501-DQB1\*0201/ DQA1\*0501-DQB1\*0201. We did not find a difference in BMI z score nor overweight/obesity rates when the group was stratified according to HLA risk genotype. The low rate of obesity in our study group (5%) may explain the difference between the two studies.

In conclusion, our study confirms that patients with high risk HLA genotypes are younger at diagnosis than those with low risk alleles. We have demonstrated an association between vitamin D level, adiposity and younger age of onset and these

factors may play a role in disease pathogenesis in this group of patients. However these factors do not differ between patients with different HLA risk.

## Chapter 5

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### **Changing characteristics of children and young people at type 1 diabetes onset over 15 years**

**Aim:** To determine if adiposity, viruses or vitamin D deficiency, are more prevalent at the diagnosis of type 1 diabetes in children diagnosed currently compared with those diagnosed 15 years earlier

## **Introduction**

As the incidence of T1D has increased without a concurrent increase in population genetic susceptibility, it appears that the environment has become more permissive for the development of the disease. Several environmental factors and changing population characteristics have been investigated to account for the increasing incidence. Vitamin D deficiency (Littorin et al. 2006; Baumgartl et al. 1991; Pozzilli et al. 2005) and EV infection (Yeung et al. 2011; Craig et al. 2013) are both associated with T1D at diagnosis. The accelerator hypothesis suggests that rising rates of obesity may be linked with changes in T1D by the induction of insulin resistance and the promotion of beta cell destruction (Verbeeten et al. 2010; Wilkin 2009). An increase in frequency of these factors over time may, in part, contribute to the rising incidence of T1D or earlier presentation in at-risk individuals.

We hypothesised that there has been a decrease in frequency of HLA class II high-risk genotypes and an increase in environmental triggers and adiposity in T1D incident cohorts over time. The aim of the study was to compare the frequency of HLA class II risk genotypes and prevalence of vitamin D deficiency, virus infection and adiposity between two incident cohorts diagnosed fourteen years apart.

## **Methods**

### **Study populations**

Cohort A consisted of two hundred and six patients (male 38.8%), median age 8.2 [IQR 4.4-11.0] years diagnosed with T1D at the Children's Hospital at Westmead between April 1997 and September 1999 as previously reported (Craig, Howard, et al. 2003a).

Cohort B comprised two hundred and thirteen children and young people aged less than 16 years and newly diagnosed with T1D between August 2010 and January 2014 at the Children's Hospital at Westmead or St George Hospital, Sutherland, New South Wales. Details of this cohort are described in chapter 4.

### **Ethnicity**

Details of country of birth and ethnicity of patients, parents and grandparents were collected by standardised questionnaire or interview. Ethnicity was defined using Australian Bureau of Statistics standardised codes and grouped as described in Chapter 3 – Methods.

### **Samples and testing methods**

#### **Biochemical and clinical measurements**

Height was measured using a Harpenden stadiometer and weight measured using digital scales as described in Chapter 3 – Methods.

Samples were collected within two weeks of diagnosis. Measurement of whole blood glucose (mmol/L), venous pH and bicarbonate was performed prior to the administration of insulin. Diabetes associated autoantibody testing was performed as described in Chapter 3 - methods and Chapter 4 - results. Different radioimmunoassays were used for testing IA2 and GAD levels in the two cohorts; the insulin autoantibody assay remained unchanged. In both cohorts, 25- hydroxyvitamin D was measured using radioimmunoassay (DiaSorin, Stillwater, MN). Vitamin D deficiency was defined as levels less than 50nmol/L (Paxton et al. 2013).

## **Virus testing**

In Cohort A, plasma and stool were collected for virus testing using monoplex EV PCR as previously described (Craig, Howard, et al. 2003a). In Cohort B, plasma or serum was tested using EV nested RT-PCR and multiplex PCR (Epstein Barr virus, cytomegalovirus, Herpes simplex virus and EV); throat swabs or stool were tested using multiplex PCR as described in Chapter 3 - methods.

## **HLA genotyping**

For Cohort A, HLA genotyping of HLA-DRB1 and -DQB1 alleles was performed on blood collected at the time of diagnosis as previously described (Craig, Howard, et al. 2003a). In Cohort B, HLA genotyping was performed on saliva samples as described in Chapter 3 - methods

Patients were divided into four groups for the analysis of genetic risk:

- HLA DRB1\*03-DQB1\*02/DRB1\*04-DQB1\*0302 (DR4-DQ2/DR4-DQ8) (having both high risk alleles)
- DRB1\*04-DQB1\*0302/x (DR4-DQ8/x) or DRB1\*03-DQB1\*02/y (DR3-DQ2/y) (either heterozygous or homozygous for risk alleles) or
- z/z (patients without either risk allele).

Ethics approval for the study was granted by the Sydney Children's Hospital Network Research Committee.

## **Statistical Analysis**

Descriptive statistics are presented as mean ( $\pm$  SD) for normally distributed continuous variables and median [interquartile range] for skewed variables. Vitamin D was analysed as a categorical variable (deficient vs not deficient) and as a continuous variable. Differences in frequency of categorical variables between patients stratified by cohort were assessed using chi-square tests. The Student t test or Mann-Whitney U test were used to assess differences in continuous variables between groups. Multivariable linear regression was used to examine the effect of multiple explanatory variables on the difference in vitamin D level between cohorts.

## **Results**

There was no difference in the proportion of patients stratified by age group in the two cohorts as shown in Figure 5.1.



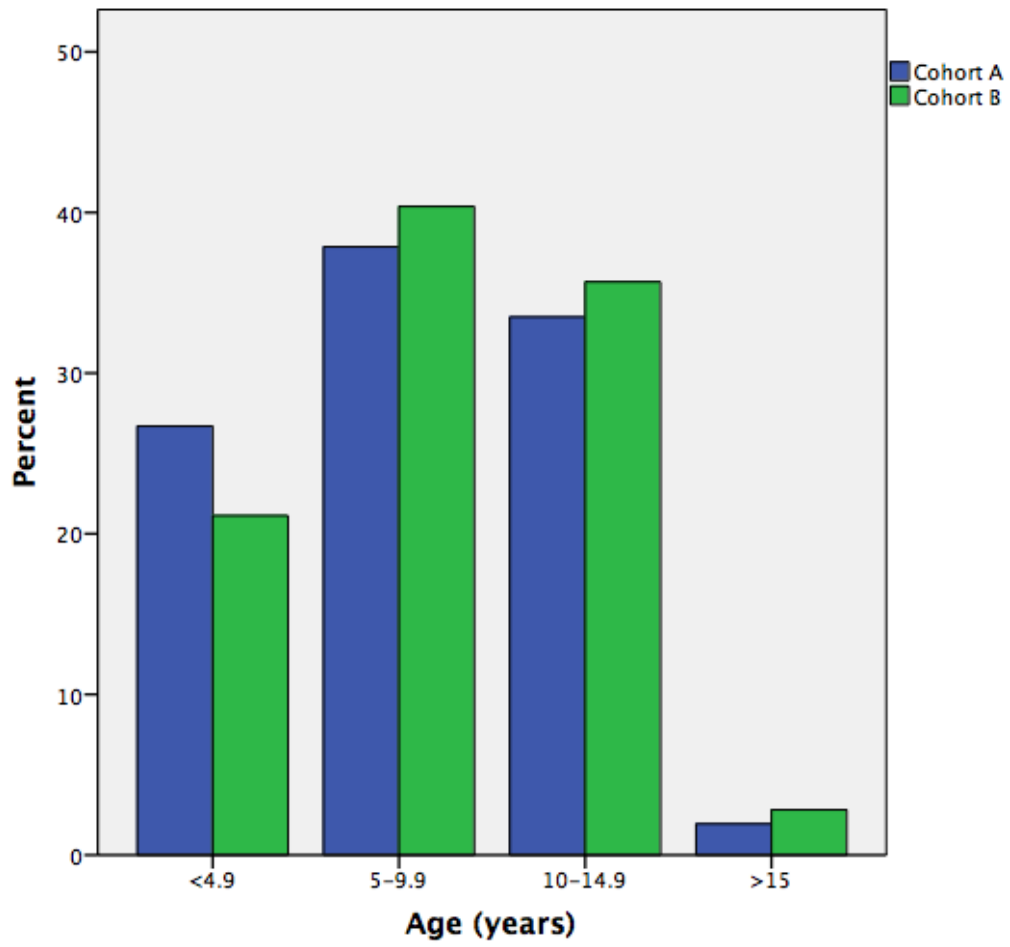


Figure 5.1 Cohorts A and B stratified by age group

Table 5.1 Comparison of Cohorts A and B

	<b>Cohort A (1997-1999)</b>	<b>Cohort B (2010-2014)</b>	<b>p</b>
Recruitment rate (%)	99%	79%	
Male (%)	39	50	0.03
Age (years)	8.1 [4.4-11.0]	8.8 [5.5-11.8]	0.14
Glucose (mmol/L)	24.8 [19.3-34.9]	25.0 [18.9-33.7]	0.40
Bicarbonate (mmol/L)	21.0 [12.0-24.0]	19.6 [9.1-24.0]	0.42
DKA (%)	35	40	0.41
1 islet cell antibody (%)	93	100	<0.001
2 islet cell antibodies (%)	69	97	<0.001
3 islet cell antibodies (%)	29	64	<0.001
Australian/European (%)	57	37	<0.001
Age less than 5y (%)	27	21	0.21
Vitamin D level (nmol/L)	65.0 [53.0-83.0]	58.3 [38.1-72.9]	0.001
Vitamin D deficiency (%)	19.9	40.5	<0.001
BMI z score	0.7±1.0	0.7±0.9	0.89
EV (%)	30	10	<0.001
Overweight or obese (%)	37	31	0.30

Characteristics of the two cohorts are shown in Table 5.1. There were a lower percentage of male patients in Cohort A. There was no significant difference in age, glucose level at diagnosis or proportion of patients with DKA. The proportion of Australian/European patients was significantly lower in the Cohort B. Islet cell autoantibody profiles between the two study groups also differed with a greater proportion of patients in cohort B having one, two or all three islet cell antibodies positive.

Median vitamin D level was lower in Cohort B compared with Cohort A (p=0.001). The difference in vitamin D level remained significant after adjusting for ethnicity and bicarbonate level using multivariable linear regression (p=0.03).

A greater proportion of patients in Cohort B were vitamin D deficient compared with Cohort A (odds ratio 2.7 (95% CI 1.6-4.6). The difference in odds of vitamin D deficiency between cohorts remained significant when adjusted for ethnicity (OR 2.1, 95% CI 1.2=3.7,  $p=0.01$ ).

A smaller proportion of patients in Cohort B had evidence of EV infection (plasma, throat swab or stool) compared with Cohort A (plasma or stool).

There was no difference in BMI z score or proportion of overweight/obese patients between the two cohorts.

HLA-DRB1 and -DQB1 testing was performed in 184/205 (89.3%) of Cohort A. The proportion of patients carrying the highest risk genotype (DR4-DQ8/DR3-DQ2) was higher in Cohort A compared with Cohort B (35% vs 23%,  $p=0.02$ ) as shown in Figure 5.2. The difference in proportion of patients with high-risk HLA genotypes was independent of ethnicity (coded as Australian/European) (OR 1.7, 95% CI 1.0-2.9,  $p=0.04$ ). There was no difference in the proportion of patients with lower risk genotypes in the two cohorts.

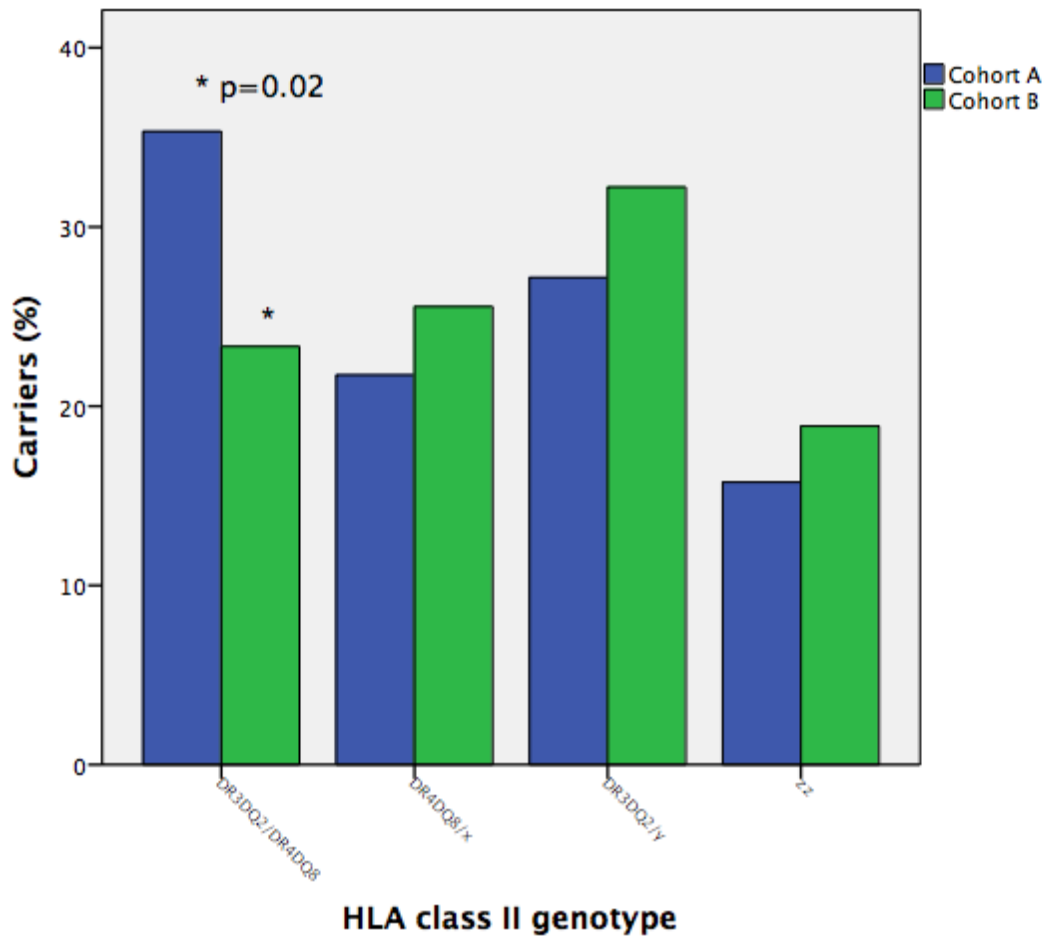


Figure 5.2 Distribution of HLA class II genotypes in Cohorts A and B

### Discussion

This study has described changes in patient characteristics and environmental factors in two cohorts of newly diagnosed T1D patients presenting to a tertiary children’s hospital in Westmead, Sydney, Australia, separated by fifteen years. A smaller proportion of the patients in Cohort B carried the highest risk HLA genotype (HLA-DRB1\*04-DQB1\*0302/DRB1\*03-DQB1\*02) compared with Cohort A, independent of ethnicity or age. In Cohort B, patients were less likely to be Australian/European, a greater proportion were vitamin D deficient and vitamin D levels were lower compared with Cohort A. Differences in vitamin D levels and odds of vitamin D deficiency were

independent of ethnicity and bicarbonate levels. Patients in cohort B were less likely to have an EV infection at diagnosis compared with Cohort A

In keeping with previous studies in Australia and internationally, the proportion of patients with the highest risk genotype is lower in cohorts diagnosed more recently compared with those diagnosed several decades earlier. Published cohorts have been predominantly of European descent, both in Australia and overseas (Kontiainen et al. 1988; Hermann et al. 2003; Mäenpää et al. 1991; Gillespie et al. 2004; Furlanos et al. 2008). Our study shows that this trend, which has been observed since the 1950s, has continued for another decade in a cohort with greater ethnic heterogeneity, adding weight to the greater influence of environmental triggers in disease pathogenesis. Although HLA typing methods differed between the two cohorts, high correlation has been demonstrated between HLA genotyping results obtained from blood and saliva samples (Juhos et al. 2014; Bahlo et al. 2010).

An increase in migration to Australia was seen in the fifteen years between recruitment of the two cohorts (Australian Bureau of Statistics, 2013). In the 1990s, 22% of the population were born overseas compared with 28% in 2013. Almost 30% of migrants are from England/New Zealand, however migrants from China, India and South East Asia are among the fastest growing migrant populations. Different T1D HLA susceptibility haplotypes have been described in these populations (Mehra et al. 2007; G. Kaur et al. 2008; Erlich et al. 2008) and this may account for the lower rates of the high-risk HLA-DR4-DQ8/DR3-DQ2 haplotype in cohort B. Previous studies have also demonstrated that T1D incidence of migrant populations is similar to that of their

adopted country and higher than their country of origin, again highlighting the role of local environmental factors (Raymond et al. 2001; Feltbower et al. 2002).

Vitamin D deficiency has been proposed as an environmental factor influencing the development of T1D. Evidence for the role of maternal vitamin D intake from diet and supplements is conflicting, with some studies reporting protection from islet autoimmunity and T1D (Brekke & Ludvigsson 2007; Fronczak et al. 2003) and others demonstrating no effect (Marjamäki et al. 2010). Vitamin D intake in early life may also influence the development of T1D (T. E. S. 2. S. Group 1999; Hypponen et al. 2001; Stene et al. 2003). Many earlier cross-sectional studies have demonstrated lower vitamin D levels in T1D patients compared with controls (Littorin et al. 2006; Baumgartl et al. 1991; Pozzilli et al. 2005; Borkar et al. 2009). We found a higher prevalence of vitamin D deficiency and lower median vitamin D levels at diagnosis in Cohort B compared with Cohort A. This suggests that vitamin D may play a role in pathogenesis around the time of diagnosis.

Huynh et al reported that vitamin D levels were influenced by bicarbonate levels or presence of DKA (Huynh et al. 2009). The difference in vitamin D deficiency between groups was independent of bicarbonate levels in our cohort.

The ethnic composition of our clinic population has changed over the past fifteen years and now a greater proportion of our patients are of non-Australian/European ethnicity. Ethnicity can determine vitamin D levels and patients with darker skin are at risk of vitamin D deficiency due to reduced ultraviolet absorption (Robinson 2005; Paxton et al. 2013). In a case series from Sydney, patients presenting with severe nutritional

vitamin D deficiency were predominantly recent immigrants or first generation offspring of immigrants from the Indian Subcontinent, Africa or the Middle East (Robinson 2005). The difference in vitamin D levels between groups remained significant after adjustment for ethnicity. Recruitment rate for cohort B was 79% and many potential participants were not approached as English was their second language and an interpreter was not available. The rate of vitamin D deficiency may have been higher in non-recruited patients, thus the 20% difference in vitamin D deficiency between cohorts may be an underestimate of the true difference.

We found a lower rate of EV infection in cohort B compared to Cohort A. The contemporary rate of 10% is consistent with some previous studies (S. Oikarinen et al. 2011), but lower than other cross-sectional studies (Clements et al. 1995; Andréoletti et al. 1997; Yeung et al. 2011). EV71 outbreaks in Australia and South East Asia were reported during the recruitment periods of both cohorts (Zander et al. 2014; Chang et al. 1999; AbuBakar et al. 1999).

EV has been linked with the development of autoimmunity, rather than clinical disease in prospective studies (Lonnrot et al. 2000), therefore it is possible that infection occurred earlier in the pathogenic process in Cohort B compared with Cohort A. Alternatively, a different virus or another agent may have acted as the trigger. As reported in results chapter 4, there was evidence of viral infection in 22% of patients, most commonly EBV or HSV. Delays in sample processing may also have contributed to the low rate of EV isolation in cohort B. Different assays were used to test samples in the two cohorts, however the RT-PCR in cohort B is a more sensitive technique and therefore unlikely to have contributed to the lower virus isolation rate.

A smaller proportion of patients in Cohort B had stool samples available for virus testing (13% vs 53% in cohort A). The difference in sample collection was in part due to changes in patient management practices in the time between recruitment of the two cohorts. Standard management of newly diagnosed patients historically included inpatient admission for up to one week for stabilisation and education, during which time blood and stool samples could be collected. More recently, an ambulatory care model has been adopted for management of appropriately selected patients, including those aged greater than 3 years without evidence of ketoacidosis. With greater community awareness of T1D, the majority of patients present early and in a metabolically stable condition and are therefore suitable for outpatient management. As such, patients in Cohort B were often rapidly discharged to the ambulatory care unit after initial assessment and collection of diagnosis bloods in the emergency department. Patients were often unwilling to undergo repeat venepuncture for study bloods or to provide a stool sample. As EV may be isolated from the stool in the absence of viraemia (Craig, Robertson, et al. 2003b), this may have also contributed to the lower rates of EV detection.

Two studies from our centre have reported a plateau in BMI z scores in newly diagnosed patients between 1976 and 2009 (Clarke et al. 2006; Islam et al. 2014). Our recruitment period immediately followed the study by Islam et al and found no difference in BMI z score or proportion of overweight/obese patients, consistent with the earlier findings. The accelerator hypothesis suggests that increasing rates of adiposity would lead to a shift to younger age of T1D onset (Wilkin 2009; Kibirige et al. 2003). Consistent with this, we did not find differences in the proportion of age groups between cohorts.



Higher rates of antibody positivity were demonstrated in cohort B compared with cohort A. This may have been influenced by the change in IA2 and GAD assays and increased test sensitivity in the later cohort (Perchard et al. 2014) or evidence of increased autoimmunity in patients at lower genetic risk.

In conclusion, we have demonstrated a lower frequency of the highest risk HLA genotype, independent of ethnicity, in a contemporary cohort compared with a cohort diagnosed fifteen years earlier. Vitamin D deficiency is more prevalent currently independent of changes in ethnic composition of our clinic population and bicarbonate levels. Measures of adiposity were unchanged between cohorts. Our findings suggest that a greater frequency of vitamin D deficiency may contribute to the increasing incidence of T1D in our population.

## Chapter 6

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### **Characteristics at type 1 diabetes diagnosis, genetic risk and their role in the development of vascular complications**

**Aim:** To determine if features at the diagnosis of type 1 diabetes (adiposity, viral trigger, vitamin D deficiency, HLA genotype) are associated with increased risk of microvascular complications in an incident cohort after 15 years diabetes duration

## **Introduction**

Microvascular complications of diabetes result in significant long term morbidity. Well-recognised risk factors include poor glycaemic control, longer diabetes duration, systolic blood pressure and dyslipidaemia (Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) Research Group 1995; Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) Research Group 2000b; Gallego et al. 2008). However these risk factors develop over time and microvascular complications may already be present when these factors are recognised. Identifying early predictive factors which increase a patient's risk of complications would allow risk stratification and targeted management.

Vitamin D deficiency has been investigated as a possible risk factor for microvascular complications. It has been linked with nephropathy and retinopathy, although mainly in studies of T2D patients (Inukai et al. 2000; Huang et al. 2012; Aksoy et al. 2000; Patrick et al. 2012; Payne et al. 2012), and it may also play a role in the development of peripheral neuropathy (P. Lee & Chen 2008; Shehab et al. 2011; Soderstrom et al. 2011). The role of vitamin D deficiency in microvascular complications of T1D has been investigated, however results are inconsistent (H. Kaur et al. 2011; Joergensen et al. 2011). Timing and duration of vitamin D deficiency, as well as varying definitions of deficiency may contribute to differences in results.

The risk of microvascular complications is also influenced by genetic predisposition, as demonstrated by familial clustering of complications (Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications

(DCCT/EDIC) Research Group 1997). In addition, the roles of HLA haplotype and other non-HLA single nucleotide polymorphisms (SNP) have been studied, although results are conflicting. The same genetic factors have been shown to be protective or increase susceptibility in different cohorts (Lipner et al. 2013; Pezzolesi et al. 2009; Fagerholm et al. 2012; Möllsten et al. 2011; Syreeni et al. 2011).

Recent analyses have suggested distinct diabetes phenotypes characterised by the pattern and timing of the development of islet autoimmunity or the number of diabetes autoantibodies present at diagnosis. In a prospective cohort of patients genetically at risk of T1D, early seroconversion to autoimmunity increased the risk of diabetes progression within ten years (A.-G. Ziegler et al. 2013). Warncke et al demonstrated that patients stratified according to the number of autoantibodies at diagnosis differed in BMI percentile, weight loss before diagnosis, fasting C-peptide and insulin sensitivity score (Warncke et al. 2013). Similarly, in an Australian study, a small group of patients who were persistently autoantibody negative three years after diagnosis had relatively preserved C-peptide compared with antibody positive patients (Hameed et al. 2010).

These studies demonstrate that groups of patients with T1D differ according to metabolic and immunologic characteristics. Immunologic factors, characterised by differences in islet cell autoantibody profile, may determine the rate of autoimmune beta cell destruction and the likelihood of DKA at diagnosis. Extending this finding, it is conceivable that different diabetes phenotypes may be associated with intrinsic differences that influence features at diabetes diagnosis and risk of microvascular complications.

We hypothesised that the risk of microvascular complications in young adults with T1D is influenced by diabetes phenotype at diagnosis.

## Method

### Study design and recruitment

An incident cohort (Cohort A, n=206) diagnosed between April 1997 and September 1999 at the Children's Hospital at Westmead was followed longitudinally until February 2014. This group was well-characterised at diagnosis, including demographic details, ethnicity, presence of diabetic ketoacidosis, vitamin D status, molecular and serological evidence of EV infection, and HLA class II markers of diabetes risk (Craig, Howard, et al. 2003a). Patients were invited for diabetes complications assessment at minimum two years diabetes duration.

The effect of factors at diagnosis on the development of microvascular complications was examined as shown in Figure 6.1.

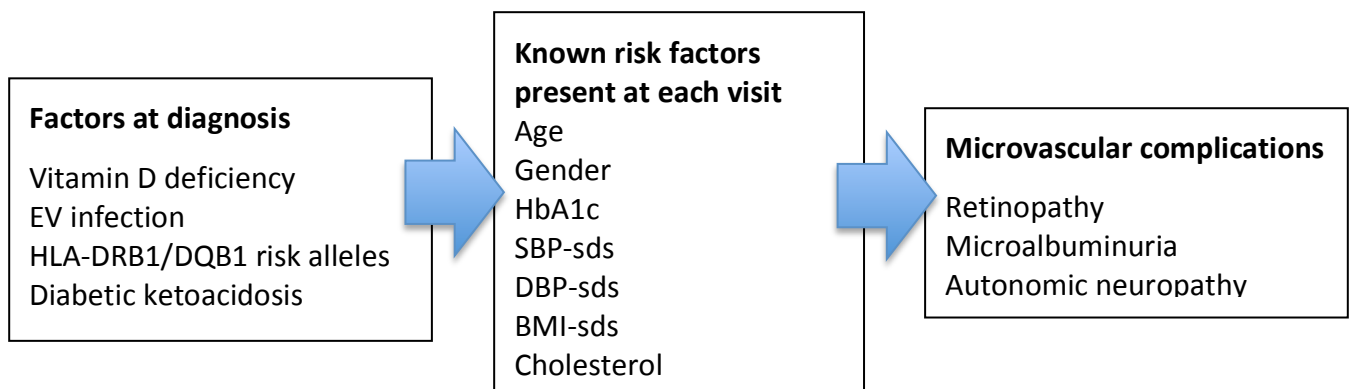


Figure 6.1 Known and proposed factors that may influence the development of microvascular complications

## **Clinical assessment**

Participants underwent a structured history and clinical examination as described in Chapter 3 - Methods. In brief, height and weight were measured to determine BMI (kg/m<sup>2</sup>) and z score for these parameters were calculated using CDC LMS standards. Blood pressure was measured in the seated patient after 5 minutes rest using an aneroid sphygmomanometer and an appropriately-sized cuff.

Retinopathy was assessed by grading of standard fundal photographs taken through a dilated pupil as previously described. All grading was performed by a single ophthalmologist blinded to the participant's diabetes complications status. Retinopathy was defined as the presence of at least one microaneurysm or haemorrhage in one eye (grade 21 or higher).

Pupillary autonomic function was assessed by measuring the pupil size before and three seconds after a light stimulus was delivered, using an infrared pupillometer (Pupilsan, Fairvill Medical Optics). Reflex pupillary amplitude and maximum constriction velocity were also measured. Pupillary abnormalities were defined as < 5th percentile for non-diabetic controls. Small resting pupil diameter, defined as <5.3mm, was also used as an outcome because we have previously shown this cut off predicts subsequent development of retinopathy and microalbuminuria (Maguire et al. 2007).

## **Biochemical measurements**

Albumin was measured using polyclonal radioimmunoassay as previously described. Early elevation of albumin excretion rate (AER) was defined as mean excretion rate >7.5µg/min. Microalbuminuria was defined as excretion rate >20µg/min or spot

albumin-creatinine ratio (ACR)  $\geq 3.5$  mg/mmol for male, ACR  $\geq 4.0$  mg/mmol for female in two out of three timed consecutive overnight urine samples.

HbA1c was measured using high performance liquid chromatography (Diamat Bio-Rad analyser, Bio-Rad, Hercules, CA; non-diabetic range 4-6%).

Diabetic ketoacidosis was defined as pH $<7.3$  or bicarbonate  $<15$ mmol/L on venous blood gas in the presence of ketosis and hyperglycaemia (Wolfsdorf et al. 2014).

### **HLA class II genotyping**

HLA class II typing of the DRB1 and DQB1 alleles was performed at the Tissue Typing Laboratories of the Red Cross Blood Bank, Sydney, and the Royal Melbourne Hospital as previously described (Tait et al. 1995). In brief, DNA was extracted from 5 mL of whole blood. Exon 2 polymorphisms were determined from PCR-amplified DNA using sequence-specific oligonucleotides. Alleles were assigned on the basis of the hybridization patterns.

HLA risk haplotypes were defined as:

-HLA DRB1\*03-DQB1\*02/DRB1\*04-DQB1\*0302 (DR4-DQ2/DR4-DQ8) (having both high risk alleles)

-DRB1\*04-DQB1\*0302/x (DR4-DQ8/x)

-DRB1\*03-DQB1\*02/y (DR3-DQ2/y) (either heterozygous or homozygous for risk alleles)

-z/z (patients without either risk allele).

Vitamin D (25-hydroxyvitamin D) was measured by radioimmunoassay (DiaSorin, Stillwater, MN).

The study was approved by the Ethics committee of the Sydney Children's Hospitals Network.

### **Statistical analysis**

Descriptive statistics were defined as mean (+ SD) for normally distributed variables and median [interquartile range] for skewed variables. The difference in frequencies between groups was assessed using chi-square tests. To compare mean values for continuous variables, the Student t test was used for variables with a normal distribution and the Mann-Whitney U test for variables without a normal distribution.

Outcome variables were retinopathy, elevated albumin excretion rate, microalbuminuria, abnormal pupillary reaction, peripheral nerve abnormality, insulin dose and mean HbA1c-SD.

Kaplan-Meier survival statistics were used to calculate differences in time to onset of each complication. The log rank test was used to compare groups stratified by presence or absence of each factor at diagnosis. Cox proportional hazard regression was used to determine the predictors of individual complications using duration of diabetes as the time variable. Explanatory variables were factors present at diagnosis (vitamin D deficiency as a categorical variable using 25OHD level <50nmol/L to define deficiency), EV infection, diabetic ketoacidosis, HLA class II risk allele/haplotype.



Longitudinal analysis of the risk of microvascular complications according to factors present at diagnosis was performed using generalised estimating equations, which uses all patient visits and accounts for correlations between repeated measures in individual patients. In addition to the explanatory variables above, age, gender, HbA1c (%), systolic and diastolic blood pressure (measured values in mmHg and converted to z-scores), duration, cholesterol level and body mass index (derived from height and weight and converted to z-scores) were also included. Univariate analysis was performed and any significant factors were included in multivariable analyses.

SPSS Statistics v21 (IBM, Armonk, NY) was used to perform the statistical analysis.

## **Results**

The follow up group consisted of one hundred fifty-six patients (40% male, 76% of original cohort). At the final visit, median age of participants was 17.1 years [15.7-18.8], duration 10.2 years [6.3-14.1] and HbA1c 8.8% [7.9-9.8]. Median number of visits was 3 and total number of visits ranged from 1 to 7.

There was no difference between patients who did or did not attend follow up (Table 6.1).

Retinopathy assessment was performed in 154 patients (98.7%) and was present in 35.4%. Urine albumin excretion was tested in 148 patients (94.9%). Microalbuminuria was present in 6 patients (4.3%). Pupillometry was performed in 147 patients (94.2%) and an abnormality was detected in 113 (76.9%) patients. Peripheral nerve testing was

performed in 155 patients (99.3%) and an abnormality was detected in 88 patients (56.8%).

Table 6.1 Characteristics of patients who did or did not attend follow up

	<b>Attended follow up</b>	<b>Did not attend follow up</b>	<b>p</b>
Number	156	50	
Male (%)	40.4	34.0	0.26
Age at diagnosis (y + SD)	7.9 + 3.8	8.1 + 4.6	0.78
EV at diagnosis (%)	28.0	30.8	0.43
Mean vitamin D at diagnosis (nmol/L)	67.8	71.1	0.48
Vitamin D deficiency at diagnosis (%)	21.1	21.6	0.94
HLA-DQB1*02 or *0302	65.0	52.3	0.13
HLA-DRB1*3 or *4	91.3	95.1	0.33
HbA1c at diagnosis	8.4 + 1.8	8.8 + 1.9	0.24

## **Factors influencing the development of microvascular complications**

### **Factors at diagnosis**

There was no association between EV infection, vitamin D deficiency and DKA at diagnosis and the subsequent development of albuminuria or abnormal pupillometry.

### **Retinopathy**

The risk of retinopathy was reduced in males and increased with age, HbA1c, BMI and duration (Table 6.2). These factors remained significant in multivariable GEE analysis, with the exception of HbA1c. EV infection at diagnosis reduced the odds of the development of retinopathy (OR 0.47 (95% CI 0.25-0.88), p=0.02) after adjusting for gender and age.

### **Elevated Albumin Excretion Rate**

In univariate analysis, the risk of elevated AER increased with age, BMI and duration of diabetes, however none of these factors were significant in multivariable analysis (Table 6.3).

### **Microalbuminuria**

The risk of microalbuminuria was increased with increasing BMI (OR 1.1, 95% CI 1.01-1.20, p=0.03) only.

### **Abnormal pupillometry**

The risk of abnormal pupillometry was reduced with age and the DRB1\*04-DQB1\*0302/x haplotype. The risk increased with BMI z score. Both age and DRB1\*04-DQB1\*0302/x remained significant factors in multivariable analysis (Table 6.4).

Systolic and diastolic blood pressures were not risk factors for development for any microvascular complications.

Table 6.2 Risk factors for retinopathy

<b>Retinopathy</b>		
<b>Factor</b>	<b>Univariate Analysis OR (95% CI), p</b>	<b>Multivariable Analysis* OR (95% CI), p</b>
Gender (M relative to F)	0.5 (0.3-0.9), 0.03	0.5 (0.3-1.0), 0.04
Age	1.2 (1.1-1.3), 0.001	1.1 (1.0-1.2), 0.04
HbA1c	1.2 (1.0, 1.3), 0.04	
BMI	1.1, (1.1-1.2), <0.001	1.1 (1.0-1.1), 0.04
Duration	1.2 (1.2-1.3), <0.001	1.2 (1.1-1.3), 0.001
EV RNA	1.1 (0.6-1.8), 0.8	0.47 (0.25-0.88), 0.02
Vitamin D deficiency	0.5 (0.2-1.2), 0.1	
DKA	0.8 (0.4-1.4), 0.4	
DR4-DQ8/DR3-DQ2	2.3 (0.6-2.3), 0.6	
DR4-DQ8/x	1.3 (0.8-2.4), 0.3	
DR3-DQ2/y	0.7 (0.4-1.3), 0.3	
z/z	1.3 (0.7-2.6), 0.4	

\* Adjusted for gender, age, BMI, duration, HbA1c

Table 6.3 Risk factors for elevated AER

<b>Elevated albumin excretion rate</b>	
<b>Factor</b>	<b>Univariate Analysis OR (95% CI), p</b>
Gender (M relative to F)	1.3 (0.8-2.3), 0.3
Age	1.1 (1.1-1.2), 0.001
HbA1c	1.2 (1.0-1.4), 0.06
BMI	1.05 (1.0-1.10), 0.04
Duration	1.1 (1.0-1.2), 0.003
EV RNA	1.1 (0.6-2.0), 0.8
Vitamin D deficiency	1.2 (0.6-2.3), 0.7
DKA	0.6 (0.3-1.1), 0.08
DR4-DQ8/DR3-DQ2	1.0 (0.6-1.8), 0.95
DR4-DQ8/x	1.4 (0.8-2.5), 0.2
DR3-DQ2/y	0.6 (0.3-1.0), 0.07
z/z	1.3 (0.7-2.7), 0.4

Table 6.4 Risk factors for abnormal pupillometry

<b>Abnormal pupillometry</b>		
<b>Factor</b>	<b>Univariate Analysis OR (95% CI), p</b>	<b>Multivariable Analysis* OR (95% CI), p</b>
Gender (M relative to F)	0.8 (0.5-1.3), 0.4	
Age	0.9 (0.86-0.98), 0.007	0.92 (0.86-0.99), 0.02
BMI SDS	1.4 (1.0-1.9), 0.03	
HbA1c	0.9 (0.8-1.1), 0.2	
Duration	0.97 (0.92-1.03), 0.3	
EV RNA	1.1 (0.7-1.9), 0.7	
Vitamin D deficiency	0.8 (0.4-1.6), 0.5	
DKA	1.0 (0.6-1.7), 0.9	
DR4-DQ8/DR3-DQ2	0.8 (0.5-1.4), 0.5	
DR4-DQ8/x	0.5 (0.3-0.8), 0.007	0.5 (0.3-0.8), 0.005
DR3-DQ2/y	1.4 (0.9-2.4), 0.2	
z/z	1.3 (0.7-2.5), 0.4	

\*Adjusted for age, DR4-DQ-8

### **Time to onset of complications**

The presence of EV infection, vitamin D deficiency and DKA at type 1 diabetes diagnosis was not associated with a difference in time to onset of retinopathy, microalbuminuria or pupillary abnormality.

Patients with HLA-DRB1\*03 or DRB1\*04 alleles had a longer time to onset of microalbuminuria compared with those without these alleles (mean time to onset 15.7 years (95% CI 15.2-16.3, p=0.02)) as shown in Figure 6.1. Patients with the HLA-DRB1\*03 allele demonstrated a trend to increased time to onset of retinopathy compared with those without this allele (mean time to onset 13.0 years (95% CI 12.1-13.9) vs 12.0 years (95% CI 10.7-13.2, p=0.05). There was no association between HLA-DR and DQ alleles and the onset of pupillary abnormality.

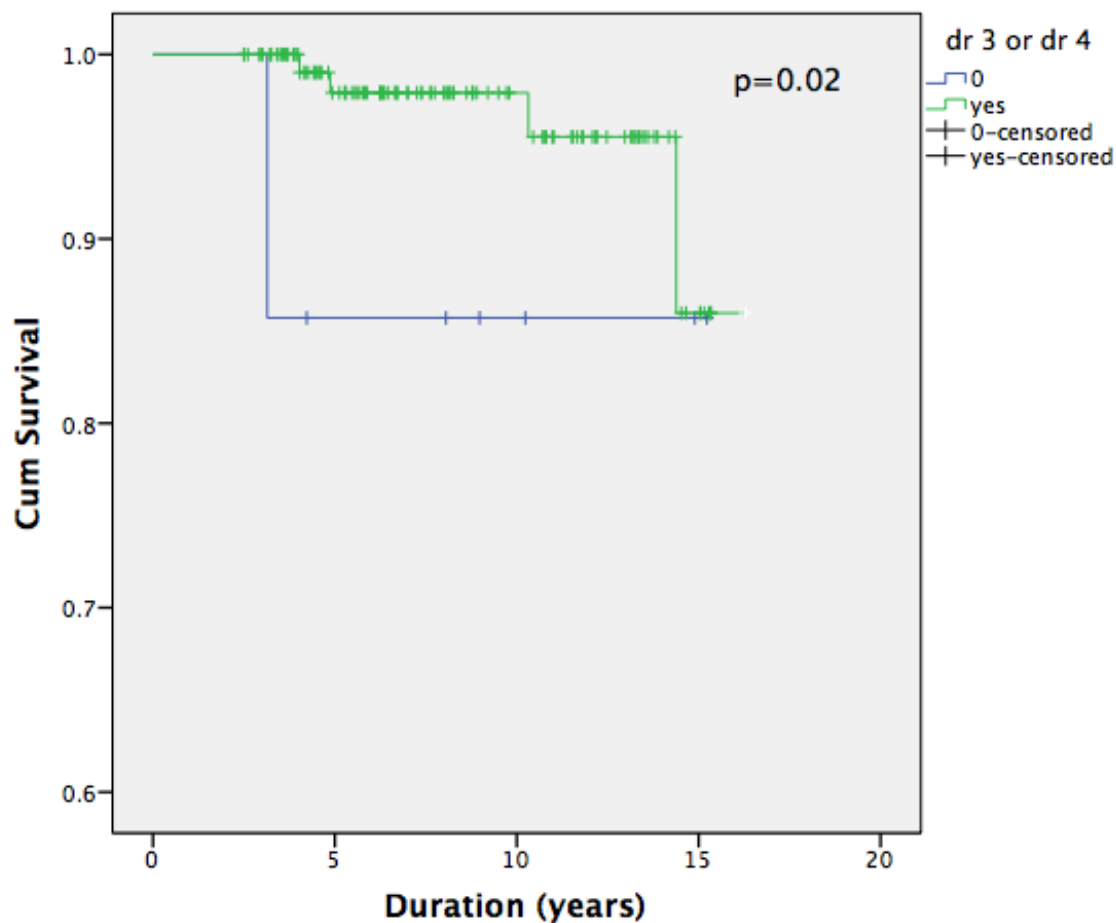


Figure 6.2 Kaplan Meier Survival Curve for onset of microalbuminuria (DR3 or DR4 positive vs DR3 or DR4 negative)

### Discussion

In this longitudinal cohort with median duration of diabetes 10.2 years at assessment, HLA genotype influenced the risk of microvascular complications. The presence of DRB1\*03 or 04 increased the time to onset of microalbuminuria compared with patients without these alleles. The presence of the DRB1\*04-DQB1\*0302/x haplotype was protective against abnormal pupillometry independent of age. HLA genotypes did not influence the risk of retinopathy. When analysing the influence of factors at diagnosis on the development of complications, EV infection reduced the risk of

retinopathy. Vitamin D deficiency and DKA did not contribute to the risk of microvascular complications.

Analysing all patient visits using GEE, the risk of retinopathy was increased with female gender, age, duration, HbA1c and BMI. While many of these are established risk factors, BMI is not typically associated with retinopathy in T1D. We previously found blood pressure was a risk factor, although it was not significant in this study. BMI was associated with an increased risk of elevated AER and microalbuminuria, as we previously observed (Stone et al. 2006). Elevated AER was also increased with age and duration.

The influence of HLA class I and II risk alleles on the development microvascular complications has been studied previously with varying results. Patients with retinopathy were more likely to carry high risk HLA class II alleles or haplotypes in some (Cruickshanks et al. 1992; D. Agardh et al. 1996), but not all studies (E. Agardh et al. 2004; Falck et al. 1997; Jensen et al. 2011; Wong et al:2002). HLA-B62, -Cw4, -DQ4 and -DR1 have also been associated with retinopathy (Mimura et al. 2003; Falck et al. 1997) In contrast, Lipner et al demonstrated that HLA-DRB1\*03 and DQA1\*0501-DQB1\*0201 were protective against retinopathy. As these are in linkage disequilibrium, the individual contribution of each risk allele/haplotype is unclear (Lipner et al. 2013). Our study has demonstrated no association between HLA-DRB1 or DQB1 alleles/haplotypes and retinopathy. The difference in findings may be due to our smaller sample size or related to younger age at diagnosis of T1D. As described in Chapter 4, patients aged less than 5 years at diagnosis were more likely to have the high

risk HLA genotype, more autoantibodies and therefore potentially less likely to have residual insulin secretion.

Fewer studies have investigated the association between HLA-class I and II risk alleles and nephropathy. One study has found no difference in the frequency of HLA-A, B, C, DR, DQA1 or DQB1 alleles in patients with and without nephropathy defined by “Albustix” positive proteinuria. The degree of albuminuria was not quantified (Chowdhury et al. 1999). In the Genetics of Kidneys in Diabetes (GoKinD) study, patients with HLA-DRB1\*04 were 50% less likely to have nephropathy compared with those negative for DRB1\*04 (Cordovado et al. 2007). We did not demonstrate a protective effect of HLA risk haplotypes in univariate analysis, however DRB1\*04 or \*03 increased the time to onset of microalbuminuria in survival analysis. The putative mechanism for this protective effect is unclear, however HLA alleles may influence the inflammatory response associated with development of diabetic nephropathy.

Differences in findings may relate to the definition or ascertainment of the presence of complications (eg. retinopathy vs proliferative retinopathy, questionnaire vs examination, “Albustix” vs quantitative assessment of albuminuria) and duration of diabetes at the time of assessment. Also different populations may have additional genetic factors in linkage disequilibrium with the HLA alleles that may interact to influence risk. The method of analysis may also explain these differences; previous studies have not reported the influence of HLA alleles on time to onset of microvascular complications in a prospective longitudinal cohort as we have here.



In this study, the HLA-DRB1\*04-DQB1\*0302/x genotype reduced the risk of abnormal pupillometry. One previous study has found that the HLA-DR3/4 genotype increased the odds of cardiac autonomic neuropathy by six times compared with other genotypes (Barzilay et al. 1992). There have been no previous studies examining the association between HLA risk alleles and other measures of autonomic neuropathy. Abnormalities in pupillometry precede changes in cardiac autonomic reflexes and this may explain the discrepancy in findings between the two studies (Pena et al. 1995).

EV infection at diagnosis reduced the risk of retinopathy. This is the first report to examine infectious state at diagnosis and complications outcomes longitudinally. In this cohort, patients with an infectious trigger were less likely to carry the high-risk HLA-DRB1\*03-DQB1\*02 haplotype (Craig, Howard, et al. 2003a). This suggests that there may be a subgroup of patients with low genetic risk and unique immunological profile, which is associated with reduced risk of microvascular complications. Alternatively, patients with EV infection at diagnosis may have a less aggressive disease, with residual beta cell function that protects from complications. However, c-peptide was not measured at the time of retinopathy assessment.

Vitamin D deficiency has been linked with microvascular complications in some studies. A cross-sectional study from our centre demonstrated that patients with vitamin D deficiency were twice as likely to have retinopathy than those with normal vitamin D levels (H. Kaur et al. 2011). However a longitudinal study from Denmark found that severe vitamin D deficiency, defined as <10th percentile (15.5nmol/L), measured at median 3 years after diagnosis did not contribute to the risk of microvascular complications including retinopathy (Joergensen et al. 2011).

In our study, vitamin D levels were measured at diagnosis. Therefore it appears that if there is a link between vitamin D and retinopathy, levels at the time of complications assessment may be more significant than levels earlier in the course of diabetes. Alternatively, the presence of retinopathy may influence time spent outdoors or factors associated with the development of retinopathy may influence vitamin D metabolism.

The main strengths of this study are the prospective, longitudinal design following up a cohort well-characterised at diagnosis. Diabetes autoantibodies were measured at diagnosis to differentiate type 1 from type 2 diabetes. Patients underwent objective measures of diabetes complications allowing us to accurately determine time to onset.

The study is limited by the relatively small cohort that may have precluded finding significant relationships between the variables at diagnosis and the development of surrogate markers of complications. In addition, only a small number of patients returned for assessment in the latter part of the study period (22%). As a result, we were unable to assess complication rates at maximum diabetes duration. Given the length of time from diagnosis, a large proportion of patients had moved and contact details were inaccurate. Nevertheless, the statistical models are permissive to variable follow up periods in the cohort and the inclusion of multiple visits per patient using GEE maximises the power of the study. Future studies incorporating data linkage may allow more comprehensive follow up of longitudinal cohorts and incorporate larger study groups.

In conclusion, our study has shown that groups stratified according to genetic risk and infection at diagnosis have different risks of microvascular complications. HLA genotype

may determine immunological features such as susceptibility to infection and inflammatory response, thereby influencing subsequent development of microvascular complications. These findings highlight the varying phenotypes of type 1 diabetes.

## Chapter 7 (Published Work)

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**Vitamin D deficiency is not associated with changes in retinal geometry parameters in young people with type 1 diabetes**

**Aim:** To determine if vitamin D deficiency is associated with adverse retinal vascular geometry parameters

## Clinical Study

# Vitamin D Deficiency Is Not Associated with Changes in Retinal Geometric Parameters in Young People with Type 1 Diabetes

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Changes in retinal geometric parameters predict risk and progression of diabetic retinopathy (DR). We have shown that vitamin D deficiency (VDD) is associated with DR. We hypothesized that VDD mediates changes in retinal geometric parameters. Retinal vascular geometric parameters were assessed using a semiautomated computer program in photographs from young people with type 1 diabetes (T1D) ( $n = 481$ ) and summarized as central retinal arteriolar and venular equivalents (CRAE, CRVE), fractal dimension, length-diameter ratio, branching angle and curvature tortuosity. Parameters were compared between those with and without DR and VDD (25-hydroxyvitamin D concentration  $\leq 50$  nmol/L). Retinal vascular geometric parameters were also compared across quartiles of vitamin D levels. Median CRVE was higher in patients with DR compared with those without (median (IQR) CRVE  $247.3 \mu\text{m}$  (31.3) versus  $238.8 \mu\text{m}$  (23.5),  $P = 0.01$ ). Fractal dimension was marginally greater in patients without VDD (1.49 (0.06) versus 1.47 (0.07)  $P = 0.03$ ). There was no difference in CRAE, CRVE, length-diameter ratio, branching angle, and curvature tortuosity between those with and without VDD and across quartiles of 25OHD. In conclusion, DR is associated with higher CRVE in young people with T1D; however, VDD is not associated with changes in retinal vascular geometric measures, suggesting an earlier role in the time course of DR pathogenesis.

## 1. Introduction

Despite improvements in treatment, diabetic retinopathy (DR) remains a significant complication of type 1 diabetes (T1D). Identification of early treatable predictors of DR, may allow more aggressive management of those at high risk. There is increasing evidence that vitamin D deficiency (VDD) may play a role in pathogenesis of DR. In adults with type 2 diabetes, lower 25-hydroxyvitamin D (25OHD) levels have been associated with proliferative DR [1, 2]. We have shown that VDD is associated with a twofold increased risk of DR (OR 2.12, 95% CI 1.03–4.33) independent of diabetes duration and HbA1c [3].

Changes in retinal microvascular geometry can be used to predict DR prior to the development of microaneurysms or hemorrhage [4, 5]. These retinal measurements include retinal arteriolar and venular calibers, vessel tortuosity, length-diameter ratio, branching angles, and fractal dimension. In the Wisconsin Epidemiology Study of Diabetic Retinopathy (WESDR), an increase in retinal venular caliber of  $10 \mu\text{m}$  was associated with higher 6-year incidence of DR, progression of DR, and incidence of proliferative DR [6]. Longitudinal studies from our group have demonstrated that larger retinal arteriolar caliber [7, 8] and changes in length-diameter ratio and tortuosity [9] predict the development of DR. In cross-sectional studies, increased vessel tortuosity [10] and

increase fractal dimension [11] have also been associated with increased risk of DR independent of known risk factors of microvascular complications.

The mechanisms underlying these changes in retinal geometry are unclear, but may relate to endothelial cell dysfunction, neovascularization or relative tissue hypoxia [9, 10]. Calcitriol, the active form of vitamin D, has been shown to inhibit retinal neovascularization, and reduce endothelial cell viability and function in animal models [12] and adults with type 2 diabetes [13].

We therefore hypothesized that VDD mediates changes in retinal vascular geometry by its effects on endothelial cell function and angiogenesis and examined differences in retinal vascular caliber, fractal dimension, length-diameter ratio, branching angle, and curvature tortuosity in young people with and without DR and VDD. Since the effect of 25OHD may be nonlinear, we investigated whether there was a level at which changes in retinal vascular geometry occur by comparing patients grouped according to quartiles of 25OHD.

## 2. Methods

We assessed 481 young people (52% male) attending the Diabetes Complications Assessment Service at the Children's Hospital at Westmead, Australia, between 2009 and 2010. Patients were defined as Caucasian/non-Caucasian according to the Australian Bureau of Statistics (ABS) standards for classifying the ethnic and cultural composition of the Australian population [14].

DR was assessed by a single ophthalmologist blinded to 25OHD status using digitised seven-field stereoscopic fundal photographs of both eyes and graded according to the Early Treatment Diabetic Retinopathy Study adaptation of the modified Airlie House classification. Retinal vascular geometric measures were performed using a semiautomated computer program (Singapore Vessel I Assessment, SIVA) as previously described [15, 16]. In brief, central retinal arteriolar and venular equivalents (CRAE, CRVE) were calculated from the calibers of the largest 6 arterioles and venules of the left eye, respectively. Previous studies have demonstrated high correlation between left and right eye measures [17]. Additional retinal vascular geometric measurements were also performed including length-diameter ratio (the distance from the midpoint of the first vessel branch to the midpoint of the second branch divided by the diameter of the parent vessel at the first branch), branching angle (the angle between two daughter vessels), curvature tortuosity (a measure of vessel shape and undulation), and fractal dimension (a measure of complexity of a fractal or self-similar structure).

Total 25OHD was measured using the LIAISON analyzer (DiaSorin Inc., Stillwater, MN), and levels were adjusted for season using correction factors derived from multiple linear regression of samples taken from 550 healthy children from Sydney, Australia, as previously described [3]. VDD was defined as 25OHD <50 nmol/L [18]. Quartiles of 25OHD levels were examined to determine if there was a level at which changes in retinal vascular caliber occurred.

TABLE 1: Retinal vascular calibers in patients with and without retinopathy.

	Retinopathy ( <i>n</i> = 43)	No retinopathy ( <i>n</i> = 404)	<i>P</i>
Central retinal arteriolar equivalent ( $\mu\text{m}$ )	168.4 (19.0)	165.9 (16.5)	0.74
Central retinal venular equivalent ( $\mu\text{m}$ )	247.3 (31.3)	238.8 (23.5)	0.01

Data presented as median (IQR).

## 3. Statistics

Descriptive statistics are presented as median and interquartile range (IQR). Differences between categorical variables were assessed using the chi-squared test. Differences between continuous independent variables were assessed using the Mann-Whitney *U* test. Analysis of variance (ANOVA) was used for multiple group comparisons. Analyses were performed using SPSS version 20 (IBM Corporation, Armonk, NY, USA).

## 4. Results

Total 25OHD was measured in 460/481 (96%). Median (IQR) diabetes duration was 6.7 years (4.3–9.4), and median HbA1c was 8.4% (7.6–9.4). Median 25OHD was 70.3 nmol/L (57.5–84.3), and VDD was present in 16%. There was a greater proportion of non-Caucasian patients in the group with VDD compared with those without VDD (64% versus 23%,  $P < 0.001$ ). DR was present in 46/470 (9.8%) for whom DR grading was available.

CRAE and CRVE were measured in 454/481 (94%) of the group. Of these, 25OHD was measured in 434 patients and DR grading available in 447. Retinal vascular caliber measurements were unable to be performed in the remainder due to unavailability or unsuitability of images. Additional retinal geometric parameters were calculated in 98% of photographs (fractal dimension 471/481, complex tortuosity 472/481, branching angle, and length-diameter ration 473/481).

Mean CRVE was greater in those with DR compared to those without (Table 1). There was no difference in mean CRAE or CRVE between those with and without VDD (Table 2).

Total 25OHD was measured in 452/481 (94%) of those with additional retinal vascular geometric measures. Fractal dimension was marginally greater in patients who were vitamin D sufficient compared with those who were VDD (1.49 (0.06) versus 1.47 (0.07)  $P = 0.03$ ). There was no significant difference between branching angle, length-diameter ratio, and tortuosity between those with or without VDD (Table 3) or with or without retinopathy (data not shown).

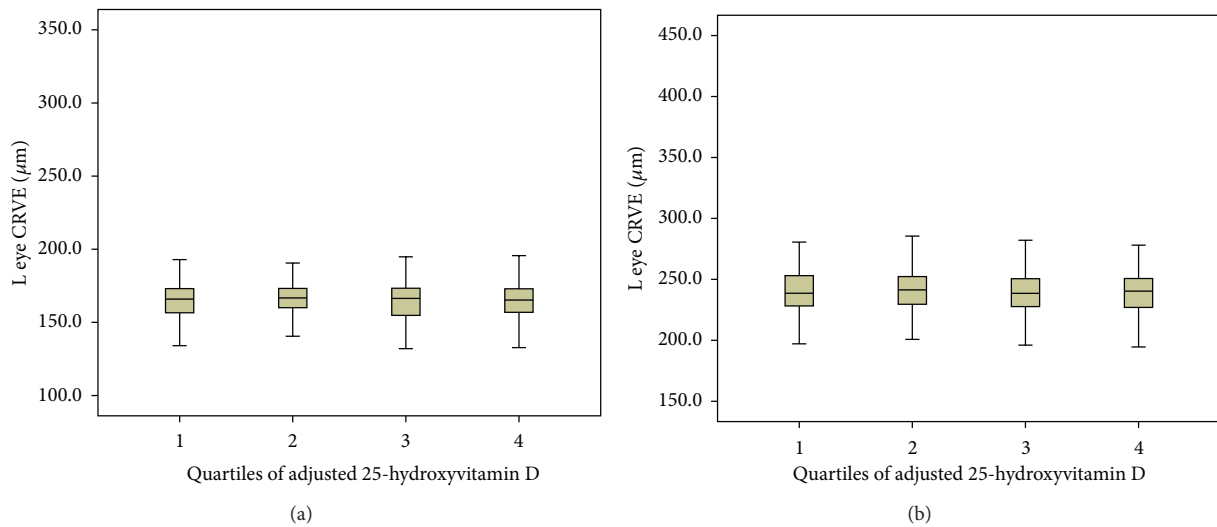


FIGURE 1: Retinal vascular calibers according to quartiles of 25-hydroxyvitamin D (25OHD). (a) Left eye central retinal arterial equivalent. (b) Left eye central retinal venular equivalent. Quartile 1: 25OHD < 57.5 nmol/L, quartile 2: 25OHD 57.6–70.3 nmol/L, quartile 3: 25OHD 70.4–84.3 nmol/L, and quartile 4: >84.3 nmol/L.

TABLE 2: Retinal vascular calibers in patients with and without vitamin D deficiency.

	Vitamin D deficient ( <i>n</i> = 72)	Nonvitamin D deficient ( <i>n</i> = 362)	<i>P</i>
Central retinal arteriolar equivalent ( $\mu\text{m}$ )	165.4 (17.3)	166.3 (15.8)	1.0
Central retinal venular equivalent ( $\mu\text{m}$ )	238.6 (27.1)	240.3 (22.8)	0.9

Data presented as median (IQR).

TABLE 3: Retinal vascular geometric parameters in patients with or without vitamin D deficiency.

	Vitamin D deficient ( <i>n</i> = 73)	Non vitamin D deficient ( <i>n</i> = 379)	<i>P</i>
Tortuosity ( $\times 10^{-5}$ )	10.1 (2.5)	10.2 (2.2)	0.55
Length-diameter ratio	13.9 (5.6)	14.4 (5.7)	0.25
Branching angle (degrees)	81.4 (9.0)	81.8 (8.4)	0.39
Fractal dimension	1.47 (0.07)	1.49 (0.06)	0.03

Data presented as median (IQR).

No difference in retinal vascular caliber (Figure 1), fractal dimension, branching angle, length-diameter ratio, or curvature tortuosity was seen with 25OHD quartiles (data not shown).

## 5. Discussion

In this cross-sectional study of 481 young people with T1D, VDD was associated with a twofold increased risk of DR. However, VDD was not associated with changes in retinal vascular caliber or with differences in branching angle, length-diameter ratio, or tortuosity. In fact, normal 25OHD levels were associated with marginally greater fractal dimension. These results suggest that VDD may exert its effect at a later stage in the pathogenesis of DR or different factors may influence changes in retinal geometric parameters.

Assessment of retinal geometry has emerged as a useful biomarker of DR as well as other microvascular complications, stroke, and coronary vascular disease. Changes in

retinal vascular caliber may underlie some of the pathophysiological changes associated with vascular complications [5]. We have demonstrated that mean CRVE was greater in those with DR compared with those without. In the WESDR, larger caliber venules were associated with increased 6-year incidence of DR, risk of DR progression, and incidence of proliferative DR [6]. It would appear that early increases in venular caliber persist throughout the development and progression of DR.

These findings are in contrast to earlier longitudinal studies from our group and others demonstrating that widening of retinal arterioles is associated with an increased incidence of DR [7, 8, 19]. Our group with DR had higher CRAE (168.4 versus 165.9  $\mu\text{m}$ ) but this was not statistically significant. The current group was older (mean age 14.9 years versus 13.5 years) and had longer diabetes duration (6.7 years versus 6.3 years) than the previous studies. It is possible that these early arteriolar changes, which may reflect early endothelial dysfunction, resolve prior to the development of features of DR.

VDD has been implicated in the development of microvascular and macrovascular diseases [20, 21]. In animal models of retinopathy, calcitriol inhibits angiogenesis and reduces retinal endothelial cell viability, processes which are thought to be involved in the pathogenesis of DR [12]. We found that fractal dimension was marginally decreased in VDD patients, suggesting reduced angiogenesis. We did not measure parathyroid hormone (PTH) levels nor 1,25-hydroxyvitamin D levels in this cohort. It is possible that normal or elevated 1,25-hydroxyvitamin D levels, associated with elevated PTH in the VDD group, resulted in inhibition of angiogenesis. An alternative explanation is that 25OHD acts as a cofactor or promoter at an earlier time point in the development of DR, possibly via increased inflammation.

Although we did not demonstrate an association between VDD (using a level of 50 nmol/L to define deficiency) and retinal vascular caliber changes, we speculated whether there might be a level at which changes in vascular caliber would be seen. This might help to establish the level of 25OHD required to prevent the development of DR. Earlier studies have used varying levels of 25OHD to define deficiency [22–24]. However, we did not find evidence of differences in retinal geometry measures in patients grouped according to 25OHD quartiles.

Changes in other measures of retinal vascular geometry, such as length-diameter ratio, tortuosity, and fractal dimension, are useful in the prediction of incident DR [9] and are also associated with the presence of DR [11, 15]. Identification of reversible factors associated with these early changes could be used for prevention of this significant microvascular complication. However, we did not demonstrate any association between VDD and length-diameter ratio, curvature tortuosity and branching angles.

A possible explanation for these findings may be the decreasing incidence of DR in our clinic [25], reducing the power of the study to detect between group differences. Therefore, although the study population was relatively large, it may not have been adequate to detect a difference in retinal vascular geometric measures between those with and without VDD. Also, duration of diabetes was relatively short compared with earlier adult studies. In addition, retinal vascular caliber measurements were performed on central arterioles and venules. It is possible that vitamin D may cause changes in peripheral retinal vasculature which would not have been detected in this study.

The strength of this study is the use of established methods in a well-characterised cohort.

One limitation is the cross-sectional nature of this study. Further studies could examine longitudinal changes in vascular geometric parameters with presence of VDD at baseline.

## 6. Conclusion

In young people with type 1 diabetes, retinal venular caliber was wider in those with DR compared with those without. However, although VDD was associated with a twofold increased risk in DR, VDD was not associated with a range of retinal vascular geometric measures. This suggests that VDD

plays an earlier role in the pathogenesis of DR, or as a cofactor influencing changes in retinal geometric parameters.

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## Chapter 8

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### **Predictors of mortality in young people with childhood onset type 1 diabetes: role of complications assessment**

**Aim:** To determine if adolescent complications and subclinical autonomic neuropathy predict mortality in young adults with type 1 diabetes 23 years later

## **Introduction**

T1D is associated with increased mortality compared with the general population despite improvements in diabetes management and targets for optimizing glycaemic control. Studies of different populations demonstrate standardised mortality rates ranging from 2.0-7.55, depending on region (Joergensen et al. 2011; Patterson et al. 2007; Skrivarhaug et al. 2005; Dahlquist & Källén 2005; Podar et al. 2000; Edge et al. 1999; Laing et al. 2003; O'Grady et al. 2012).

Autonomic neuropathy is a well-recognised microvascular complication of T1D in both adults and young people. Symptoms of autonomic dysfunction may be subtle and remain unrecognized until late in the disease course. Subclinical autonomic neuropathy may be detected using relatively simple and non-invasive tests, even in young people. In a recent meta-analysis, the prevalence of cardiovascular autonomic dysfunction and abnormal pupillometry was 28% and 42%, respectively (Tang et al. 2013).

Excess mortality may be attributable to autonomic nerve dysfunction. This association was initially reported by Ewing, with mortality rates of 50% in those with abnormal autonomic tests compared with 15% with normal tests (Ewing et al. 1980). Since then numerous studies have confirmed significantly increased mortality associated with autonomic neuropathy (O'Brien et al. 1991; Wheeler et al. 2002; Veglio et al. 2000). In a Danish population study of older adults with T1D, several screening tests of cardiovascular autonomic neuropathy predicted mortality (May & Arildsen 2011). A meta-analysis of mortality and autonomic dysfunction in both type 1 and type 2

diabetes patients reported a relative risk for mortality of 3.45 in diabetes patients with two or more abnormal cardiovascular autonomic tests (Maser et al. 2003).

The majority of previous studies of autonomic dysfunction and mortality have only included adults with relatively short follow up. We aimed to determine if measures of subclinical autonomic dysfunction, microvascular complications or glycaemic control in adolescence predict mortality in young people with T1D 23 years later.

## **Methods**

### **Study Population**

The Children's Hospital at Westmead, Sydney, Australia conducts a Diabetes Complications Assessment Service for patients with T1D with minimum duration 2 years. A longitudinal cohort of four hundred thirteen young people diagnosed with T1D between 1973 and 1993, who attended for their first diabetes complications assessment between 1990 and 1995 were followed until February 2014. Results from two subsets of the cohort have been previously described (Maguire et al. 2007; Donaghue et al. 2003).

### **Diabetes complications assessment**

Participants were assessed using a structured interview and clinical examination as described in Methods (Chapter 3).

Cardiovascular autonomic nerve function was assessed by three tests of heart rate variation (maximum-minimum heart rate during deep breathing (DBT) – abnormal <22, heart rate change during a Valsalva manoeuvre (Valsalva ratio, mean of 3 manoeuvres) and lying to standing heart rate change (30:15 ratio) – abnormal <1.08 (PHR). In

addition, change in blood pressure from lying to standing (PBPT) was assessed with abnormal defined as systolic blood pressure fall of  $>13\text{mmHg}$ . Patients were classified as having subclinical cardiac autonomic neuropathy if an abnormality was detected in at least one test. Cardiac autonomic neuropathy ranges used were derived from 75 control subjects as previously described (Schwingshandl et al. 1993).

Pupillary autonomic function was assessed using an infrared pupillometer (Pupilsan, Fairvill Medical Optics) as previously described (Schwingshandl et al. 1993). Parameters included were pupil diameter before and three seconds after a light stimulus was delivered, reflex pupillary amplitude and maximum constriction velocity. Reference ranges were derived from 122 non-diabetic control subjects (Donaghue et al. 1993; Schwingshandl et al. 1993). Abnormal cardiovascular and pupillary tests were defined as less than the fifth percentile of the reference range.

Retinal screening was performed using photographs taken by a Topcon Fundus camera (TRC 50-VT, Tokyo Optical Co., Tokyo) after dilatation of the pupils with cyclopentolate 1% and phenylephrine 2.5% as described in methods (chapter 3). The photographs were graded by the same ophthalmologist, blinded to the participant's complications status, according to the Early Treatment Diabetic Retinopathy Study (ETDRS) adaptation of the modified Airlie House classification of diabetic retinopathy.

Retinopathy was defined as the presence of at least one micro aneurysm or haemorrhage (grade 21 or higher).

Peripheral nerve function was assessed by hot and cold thermal threshold testing of the left foot using the TSA-II Neurosensory Analyzer (Medoc Ltd). Vibration threshold at the left medial malleolus and left great toe was measured with the VAS-3000 Vibratory Sensory Analyzer (Medoc Ltd). Peripheral nerve abnormalities were defined as < 95% of the normal range in a nondiabetic adolescent control group.

### **Biochemical measurements**

Albumin was measured using polyclonal radioimmunoassay (Pharmacia RIA, Beckman Coulter, Australia) prior to 2000, nephelometric assay using an IMMAGE analyser ( $\text{IMMAGE} = (0.8734 \times \text{radio-immunoassay value}) - 0.501$ ;  $r = 0.99$ ) from 2000 to 2003 and competitive chemiluminescence immunoassay using the IMMULITE analyser (Diagnostic Products, Los Angeles, CA) thereafter. Creatinine was measured by Jaffe reaction, Dimension ARX (Dade Behring, Newark, DE). Early elevation of albumin excretion rate (AER) was defined as mean excretion rate  $> 7.5 \mu\text{g}/\text{min}$ . Microalbuminuria was defined as AER  $> 20 \mu\text{g}/\text{min}$  or spot albumin-creatinine ratio (ACR)  $\geq 3.5 \text{ mg}/\text{mmol}$  for males or ACR  $\geq 4.0 \text{ mg}/\text{mmol}$  for females in two out of three timed consecutive overnight urine samples.

HbA1c was measured using high performance liquid chromatography (Diamat Bio-Rad analyser, Bio-Rad, Hercules, CA; non-diabetic range 4-6%).

### **Assessment of mortality rates**

Mortality data were obtained by linkage with the National Death Index (NDI) (maintained by the Australian Institute of Health and Welfare) in February 2014, including all deaths from date of diabetes onset until the linkage date. Records at that

time were current until mid-December 2013. Secondary ascertainment of mortality rates was performed by crosschecking with records held by Diabetes Australia.

Cause of death was available from the NDI for the majority of patients and coded according to the International Classification of Diseases (ICD)-9 for deaths prior to the end of 1996 and by ICD-10 thereafter. National standardized death rates from the Australia Bureau of Statistics for the relevant age groups were used to calculate the number of expected deaths in the cohort.

The study was approved by the Sydney Children's Hospitals Network Human Research Ethics committee.

### **Statistical Analysis**

Descriptive statistics are reported as frequency (percentage) for categorical variables, mean  $\pm$  standard deviation for normally distributed variables and median (interquartile range) for continuous variables. Differences in proportions were calculated using chi-square tests and differences in median using the Mann-Whitney U test.

Standardised mortality ratio was calculated as observed deaths/expected deaths for age- and sex-matched population over the time period studied and 95% confidence intervals were calculated using a Poisson distribution.

Generalised estimating equations were used to assess the association between the outcome (mortality at data linkage) and potential explanatory variables measured at all subject visits. The predictors considered were retinopathy, elevated albumin excretion

rate, microalbuminuria, cardiovascular nerve abnormality and pupillary abnormality (in either one, two or three parameters) as categorical variables and HbA1c as a continuous variable. Significant predictors in univariate analysis were included in the multivariable model. Results are reported as odds ratios (OR) and 95% CIs.

We calculated that a sample size of 300 would give at least 80% power at 5% significance to detect a two-fold difference in mortality between patients with and without autonomic neuropathy, assuming that 20% of the total cohort had at least one abnormal test of autonomic function(Maser et al. 2003).

Analysis was performed using SPSS v21 (IBM statistics, Armonk, NY).

## **Results**

Baseline characteristics at first visit are documented in Table 8.1. The proportion of patients with a cardiovascular nerve abnormality was higher in the deceased group compared with the group still alive at follow up (50% vs 19.4%,  $p=0.01$ ). There was no significant difference between the two groups in other parameters.



Table 8.1 Characteristics at baseline for whole group and alive vs deceased

	<b>Whole group</b>	<b>Alive</b>	<b>Deceased</b>	<b>p</b>
n	413	399	14	
Male	198 (47.9)	190 (47.6)	8 (57.1)	0.59
Age (years)	14.4 [12.8-16.1]	14.4 [12.9-16.2]	14.7 [13.2-16.7]	0.58
Duration (years)	6.2 [3.6-9.2]	6.2 [3.7-9.27]	5.60 [3.47-9.26]	0.64
HbA1c	8.4 [7.5-9.3]	8.4 [7.5-9.4]	8.19 [7.2-9.6]	0.86
SBP sds	0.2 [-0.2-0.95]	0.2 [-0.2-0.95]	0.19 [-0.3-0.7]	0.47
DBP sds	0.7 [-0.03-1.2]	0.7 [-0.02-1.3]	0.77 [-0.6-1.2]	0.68
Retinopathy	120/323 (37.2)	118/314 (37.6)	2/9 (22.2)	0.49
Elevated AER	84/253(33.2)	79/245 (32.2)	5/8 (62.5)	0.12
Microalbuminuria	16/219 (6.8)	16/227 (7.0)	0 <sup>#</sup>	1.00
Peripheral neuropathy	62/415 (15.0)	59/398 (14.8)	3/14 (21.4)	0.45
Cardiovascular autonomic neuropathy	84/410 (20.5)	77/396 (19.4)	7/14 (50.0)	0.01
Abnormal pupillary response	59/240 (24.6)	56/232 (24.1)	3/8 (37.5)	0.41

Data presented as n (%) or median[IQR]

<sup>#</sup>Urine sample not available for 6 of deceased group at first visit

Diabetes data totaling 11782 person-years of follow up were analyzed. Median duration of follow up was 28.0 years [IQR 25.6-31.5] at February 2014. Mean age at February 2014 was 36.8 ± 2.8 years.

Fourteen patients were deceased (3.4% of cohort, 57% male, median age 28.3yrs [24.8-32.9]. Median duration of diabetes at death was 19.6yrs [13.5-26.0] and median HbA1c

8.2% [7.2-9.6]. Retinopathy was present in 2/9 (22.2%), elevated AER in 5/8 (62.5%) and peripheral neuropathy in 3/14 (21.4%). None of the deceased patients had microalbuminuria at the first visit. Evidence of early autonomic neuropathy was present in a significant proportion - cardiovascular abnormality 50% and pupillary abnormality in 37.5%. All-cause mortality was 1.19/1000 person-years of diabetes.

Acute complications of diabetes were the primary cause of death (COD) for 4 patients (25%). One of these deaths was associated with hypoglycaemia and the remainder were classified as due to diabetes with or without ketoacidosis. The three latter patients were all female and mean HbA1c was  $9.9 \pm 1.7\%$  at the first complications assessment visit. Mean age at death was  $26.3 \pm 8.7$  years and duration  $19.1 \pm 10.7$  years. All three patients had elevated AER.

Chronic kidney disease was the primary COD for one patient (6%). Urine albumin excretion rate was not available for this patient at the first visit. Three patients died in road traffic accidents. Further details, such as whether the deceased was the driver or passenger, were not available, however all three patients were aged over 18 years at the time of death. Malignancy was the cause of death in three patients. These malignancies were chronic myeloid leukaemia, skin and connective tissue malignancies with secondary malignancies in the lung. Intracranial hemorrhage and opioid dependence accounted for two deaths. COD was not available for 1 patient.

Table 8.2 Cause of death

<b>Cause of death</b>	<b>N</b>
<i>Acute complications of diabetes</i>	
Diabetes with DKA	2
Diabetes without complications	1
Hypoglycaemia	1
<i>Chronic complications of diabetes</i>	
Chronic kidney disease	1
<i>Other causes of death</i>	
Malignancy	3
Intracranial haemorrhage	1
Car accident	3
Opioid dependence	1
Unknown	1
<b>Total</b>	<b>14</b>

The overall standardized mortality ratio (SMR) was significantly increased at 2.49 (95% CI 1.4-4.2). The SMR for females was also significantly increased at 3.50 (95% CI 1.3-7.8). The SMR for males was 2.1 (95% CI 0.9-4.0).

In univariate analysis, mortality was predicted by cardiac autonomic neuropathy (OR 1.47 [1.12-1.93], p=0.005 and elevated AER (OR 1.24 [1.02-1.5], p=0.031). Mortality was not predicted by HbA1c, duration or age. Systolic blood pressure SDS reduced mortality (OR 0.89 [95% CI 0.82-0.97], p=0.009).

In multivariable analysis, cardiac autonomic neuropathy, elevated AER and pupillary abnormality were significant predictors of mortality. Systolic blood pressure again reduced mortality.

Table 8.3 Univariate and multivariable analysis of predictors of mortality

<b>Risk factor</b>	<b>Odds ratio (95% CI)</b>	<b>p</b>
<i>Univariate analysis</i>		
Retinopathy	0.91 (0.72, 1.14)	0.40
Cardiovascular abnormality	1.47 (1.12, 1.93)	0.005
Peripheral nerve abnormality	1.15 (0.93, 1.42)	0.20
Pupillary abnormality	1.67 (0.96, 2.90)	0.070
2 or 3 pupillary abnormalities	1.60 (0.72, 3.56)	0.25
Elevated AER	1.24 (1.02, 1.50)	0.031
Microalbuminuria	1.02 (0.65, 1.59)	0.94
HbA1c	1.02 (0.95, 1.09)	0.69
BMI SDS	0.93 (0.82, 1.06)	0.29
Duration	0.99 (0.98, 1.00)	0.20
Age	0.997 (0.99, 1.00)	0.28
Systolic blood pressure SDS	0.89 (0.82, 0.97)	0.009
Diastolic blood pressure SDS	0.97 (0.89, 1.07)	0.59
<i>Multivariable analysis</i>		
Systolic blood pressure SDS	0.46 (0.27, 0.79)	0.005
Elevated AER	4.54 (1.23, 16.80)	0.023
Pupillary abnormality	4.27 (1.20, 15.22)	0.025
Cardiovascular abnormality	4.65 (1.03, 20.98)	0.045

## **Discussion**

This prospective study of mortality rates in a prospective cohort of young people with median diabetes duration of 28 years demonstrates that mortality rates are 2.5 fold higher compared with the age-matched population. In addition, mortality rates for females are 3.5 fold higher. Mortality was predicted by subclinical cardiac autonomic dysfunction and early elevated AER (AER >7.5 µg/min). Systolic blood pressure SDS reduced mortality.

In previous studies, many deaths have been attributed to cardiovascular and renal disease in older age groups (Morrish et al. 2001; Laing et al. 1999; Laing et al. 2003); acute metabolic complications account for a greater proportion in patients aged less than 40 years (Laing et al. 1999). Recent studies report a trend of decreasing mortality rates over time (Harding et al. 2014; Lind et al. 2013), however life expectancy is still reduced by eleven to thirteen years compared with the age-matched population (Livingstone et al. 2015).

Acute and chronic complications of diabetes accounted for approximately one third of deaths. Some of the remaining deaths may be attributable to diabetes. In the case of patients who died in road traffic accidents, it was not possible to determine whether the patient was the driver or passenger and whether the accident could have been related to diabetes. Three patients died of malignancies, which included chronic myeloid leukaemia and skin/connective tissue malignancies. There may be an association between acute leukaemia and T1D (Feltbower et al. 2004; Hemminki et al. 2012) and a large European study reported an increased risk of B cell chronic lymphocytic leukaemia in males with type 2 diabetes (Khan et al. 2008), however no association with chronic myeloid leukemia has been reported. The remaining deaths are unlikely to be directly related to diabetes.

A recent Swedish study has documented an increased risk of unnatural deaths (accident, suicide, homicide or iatrogenic effects) in patients with diabetes compared with the general population. Age less than 40 years at registration was used as a proxy measure for and accounted for only 6% of cases in the study. In this group, the risk ratio for suicide was 6.7 (95% CI 4.27-10.50) and 7.57 (95% CI 2.88-3.68) for accidents when

compared with the general population. This group was also over-represented among cases of poisoning by unspecified drugs or medication. In the subgroup of patients who died by suicide, 30% were poisonings with insulin or oral hypoglycaemic agents (Webb et al. 2014). Earlier studies have also reported an increased risk of suicide attempts, suicidal ideation and violent deaths (Wibell et al. 2001; Dahlquist & Källén 2005; A. Roy et al. 2010). In our cohort we were unable to determine whether deaths were by suicide, however it is possible that this was the case in the deaths due to hypoglycaemia or opioid dependence. Approximately one-fifth of deaths were due to accidents.

Our findings are consistent with the EURODIAB study which demonstrated macroalbuminuria, peripheral and autonomic neuropathy as the most important risk markers for mortality, with the latter associated with 3 times the risk of death. However this population was significantly older at baseline (Soedamah-Muthu et al. 2008). In our study, we documented evidence of increased mortality risk even with early signs of microvascular complications in a young adult cohort.

The association between diabetic nephropathy, cardiovascular disease and mortality has been extensively documented. Orchard et al described a cohort of young adults with cardiac autonomic neuropathy in which increased mortality was related to the presence of nephropathy. This cohort was diagnosed in an earlier time period (1950-1980) and only one test of cardiac autonomic neuropathy was performed (Orchard et al. 1996). Another small study demonstrated an association between autonomic dysfunction, characterized by reduced 24 hour RR interval variability, microalbuminuria and macroalbuminuria, supporting the relationship between these two complications (Mølgaard et al. 1994). These findings represent a reduction in parasympathetic activity

and possible relative increase in sympathetic activity, a pattern which is associated with increased risk of sudden death in ischaemic heart disease (Farrell et al. 1991; Kleiger et al. 1987).

In our study, although early elevation of albumin excretion rate predicted mortality, the presence of microalbuminuria did not. However the rate of microalbuminuria was only 6.8% and therefore the small numbers of patients with this complication is likely to have contributed to this finding.

Two recent studies have documented higher mortality rates with increasing HbA1c (Orchard et al. 2015; Lind et al. 2014). In our study, mortality was not predicted by glycaemic control. This may reflect the younger age of our cohort at the time of follow up.

Autonomic dysfunction may be associated with a prolonged QTc (Bellavere et al. 1988) and increased mortality (Veglio et al. 2000). Autonomic dysfunction and hypoglycaemia has been proposed as a mechanism underlying the “dead in bed” syndrome (Weston et Gill 1999). We were unable to determine whether the two deaths attributed to hypoglycaemia and acute diabetes complication could have been classified as “dead in bed”. However this devastating complication remains a concern for young people with T1D.

An unexpected finding was the reduction of mortality with systolic blood pressure SDS. We have shown a trend of lower systolic blood pressure SDS in our centre over time (Downie et al, 2011). The elevated systolic blood pressure in our cohort may have been

a result of anxiety during examination in patients who may have been more concerned about their diabetes control and overall health.

One limitation of this study is that this is a clinic-based population, raising the possibility of selection bias towards patients who are more compliant with their diabetes management. However in our centre, all patients are routinely recommended for complications testing after diabetes duration of two or five years for post- and pre-pubertal diabetes onset, respectively (Donaghue et al. 2014). In addition, we did not find an association between glycaemic control and risk of mortality. Our mortality rates are comparable with a population-based Swedish study (Dahlquist & Källén 2005).

In conclusion, we have demonstrated that mortality is particularly increased in females with and is associated with increased albumin excretion, subclinical cardiac autonomic dysfunction and abnormal pupillary response. These findings may assist in early-targeted treatment in patients at significant increased risk of mortality. A significant proportion of deaths in the cohort may have been unrelated to diabetes, but reflect similar findings of excess mortality due to accidents and other unnatural causes in other studies.



## Chapter 9 Conclusion and future directions

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This thesis comprises five studies describing the course of T1D from diagnosis, through the development of microvascular changes, to mortality. The aim of the thesis was to explore the heterogeneity of T1D by examining features of four cohorts diagnosed over a period of forty years.

Several groups have reported a lower proportion of high-risk HLA genotypes in contemporary cohorts compared with earlier cohorts (Kontinen et al. 1988; Mäenpää et al. 1991; Hermann et al. 2003; Gillespie et al. 2004; Furlanos et al. 2008) and we have similarly demonstrated a lower proportion of the highest risk T1D HLA genotype in our comparison of two cohorts diagnosed fifteen years apart (Cohorts A and B) (Figure 9.1). Rates of migration to Australia increased in the period between recruitment of the two cohorts and a greater proportion of patients were of non-Australian/European ethnicity in the later cohort. The reduction in proportion of high-risk HLA genotypes was independent of ethnicity. Migrants from Asia and India are among the fastest growing migrant populations in Australia and these ethnic groups have been shown to have unique HLA susceptibility genotypes (Mehra et al. 2007; Erlich et al. 2008). Migration from these countries may have contributed to our findings and demonstrate that the trend to T1D diagnosis in young people at lower genetic risk is evident even in a more ethnically diverse population.

We hypothesised that increasing rates of environmental triggers (vitamin D deficiency, virus infection and adiposity) in children recently diagnosed with T1D contribute to the increasing incidence of T1D in those at lower genetic risk. Increased rates of vitamin D deficiency and lower vitamin D levels were found in Cohort B, however rates of virus infection were lower. There was no difference in rates of overweight or obesity. Cohort

B were more ethnically diverse, with a lower proportion of non-Australian/European children, who may be predisposed to lower vitamin D levels. However the difference in vitamin D deficiency rates was independent of ethnicity. Therefore this supports the hypothesis that lower vitamin D levels may contribute to T1D diagnosis in groups at lower genetic risk.

In the 1990s and preceding decades, the incidence of T1D increased by 3% per year. A plateau in incidence from 2005 has been recently reported in both Finnish and Swedish high-risk populations (Berhan et al. 2011; Harjutsalo et al. 2013). In New South Wales, Australia, there also appeared to be a plateau in incidence from 2005 to 2007, however incidence increased subsequently in 2008 (Tran et al. 2014). More recent incidence rates have not been published to determine if the Scandinavian trend to stabilisation in T1D rates is reflected locally. This could have contributed to our observation that there has not been an increase in virus-triggered T1D in cohort B.

The accelerator hypothesis proposes that weight is a modifying factor resulting in earlier age of T1D diagnoses. Increasing adiposity rates have been reported in T1D cohorts in other countries (Evertsen et al. 2009; Betts et al. 2005; Kordonouri & Hartmann 2005; Kibirige et al. 2003). In our centre, we have demonstrated a stabilisation in adiposity rates (Clarke et al. 2006; Islam et al. 2014) and our findings were consistent with no change in BMI SDS or proportion of overweight or obese children between Cohorts A and B.

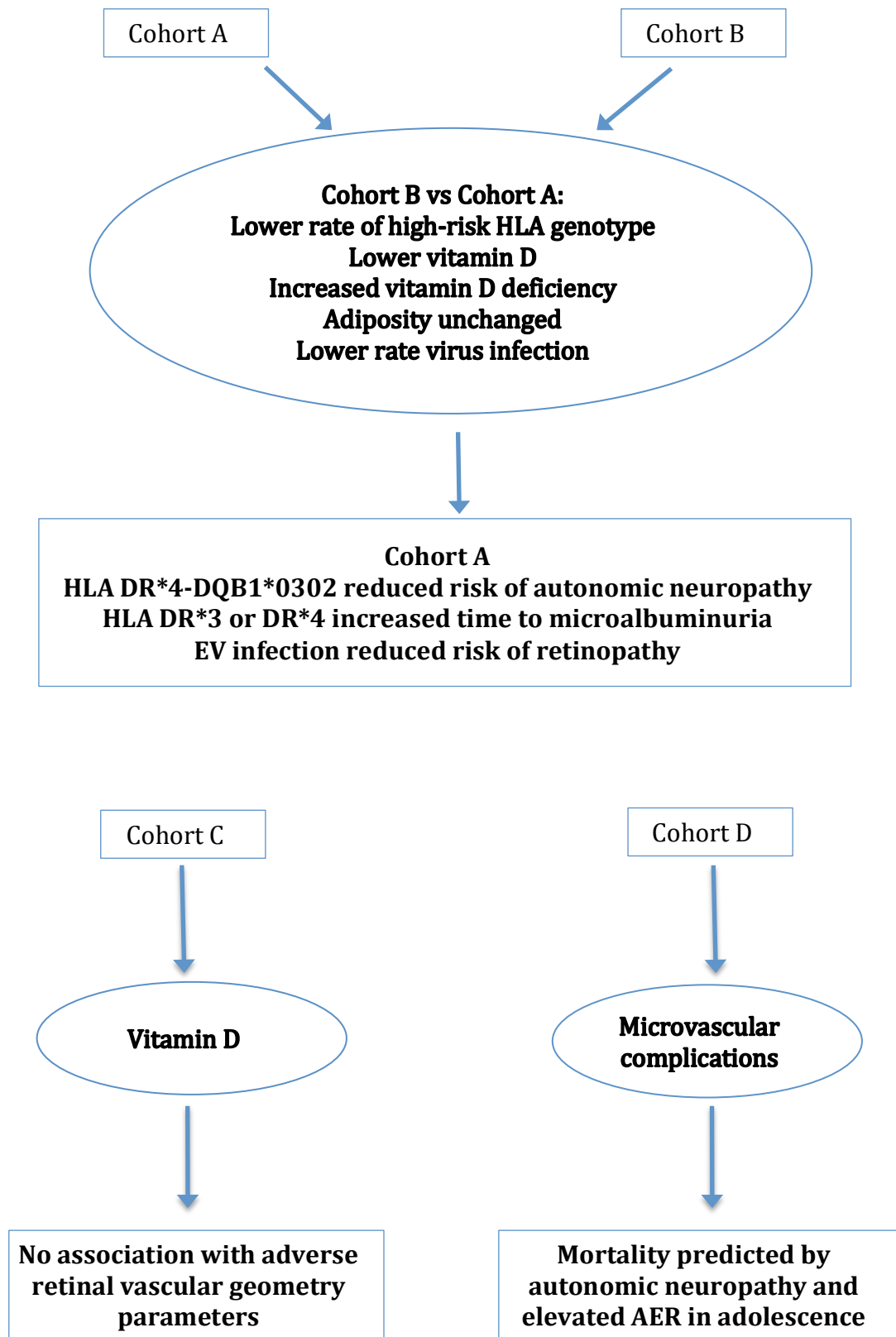


Figure 9.1 Overview of studies and major findings

The participation rate in Cohort B was lower than Cohort A and non-participants were likely to be of non-English speaking background. The effect of socioeconomic status was not examined. These groups may be at greater risk of vitamin D deficiency or virus infection and be less likely to carry the high-risk HLA genotype; therefore our findings may underestimate the true difference in these factors.

In Cohort B, patients aged less than five years at diagnosis were more likely to carry the highest-risk HLA genotype consistent with our finding that patients at higher genetic risk were younger than those at lower risk. Younger patients were more likely to have three positive islet autoantibodies, and had greater height and weight z scores and lower vitamin D levels than older patients. This suggests that the autoimmune process is more aggressive in younger patients and/or those at greater genetic risk. In younger patients, environmental factors such as vitamin D deficiency or rapid weight or height gain from birth may act as modifiers, accelerating the autoimmune process. In analysis of the entire cohort, there were no differences in rates of vitamin D deficiency, virus infection or adiposity when stratified according to HLA risk genotype suggesting that the effect of environmental modifiers is restricted to younger patients. Further investigation of cohorts A and B could include review of growth trajectory from birth, breastfeeding and dietary history or past infection.

Study 3 examined our hypothesis that HLA genotype and features at onset (vitamin D level, virus infection) predict the development of microvascular complications (Figure 9.1). A novel finding was a reduction in risk of abnormal pupillometry with HLA-DRB1\*04-DQB1\*0302 independent of other risk factors. The presence of DR3 or DR4 was protective against the development of microalbuminuria. EV infection at diagnosis

was protective against the development of retinopathy. In this cohort, patients with an infectious trigger (EV infection) were less likely to carry to high risk HLA haplotype - DRB1\*03-DQB1\*02 (Craig, Howard, et al. 2003a). Therefore patients with an infectious trigger or with high-risk HLA alleles may represent a subgroup with lower risk of microvascular complications, supporting the concept of different T1D phenotypes. This study contributes to knowledge of risk stratification and appropriate targeting of risk-modification strategies to patients with specific HLA alleles. The role of additional factors at diagnosis or longitudinally, such as markers of autoimmunity or variations in vitamin D levels, should be investigated in future studies. Further exploration of the association between EV and reduction in retinopathy could include examination of retinal vascular geometry from diagnosis.

Study 3 was limited by the low follow up rates at maximum diabetes duration and there may have been a bias towards participation of young people with greater treatment adherence and lower risk of microvascular complications, thus reducing the chance of identifying additional significant associations. However it may increase the significance of our positive findings. Future studies using data linkage across regions would allow examination of larger groups and improve long-term follow up rates.

In Cohort C, vitamin D deficiency was shown to double the odds of retinopathy (H. Kaur et al. 2011). Although vitamin D deficiency has been associated with microvascular complications of T2D, fewer studies have investigated the role of vitamin D in microvascular disease in T1D (Figure 9.1). Changes in retinal vascular geometry are associated with risk factors for microvascular complications, and can predict risk and progression of retinopathy and nephropathy. We hypothesised that vitamin D

deficiency was associated with adverse changes in retinal vasculature. We found that retinopathy was associated with increased venular width (measured as central retinal venular equivalent). Fractal dimension was marginally greater in those without vitamin D deficiency, however no other changes in retinal vascular geometry were associated with vitamin D status. This suggests that the mechanism underlying the association between vitamin D deficiency and retinopathy is not reflected in adverse changes in retinal vascular geometry. In the longitudinal study of Cohort A, vitamin D at T1D diagnosis was not associated with retinopathy development. Future studies to measure vitamin D at time points between diagnosis and onset of retinopathy may help to elucidate the mechanism underlying the demonstrated association.

It is well documented that mortality rates are increased in patients with T1D, predominantly due to increased risk of cardiovascular disease and microvascular complications. Of the latter, early studies have demonstrated significant associations between autonomic neuropathy, nephropathy and increased risk of death. We hypothesised that complications during adolescence, including subclinical changes in autonomic reflexes predict mortality in young people with T1D followed up for 23 years. We found that mortality was particularly increased in females and was also associated with increased albumin excretion, subclinical cardiac autonomic dysfunction and pupillary abnormality (Figure 9.1). Examination for microvascular complications during adolescence, together with identification of HLA genotype, may improve risk stratification and institution of early, targeted treatment of patients to improve mortality. There is a paucity of studies examining the role of HLA risk genotype and mortality. HLA risk genotype was not available for this cohort, however ongoing

longitudinal study of Cohorts A and B and future linkage with the National Death Index will allow investigation of long term outcomes for these well-characterised groups.

Future studies of Cohorts A and B could include further investigation of the role of dietary and environmental factors in early life and longitudinal examination, including retinal vasculature, prior to the onset of microvascular complications. Data linkage across regions may allow examination of larger cohorts and more comprehensive testing.

In conclusion, this thesis has described characteristics of four T1D cohorts and examined changes in genetic and environmental triggers over time. The findings support the concept of different phenotypes within T1D characterised by inherent genetic risk, metabolic features, environmental triggers and their interaction to influence the development of autoimmunity and age at T1D diagnosis. Further longitudinal studies of incident and birth cohorts to correlate genetic risk, growth trajectory, evidence of autoimmunity, infection risk and frequency, and long-term outcomes including mortality, are needed to clarify these different phenotypes.



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