Title	Maturity onset diabetes of the young: novel insights into the diagnosis, optimal treatment and clinical progression of the condition using biomarkers and new technology.
Authors(s)	Bacon, Siobhan
Publication date	2016
Publisher	University College Dublin. School of Medicine
Link to online version	http://dissertations.umi.com/ucd:10127
Item record/more information	http://hdl.handle.net/10197/8614

Maturity Onset Diabetes of the Young: Novel Insights into the diagnosis, optimal treatment and clinical progression of the condition using biomarkers and new technology.

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A thesis submitted for the degree of Doctor of Philosophy

2013-2016

Maturity Onset Diabetes of the Young: Novel Insights into the diagnosis, optimal treatment and clinical progression of the condition using biomarkers and new technology.

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This thesis is submitted to University College Dublin in fulfilment of the requirement for the degree of Doctor of Philosophy.

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May, 2016

Statement of authentication

I hereby certify that the submitted work is my own work, was completed while registered as a candidate for the degree stated on the title page and I have not obtained a degree elsewhere on the basis of the research presented in the submitted work.

Acknowledgements

Firstly, I want to acknowledge my clinical supervisor, Dr. Maria Byrne whose passion and dedication to the care of the patient with genetic forms of diabetes is undeniable. A sincere thanks for the advice, encouragement and endless cups of coffee over the years. To Prof. Prehn, my academic supervisor, many thanks for your patience and wisdom over the duration of this thesis. You have been a driving force in the completion of my research and it has been greatly appreciated.

I have been fortunate to work with some of the best diabetes nurses this country has to offer both in the research and clinical arena. In particular, I wish to mention the research nurses; Marie Burke, Ailbhe McCarthy, Blathnid Connolly and last but not least Eilish Donnelly. I would like to acknowledge their support and encouragement. The tea-room in the diabetes day centre of the Mater Hospital has been a great source of comfort eating and much banter.

I want to thank all the patients who have participated in this study who have been so generous with their time. In particular I would like to recognise the MODY cohort, over the years I have learned a great deal from them and it has been a fantastic experience.

Finally, I want to thank my friends and family who have put up with me over these, at times trying years. This thesis is dedicated to my husband, Paul and my little boy, Finn who made an appearance in the middle of this work. Paul, thanks for being you, the love of my life and being there whenever I needed a hug, a cry or a laugh. Just to mention, this time last year I added the best title to my name; that of being a “Mammy”.

Abstract

Our improved understanding of the pathophysiology of diabetes has resulted in an increased complexity in diagnosing the disorder. Maturity Onset Diabetes of the Young (MODY) is an autosomal dominant disorder which represents 2-3% of all cases of diabetes. Traditionally, MODY was associated with a lean phenotype, a lack of pancreatic auto-antibodies and absent features of the metabolic syndrome. However, it is now appreciated that clinical features both between and within MODY pedigrees are heterogeneous. The presence of novel spontaneous mutations further complicates the diagnosis. The description of MODY as a clinical entity is a relatively new phenomenon; as a result the natural progression of the disorder remains ambiguous. In this candidature, I study aspects of the natural progression and clinical management of MODY from the fetal state through the neonatal period into adolescence and finally look at the development of micro and macrovascular complications including atherosclerotic burden in this cohort. The challenge of differentiating MODY from the more common diabetes forms has resulted in much research activity seeking a biomarker which can accurately diagnose MODY. miRNAs offer an exciting possibility in biomarker development. In this thesis I also present the novel findings of urinary miRNAs in MODY mutation carriers. The overarching aim of this thesis is to improve our understanding and the clinical management of this unique population.

Publications:

1. **Bacon. S**, Kyithar. MP, Condron. EM, Vizzard.N , Burke.M , Byrne. MM *"Prolonged episodes of hypoglycaemia in HNF4A-MODY mutation carriers with IGT. Evidence of persistent hyperinsulinism into early adulthood"*. Acta Diabetologia 2016 Dec :53 (6):965-972. PMID 27552834.

2. **Bacon S**, M.P. Kyithar, S.R. Rizvi, E. Donnelly , A. McCarthy , M. Burke, K. Colclough , S. Ellard, M.M. Byrne. *"Successful maintenance on sulphonylurea therapy and low diabetes complication rates in a HNF1A-MODY cohort"*. Diabet Med. 2015 Oct 19. PMID 26479152

3. **Bacon. S**, Schmid. J, McCarthy. A, Edwards. J, Fleming. A, Kinsley,BT Firth. RG, Byrne. B, Gavin. C, Byrne, MM. *"The clinical management of hyperglycaemia in pregnancy complicated by maturity-onset diabetes of the young"*.Am J Obstet Gynecol. 2015 Aug; 213 (2):236. PMID 25935773

4. **Bacon S**, Engelbrecht B, Schmid J, Pfeiffer S, Gallagher R, McCarthy A, Burke M, Concannon C, Prehn JHM, Byrne MM. *"MicroRNA-224 is Readily Detectable in Urine of Individuals with Diabetes Mellitus and is a Potential Indicator of Beta-Cell Demise"*.Genes (Basel). 2015 Jun 23;6(2):399-416. PMID 26110317.

5. **Bacon S**, Kyithar MP, Schmid J, Costa Pozza A, Handberg A, Byrne MM. *"Circulating CD36 is reduced in HNF1A-MODY carriers"*. PLoS One. 2013 Sep 12;8(9). PMID: 24069322

6. Bonner C., Nyhan K.C., **Bacon S.,** Kyithar M.P., Schmid J., Concannon C.G., Bray I.M., Stallings R.L., Prehn J.H., Byrne M.M. *"Identification of circulating microRNAs in HNF1A-MODY carriers"*. Diabetologia. 2013;56:1743–1751. PMID 23674172

7. Kyithar MP, Bonner C, **Bacon S**, Kilbride SM, Schmid J, Graf R, Prehn JH, Byrne MM. *Effects of hepatocyte nuclear factor-1A and -4A on pancreatic stone protein/regenerating protein and C-reactive protein gene expression: implications for maturity-onset diabetes of the young*.J Transl Med. 2013 Jun 26;11:156. PMID: 23803251

8. **Bacon S**, Kyithar MP, Schmid J, Rizvi SR, Bonner C, Graf R, Prehn JH, Byrne MM. *Serum levels of pancreatic stone protein (PSP)/reg1A as an indicator of beta-cell apoptosis suggest an increased apoptosis rate in hepatocyte nuclear factor 1 alpha (HNF1A-MODY) carriers from the third decade of life onward.*BMC Endocr Disord. 2012 Jul 18;12:13. PMID:22808921

9. Kyithar MP, **Bacon S,** Pannu KK, Rizvi SR, Colclough K, Ellard S, Byrne MM.*Identification of HNF1A-MODY and HNF4A-MODY in Irish families: phenotypic characteristics and therapeutic implications.*Diabetes Metab. 2011 Dec;37(6):512-9. PMID:21683639

10. Bonner C*, **Bacon S*,** Concannon CG, Rizvi SR, Baquié M, Farrelly AM, Kilbride SM, Dussmann H, Ward MW, Boulanger CM, Wollheim CB, Graf R, Byrne MM, Prehn JH. *INS-1 cells undergoing caspase-dependent apoptosis enhance the regenerative capacity of neighboring cells.*Diabetes. 2010 Nov;59(11):2799-808. PMID:20682686. *Shared co-authorship

11. **Bacon S**., Kyithar M.P , Prehn JHM, and Byrne MM et al. *Serum levels of Pancreatic Stone Protein (PSP)/reg1a as an indicator of beta-cell apoptosis suggest an increased apoptosis rate in Hepatocyte Nuclear Factor 1 alpha (HNF1a-MODY) carriers from the third decade of life onward.* BMC Endocrine Disorders. 2012. 12 (13) PMID:22808921

Published Abstracts (First Author only) produced during thesis

1. ***The clinical management of hyperglycemia in pregnancy complicated by maturity-onset diabetes of the young***
   Bacon S, Byrne MM et al .*Diabetologia,* September 2015, Volume 58, Supplement 1

2. ***MicroRNA-224 is readily detectable in urine of individuals with Diabetes Mellitus and is a potential indicator of beta-cell demise***
   Bacon S, Byrne MM et al. *Diabetologia,* September 2015, Volume 58, Supplement 1

3. ***Diabetes-Associated MiRNA are readily detectable in urine of Individuals with Type 1 Diabetes Mellitus, Type 2 Diabetes Mellitus and HNF1A-MODY.***
   Bacon S, Byrne, MM et al. *Endocrine Reviews*, 2015, Vol 36, Issue 2

Oral presentations both national and international

(First Author Only)

1. ***The clinical management of hyperglycemia in pregnancy complicated by maturity-onset diabetes of the young***
   Bacon S, Byrne, MM et al. Oral Scientific Presentation, NASOM, Banff, Canada, 2015

2. ***The clinical management of hyperglycemia in pregnancy complicated by maturity-onset diabetes of the young***
   Bacon, S, Byrne, MM et al. ENDO, 2015, San Diego.

3. ***Less Favourable Outcomes in Women with Type 2 Diabetes Mellitus than Type 1 Diabetes Mellitus. Findings from a large Multicentre Cohort.***
   Bacon, S, Kinsley, BT et al.
   Oral Scientific Presentation, NASOM, Banff, Canada, 2015

4. ***The potential use of Serum Pancreatic Stone Protein (PSP/reg) as a Biomarker of beta-cell regeneration in humans with diabetes***
   Bacon, S, Kyithar MP, Byrne, MM et al. Irish Endocrine Society, 2009

5. ***The Relevance of one abnormal value***
   Bacon S, Kinsley BT et al. Irish Endocrine Society, 2009

Poster Presentations (International only)

1. ***Diabetes-Associated miRNA are readily detectable in urine of Individuals with Type 1 Diabetes Mellitus, Type 2 Diabetes Mellitus and HNF1A-MODY.***

   Bacon, S, Byrne, MM et al. Late-Breaking Abstracts, ENDO, 2015, San Diego.

2. ***miRNA 224 is readily detectable in urine of individuals with diabetes mellitus and a potential indicator of beta-cell demise.***

   Bacon S, Engelbrecht B, Schmid J, Pfeiffer S, Concannon CG, Mc Carthy A, Burke M, Prehn JHM, and Byrne MM. EASD, Stockholm 2015.

3. ***The clinical management of hyperglycemia in pregnancy complicated by maturity-onset diabetes of the young***

   Bacon S, Byrne, MM et al. EASD, Stockholm 2015.

4. **Serum levels of Pancreatic Stone Protein/reg 1A in HNF1A-MODY may indicate increased beta-cell destruction during disease progression**

   Bacon, S, Kyithar MP, Byrne, MM et al. ADA Scientific Sessions 2011

5. **The potential use of Serum Pancreatic Stone Protein (PSP/reg) as a Biomarker of beta-cell regeneration in humans with diabetes**

   Bacon, S, Kyithar MP, Byrne, MM et al. 69th ADA Scientific Sessions, 2009

Awards & Grants

- Health Professional Fellowship Research Award 2013 for 178,000 euro from the Health Research Board of Ireland.

- Peter Garner Young Investigator award, North American Society of Obstetric Medicine, Canada 2015

- Awarded the MSD Research Bursary fellowship 2012-2013 for 45,000 euro.

- Irish Endocrine Society Eli Lilly Fellowship 2014 for 20,000 euro.

- Supervisor Irish Endocrine Student Scholarship 2014 for 2,000 euro.

- Eli Lilly Travel Fellowship 2015 for 2,000 euro.

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Introduction

1 Chapter 1: Introduction

1.1 Context:

It is now well recognised that Diabetes Mellitus is a heterogeneous disorder. The term "diabetes" can no longer be simplified into type 1 diabetes mellitus (T1DM) or type 2 diabetes mellitus (T2DM), but rather encompasses a broad spectrum including the monogenic forms of diabetes. The prevalence of diabetes in Ireland is approximately 9.6% (Institute of Public Health), with 15,000 new individuals being diagnosed per annum.

The principal pathology in all forms of diabetes is insufficient insulin supply to meet glucose demand. Insulin is the principal hormone involved in the maintenance of glucose levels within the physiological range of 4-8 mmol. The ß-cells of the islets of Langerhans of the pancreas are responsible for the secretion of insulin. T1DM results from an absolute deficiency in insulin production and secretion. Individuals with T1DM typically have a lean phenotype, are ketotic when hyperglycaemic and are auto-antibody positive (Glutamic Acid Decarboxylase (GAD)/ Islet Cell Antibody (ICA)). A relative insulin deficiency in addition to insulin resistance are key factors in the development of T2DM (Efendic, Luft et al. 1984). Typically, insulin resistance and consequential T2DM are associated with an overweight or obese phenotype. However, in approximately 20% of cases with T2DM a lean phenotype is observed (Nguyen, Nguyen et al. 2011). The acronym MODY stands for Maturity Onset Diabetes of the Young. It is an autosomal dominant monogenic form of diabetes. The traditional teaching states that individuals with MODY also develop a relative insulin deficiency in the absence of insulin resistance. They are classically described as having a lean body habitus and lacking features of the metabolic syndrome (Bellanne-Chantelot, Levy et al. 2011). As a result of the overlap in clinical characteristics between the diabetes sub-types, making an accurate diagnosis of a particular form of diabetes can be a challenge. Inherent to all diabetes forms albeit T1DM, T2DM or MODY is a reduction in overall ß-cell mass (Kloppel, Lohr et al. 1985, Butler, Janson et al. 2003, Vesterhus, Haldorsen et al. 2008). The

determination of ß-cell mass is confined to functional assessment since imaging or histological studies of the pancreas in humans remain inaccurate or infeasible.

The subject matter of the current body of work focuses on MODY which accounts for 1-3% of all diabetes (Fajans, Bell et al. 2001). MODY comprises of a number of single gene disorders affecting both ß-cell function and mass (Byrne, Sturis et al. 1996, Vesterhus, Haldorsen et al. 2008). In the current chapter, I will present the historical milestones pertinent to the current understanding of the MODY subtypes. Subsequently, I will discuss the pathophysiology of a MODY mutation and detail the MODY subtypes, their clinical phenotype and consequence. Finally, the aims of this candidature are discussed in detail.

1.2 Historical Prospective:

The seminal work of Fajans and Tattersall led to the description of MODY, initially coined in 1964 with the description of the first pedigree; the "RW pedigree" (Fajans 1990). Serial testing of the pedigree demonstrated a relatively slow progression from glucose tolerance to intolerance amongst mutation carriers (Fajans and Brown 1993). Throughout the 1980s and 1990s the various research groups in Europe and the United States discovered the multiple genetic entities responsible for the various MODY subtypes. To date, 13 different mutation subtypes of MODY have been identified. Table 1.1 contains details of all 13 MODY subtypes, their gene function and linked chromosomes. The majority are secondary to transcription factor defects with the most common being a defect in the hepatocyte nuclear factor 1 alpha (HNF1A) accounting for 52% of all MODY cases in Northern Europe (Shields, Hicks et al. 2010). A defect in the enzyme glucokinase which catalyses the conversion of glucose to glucose-6-phosphate, the initial step in glucose metabolism in the ß-cell, accounts for approximately 30% of MODY cases (Shields, Hicks et al. 2010). A mutation in the transcription factor hepatocyte nuclear factor 4 alpha (HNF4A) accounts for 6-10% of all MODY (Shields, Hicks et al. 2010). The mutations in HNF1ß (Edghill, Bingham et al. 2006), pancreas-duodenum homeobox protein 1(PDX 1) (Stoffers, Ferrer et al. 1997) , neurogenic differentiation 1 (NEUROD1) (Malecki, Jhala et al. 1999) , CEL and INS are exceedingly rare and are therefore not included in this thesis (Edghill, Flanagan et al. 2008, Torsvik, Johansson et al. 2010). ABCC8 and KCN J11

are responsible for neonatal diabetes and are not discussed in this candidature (Yorifuji, Nagashima et al. 2005, Bowman, Flanagan et al. 2012). Co-segregation with diabetes in a significant number of pedigrees is withstanding for mutations of Krupel like factor2 (KLF 2) (Neve, Fernandez-Zapico et al. 2005), paired box gene 4 (PAX 4) (Plengvidhya, Kooptiwut et al. 2007) and tyrosine kinase B lymphocyte specific (BLK) (Borowiec, Liew et al. 2009). Therefore, for the purpose of this body of work I will focus my attention on the most common subtypes of MODY; HNF1A, GCK and HNF4A-MODY.

MODY subtype	Gene name	Gene function	Reported Prevalence	Chromosome
HNF4A	Hepatocyte Nuclear Factor 4 alpha	Transcription Factor	6-10%	20
GCK	Glucokinase	Glycolytic Enzyme	30%	7
HNF1A	Hepatocyte Nuclear Factor 1 alpha	Transcription Factor	52%	12
IPF/PDX	Pancreas-Duodenum Homeobox Protein 1	Transcription Factor	-	13
HNF1b	Hepatocyte Nuclear Factor 4 alpha	Transcription Factor	1-2%	17
NEUROD1	Neurogenic Differentiation 1	Transcription Factor	-	29.32.2
KLF II	Krupel Like Factor2	Transcription Factor	-	29.25.12
CEL	Carboxyl-Ester Hydrolase	Transcription Factor	-	9q34
PAX4	Paired Box gene	Transcription Factor	-	7q32
INS	Insulin	-	-	11p15
BLK	tyrosine kinase B lymphocyte specific	Transcription Factor	-	8p23

Table 1-1: MODY mutation subtypes

1.3 Pathophysiology: The Insulin Secretory Defect associated with MODY.

As aforementioned, typically, MODY is associated with an insulin secretory defect in the absence of insulin resistance. Insulin secretion involves a complex array of factors. In brief, glucose enters the ß-cell via the glucose transporter GLUT2, the enzyme glucokinase subsequently phosphorylates glucose yielding glucose 6 phosphate (Morita, Yano et al. 1994, Sweet, Li et al. 1996, Roche, Assimacopoulos-Jeannet et al. 1997). Glycolysis yields the substrate pyruvate which enters the tricarboxylic acid (TCA) cycle in the mitochondrion. Pyruvate is metabolised by pyruvate dehydrogenase generating adenosine triphosphate (ATP) in the respiratory chain. ß-cells contain ATP-sensitive potassium channels which close on elevation of the ATP/Adenosine diphosphate ratio. Closure of these channels causes membrane depolarisation resulting in opening of the voltage dependent calcium channels. The influx of calcium results in insulin exocytosis (Henquin, Ravier et al. 2003, MacDonald and Wheeler 2003) . It is established that HNF1A, HNF4A and GCK are involved in multiple aspects of glucose homeostasis. HNF1A and HNF4A play an important role in the embryonic development of the pancreas (Ryffel 2001) . They are also important in the maintenance of ß-cell function throughout life. HNF4A is known to act as an upstream regulator of HNF1A (Tian and Schibler 1991, Kuo, Conley et al. 1992). Together the transcription factors form a regulatory loop in the ß-cell .It has been demonstrated that HNF1A and 4A influence not only the expression of the insulin gene, but also GLUT2 and several other important proteins involved in glucose mitochondrial metabolism (Stoffel and Duncan 1997, Dukes, Sreenan et al. 1998, Wang, Maechler et al. 1998, Shih, Screenan et al. 2001). Molecular studies have demonstrated that HNF4A-regulated gene expression profiles are remarkably similar to that of its' downstream transcription regulatory protein; HNF1A and that both factors may activate transcription in a synergistic fashion (Eeckhoute, Formstecher et al. 2004). It is now believed that HNF4A has an effect not only on the ß-cells but also on the α-cells of the islet (Bonner, Kerr-Conte et al. 2015). Indeed, HNF4 and HNF1A mutations may have a global effect on both islet structure and function (Ilag, Tabaei et al. 2000).

GCK functions as a glucose sensor controlling the rate of entry of glucose into the cell and the glycolytic pathway (Velho, Froguel et al. 1992). GCK is expressed not only in the pancreatic ß-cells but also the liver where it is involved in the storage

of glycogen post-prandially (Velho, Petersen et al. 1996). Unlike T2DM, insulin resistance is not generally a feature of MODY (Byrne, Sturis et al. 1996, Herman, Fajans et al. 1997, Lehto, Bitzen et al. 1999). Rather, decreased insulin secretory response is demonstrated both in diabetic and non-diabetic mutation carriers. Byrne et al reported that insulin secretory rates are normal until a certain glucose threshold is reached at which point insulin secretion is diminished when plasma glucose is >7/8 mmol/mol in HNF4A/1A respectively (Byrne, Sturis et al. 1995, Byrne, Sturis et al. 1996). In GCK-MODY, first phase insulin secretion is preserved (Byrne, Sturis et al. 1994). Figure 1.1 is an illustration if the ß-cell and the proteins implicated in MODY.

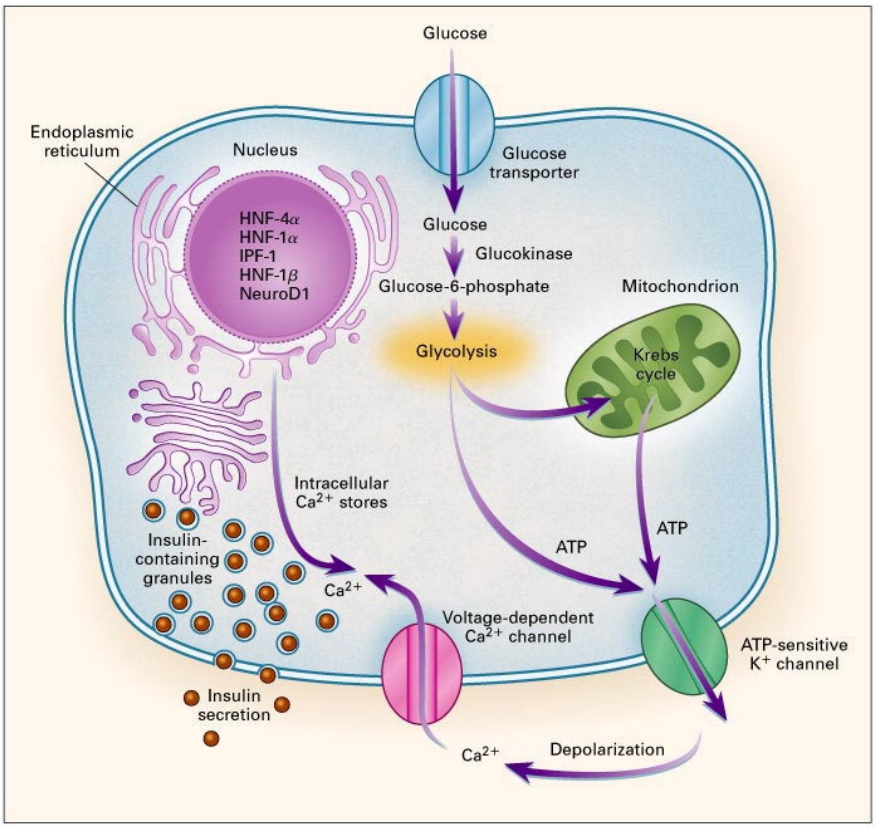

Figure 1-1: Model of the pancreatic ß-cell and the proteins implicated in MODY. (Adopted from Fajans, SS NEJM 2001).

1.4 Description of the MODY subtypes:

1.4.1 HNF1A-MODY

The HNF1A MODY transcription factor is expressed in the liver, kidney, intestine as well as the pancreas (Frain, Swart et al. 1989, Emens, Landers et al. 1992). The mutation is mapped to chromosome 12 (Yamagata, Oda et al. 1996). The HNF1A gene contains 10 exons, spanning 23kb, codes for 628 amino acids and is composed of 3 functional domains. The domains are as follows; a short N terminal dimerization domain encoded by exon 1, a homeobox DNA binding domain encoded by exons 2,3,4 and a C-terminal transactivation domain encoded by exons 5-10 (Frain, Swart et al. 1989, Nicosia, Monaci et al. 1990, Kaisaki, Menzel et al. 1997). HNF1A mutations are most frequently missense with frameshift, nonsense, splice site promotor region, in frame deletions, insertions and duplications occurring with less frequency. The most common mutation reported for HNF1Ais c.872dupC (p.G292fs; previously denoted p291fsinsC). The following clinical guidelines have been established to assist in diagnosis of HNF1A-MODY (Ellard, Bellanne-Chantelot et al. 2008);

1. A young age at onset of diabetes (typically diagnosed before the age of 25 years)
2. Non-insulin dependence beyond the “honeymoon period” of 3 years
3. An absence of islet autoantibodies (GAD/ ICA/IA-2)
4. A family history of diabetes (at least 2 generations affected)
5. A sensitivity to sulphonylurea therapy
6. Glycosuria at low blood glucose levels (<10mmol/l)
7. An absence of features of the metabolic syndrome

The advent of time and larger observational studies has resulted in a modification of the above mentioned features. Notably, more recent literature describes a positivity for islet cell auto-antibodies in MODY. The incidence varies depending upon the population studied but ranges from 1-

25% (McDonald, Colclough et al. 2011, Urbanova, Rypackova et al. 2013). One particular studied noted transient kinetics in relation to autoantibody positivity with a reduction with improved glycaemic control. In addition, a lean phenotype is not an absolute necessity for clinical recognition of MODY. On observation of our MODY cohort, in particular in individuals with a bilinear transmission of diabetes, features of the metabolic syndrome may indeed be present. Of late, two studies using a large paediatric and adult HNF1A-MODY cohort have described additional clinical criteria to assist in diagnosing MODY (Chambers, Fouts et al. 2015, Grzanka, Matejko et al. 2016). With the recognition of de novo mutations, a family history of diabetes is no longer an essential pre-requisite of a MODY diagnosis (Stanik, Dusatkova et al. 2014). The expanded criteria also suggest to differentiate MODY from T1DM features such as a low total daily insulin rate (TDIR) of <0.3 u/kg and the percentage of basal insulin being >30% of the TDIR. In addition, to assist in differentiating from T2DM, a prolonged period of sulphonylurea monotherapy and a BMI<30kg/m^2 are proposed. Recognition of the heterogeneity of the disorder can result in bewilderment amongst health care professionals. Therefore, as an adjunct to clinical criteria, an on-line MODY probability calculator has been developed to assist clinicians in their diagnosis of MODY (Shields, McDonald et al. 2012).

1.4.2 HNF4A-MODY

HNF4A is mapped to chromosome 20, is 30kb in size and contains 13 exons (Bell, Xiang et al. 1991). It is an orphan member of the nuclear receptor family of ligand activated transcription factors. As aforementioned, HNF4A shares similarities in phenotype with HNF1A-MODY as a result of their mutual control of transcription. The clinical criteria for determining who should be selected for HNF4A screening are similar to with the exception of glycosuria. An additional difference between HNF4A and HNF1A is the development of macrosomia and the associated transient neonatal hypoglycaemia (Fajans and Bell 2007, Pearson, Boj et al. 2007). It is now recognised that HNF4A-MODY is associated with a "biphasic" phenotype. In a proportion of mutation carriers there is an initial period of neonatal hyperinsulinemic hypoglycaemia, this is followed typically by a period of "wellness" during adolescence and finally diabetes develops in adulthood (Fajans and Bell 2007).

1.4.3 GCK –MODY

As a result of its role in glucose sensing, mutations in GCK are associated with an increase in the threshold concentration of glucose necessary to stimulate insulin secretion from a normal of 5 mmol/l to 6-7 mmol/l (Velho, Froguel et al. 1992). It is reported, in general, that GCK mutations result in stable hyperglycaemia with no progression in the disorder (Pearson, Starkey et al. 2003, Steele, Shields et al. 2014). However, an exception to this rule is in pregnancy, where insulin may be required to reduce the incidence of fetal macrosomia (birth weight > 4.5kg at any gestation).

1.5 Clinical Heterogeneity is associated with MODY

As previously stated, the differentiation of MODY from other diabetes subtypes, in particular T2DM can be clinically challenging (Cuesta-Munoz, Tuomi et al. 2010, Gardner and Tai 2012) .The penetrance of diabetes with a MODY mutation is 98.7% by the age of 75 years. The clinical recommendations preceded the current

obesity academic affecting patients at a young age, and it is likely that additional superimposed environmental factors supervene to cause an earlier manifestation of the disorder. There can be clinical heterogeneity both between and within family pedigrees with regards to phenotypic expression. A number of factors are also reported to influence the variable clinical expression of MODY including ethnicity, molecular characteristics, parent-of –origin effect and in-utero hyperglycaemia (Klupa, Warram et al. 2002, Bellanne-Chantelot, Carette et al. 2008, Bellanne-Chantelot, Levy et al. 2011). These factors are detailed further below.

1.5.1 Ethnicity

A publication by Bellanne-Chantelot demonstrated that the likelihood of HNF1A-MODY amongst clinically selected cases were higher amongst Caucasian individuals that were of European origin (Bellanne-Chantelot, Levy et al. 2011).

1.5.2 Molecular characteristics of the HNF1A-MODY gene

It is hypothesized that the molecular characteristics of the HNF1A gene mutation affect age of onset of diabetes. Age is lower in patients with truncating mutations when compared to patients with missense mutations and missense mutations affecting the dimerization DNA binding domains are associated with a lower age at diagnosis than those affecting transactivation domains (Bellanne-Chantelot, Carette et al. 2008).

1.5.3 In-utero hyperglycaemia

In utero hyperglycaemia is associated with an earlier diagnosis of T2DM in Pima Indians. It has also been demonstrated that the incidence increases if the mother is diagnosed pre-pregnancy because the offspring is exposed to a hyperglycaemic milieu for the duration of the gestation (Knowler, Bennett et al. 1978).The mean age of diagnosis of MODY in the UK is 20.4 years and in Ireland is 36 ±3 years (Kyithar, Bacon et al. 2011) however the age of diagnosis varies widely between 4-74 years (Frayling TM, Diabetologia Suppl 50;s94,2001). It has been reported

that similar to that noted in the Pima Indians with T2DM, the HNF1A-MODY gene causes a reduced age at diagnosis when associated with exposure to hyperglycaemia in utero (Klupa, Warram et al. 2002). There is somewhat limited data available as to causative factors for the increased penetrance of the HNF1A mutation in offspring to mothers with diabetes during pregnancy, the fact that HNF1A is important in the early development of the pancreas may be the cause of a more severe form of ß-cell defect.

Caveats to note in these studies must be considered in that the studies were retrospective and the offspring of the mothers with pre-pregnancy diabetes had a higher BMI. One also has to consider a heightened awareness amongst mothers with diabetes for the detection of signs and symptoms of hyperglycaemia in their offspring.

1.5.4 Parent-of-origin

In the study by Kulpa et al a more severe phenotype was noted in HNF1A mutation inherited from the mother than from the father. Diabetes tended to be diagnosed at a younger age in addition to an increased likelihood for insulin requirements (Klupa, Warram et al. 2002).

As a result of clinical heterogeneity it can be difficult to ascertain who warrants MODY screening. It is estimated that up to 80% of MODY cases remain undiagnosed worldwide (Shields, Hicks et al. 2010). The majority of individuals remain misclassified as having T1DM or T2DM. A further reason for the under diagnosis of MODY is cost. Currently, to definitively diagnose MODY a genetic confirmatory test is required, which although there is a reduction in cost with increased demand, it remains an expensive process. To alleviate the possibility of a negative test in an individual suspected of MODY a number of biomarkers have been developed to support a clinical suspicion. The biomarkers which have been studied to date including the novel biomarkers investigated by our research group will be further discussed in chapter 7.

1.6 The clinical implications of an accurate MODY diagnosis are as follows:

1.6.1 Pharmacogenetics

An important lesson arising from the study of monogenic diabetes is the discipline of pharmoacogenetics; the tailoring of treatment to the pathophysiology of the disorder. A MODY diagnosis has facilitated patients being switched from insulin or alternative oral hypoglycaemic agents to sulphonylurea therapy, which is the most appropriate therapy available. In vitro studies in MODY models have demonstrated a reduction in the mRNA levels of proteins involved in glucose metabolism as previously documented. These proteins include GLUT2, L-pyruvate kinase and mitochondrial metabolism (Stoffel and Duncan 1997, Dukes, Sreenan et al. 1998, Wang, Maechler et al. 1998, Shih, Screenan et al. 2001).

Figure 1.2 is a schematic representation of the cellular defects; as demonstrated the defects occur upstream to the sulphonylurea receptor therefore it was postulated that sulphonylurea therapy would bypass the defects and improve ß-cell function in HNF1A and HNF4A (Pearson, Starkey et al. 2003).

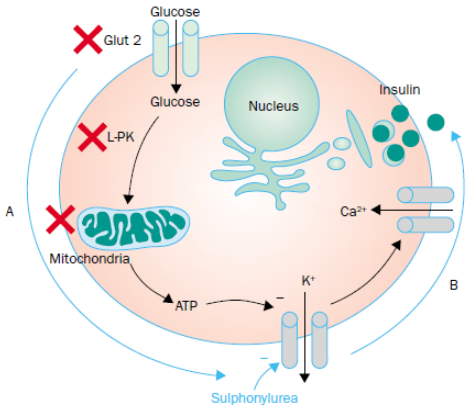

Figure 1-2: Schematic representation of defects noted in-vitro models of HNF1A/4A MODY (adapted from Pearson, ER et al, Lancet, 2003).

In GCK-MODY patients it is believed that pharmacological intervention does not improve or alter glycaemic control, therefore it is recommended not to treat hyperglycaemia attributed to a GCK defect (Stride, Shields et al. 2014).

1.6.2 Determination of the natural progression of MODY

The rate of micro- and macrovascular complications in MODY remains disputed. There is limited literature available pertaining to the subject as no longitudinal prospective study has been conducted to date. It is reported that micro and macrovascular complications occur with a similar frequency to that of T1DM and T2DM. The data that is available, however, is quite conflicting in relation to its findings (Velho, Vaxillaire et al. 1996, Isomaa, Henricsson et al. 1998, Steele, Shields et al. 2010) and much of the studies were performed in an era prior to the widespread usage of prophylactic statin therapy and stringent glycaemic target goals. Determination of the natural progression of the disorder would facilitate accurate counselling and appropriate use of intervention in MODY patients.

1.6.3 Familial Screening

A further important merit of a MODY diagnosis in an index case is to facilitate familial screening. We frequently detect additional previously undiagnosed cases of impaired glucose tolerance or diabetes when screening is performed. In those who remain normoglycaemic annual screening with an oral glucose tolerance test (OGTT) is recommended. An early diagnosis of diabetes is important to alleviate the damage caused by hyperglycaemia in HNF1A/4A, thereby preventing micro and macrovascular complications.

1.6.4 Management of MODY during pregnancy

Hyperglycaemia in pregnancy represents a unique challenge given the requirement for optimal glucose control and the constraints on therapeutic

options. Maternal genetics in GCK and HNF4A-MODY play an important role in determining fetal size. Both mutations are associated with a significant incidence of macrosomia. Macrosomia is associated with both maternal and fetal morbidities. Much clinical dilemma exists as to the use of insulin in pregnancies complicated by GCK. There is agreement however amongst all research groups that serial antenatal ultrasound scans are a requirement in the appropriate management of GCK and HNF4A-MODY
(Spyer, G., Macleod et al. 2009, Pearson, Boj et al. 2007). Pre-conception knowledge of a MODY mutation assists greatly in providing optimal care during pregnancy.

1.6.5 Economic benefit of a MODY diagnosis

The benefit in monetary terms of the appropriate medication being utilised in MODY patients has been studied (Naylor, John et al. 2014). Naylor et al demonstrated that testing for MODY in a select population is cost effective. In monetary terms, a diagnosis of HNF1A/4A-MODY facilitates a transfer of most individuals from insulin to OHA therapy. In our clinic we have transferred a number of individuals with HNF1A and HNF4A-MODY from CSII therapy to OHAs. This has resulted in a significant cost benefit not only in relation to cessation of insulin therapy but also a reduction in the prescription of glucose test strips, lancets etc. A diagnosis of GCK can result in the successful discontinuation of OHA and or insulin therapy without deterioration in glycaemic control (Pearson, Starkey et al. 2003, Steele, Shields et al. 2014). A recent publication reported inappropriate use of OHA/insulin therapy preceding molecular diagnosis of GCK-MODY (Carmody, Naylor et al. 2016). The discharge of the non-pregnant GCK-patient from frequent clinic surveillance as recommended has significant cost savings, particularly if the individual is diagnosed at a young age (Pinelli, Acquaviva et al. 2013, Schnyder, Mullis et al. 2005).

1.6.6 Psychosocial benefit of a MODY diagnosis

The psychosocial aspect of a MODY diagnosis warrants attention. Qualitative studies exploring the experiences of people with a molecular diagnosis of MODY

have been performed (Shepherd, M. 2010, Shepherd, M. and A. T. Hattersley 2004, Bosma, A. Rigter, S. 2015). These studies provide a fascinating insight into the realistic implication of such a diagnosis for the individual. Overall, it was felt that a diagnosis of MODY was beneficial; however a number of concerns were also highlighted. A primary concern expressed was that of a lack of knowledge of MODY amongst healthcare providers. There was also a frustration expressed at the length of time that it took to accurately diagnose MODY. A fear of genetic discrimination was detailed not only for the individual but also for their offspring. With the availability of next generation sequencing techniques the time to a positive MODY diagnosis will be significantly decreased. An increased awareness of the entity MODY is required through public seminars, educational lectures with relevant health care professionals. In addition, there is a necessity to address the concerns in relation to insurance charges and reimbursement policies.

1.6.7 MODY as a model for the study of the ß-cell

The wider scale impact of MODY research is that monogenic forms of diabetes such as MODY provide an ideal model for the study of the ß-cell. This is an invaluable research tool since there is a lack of available pancreatic specimens and imaging techniques to determine ß-cell function. Common variants in several genes involved in MODY are associated with an increased risk of development of T2DM (Jonasson, Wicklow et al. 2015). Elucidating the pathophysiology behind monogenic diabetes may improve understanding of the more common diabetes forms.

This thesis is a collection of published work studying the natural progression and clinical management of MODY. It encompasses studies exploring the MODY mutation from the fetal state through to adolescence and into adulthood. I also present the use of novel microRNAs as biomarkers of a MODY mutation. The objectives of each study are outlined below and detailed in subsequent chapters. Chapter 8 contains a summation of each chapter and highlights the clinical significance of our findings.

1.7 Research Hypothesis, Aims and Objectives

1.7.1 The clinical management of hyperglycaemia in pregnancy complicated by MODY.

Hypothesis: The current management of pregnancies complicated by MODY as per pre-gestational diabetes guidelines is appropriate.

Aim: To determine the optimal clinical management of diabetes in pregnancies complicated by MODY through clinical observation and the utilization of home blood glucose monitoring (HBGM) profiles.

1.7.2 Persistent fasting and post prandial hypoglycaemia in young adult subjects with HNF4A-MODY.

Hypothesis: In a proportion of individuals with HNF4A-MODY a "bi-phasic" phenotype is observed with initial neonatal hyperinsulinaemic hypoglycaemia progressing to diabetes in adulthood. The insulin secretory pattern in the intermediate adolescent state is poorly defined. I have clinically observed fasting hypoglycaemia in HNF4A-MODY mutation carriers with IGT. I propose that hyperinsulinaemic hypoglycaemia persists beyond the neonatal period in HNF4A-MODY mutation carriers.

Aims:

1. To determine the "switch-point" from hyperinsulinaemic hypoglycaemia to diabetes through the use of serial OGTT.

2. To ascertain the 72 hour glucose profile of HNF4A utilizing a continuous glucose monitoring system (CGMS).

1.7.3 Circulating CD36 is reduced in HNF1A-MODY carriers.

Hypothesis: The incidence of cardiovascular disease amongst cohorts with HNF1A-MODY is reported to be equivocal to that of a T1DM population. However, individuals with HNF1A-MODY have a favourable metabolic profile. The scavenger protein CD36 is a transmembrane glycoprotein and has been shown to be associated with lipid metabolism, atherosclerosis and platelet activation. Increased plasma levels of soluble form of CD36 have also been found to correlate with insulin resistance, diabetes and plaque instability. I propose that sCD36 levels would be altered in a HNF1A-MODY cohort.

Aim: To evaluate the levels of sCD36 in HNF1A-MODY and to compare this to individuals with T2DM and HNF1A-negative normoglycaemic controls. Levels of sCD36 are correlated with phenotypic and biochemical parameters.

1.7.4 Successful maintenance on sulphonylurea therapy and low diabetes complications rate in a HNF1A-MODY cohort.

Hypothesis: Clinical guidelines have been developed that recommend sulphonylurea medication as first line treatment for individuals with HNF1A-MODY. A micro and macrovascular complication rate similar to the T1DM population is reported in the literature. However, these studies were conducted in an era prior to statin therapy and stringent glycaemic targets. I hypothesized therefore that the current treatment of HNF1A-MODY mutation carriers with sulphonylurea therapy is clinically optimal and that complication rates (micro and macrovascular) are low in this patient population.

Aim: To determine the natural progression and clinical management of a large HNF1A-MODY cohort in a dedicated tertiary referral centre.

1.7.5 MicroRNA-224 is readily detectable in urine of individuals with diabetes mellitus and is a potential indicator of ß-cell demise.

Hypothesis: In recent years microRNA (miRNA) research has attracted much attention, in particular in biomarker development. miRNA have emerged as potent regulators of glucose homeostasis. They are differentially expressed in tissues affected by diabetes when compared to normal controls. Our research group has published on the induced suppression of endogenous HNF1A function in INS-1 cells, a cellular model of HNF1A-MODY, increasing the levels of two specific miRNA; miR-224 and miR-103 (Bonner, Nyhan et al. 2013). In the clinical setting, urine is a proven stable and useful biofluid. We subsequently hypothesized that the diabetes associated; miR-224 would be detectable in urine of participants with diabetes. In addition we proposed that the expression levels of miR-224 would differentiate between diabetes sub-types.

Aim: The aim of this study was to obtain proof-of-concept that miR-224 is detectable in the urine of HNF1A-MODY mutation carriers, and to determine whether this diabetes-associated miRNA is also elevated in the urine of patients with T1DM and T2DM. We also sought to correlate urinary levels of miRNA with serum levels, and with clinically relevant indices of renal disease.

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Chapter 2

Materials & Methods

2 Chapter 2: Methodology

2.1.1 General methodology for the establishment of the Mater MODY cohort

The MODY cohort was initially established in 2007. The referral system to the MODY clinic is described in the following paragraph.

2.1.2 Referral System:

Physicians/medical personnel from all disciplines and centres throughout Ireland can refer to the Mater MODY Clinic. A series of lectures and information evenings are conducted annually to promote awareness of MODY amongst healthcare professionals. In addition, specialist endocrinology days for current fellows and peers are held throughout the year to foster an understanding of the disorder and to provide updates on any new developments in the field. I developed a referral pathway, form and information leaflet which can be accessed through the Mater hospital website (see Appendix 3). On receipt of a referral, the individual will be considered for MODY genetic testing. If deemed suitable for the MODY study the individual is contacted and, if agreeable arrangements are made for attendance at the Diabetes Day Centre in the Mater Hospital.

Individuals are deemed clinically suitable depending on the following inclusion/exclusion criteria being met;

2.1.3 Inclusion Criteria

- a proband with diabetes or impaired glucose tolerance, diagnosed between the ages of 10 and 59 years
- A family member diagnosed with diabetes or IGT aged between 10 and 59 years
- Evidence of endogenous insulin secretion such as detectable C-peptide

- Males and females age ≥ 10 and ≤ 80 years
- Body mass index ≤ 32 kg/m^2

2.1.4 Exclusion Criteria

- Known psychiatric illness
- Inadequate capability to communicate or to cooperate,
- Legal incompetence,
- Patients whose command of English is insufficient to understand the study instructions.

2.1.5 Phenotyping of the MODY cohort:

At initial assessment phenotyping of the cohort is completed. A full medical history and physical examination is performed. A history of microvascular (i.e. retinopathy, nephropathy or neuropathy) and macrovascular disease (i.e. coronary heart disease; myocardial infarction/angina/coronary bypass grafting or percutaneous coronary intervention), cerebrovascular disease (ischaemic stroke) or peripheral vascular disease) is recorded.

Anthropometric measurements for waist/hip ratio and body mass index (BMI) were recorded. Hypertension is defined as a systolic blood pressure of >140mmHg and/or a diastolic of >90mmHg on 2 or more occasions or the use of antihypertensive agents. A 12 lead resting electrocardiogram is completed on all participants at initial assessment. Peripheral vascular disease (PVD) is defined by both clinical diagnosis and measurement of arterial brachial index/ toe brachial indices. Peripheral neuropathy is assessed using vibration proprioception threshold. Cutaneous perception is assessed using a 10g Semmes-Weinsten monofilament. Blood samples are drawn for the measurement of HbA_{1c}, fasting lipids, full blood count, thyroid function, renal and liver profiles, glutamic acid decarboxylase (GAD) and islet cell antibody (ICA) and high-sensitivity C-reactive protein (hsCRP). A first void fasting urine sample is requested for estimation of the Urinary Albumin Creatinine Ratio (UACR). Microalbuminuria is said to be

present when the UACR is 3.4-33.9 g/mol and overt albuminuria when the UACR was >34 g/mol. Persistent microalbuminuria is defined as a UACR of 3.4-33.9 g/mol on at least 2 separate occasions. The CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration, http://www.qxmd.com/calculate-online/nephrology/ckd-epi-egfr) creatinine equation was used for glomerular filtration rate (GFR) estimation. CKD was defined as an eGFR <60 mL/min/1.73 m^2

2.1.6 Oral Glucose Tolerance Test (OGTT)

A 75 g oral glucose tolerance test (OGTT) is performed on participants after a 12-h overnight fast with measurement of glucose, insulin and C-peptide at baseline and at 30 minute intervals for 120 minutes to determine the degree of glucose tolerance and insulin secretory response. Oral hypoglycaemic agents are stopped at least 48-h before the OGTT while, in those taking insulin, long-acting insulin therapy is stopped for 24-h and short-acting insulin stopped for 12-h prior to OGTT. The diagnostic criterion for the American Diabetes Association was used to define the degree of glucose tolerance. The oral glucose insulin sensitivity (OGIS) is calculated as previously described (Mari, Pacini et al. 2001).

2.1.7 Recruitment for other study populations

Recruitment for the T1DM and T2DM study populations occurred through the general diabetes outpatient clinics and the Diabetes Day Centre of the Mater Misericordiae University Hospital.

2.1.8 The Type 1 diabetes mellitus study population:

The T1DM cohort was selected based on the permanent usage of insulin from diagnosis and evidence of ß-cell dysfunction such as undetectable C-peptide and positive ICA+/GAD antibodies.

2.1.9 The Type 2 diabetes mellitus study population:

The T2DM cohort was selected based on the absence of a significant family history, an older age at diagnosis of diabetes and having no documented evidence of ketosis.The lean T2DM cohort had a higher OGIS value than that typically seen in a T2DM cohort. OGIS provides an index which is analogous to the index of insulin sensitivity obtained from a glucose clamp (Mari, Pacini et al. 2001). If a MODY diagnosis was suspected in this cohort, molecular genetic analysis for HNF1A, HNF4A and GCK-MODY was completed.

2.1.10 The normal control study population:

The normal control study population was attained through internal advertisement in addition to a staff health awareness day held bi-annually in the Mater Hospital. The cohort was selected based on being normoglycaemic, normotensive and an absence of medical co-morbidities. Individuals were excluded if taking prescribed medication for hypertension or hyperlipidaemia.

2.1.11 Mutation negative familial controls:

This cohort consists of those participants who were screened as a result of an index case within the family being identified. These individuals were genetically confirmed as not being carriers of a MODY mutation.

2.2 Ethical Approval

Institutional ethical board review approval was granted for each of the studies by the Research Ethics Committee of the Mater Misericordiae University Hospital and is updated on an annual basis. Informed, written consent is attained in triplicate, a copy is kept in the patient file as a permanent record, a second copy is kept in the study file and a third is given to the patient. For the study entitled “The clinical management of hyperglycaemia in pregnancy complicated by MODY”

ethical approval was attained from the Research Ethics Committees of each of the three Dublin maternity hospitals; the National Maternity Hospital, Holles St., The Women and Children's Hospital, the Coombe and the Rotunda Maternity Hospital. The ethical approval notification letters are contained in Appendix 2.

2.3 Biochemical Assays for the phenotyping of all cohorts

All laboratory analyses were performed with commercially available standardized methods. The plasma glucose concentration was measured using Beckman Synchron DXC800 (Beckman Instruments Inc, Brea, USA). HbA_{1c} was determined using high performance liquid chromatography (Menarini HA81-10, Rome, Italy). Insulin and C-peptide were analyzed using Immulite 2000 immunoassay (Siemens Healthcare Diagnostics, Deerfield, IL, USA). Anti-GAD_{65} antibodies were analysed by ELISA (Euroimmun, Luebeck, Germany). ICA was performed by indirect immunofluorescence test by the supra-Regional Protein Reference Unit and Dept. of Immunology in Sheffield, UK.

2.4 Genetic Analysis of HNF1A/HNF4A/GCK:

Mutation analysis was performed by polymerase chain reaction (PCR) amplification of genomic DNA, followed by semi-automated bidirectional DNA sequencing of all exons and the highly conserved flanking intronic sequences of the exon-intron splice junctions. Genetic analysis was performed by integraGen GmbH (Bonn, Germany) in 2000-2007 and the Molecular Genetics Laboratory (Exeter) from 2008-2015.

2.5 Establishment and maintenance of a Biobank

Encrypted blood, serum and urine samples are biobanked in the Metabolic Research Unit. These samples are held for a period of not less than ten years with

the consent of the individual concerned. The biobank is maintained in association with the Clinical Research Centre, UCD.

The specific methodology employed for a particular study is detailed in the relevant chapter.

2.6 References:

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Chapter 3

The clinical management of hyperglycaemia in pregnancy complicated by MODY.

The contents of this chapter are published as: *"The clinical management of hyperglycaemia in pregnancy complicated by MODY"* , Bacon S, Schmid J, McCarthy A, Edwards J, Fleming A, Kinsley B, Firth R, Byrne B, Gavin C, Byrne MM. Am J Obstet Gynecol. 2015 Apr 30 PMID:25935773.

3 Chapter 3: The clinical management of hyperglycaemia in pregnancy complicated by MODY.

3.1 Abstract

Introduction:

Women with Maturity Onset Diabetes of the Young (MODY) are often first identified and diagnosed with diabetes during pregnancy. Genetics and hyperglycaemia play an important role in determining fetal size in MODY pregnancies. The principal objective of the current study is to determine the outcomes and clinical management of hyperglycaemia in pregnancies complicated by glucokinase (GCK) and hepatocyte nuclear factor-1 alpha (HNF1A) MODY mutations.

Methods:

A retrospective chart review of 37 women with a GCK/ HNF1A-MODY mutation was conducted. Data on variables such as birth weight, mode of delivery and the treatment of hyperglycaemia was available on 89 pregnancies.

Results:

The birth weight in non-affected GCK offspring was significantly higher than in the affected GCK offspring (4.8 kg (4.1-5.2) vs. 3.2 kg (3.1-3.7), p=0.01). Seven point home blood glucose monitoring (HBGM) over a seven day period in each trimester demonstrated higher fasting and post prandial glycaemic excursions in the 1st trimester of GCK pregnancies when compared to HNF1A-MODY pregnancies (Fasting; 5.7 mmol/L (4.9-6.4) vs. 4.7 mmol/L (4.3-4.9), p=0.01 and Post-prandial: 8.5 mmol/L (7.5-10.8) vs. 6.2 mg/dL (5.5-7.3), p=0.04) despite insulin treatment. There was a higher percentage of miscarriages in the GCK group when compared to the HNF1A-MODY group (26.8% vs. 14%, p=0.1),however this did not reach significance. Insulin initiated at an early gestation appeared to lower the incidence of macrosomia in GCK non-affected offspring.

Conclusions:

Hyperglycaemia in HNF1A-MODY pregnancies is easily managed with current insulin protocols; in contrast glycaemic excursions are difficult to manage in GCK-MODY pregnancies. There were an increased percentage of miscarriages in GCK-

MODY pregnancies highlighting the importance of a diagnosis of GCK-MODY in women prior to conception and the necessity for pre-conception care.

3.2 Introduction:

Maturity onset diabetes of the young (MODY) is a monogenic autosomal dominant form of diabetes with the most common causative mutations being those in hepatocyte nuclear factor 1 alpha (HNF1A) and a mutation in the glucokinase gene (GCK) . GCK is the enzyme responsible for converting glucose to glucose-6-phosphate, the initial step in glucose metabolism in the ß-cell. In contrast to T1DM, MODY mutation carriers are usually auto-antibody negative (islet cell antibody/ anti-glutamic acid decarboxylase), non-ketotic and have detectable C-peptide suggesting preserved ß-cell function. Clinically, they differ from those with T2DM and gestational diabetes (GDM) by being lean and lacking features of the metabolic syndrome. GCK-MODY, is specifically associated with persistent fasting hyperglycaemia (Ellard, Bellanne-Chantelot et al. 2008). GCK is proposed to be a stable state of hyperglycaemia outside of pregnancy (Pruhova, Dusatkova et al. 2013, Steele, Shields et al. 2014). The reported prevalence of MODY amongst women with GDM varies between 0.1 and 6% with a higher prevalence amongst a Caucasian population (Stoffel, Bell et al. 1993, Zouali, Vaxillaire et al. 1993, Saker, Hattersley et al. 1996, Ellard, Beards et al. 2000, Weng, Ekelund et al. 2002, Zurawek, Wender-Ozegowska et al. 2007, Chakera, Spyer et al. 2014, Flack, Ross et al. 2015). Due to its relative rarity it has not been possible to perform a prospective study on the clinical management of MODY pregnancies. The literature available is therefore, based primarily on case reports or retrospective analysis of diabetes cohorts in pregnancy.

Clinical experience of treating MODY in pregnancy is also limited as currently the majority of women with GCK and HNF1A-MODY remain undiagnosed during pregnancy .In GCK it has been reported that fetal genetics are at least as important as maternal glucose control in determining fetal size and hence complications. If the infant has a glucokinase deficiency then they are on average

540 g lighter at birth (Hattersley, Beards et al. 1998). If the infant does not inherit the mutation and is exposed to untreated hyperglycaemia *in utero* then birth weight can be increased by up to 600 g (Hattersley, Beards et al. 1998). The question arises therefore, is it more harmful to treat all women with GCK with insulin or is it more prudent to withhold insulin treatment. The initiation of insulin treatment in early gestation in pregnancies complicated by GCK has not been studied. To date, there is but one case report suggesting the harmful consequence of insulin in GCK pregnancies. In this report, the author suggested that insulin resulted in a small for gestational age (SGA) offspring [11].

The principal utility of insulin during pregnancy is to achieve euglycaemia and prevent excessive fetal growth with its' inherent complications. In women with GCK, fasting hyperglycaemia pre-dates the pregnancy and should theoretically increase the risk of congenital anomalies and rates of miscarriage. Hyperglycaemia in the first trimester is also a risk factor for macrosomia (Walsh, Mahony et al. 2011). These women are exposed to hyperglycaemia both pre-conception and indeed throughout all three trimesters.

Treatment of HNF1A-MODY in pregnancy is not fraught with the same dilemma as the management of GCK pregnancies as birth weight is reported not to be affected by mutation status of the offspring. The congenital risk in HNF1A-MODY pregnancies has not been reported nor has the incidence of neonatal hypoglycaemia although a recent case of a dizygotic twin pregnancy reported transient neonatal hypoglycaemia in the twin affected with HNF1A-MODY (Bitterman, Iafusco et al. 2016).

Studies performed pertaining to pregnancy and MODY to date (Spyer, Hattersley et al. 2001, de Las Heras, Martinez et al. 2010, Chakera, Spyer et al. 2014), have largely focused on birth weight and predicting gene expression in offspring of MODY carriers. The aim of the current study is to determine the outcomes and clinical management of hyperglycaemia using home blood glucose monitoring (HBGM) in pregnancies complicated by GCK and HNF1A mutations.

3.3 Methods:

3.3.1 Subjects

A total of 37 (n=12 GCK, n=25 HNF1A-MODY) women participated in the study; these women were identified as part of the MODY cohort screening study in the Mater Hospital (Kyithar, Bacon et al. 2011). The GCK mutations were p.D311fs, A378V, p.I293R, L146fs,D160N and p.Y61X (6 pedigrees). The HNF1A-MODY mutations P379T, p.L502fs, V351fsdelG, c.1502_6 G>A, p.G292fs, S335X, Ser352fs, p.R159Q, p.R200Q and P.E230fs (11 pedigrees).

We retrospectively studied the pregnancy outcome in these 37 women. The mutation status was known pre-pregnancy in 18.9% of the women studied. Therefore, the majority of the women were diagnosed with GDM and treated as such. This analysis involved both review of maternal charts and maternal recall. There were 132 pregnancies in these women. Data available in 89 cases included birth weight, gestation at delivery and mode of delivery. Complications which included congenital anomaly, birth injury, admission to neonatal intensive care unit (NICU) and prolonged neonatal hypoglycaemia were recorded. Genotyping of 36 offspring from these 37 women was known and a presumptive diagnosis of a GCK mutation was made in an additional 5 of the offspring of GCK mothers based on a fasting blood glucose of >5.6mmol/L on 2 separate occasions. The presumptive diagnosis was made in offspring aged 10 years and older.

Diabetes care in Dublin is delivered exclusively by a single team of endocrinologists, diabetes nurse specialists and specific obstetricians at each clinical site. All women who are diagnosed with pre-gestational or gestational diabetes not responding to dietary modification are treated with insulin. Women are treated with short acting insulin analogues pre-meals and NPH insulin up to 4 times daily (pre-meal and bed time). All women perform HBGM using glucometers 7 times daily (before and 1 hour post-meal and at bed). Glucose targets are fasting pre-meal and at bed of <5mmol/L and one hour post-meal readings of <7mmol/L. The same principals were applied to the clinical management of women diagnosed with GCK or HNF-1α MODY.

The HBGM recordings of 15 women, who were all managed according to our current guidelines for diabetes in pregnancy, with a GCK or HNF1A-MODY mutation referred to one of the three tertiary centres were analysed. The median of the highest and lowest blood glucose value for each day, at each time point, was averaged over a seven day period. The relative use of each insulin was determined. In addition, data on severe hypoglycaemic episodes was recorded. A severe hypoglycaemic episode was defined as one necessitating third party assistance. Serum fructosamine corrected for albumin was checked at clinic visits. Fructosamine is a glycated serum protein complex which reflects the average blood glucose concentration over the preceding 1-3 weeks. Fructosamine is therefore particularly useful given the rapid fluctuations in insulin dosage required during pregnancy. The treatment target for fructosamine is <250 µmol/l. Data on birth weight in the general gestational diabetes cohort was available through the annual report for 2013 (Rotunda annual report). Outcome variables recorded were rate of 1st trimester spontaneous abortion (miscarriage rates) following booking to clinic, major congenital anomalies rate (CAR) classified as per EUROCAT guide, pre-term delivery (<37 weeks) and infant birth weight. Centile growth was estimated using standard centile growth charts to eliminate the confounding factor of fetal sex and gestational age at delivery. Macrosomia was defined as being a birth weight greater than the 95th centile for that sex and gestation. Small for gestational age (SGA) was defined as a birth weight <10th centile.

3.4 Assays

All laboratory analyses were performed with commercially available standardized methods. The HBGM was performed on Ascensia Contour (Bayer, Germany) glucometers. Fructosamine was determined using a colorimetric assay (Roche Diagnostics, Belgium).

3.5 Genetic Analysis

Analysis of the GCK and the HNF1A gene was performed as described in section 2.4.

3.6 Statistical analysis

Quantitative variables were expressed as median and inter quartile range (IQR); qualitative variables were expressed as absolute number or percentages. Due to the study population number normal distribution could not be determined and non-parametric tests were applied. Mann-Whitney U test was used to compare quantitative variables and the chi square test was used for frequency comparisons. Only 2 tailed p values <0.05 were considered significant.

3.7 Results

3.7.1 Clinical parameters

The clinical characteristics of the participants are contained in Table 3.1.

	GCK-MODY	HNF1A-MODY	P value
Participants	12	25	n/a
Age	22±6	24±8	n/a
No. of pregnancies (n)	56	76	n/a
No. of miscarriages (n)	15	14	0.1
No. of live births (n)	41	65	n/a
Miscarriage gestation (weeks)	7.5 (6.2-8.7)	12 (10-14)	0.003
Weight 1st Trimester(kg)	76.4(59.4-81)	75.3 (62-82)	0.9
Weight 3rd Trimester (kg)	94 (76-97)	85.9 (73-91)	0.5
Fructosamine 1st Trimester (micro mol/l)	275 (250-306)	251 (231-283)	0.5
Fructosamine 3rd Trimester (micro mol/l)	238 (226-252)	230 (208-279)	0.8
No. of pregnancies Insulin commenced (n)	12	20	0.9

Gestation insulin commenced (weeks)	14 (8-29	15 (7-28)	0.8
Insulin dose (units per kg)			
Trimester 1	0.3(0.1-0.5)	0.4(0.2-0.6)	0.4
Trimester 2	0.4(0.2-0.7)	0.6 (0.4-0.7)	0.7
Trimester 3	0.8(0.8-1.5)	0.8(0.6-0.9)	0.7
Delivery gestation offspring (weeks)	40 (39-40)	39 (38-40)	0.02
Birth weight offspring kg/ centile	3.9 (3.2-4.5)/88th (33rd-99th)	3.6(3.1-4)|60th (19th-88th)	0.4
Macrosomia (>95th centile) (n)	12	7	0.01
C-section (insulin treated)(n)	5	5	0.8
C-section (non-insulin treated) (n)	4	2	0.7

Table 3-1: Clinical Characteristics GCK vs. HNF1A-MODY. n/a=not applicable

There was no significant difference in birth weight in GCK pregnancies when compared to HNF1A-MODY pregnancies (3.9 kg (3.2-4.5) vs. 3.6 kg (3.1-4), p=0.4). Insulin doses expressed in units per kg did not significantly differ between the groups throughout the trimesters (see figure 3.1).

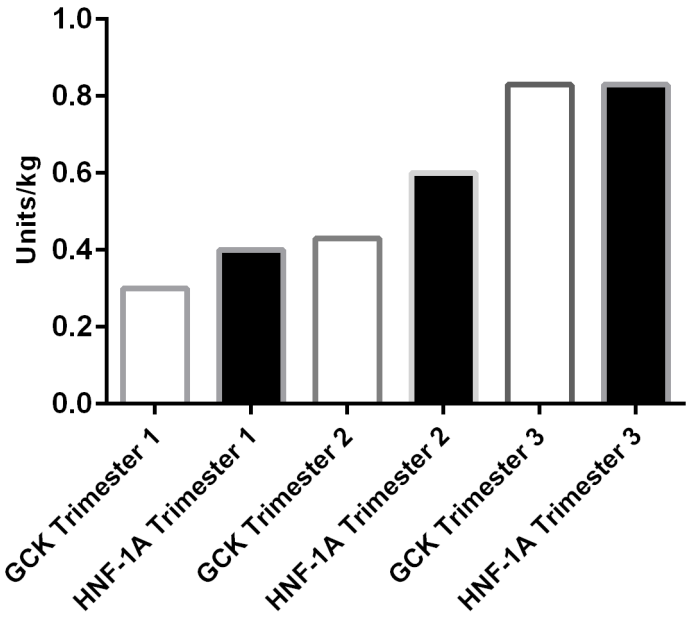

Figure 3-1: Units of Insulin per kg during each trimester. (n=5 GCK-MODY, n=10 HNF1A-MODY).

In both the GCK and the HNF1A-MODY group 7 injections (3 short-acting insulin analogues and 4 NPH injections) were required in the subgroup analysed. There was no significant difference in fructosamine levels between the GCK and the HNF1A-MODY group. The fructosamine at presentation in both the GCK and the HNF1A-MODY groups was higher than that noted in our general gestational diabetes mellitus cohort (236 µmol/l ±15).

The rate of miscarriage in the GCK group was 26.8% compared to 18.4% in the HNF1A-MODY group, which is comparable to the background population miscarriage rate of 15% (www.irishhealth.com). The miscarriages occurred at an earlier gestation in the GCK group than that noted in the HNF-1α group (7.5 weeks (6.2-8.7) vs. 12 weeks (10-14), p=0.003)

3.7.2 Home Blood Glucose Monitoring

In 15 of the pregnancies (n=5 GCK-MODY, n=10 HNF1A-MODY) HBGM data was studied. Figure 3.2 demonstrates the average glucose recordings for each of these groups of women respectively for one week during the 1st and 3rd trimester. Women with GCK have more dramatic glycaemic excursions than that noted for HNF1A-MODY pregnancies particularly in the first trimester. These excursions were significantly higher in the morning and early afternoon in the GCK group in the first trimester when compared to the HNF1A-MODY group (table 3.2). In the third trimester the pre-bedtime and fasting HBGM readings were significantly higher in the GCK group when compared to the HNF1A-MODY group (table 3.2).

First Trimester HBGM	**GCK-MODY (median [IQR]) mmol/mol**	**HNF1A-MODY (median [IQR]) mmol/mol**	**P value**
Pre-Breakfast	5.8 [5-6.4]	4.7 [4.3-4.9]	**0.01**
Post-Breakfast	8.6 [7.5-10.9]	6.2 [5.9-7.3]	**0.01**
Pre-Lunch	5.7 [4.8-6.8]	4.5 [4-5]	**0.05**
Post-Lunch	7.3 [6.6-8.9]	6.8 [6-7.7]	ns
Pre-Dinner	5.9 [5.2-5.9]	5.4 [4.7-6.5]	ns
Post-Dinner	6.6 [5.4-8.7]	6.8 [4.8-8.7]	ns
Pre-Bedtime	6.4 [5.5-7]	5.3 [4.8-6.4]	ns
Third Trimester HBGM	**GCK-MODY** mmol/mol	**HNF1A-MODY** mmol/mol	
Pre-Breakfast	5.5 [4.8-5.9]	4.82 [4-4.9]	**0.03**
Post-Breakfast	8 [6-8.5]	6.9 [6.6 -8.5]	ns
Pre-Lunch	4.8 [3.9 -6.5]	4.8 [4.7-6.3]	ns
Post-Lunch	7.4 [6.5-8.6]	6 [5.9-7]	ns
Pre-Dinner	5.2 [4.8-8]	5 [4.8-5.9]	ns
Post-Dinner	6.6 [6-7.7]	7 [6.5-7.4]	ns
Pre-Bedtime	6.8 [6-7.7]	5 [4.7-5]	**0.05**

Table 3-2: HBGM in first and third trimester in GCK and HNF1A- MODY pregnancies.

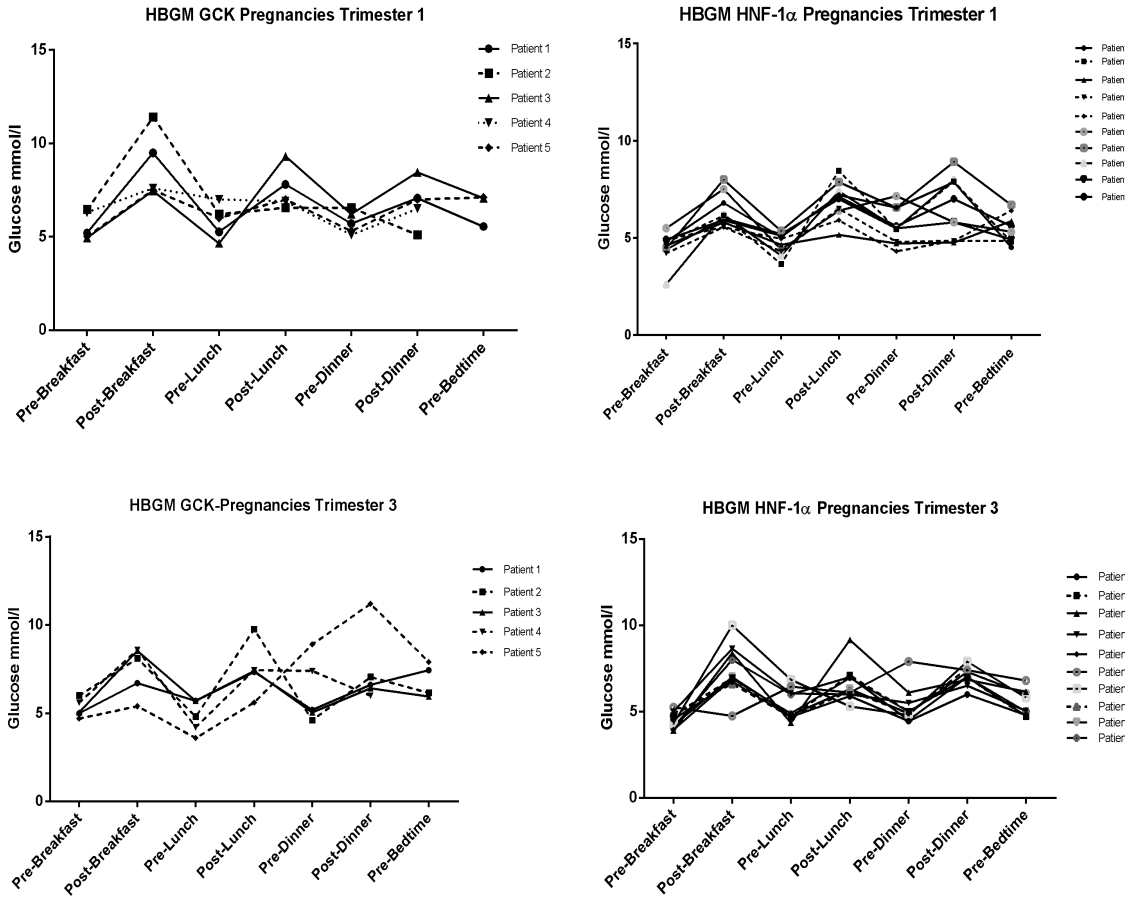

Figure 3-2: First Trimester HBGM and Third Trimester HBGM. All patients are treated with insulin therapy.

3.7.3 Effect of mutation status on birth weight of offspring in the GCK and HNF1A-MODY group

Birth weight in the GCK offspring who were non-affected (GCK N/N) was significantly higher than in the GCK affected (GCK N/M) group (4.8 kg (4.1-5.2) vs3.2 kg (3.1-3.7), p=0.01). There was no difference in the birth weight of HNF1A-MODY group between those offspring who were affected and those not carrying the mutation.

3.7.4 Effect of insulin treatment on outcome in GCK pregnancies.

There was no difference between the insulin treated (n=6) and non-insulin (n=17) treated groups with regards to mean birth weight in GCK pregnancies when mutation status of the offspring was not considered. In addition there was no statistically significant difference in the incidence of macrosomia in the offspring of mothers who were treated with insulin (see table 3.3).

	GCK-Offspring without mutation (GCK N/N) N=10		GCK-Offspring with mutation (GCK N/M) N=13	
	No insulin N=7	Insulin N=3	No insulin N=10	Insulin N=3
Birth weight (kg)	4.1 (3.3-4.9)	4 (3.8-4.2)	3.2 (3.1-3.7)	3.3 (3-3.9)
Gestational Age (weeks)	40 (38-40)	38 (38-40)	40 (39-40)	39 (38-40)
Week insulin commenced	n/a	13 (10-28)	n/a	30 (6-30)
Macrosomia (n)	4	1	0	0
SGA (n)	0	0	10	0
Complication rate (n)	3	0	10	0

Table 3-3: The effect of Insulin treatment on pregnancies complicated by GCK-MODY. n/a=not applicable

In the GCK-MODY N/M offspring group (n=13) treatment with insulin did not result in SGA (birth weight <10th centile) in any infant.

There was an increased rate of complications detected in 3/7 (37.5%) of offspring of the non-insulin treated GCK-MODY N/N group. These complications included a congenital anomaly (neural tube defect), shoulder dystocia and prolonged neonatal hypoglycaemia. There were no complications noted in the insulin treated GCK-MODY N/N group albeit a small group studied.

3.7.5 Outcome in pregnancies complicated by HNF1A- MODY

In the HNF1A-MODY group there was no difference between the insulin and non-insulin treated groups with regards to mean birth weight (3.2 kg (3.2-3.9) vs. 3.6 kg (3.2-4), p=0.5). There was a higher incidence of macrosomia amongst the HNF1A-MODY N/M offspring that were not treated with insulin , likewise there was a higher incidence of complications (16%; prolonged hypoglycaemia) amongst HNF1A-MODY N/N offspring not treated with insulin but these differences did not reach statistical significance.

There was no incidence of severe hypoglycaemia reported in either the GCK or the HNF1A-MODY group during all 3 trimesters.

3.8 Conclusions

To date, publications on MODY in pregnancy have focused largely on fetal size as dictated by mutation status or the mode of treatment used to treat hyperglycaemia during the pregnancy. To our knowledge, this is the first paper to observe glycaemic variability and detail fetal and neonatal outcomes in pregnancies complicated by MODY.

This is the first report on the clinical management of pregnancies complicated by HNF1A-MODY. We report that HNF1A-MODY is a relatively easy disorder to manage during pregnancy. The birth weight of the offspring was similar to that of the background population as has been previously reported (Estalella, Rica et al. 2007, Pearson, Boj et al. 2007). The current conventional clinical management of HNF1A-MODY during pregnancy utilising protocols as for pre-gestational diabetes does appear to be appropriate. In contrast, GCK-MODY is subject to hyperglycaemic variability and in clinical practice it can be quite difficult to achieve euglycaemia in this unique population. Individuals with GCK also maintain elevated glucose by mounting counter-regulating responses at higher glucose levels. The data from the current study demonstrate significant hyperglycaemic excursions in particular in the first trimester in the subset of GCK pregnancies examined with HBGM readings.

The fructosamine levels at the booking visit in both the HNF1A and GCK-MODY groups also reflect significant hyperglycaemia pre-pregnancy. The fructosamine level at presentation for both the GCK and HNF1A-MODY groups is higher than that of the general gestational diabetes mellitus cohort (236 µmol/l) and should alert the care provider to the possibility of pre-gestational diabetes and prompt screening for MODY in these cases. The fructosamine levels in both the HNF1A and GCK-MODY groups do decrease throughout the trimesters reflecting an improvement in glycaemic control.

We highlight, (see figure 3.2) that hyperglycaemia in GCK pregnancies is difficult to control, in particular, in the first trimester even in a specialised antenatal diabetes unit. Insulin initiated during the first trimester did not significantly decrease overall birth weight. We propose that pre-conception detection of a GCK mutation and pre-conception care for women with GCK is crucial to improve glycaemic control. Pre-conception counselling is known to improve early glycaemic control and reduce the risk of poor pregnancy outcomes (Kitzmiller, Block et al. 2008, Jensen, Korsholm et al. 2009). The attendance at a pre-pregnancy diabetes clinic facilitates counselling on diet, the initiation of high dose folic acid, discontinuation of potential teratogenic medication and, if necessary an optimal insulin dose regime pre conception. All of these measures may assist in the prevention of miscarriages and congenital anomalies.

We report a higher miscarriage rate (26.8%) in the GCK group when compared to the background population rate of 15%. As aforementioned in women with GCK-MODY, fasting hyperglycaemia pre-dates the pregnancy and should theoretically increase the risk of congenital anomalies and rates of miscarriage. The very high fructosamine levels at the booking visit in the GCK pregnancies reflect hyperglycaemia pre-conception. Hyperglycaemia, even in the first trimester is also an established risk factor for macrosomia (Walsh, Mahony et al. 2011). As stated by Spyer et al. the fetal insulin hypothesis postulates that unaffected offspring of mothers with a GCK mutation produce excess insulin in response to raised maternal blood glucose with resultant macrosomia (Spyer, Macleod et al. 2009). We report an increased macrosomic rate in GCK N/N group not treated

with insulin (62.5%) and consequently an increased rate of neonatal co morbidities (37.5%).

In order to appreciate the most appropriate treatment of GCK pregnancies one would have to complete a RCT comparing the early initiation of insulin therapy to diet therapy or indeed no intervention to determine the effect on fetal size. Due to the relative rarity of GCK-MODY it is not clinically possible to perform a prospective study. Of late, it has been recommended to treat pregnant women with GCK with insulin only if serial fetal ultrasound monitoring suggests the development of macrosomia i.e. if the abdominal circumference (AC) >75th when it is assumed that the fetus has not inherited the mutation (Buchanan, Kjos et al. 1994, Spyer, Hattersley et al. 2001).However, data on the performance of this recommendation are lacking and we would suggest that initiation of insulin at a late gestation is unlikely to prevent macrosomia in GCK offspring. The principal problem is that fetal genotype cannot be safely predicted. It is not justifiable to use chorionic biopsy or amniocentesis for this purpose given the inherent risk of miscarriage with the procedure (Colom and Corcoy 2010).

The lack of available clinical studies on pregnancies complicated by GCK and HNF1A-MODY mutations necessitates the use of guidelines designed for the management of gestational diabetes. For optimal glycaemic control in pregnancies complicated by pre-existing diabetes the ADA recommend provision of basal and prandial insulin needs with intensified insulin regimes (Kitzmiller, Block et al. 2008). In the current study, we did not note any adverse effect from insulin therapy. The insulin doses required did increase as the pregnancies progressed however there was no incidence of severe hypoglycaemia. We did not note any incidence of SGA. This finding was similar to that noted in the literature (Estalella, Rica et al. 2007). Fetal size and placental weight are correlated in the third trimester (Molteni, Stys et al. 1978). The effect of mutation status on placental weight was studied by Shields et al. (Shields, Spyer et al. 2008). There was no difference in placental weight in both the GCK N/N and GCK N/M groups between those treated with insulin and those not treated with insulin (680 g vs. 680g, respectively, p=0.69)

We recognize that there are limitations to this study including the relative, although unique, small study population. In addition, retrospective analysis of chart data was required for a proportion of the pregnancies. Reassuringly, however, the findings of the study are comparable to those previously reported. In addition, this is the first study to our knowledge that contains HBGM throughout the pregnancies of women with GCK and HNF1A-MODY. The uniform management of pregnancies complicated by diabetes according to the current guidelines is a further merit of the study.

The data on the clinical management of pregnancies complicated by MODY is very limited. Relative to the incidence of other forms of diabetes, GCK and HNF1A-MODY are rare disorders. This study demonstrates that the current management of HNF1A-MODY pregnancies appears to be appropriate. In GCK pregnancies, however, the glycaemic variability that we have clinically noted warrants attention. Preconception knowledge of a GCK mutation and subsequent planning in a GCK pregnancy is important, reiterating the necessity for diagnosing female family members of a GCK pedigree. At present, given the limitations of ultrasound surveillance and the lack of safety data supporting predictive genetic testing we cannot safely predetermine the fetal genotype. To date, this is the largest study performed specifically looking at glycaemic excursions in pregnancies complicated by GCK and HNF1A-MODY.

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Chapter 4

Prolonged episodes of hypoglycaemia in HNF4A-MODY mutation carriers with IGT. Evidence of persistent hyperinsulinism into early adulthood.

The contents of this chapter are published as: *"Prolonged episodes of hypoglycaemia in HNF4A-MODY mutation carriers with IGT. Evidence of persistent hyperinsulinism into early adulthood*", S. Bacon, M P Kyithar, E M Condron, Vizzard , M. Burke , M.M. Byrne. Acta Diabetologia 2016 Dec :53 (6):965-972. PMID 27552834.

4 Chapter 4: "Prolonged episodes of hypoglycaemia in HNF4A-MODY mutation carriers with IGT. Evidence of persistent hyperinsulinism into early adulthood.

Abstract:

Introduction:

HNF4A is an established cause of Maturity Onset Diabetes of the Young (MODY). Congenital hyperinsulinism can also be associated with mutations in the *HNF4A* gene. A dual phenotype is postulated in *HNF4A* mutation carriers with hyperinsulinaemic hypoglycaemia in the neonatal period progressing to diabetes in adulthood. The nature and timing of the transition to diabetes remains poorly defined. In the current study we aimed to establish changes in glycaemia and insulin secretion in response to oral glucose over a 6 year period. In addition, we investigated glycaemic variability and hypoglycaemia in HNF4A-MODY mutation carriers using a continuous glucose monitoring system (CGMS).

Methods:

A 120 minute oral glucose tolerance test (OGTT) with measurement of glucose, insulin and C-peptide at 30 minute intervals was performed in n=14 *HNF4A* participants with diabetes mellitus (DM), n=7 *HNF4A*-IGT and n=10 age-and BMI-matched MODY negative family members. Serial assessment was performed in the *HNF4A*-IGT cohort over a 6 year interval. In a subset of HNF4A-MODY mutation carriers, CGMS was applied over a 72 hour period.

Results:

We did not detect a deterioration in glycaemic control in a *HNF4A*-IGT cohort over a 6 year period (initial AUC glucose: 29.5 mmol/L/120min (27-42) vs. AUC glucose @ 6 yr. follow-up: 34.8 mmol/L/120min (28.7-38), p=0.9). Likewise, there was no change in AUC insulin over the study period (888 mmol/L/120min (612-1883) vs. 932 (663-1117) mmol/L/120min, p=0.7). CGMS profiling demonstrated prolonged periods of hypoglycaemia in the *HNF4A*-IGT group when compared to the *HNF4A*-DM group (432 mins. vs. 138 mins. p=0.04).

Conclusion:

In a young adult *HNF4A*-IGT cohort, we demonstrate preserved glucose, insulin and C-peptide secretory responses to oral glucose over a 6 year period. Utilising CGMS, prolonged periods of hypoglycaemia are evident despite a median age of 21 years. We have proposed that a prolonged hyperinsulinaemic phase into adulthood is responsible for the notable hypoglycaemic episodes.

4.1 Introduction:

Maturity onset diabetes of the young is an autosomal dominant form of diabetes which accounts for 1-2% of all diabetes (Frayling, Evans et al. 2001) . The most common form of MODY in the UK and Ireland is attributed to a mutation in the transcription factor; hepatocyte nuclear factor 1 alpha (*HNF1A*-MODY) (Frayling, Evans et al. 2001, Kyithar, Bacon et al. 2011). In comparison, *HNF4A*-MODY is a relatively rare disorder accounting for approximately 6-10% of all MODY cases in the UK and Ireland (Shields, Hicks et al. 2010). The HNF4A protein is an orphan member of the nuclear receptor family of ligand-activated transcription factors. Impaired beta-cell function is a known feature of both *HNF4A*-MODY mutation carriers with and without clinically diagnosed diabetes (Herman, Fajans et al. 1994, Tripathy, Carlsson et al. 2000). An annual deterioration of 1-4% in glucose stimulated insulin secretion in the *HNF4A*/RW pedigree is reported in the literature (Fajans and Brown 1993). It has also been demonstrated that deletion of *hnf4a* in the beta-cells of animal models results not only in impaired glucose stimulated insulin secretion but interestingly also in fasting hyperinsulinism (Gupta, Vatamaniuk et al. 2005, Pearson, Boj et al. 2007). Of late, heterozygous mutations in the *HNF4A* gene have been shown to be a cause of congenital hyperinsulinism (Pearson, Boj et al. 2007, Flanagan, Kapoor et al. 2010). It has been postulated, therefore, that a "biphasic phenotype" exists in a subset of *HNF4A*-MODY carriers with a period of hyperinsulinaemic hypoglycaemia as a neonate and subsequent diminished insulin secretion and diabetes in later adulthood. In those affected with congenital hyperinsulinism, macrosomia is a consistent finding. The hyperinsulinism attributed to *HNF4A*-MODY can be

transient or persistent. It has a tendency to be responsive to diazoxide treatment with withdrawal of same ranging from hours to 12 years (Kapoor, Locke et al. 2008, Flanagan, Kapoor et al. 2010, McGlacken-Byrne, Hawkes et al. 2014).

The *HNF4A*-MODY cohort in the Mater Hospital are diagnosed and clinically managed at a dedicated MODY centre enabling the longitudinal study of the pathophysiology of the mutation. The penetrance of *HNF4A*-MODY is 95% by the age of 40 years; therefore it is recommended that genetically confirmed normoglycaemic *HNF4A*-MODY mutation carriers have an annual oral glucose tolerance test (OGTT) to detect the development of diabetes (Pearson, Boj et al. 2007). In performing OGTTs on the *HNF4A* cohort we have previously noted fasting hypoglycaemia in a proportion of adult mutation carriers. This would suggest that the described so-called "biphasic phenotype" associated with *HNF4A*-MODY persists beyond the neonatal period. To this end, in the current study we aimed to establish changes in glycaemia and insulin secretion in response to oral glucose over a 6 year period. We have also sought to elucidate this phenomenon further through the use of a continuous glucose monitoring system (CGMS) to detect glycaemic variability and , in particular, episodes of hypoglycaemia.

4.2 Research Design and Methods:

4.2.1 Phenotyping of the *HNF4A*-MODY cohort

The current study included 14 individuals with *HNF4A*- DM, and 7 *HNF4A*-MODY individuals with IGT. Further details of the mutations of this cohort are contained in the Table 4.1. A group of 10, age- and BMI-matched normoglycaemic mutation negative family members formed a control group (hereafter referenced to as normal controls) to the IGT group for comparative purposes. The clinical

characteristics of all three groups are detailed in Table 4.2. As part of the MODY screening programme, a full medical history and physical examination is performed. Anthropometric measurements including BMI were recorded. A 75 g oral glucose tolerance test (OGTT) is performed on participants with *HNF4A*-MODY after an overnight fast with measurement of glucose, insulin, C-peptide and glucagon at baseline and 30 min intervals to determine degree of glucose intolerance and insulin secretory response. If applicable, oral hypoglycaemic agents are stopped at least 48-h prior to testing while long acting insulin therapy is stopped for 24-h and short acting insulin for 12-h before OGTT. The oral glucose insulin sensitivity (OGIS) is calculated as previously described (Mari, Pacini et al. 2001). Blood samples are drawn for the measurement of HBA_{1c}, fasting lipids, full blood count, thyroid function, renal and liver profiles, glutamic acid decarboxylase (GAD65) auto antibodies, and pancreatic islet cell auto antibodies (ICA). Routine clinical follow up involves annual OGTTs in the *HNF4A*- IGT group. Details of the particular class of medication used in the group are detailed in Table 4.2. The majority of the *HNF4A*- DM group were treated with a sulphonylurea (gliclazide). The median dosage of gliclazide required to maintain euglycaemia in the *HNF4A*- DM group was 180 mg (40-290) per day. Of the 5 *HNF4A*- DM individuals who participated in the CGMS arm, 4 were treated with a sulphonylurea and 1 was treated with metformin (intolerant of gliclazide secondary to hypoglycaemic episodes). The 5 *HNF4A* –IGT individuals were treated with diet alone. The study was approved by the Research Ethics Committee at the Mater Misericordiae University Hospital Dublin and all participants gave informed written consent.

Location in HNF4A	cDNA level	Protein level	Protein effect	No. of patients N=21	No. of families N=8
Exon 1	c. 3G>A	p.Met1?	Missense	3	1 (2DM, 1 IGT)
Exon 4	c.340C>T	p.Arg114Trp	Missense	1	1 (DM)
Exon 4	c.460delC	p.Arg154fs	Frameshift	1	1 (DM)
Exon 8	c.868C>T	p.R290C	Missense	8	1 (4 DM, 4 IGT)
Exon 8	c.869G>A	p.Arg290His	Missense	2	1 (DM)
Exon 9	c.1117C>T	p.Gln373Ter	Nonsense	1	1 (DM)
Intron 7	c.872-1G>T	p.0?	Splice site	4	1 (3 DM, 2 IGT)
P2 Promoter	c.-179T>C	p.0?	Aberrant gene transcription	1	1 (DM)

Table 4-1: Mutation details of cohort. DM= diabetes mellitus, IGT=impaired glucose tolerance.

4.2.2 Data on Continuous Glucose Monitoring System (CGMS)

A subset of 10 individuals with *HNF4A*-MODY consented to CGMS profiling. Five *HNF4A*- DM and five *HNF4A*-IGT individuals participated in this study arm. A blinded iPro 2 Continuous Glucose Monitor (Medtronic Inc.) was inserted by a single researcher. Participants were provided with full instructions on CGM device care and calibration. Participants were instructed to wear the device for a minimum period of 72 hours. They were trained on using One Touch Contour glucometer and advised to facilitate calibration of the CGM device by performing a capillary blood glucose check 1 hour and 3 hours following device insertion. Thereafter, a minimum of 4 glucometer recordings ideally pre-meals and bed-time were advised. Participants were encouraged to maintain their current dietary and

activity habits for the study duration. A simple log of dietary intake, activity levels, meal times and sleep/wake times were recorded. On completion of the minimum 72 hour study period, the participant returned to the metabolic research unit in the Mater Misericordiae University Hospital where the device was removed by the same researcher. Using the iPro2 recorder package, sensor readings were converted into excel format for each subject and each individual trace was analysed.

4.2.3 CGM parameter calculations

The parameters of glycaemic variability utilised were mean blood glucose (MBG), standard deviation of blood glucose (SDBG) and mean amplitude of glycaemic excursions (MAGE). The MBG was calculated for the arithmetic average of all glucose readings. The SDBG represented the SD calculated for the overall 72 hour period of CGM readings. MAGE calculation was made by measuring the arithmetic mean of the difference between consecutive peaks and nadirs provided that the difference was greater than the SD around the mean glucose values. An asymptomatic hypoglycaemic episode was defined as per ADA criteria as a blood glucose level of <3.9 mmol/L. Since the glycaemic threshold for activation of glucagon and epinephrine secretion as glucose levels decline is normally 3.9 mmol/L and since the antecedent plasma glucose concentrations of <3.9mmol/L reduce sympathoadrenal responses to subsequent hypoglycaemia, this criterion sets the lower limit for the variation in plasma glucose in non-diabetic, non-pregnant individuals as the conservative lower limit for individuals with diabetes.

4.2.4 Biochemical Analysis

All laboratory analyses were performed with commercially available standardized methods. The plasma glucose concentration was measured using Beckman Synchron DXC800 (Beckman Instruments Inc, Brea, USA).HbA_{1c} was determined using high performance liquid chromatography (Menarini HA81-10, Rome, Italy). Insulin and C-peptide were analysed using Immulite 2000 immunoassay (Siemens Healthcare Diagnostics, Deerfield, IL, USA). Anti-GAD_{65} antibodies were analysed by ELISA (Euroimmun, Luebeck, Germany). ICA was performed by indirect immunofluorescence test by the supra-Regional Protein Reference Unit and Dept. of Immunology in Sheffield, UK. Glucagon was measured using a radioimmunoassay specific for pancreatic glucagon (Eurodiagnostica, Sweden).

4.2.5 Genetic Analysis

Analysis of the HNF4A gene was performed by direct sequencing of the P2 promoter, exon 1d and exons 2-10 (HNF4A sequence accession number NM 175914.3). Sequencing of the HNF4A gene was performed by IntegraGen (Bonn, Germany) in 2006-2007 and the Molecular Genetics Laboratory (Exeter, UK) in 2008-2014.

4.2.6 Statistical Analysis:

Statistical analyses were performed using Prism/MatLab and its statistical toolbox (The MathWorks Inc.)Natick, USA). Parametric data are given as mean and SEM and compared using student T tests. Non-parametric data are given as median and interquartile range (IQR) and were compared by Mann-Whitney U test. Hypotheses tests were considered statistically significant if $p<0.05$.

4.3 Results:

The clinical characteristics of the *HNF4A*- DM, *HNF4A*-IGT and the normal controls are contained within Table 4.2. The *HNF4A*- DM group were older with a higher BMI (although still within the normal range) than both the *HNF4A*- IGT and the normal controls. The median birth weight of the *HNF4A*-DM cohort was 3840g (3665-4728) compared to 4280g (3855-5018) in the *HNF4A*-IGT group (p=0.4). Only one participant with *HNF4A*-IGT had prolonged hypoglycaemia that occurred at birth and lasted 8 days in total.

Parameter	*HNF4A*-DM (1)	*HNF4A*-IGT (2)	Control (3)	P values	P values	P values
	N=14	N=7	N=10	(1 vs. 2)	(1 vs. 3)	(2 vs. 3)
Current Age (yrs.)	44(37-54)	21 (14-29)	21 (13-26)	<0.0001	<0.0001	0.2
Age @ diagnosis of diabetes/IGT (yrs.)	28 (23-36)	13 (12-19)	N/A	0.003	N/A	N/A
Age @ diagnosis of MODY (yrs.)	37 (32-43)	19.5 (14-23)	N/A	0.0002	N/A	N/A
BMI (kg/m^2)	24.3 (22.7-26)	20 (17-21)	20.4 (18-23)	0.0009	0.003	0.3
Treatment	SU=9,Met=2 Diet =1,Insulin=1	Diet	N/A	N/A	N/A	N/A
HbA_{1c} %/(mmol/mol)	6.9 (6.2-7.9) (52(44-63))	5 (4.8-5.5) (31 (29.7-37))	5.2 (4.9-5.3) (33 (30-34))	0.001	<0.0001	0.7
2 hr glucose (mmol/L)	16.7 (12.8-22)	8.2 (7.9-9.1)	5 (3.7-5.6)	<0.0001	<0.0001	0.0001
Fasting Insulin (pmol/L)	25(14.4-39.1)	31.7 (14.4-85.3)	16.9 (14.4-40)	0.5	0.8	0.2
Fasting glucagon (pmol/L)	125 (112.5-125)	125(121.3-128.8)	125 (115-135)	0.9	0.4	0.6

Table 4-2: Clinical Characteristics of all groups studied.

N/A=not applicable..SU=sulphonylurea. Met=metformin

4.3.1 Insulin & C-peptide responses to oral glucose in the HNF4A-DM, IGT and normal control groups.

The glucose, insulin and C-peptide responses to oral glucose in each group studied are illustrated in Figure 4.1. (The AUC values for glucose, insulin and C-peptide are contained in Table 4.3).There was, as expected, a significant difference in the AUC glucose between the *HNF4A*- IGT and normal control groups (29.5 mmol/L/120 min (27-42) vs. 24.5 mmol/L/120 min (21-25), p=0.001). There was no difference in the AUC insulin or AUC C-peptide between the *HNF4A*- IGT and normal controls. In fact, a significantly higher insulin level was noted at 90 mins. in the HNF4A-IGT group when compared to the normal controls (90 mins: 278.5 pmol vs. 207 pmol, p=0.05). Glucagon levels at baseline were similar in all groups studied.

In accordance with previous publications, we note a mean incremental glucose response (0-120 mins) of 10 mmol/L in the *HNF4A*-DM group (Stride, Vaxillaire et al. 2002). The OGIS value, reflecting insulin sensitivity, did not differ between *HNF4A*-DM group and the *HNF4A*-IGT group (356 ml/min/m^2 (305-427) vs. 503 ml/min/m^2 (415-541), P=0.08). There was no difference in the OGIS value between the normal control group and the *HNF4A*-IGT group (499 ml/min/m^2 (482-511) vs. 503 ml/min/m^2 (415-541), p=0.9).

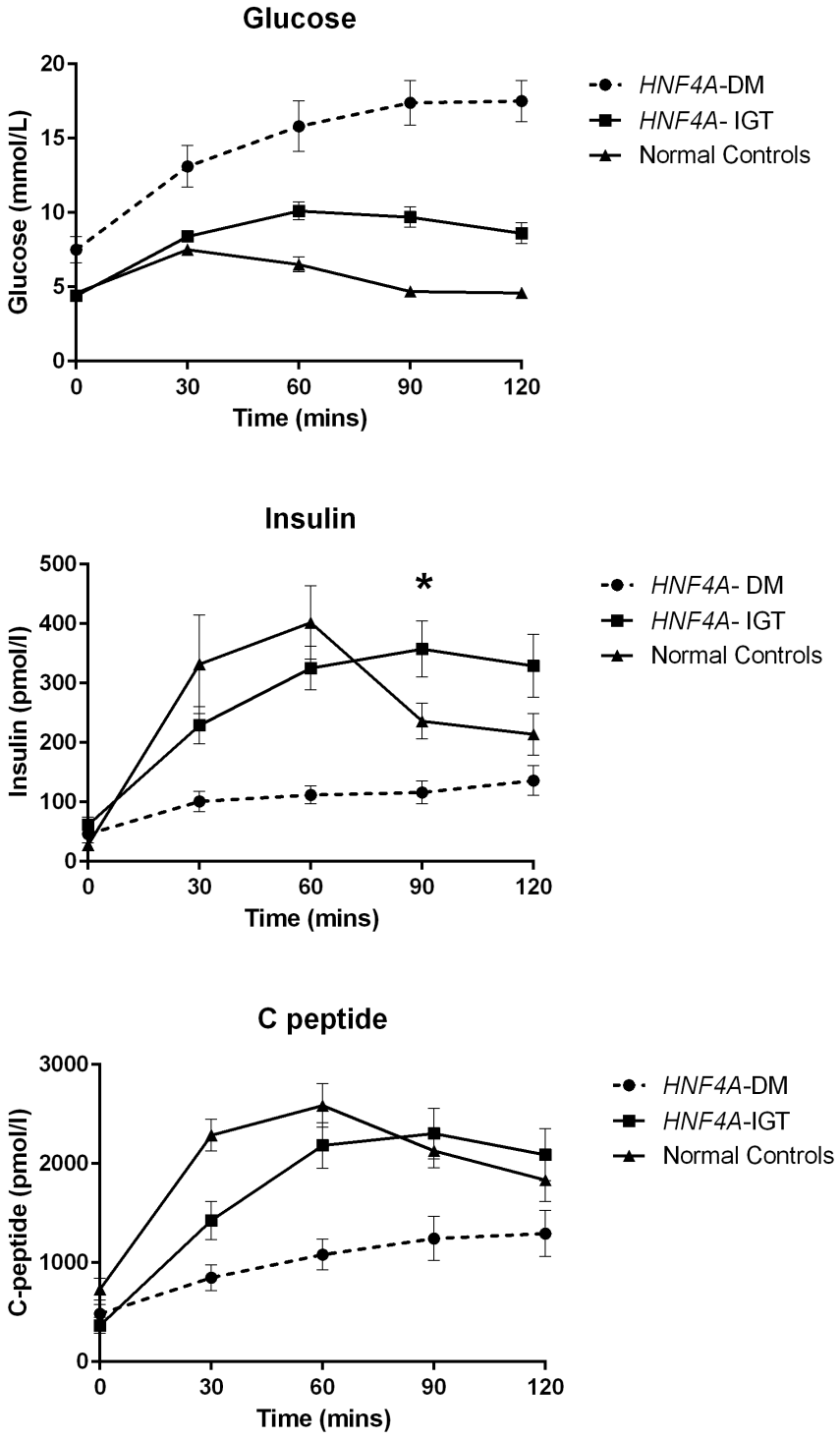

Figure 4-1 a,b,c: OGTT profiles demonstrating glucose (a), insulin (b) and C-peptide (c) secretory response to oral glucose in the *HNF4A*- DM, *HNF4A*- IGT and normal control groups at initial diagnosis of MODY. * denotes significant p value of <0.05

Parameter	HNF4A-DM (1) N=14	HNF4A-IGT (2) N=7	Negative controls (3) N=10	P values (1 vs. 2)	P values (1vs. 3)	P values (2 vs.3)
AUC glucose mmol/L/120min	58 (40.8-70)	29.5 (27-42)	24.5 (21-25)	0.0003	<0.0001	0.001
AUC insulin pmol/L/120min	415 (225-574)	995 (799 -1230)	960 (679-1487)	<0.0001	0.0002	0.9
AUC C peptide pmol/L/120min	3608 (2324-5926)	7834 (5348-9962)	7928 (7048-9129)	0.05	0.0009	0.4

Table 4-3: The AUC values for glucose, insulin and C-peptide in the *HNF4A*-DM, IGT and normal control groups.

4.3.2 Preservation of insulin and C-peptide response on serial oral glucose tolerance testing over a 6 year period

Preservation of insulin and C-peptide secretion is observed over the duration of screening in the *HNF4A*- IGT cohort. Serial oral glucose tolerance testing in the *HNF4A*- IGT cohort over a period of 6 years is illustrated in Figure 4.2. There was no difference in the serial assessments of AUC for glucose (initial AUC glucose: 29.5 mmol/L/120min (27-42) vs. AUC glucose @ 6 yr. follow-up: 34.8 mmol/L/120min (28.7-38), p=0.9) or for AUC insulin over the study period (888 mmol/L/120min (612-1883) vs. 932 mmol/L/120min (663-1117), p=0.7). Likewise, there was no change in AUC C-peptide over the study duration (6043 pmol/L/120min (3806-10919) vs. 6043 pmol/L/120min (3806-10919), p=0.8). Over the study period, there was no difference in HbA_{1c} (initial $HbA_{1c;}$ 5.1% (4.9-5.3) (32 mmol/mol (30-34)) vs. 6 year follow-up HbA_{1c}; 5.1% (4.9-5.4) (32mmol/mol (31-34.5)), p=0.9).

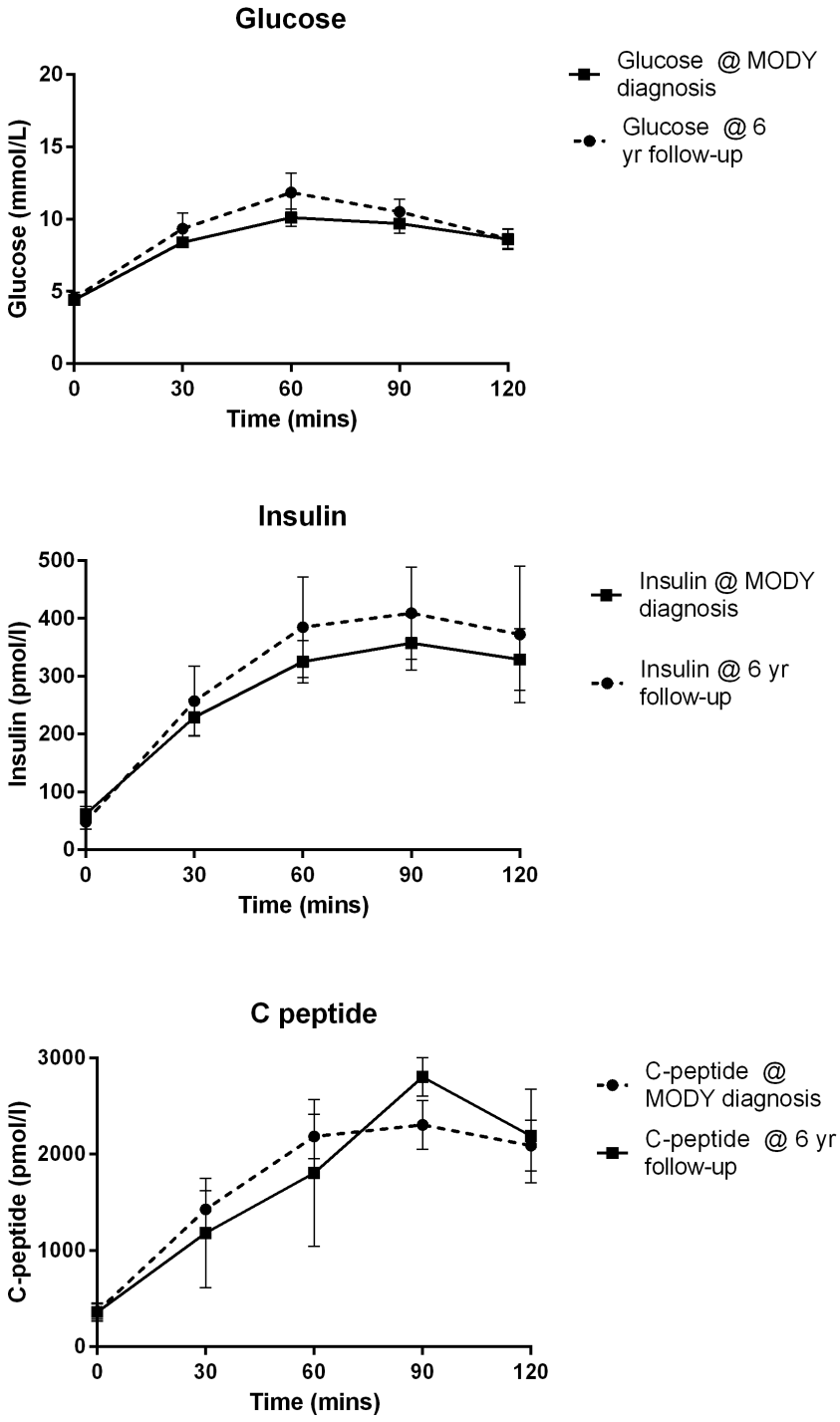

Figure 4-2 a,b,c : Serial OGTTs in the *HNF4A*- IGT cohort over a period of 6 years. 2a=Glucose profile, 2b=Insulin profile, 2c=C-peptide profile.

4.3.3 Minimal glycaemic variability and prolonged hypoglycaemia is observed amongst HNF4A-MODY mutation carriers using CGMS.

Parameters to evaluate the glycaemic variability were MAGE and SD of the arithmetic average of interstitial glucose. The MAGE reference range in a normal non-diabetic population is given as 0.0-2.8 mmol/L (Zhou, Li et al. 2011). We note minimal glycaemic variability as demonstrated by a low MAGE in both the *HNF4A*-DM was 3.2±0.5mmol/L and in the *HNF4A*-IGT group; 2.2±0.2mmol/L.

The mean blood glucose recorded using CGMS was 7.5±0.2 mmol/L in the *HNF4A*-DM group and 4±0.8 mmol/L in the *HNF4A*- IGT group (p=0.0007).The averages of 11,729 interstitial glucose values retrieved from CGMS compared well with their corresponding finger stick capillary blood glucose with a positive correlation of r = 0.8 between these two values. In the *HNF4A*-DM group the mean duration of hypoglycaemia was 3.2%±0.7 (138 mins. ±30.2) over a 72 hour period. In the diet-treated *HNF4A*- IGT group the mean duration of hypoglycaemia over the 72 hour period was 10.6%±2.9 (432 mins.±125.5) over a 72 hour period. These episodes of hypoglycaemia occurred predominantly in the nocturnal and late post-prandial period (120-180 mins. post prandial) and were asymptomatic. A representative CGMS profile of a HNF4A-*IGT* participant over a 72 hr. period is illustrated in Figure 4.4. Prolonged episodes of nocturnal hypoglycaemia are clearly illustrated.

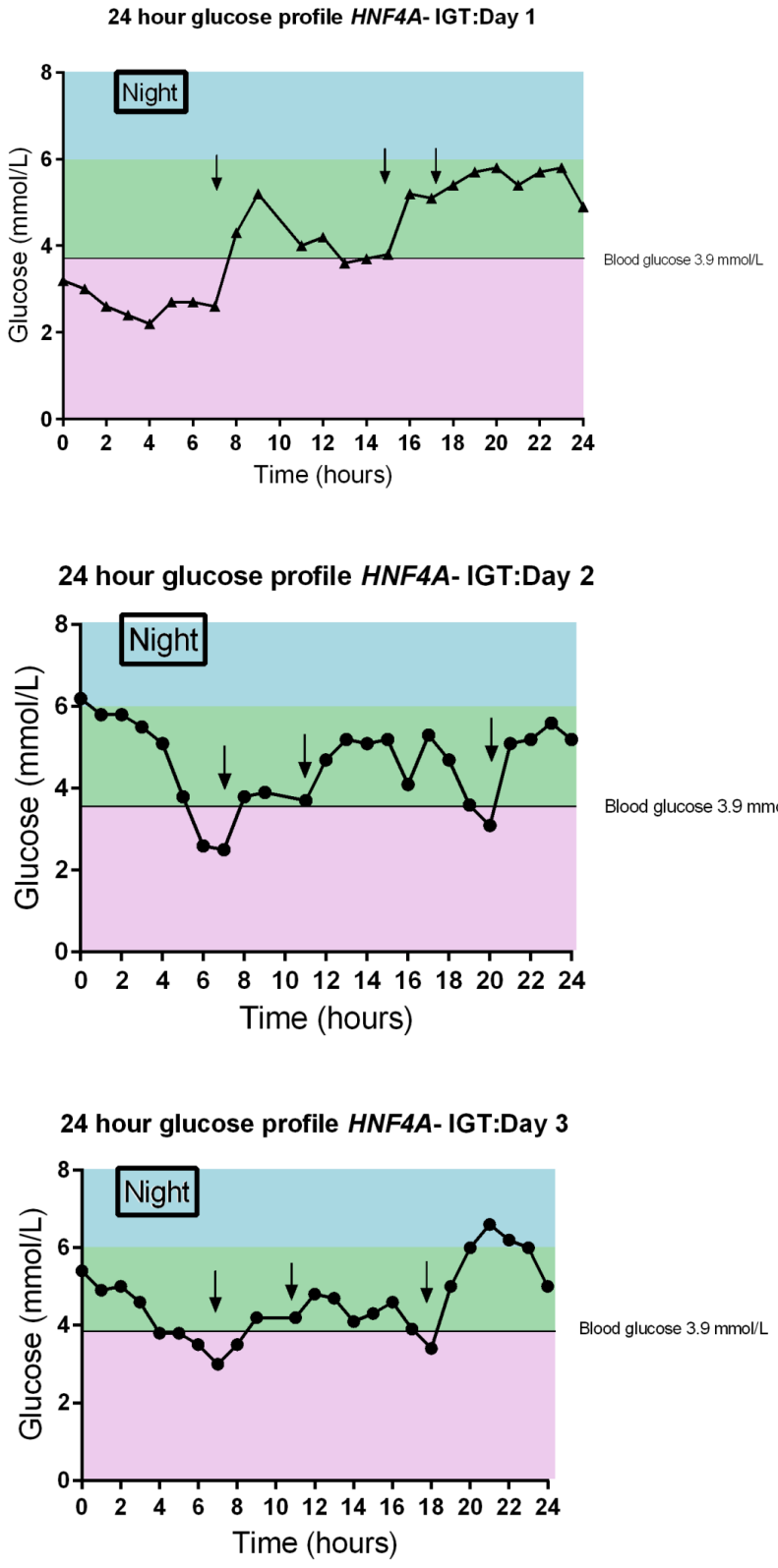

Figure 4-3: 72 hour continuous glucose monitoring profile of a representative individual with *HNF4A*-IGT. Meal times are denoted by the downward arrow. Pink colour represents glucose levels in the hypoglycaemic range <3.9 mmol/L. Green colour represents glucose levels in the range of 3.9-6.0 mmol/L. Blue represents glucose levels >6.0 mmol/L.

4.4 Conclusions

Previous literature describes a 'biphasic phenotype' in individuals with HNF4A-MODY, with an initial period of hyperinsulinaemic hypoglycaemia progressing ultimately to relative insulin deficiency and diabetes in adulthood, with an average age of onset of diabetes at 25 years of age (Pearson, Boj et al. 2007) . There is a paucity of literature available on glycaemic variability in late childhood through adolescence and into early adulthood in non-diabetic HNF4A-MODY carriers. In the current study, using serial OGTT assessment, we note no deterioration in glucose control into adolescence and beyond in a *HNF4A*- IGT group over a 6 year period. Despite a median age of 21 years we did not determine the gradual decline in insulin secretion as previously reported nor did we observe the so-called "switch point" to diabetes in this group (Fajans and Bell 2007). Interestingly, this cohort maintained a relatively low fasting blood glucose levels and a persistent hyperinsulinaemia in response to oral glucose as demonstrated by the significant elevation in insulin at 90 minutes on the OGTT.

CGMS profiling demonstrated prolonged episodes of hypoglycaemia in particular in the diet-only treated *HNF4A*-IGT cohort. The mean duration of hypoglycaemia over the 72 hour period was significantly higher in the *HNF4A*-IGT group when compared to the *HNF4A*-DM group (432 mins. vs. 138 mins. p=0.04). The studies available on CGMS in a non-diabetic population describe an average time spent in hypoglycaemia of 30±26 mins. over a 72 hour period and 47 mins. over a 66 hour period (Zhou, Li et al. 2011, Hill, NR et al. 2011). Therefore, the *HNF4A*-IGT group experienced up to a 13-fold increase in the period of time spent in hypoglycaemia when compared to a non-diabetic population. An additional finding on CGMS monitoring was evidence of minimal glycaemic excursions in the

HNF4A-MODY cohort. The MAGE values in both groups were similar to that reported in a normal non-diabetic population (Zhou, Li et al. 2011). This finding perhaps reflects the preservation of insulin secretion or a good response to sulphonylurea in the DM group or to diet therapy in the IGT cohort.

Deletion of *hnf4a* in the beta-cells of mice results in a mildly reduced blood glucose level associated with hyperinsulinism in both the fasting and fed state at 3-5 months of age (Gupta, Vatamaniuk et al. 2005). A study by Pearson et al also demonstrated low blood glucose levels secondary to hyperinsulinism at the earlier neonatal period in a beta-cell specific *hnf4a* knock out mouse model (Pearson, Boj et al. 2007). In human observational studies, it is apparent that there are dual opposing roles of HNF4A during the lifetime of a mutation carrier (Arya, Rahman et al. 2014). The initial beta-cell hypersecretory phase in the fetal and neonatal period results from islet cell hyperplasia and macrosomia. In the subsequent phase, beta-cell exhaustion results in altered glucose homeostasis and ultimately diabetes. The temporal pattern of HNF4A in humans is heterogenous, with the initial phase of neonatal hypoglycaemia requiring diazoxide for up to 1 year. The longest reported case of neonatal hypoglycaemia requiring treatment in a HNF4A-MODY mutation carrier is 12 years (Flanagan, Kapoor et al. 2010). Our study demonstrates a persistent low fasting blood glucose and HbA_{1c} over time in a HNF4A-IGT cohort with preserved insulin secretion levels and a similar basal glucagon level to a non-diabetic population. Therefore, we propose that the hypoglycaemia demonstrated by CGMS in the *HNF4A*- IGT cohort maybe due to a prolonged hyperinsulinaemic hypoglycaemic phase into adulthood.

A previous publication noted diminished glucagon secretory responses to arginine in non-diabetic HNF4A-MODY mutation carriers (Herman, Fajans et al. 1997). Of late, literature has also demonstrated the localisation of HNF4A to the

alpha cell in human islet studies and has proposed a role for HNF4A in the expression of SGLT2 (the sodium glucose co-transporter) (Bonner, Kerr-Conte et al. 2015) .These findings would support a role for an alteration in glucagon secretion in the development of asymptomatic hypoglycaemia in this cohort. However, in the current study, we did not appreciate a significant difference in the basal glucagon profile between the groups.

A further postulated mechanism regarding the switch from hypo to hyperglycaemia in animal models involves a loss of interaction between HNF4A and other nuclear factors such as peroxisome proliferator activated receptor α (PPARα)(Rahman, Nessa et al. 2015) . The expression of PPARα is reduced in *hnf4a* deficient beta-cells. PPARα plays a role in the beta-oxidation of fatty acids. It has been postulated, therefore, that insulin levels are increased in the *hnf4a* mutant mouse model as a result of the lipid accumulation in the cytoplasm (16). Overall, our current findings support the findings of the animal studies with reduced fasting blood glucose and a prolongation of the hyperinsulinaemic phase beyond the neonatal period into early adulthood.

In conclusion, young *HNF4A*-IGT adults demonstrate preserved glucose, insulin and C-peptide secretory responses to oral glucose over a 6 year period. In this cohort, utilising CGMS, prolonged periods of hypoglycaemia are evident despite a median age of 21 years. We have proposed mechanistic theories that a prolonged hyperinsulinaemic phase into adulthood is responsible for the notable hypoglycaemic episodes. The incorporation of such information into the clinical management of *HNF4A*-MODY enables increased detection and self-awareness of hypoglycaemia and ultimately, improved patient care.

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Chapter 5

Circulating cd36 is reduced in HNF1A-MODY carriers.

The contents of this chapter are published as: *"Circulating cd36 is reduced in HNF1A-MODY carriers"*. Bacon S, Kyithar MP, Schmid J, Costa Pozza A, Handberg A, Byrne MM. PLoS One. 2013 Sep 12;8(9):e74577. doi: 10.1371/journal.pone.0074577. PMID: 24069322

5 Chapter 5; Circulating cd36 is reduced in HNF1A-MODY carriers.

5.1 Abstract

Premature atherosclerosis is a significant cause of morbidity and mortality in type 2 diabetes mellitus (T2DM). Maturity onset diabetes of the young (MODY) accounts for approximately 5 % of all diabetes, with mutations in the transcription factor; hepatocyte nuclear factor 1 alpha (HNF1A) accounting for the majority of MODY cases. The literature available states that the incidence of both micro and macrovascular disease in HNF1A-MODY is similar to that noted in a type 1 diabetes mellitus (T1DM) population. However, HNF1A-MODY carriers generally have a favourable cardiovascular metabolic profile. The scavenger protein CD36 has been shown to play a substantial role in the pathogenesis of atherosclerosis, largely through its interaction with oxidised LDL. Higher levels of monocyte CD36 and plasma CD36 (sCD36) are seen to cluster with insulin resistance and diabetes. We hypothesised a differential expression level of sCD36 in a HNF1A-MODY cohort when compared to a T2DM cohort. The aim of this study was therefore to determine levels of sCD36 in participants with HNF1A-MODY diabetes and to compare with unaffected normoglycaemic family members and participants with T2DM.

Methods:

We recruited 37 participants with HNF1A-MODY diabetes and compared levels of sCD36 with BMI- matched participants with T2DM and normoglycaemic HNF1A-MODY negative family controls. Levels of sCD36 were correlated with phenotypic and biochemical parameters.

Results:

HNF1A-MODY participants were lean, normotensive, with higher HDL and lower triglyceride levels when compared to controls and participants with type 2 diabetes mellitus. sCD36 was significantly lower in HNF1A-MODY participants when

compared to both the normoglycaemic family controls and to lean participants with type 2 diabetes mellitus.

Conclusion:

In conclusion, sCD36 is significantly lower in lean participants with HNF1A-MODY diabetes when compared to weight-matched normoglycaemic familial HNF1A-MODY negative controls and to lean participants with T2DM. Lower levels of this pro-atherogenic marker may result from the higher HDL component in the lipid profile of HNF1A-MODY participants.

5.2 Introduction

Cardiovascular disease is the major cause of morbidity and mortality in diabetes and is the largest contributor to the direct and indirect cost of managing subjects with type 1 (T1DM) and type 2 diabetes mellitus (T2DM). An individual with HNF1A-MODY is typically diagnosed with diabetes during adolescence and is therefore exposed to a long duration of dysglycaemia over their lifetime. A long duration of diabetes is a well-established risk factor for cardiovascular morbidity and mortality in diabetes. (Soedamah-Muthu, Fuller et al. 2006). However, whereas T2DM is characterised by insulin resistance and ß-cell failure, individuals with HNF1A-MODY have been shown to have normal insulin sensitivity (Tripathy, Carlsson et al. 2000, Stride, Ellard et al. 2005) . The insulin resistance in T2DM is associated with a specific dyslipidaemia; typically of hypertriglyceridaemia, low high density lipoprotein (HDL) and relatively normal low density lipoprotein (LDL). In contrast, previous studies assessing the lipid profiles of HNF1A-MODY patients have demonstrated a favourable profile from a cardiovascular aspect with lower fasting triglyceride levels and comparable HDL levels to normoglycaemic controls (Owen, Shepherd et al. 2002, Pearson, Badman et al. 2004). There has been no prospective study performed to date on subjects with HNF1A-MODY in regard to macrovascular complications. The data available is therefore limited to retrospective analysis of macrovascular disease in these individuals (Isomaa, Henricsson et al. 1998, Steele, Shields et al. 2010). These studies have reported similar rates of cardiovascular disease amongst a HNF1A-MODY cohort to a T1DM population.

The formation of an atherosclerotic plaque is the primary pathological process undermining cardiovascular disease (Scott 2004). Hyperglycaemia, hyperlipidaemia and insulin resistance are significant risk factors in the development of atherosclerotic plaque. CD36 is a transmembrane glycoprotein that has been shown to play a crucial role in the formation of such atherosclerotic plaques (Febbraio, Hajjar et al. 2001). CD36 is expressed in many cell types including macrophages. Macrophage CD36 is believed to play a critical role in the initiation and progression of atherosclerosis through its ability to bind and internalize low-density lipoprotein (LDL), thereby facilitating in the formation of foam cells (Kunjathoor, Febbraio et al. 2002, Podrez, Poliakov et al. 2002). This interaction between CD36 and oxLDL also induces the secretion of cytokines which

recruit immune cells to the arterial intima wall thereby promoting plaque development (Jiang, Wang et al. 2012) (see Figure 1). A further critical process in the development of cardiovascular disease is plaque rupture and thrombus formation. CD36 activates platelets which form thrombus at the plaque rupture site (Podrez, Byzova et al. 2007). In human studies, non-cell bound CD36 or soluble CD36 (sCD36) parallels the expression of CD36 in cells (Handberg, Levin et al. 2006). sCD36 clusters with markers of insulin resistance and is progressively related to the severity of insulin resistance and atherosclerosis in the human population (Eitzman, Westrick et al. 2000, Handberg, Levin et al. 2006, Handberg, Skjelland et al. 2008, Handberg, Norberg et al. 2010). It is believed that sCD36 is released into the circulation during low grade inflammatory states such as obesity and diabetes (Handberg, Levin et al. 2006, Glintborg, Hojlund et al. 2008). In this study we hypothesized a differential sCD36 expression in a HNF1A-MODY cohort when compared to a T2DM group. We also sought to compare sCD36 levels in a normoglycaemic HNF1A-mutation negative family member cohort. We subsequently correlated sCD36 levels with insulin sensitivity, glycaemic control, hsCRP, phenotypic characteristics and biochemical parameters.

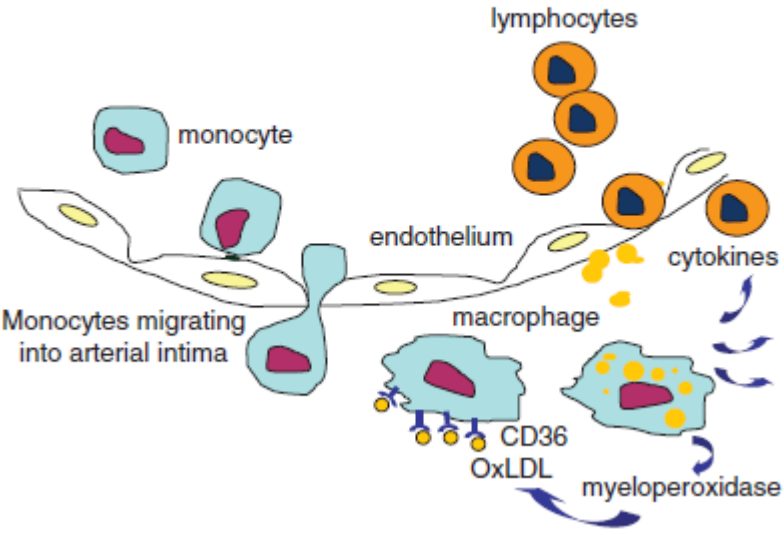

Figure 5-1: Adopted from Y.M Park Experimental and Molecular Medicine 2014.

The mechanism of atherosclerosis: Macrophages internalize oxidised LDL (oxLDL) through the scavenger receptor protein CD36. As a result, the macrophages become trapped in the arterial intima, promoting a further inflammatory insult by secreting cytokines and recruiting immune cells to the lesion. The progression of the atherosclerotic inflammatory process results in arterial narrowing.

5.3 Methods

5.3.1 Study population:

37 individuals with HNF1A-MODY diabetes participated in the study. HNF1A mutations included L17H, G207D, P291finsC, S352fsdelG, F426X, P379T, IVS7-6G>A, R200Q/N and E230fsdelGA. In addition, 21 participants with T2DM, who were BMI-matched with the HNF1A-MODY group were recruited. A further 11 participants, who were family members of the HNF1A-MODY group but negative for the mutation formed a control group. Details on the recruitment and phenotyping of these cohorts are as per sections 2.1.5, 2.1.9, 2.1.11.

5.3.2 Plasma sCD36

sCD36 was measured using an in-house enzyme-linked immunoassay (ELISA) . A pool of EDTA plasma was applied in seven dilutions and used to produce a standard concentration curve. Internal controls were run in quadruplicate on each plate. Runs were accepted if the controls were within ±2 SD from mean, and most were within 1 SD. The intra-assay coefficient of variation (CV) was 6%. Log-transformed standard curves were linear. A few measurements were outside the standard curve range and were calculated by extrapolation. sCD36 was measured in fasting samples.

5.3.3 Measurement of serum hsCRP levels

Serum hsCRP levels were measured using particle enhanced immunonephelometry assay (CardioPhase® hsCRP, Siemens) on a Siemens BN II analyzer (Siemens Healthcare Diagnostics, Deerfield, IL, USA). A typical limit for detection of hsCRP was 0.175mg/L for measurements performed using a sample dilution of 1:20. A coefficient of variation at the concentration 0.41mg/L was 7.6%. We considered that hsCRP values >10mg/l were likely to represent an inflammatory response in line with previous studies (Ridker 2003, Lee, Adler et al. 2009, Owen, Thanabalasingham et al. 2010). We therefore performed two separate analysis

approaches, one in which we included (termed ‘all patients’), and one in which we excluded (termed ‘without extreme CRP’) the 2 HNF1A-MODY patients with serum hsCRP values of >10 mg/l.

5.3.4 Statistical analysis

Clinical and biochemical data are expressed as mean ± SD. Statistical analysis was performed using SPSS statistical software package for Windows, version 20.0 (SPSS, Chicago, IL, USA). The significance of the difference between 2 groups was determined by Mann-Whitney U test (non-parametric clinical data) or t-test. For comparisons of more than 2 groups, Kruskal-Wallis test was applied. Differences were considered significant at $p \leq 0.05$. Fisher exact test was applied for aspirin and statin use.

5.4 Results:

The clinical characteristics of the lean HNF1A-MODY, the BMI-matched T2DM and normoglycaemic HNF1A-mutation negative family member groups are shown in Table 5.1.

	HNF1A-MODY	*Normoglycaemic HNF1A-MODY negative*	*T2DM*	*HNF1A-MODY vs HNF1A-MODY negative controls*	*HNF1A-MODY vs T2DM*
n=	37	11	21		
Age (yrs.)	38 (± 16)	30 (±14)	50 (±16)	0.4	0.01
Duration (yrs.)	12.3 (±11.5)	N/A	3.8 (±3.3)	<0.001	0.01
BMI (kg/m^2)	24.9 (±6.8)	25.4 (± 2.6)	24.2 (±5.3)	0.9	0.9
SBP (mmHg)	121.6 (±16)	118.9 (± 14.6)	134.1 (±19.4)	0.6	0.01
DBP (mmHg)	71.6(±16)	71 (12.4)	77.8(11.4)	0.8	0.03
Total Cholesterol (mmol/l)	4.3 (± 0.9)	4.7 (± 0.9)	4.1 (± 0.9)	0.1	0.5
HDL (mmol/l)	1.4 (±0.5)	1.2 (±0.4)	1.1 (± 0.3)	0.3	0.03
TG (mmol/l)	1.0 (± 0.7)	1.0 (± 0.5)	1.5 (± 1.0)	0.3	0.008
LDL (mmol/l)	2.4 (±0.7)	3(±0.9)	2.2(±0.9)	0.03	0.3
CD36 (arbitary units)	**0.7 (±0.4)**	**1.1(±0.5)**	**1.0(±0.5)**	**0.03**	**0.05**
OGIS ($ml/min/m^2$)	359(±91)	471 (±53.3)	310 (±71)	0.003	0.2
HbA1c (%) mmol/l	7.2(±1.1)	5.2(±0.2)	7.4(±1.3)	<0.0001	0.8
Fasting insulin (pmol/l)	26.9(±33)	37.6(±22)	67.9(±42)	0.06	0.002
hs CRP (mg/L)	0.4(±0.6)	0.8(±0.7)	1.9(±2.2)	0.7	<0.0001

Table 5-1: Clinical characteristics of subjects.Table footnote; BMI=Body Mass Index, SBP=Systolic Blood Pressure, DBP=Diastolic Blood Pressure, HDL=High density Lipoprotein, TG=Triglycerides, LDL=Low Density Lipoprotein, OGIS=Oral Glucose Insulin Sensitivity Index. hsCRP=high sensitivity C-reactive protein. ns=not significant.N/A=not applicable

Of the 37 HNF1A-MODY carriers with diabetes, 9 subjects (24.3%) had diabetic retinopathy, with proliferative retinopathy in one subject (2.7%). Only one of the subjects had evidence of microalbuminuria.

There was no personal history of myocardial infarction or ischaemic stroke in the HNF1A-MODY subjects, 3 had significant peripheral vascular disease, 2 requiring femoral popliteal by-pass grafting.

Levels of sCD36 were significantly lower in participants with HNF1A-MODY when compared to normoglycaemic HNF1A-MODY negative family controls (0.7 (±0.4) vs. 1.1 (±0.5), p=0.03). Similarly, levels of sCD36 were significantly lower when compared to participants with T2DM (0.7 (±0.4) vs. 1.0 (±0.5), p=0.05). This is further illustrated in Figure 5.2. When multivariate analysis was performed accounting for age and duration of diabetes status the levels of sCD36 remain significantly different between HNF1A-MODY participants and normoglycaemic HNF1A-MODY negative controls. Insulin sensitivity as determined using OGIS was higher in participants with HNF1A-MODY when compared to the BMI-matched participants with T2DM; however, this did not reach statistical significance (359 ±91 vs. 310±71 ml/min/m^2)

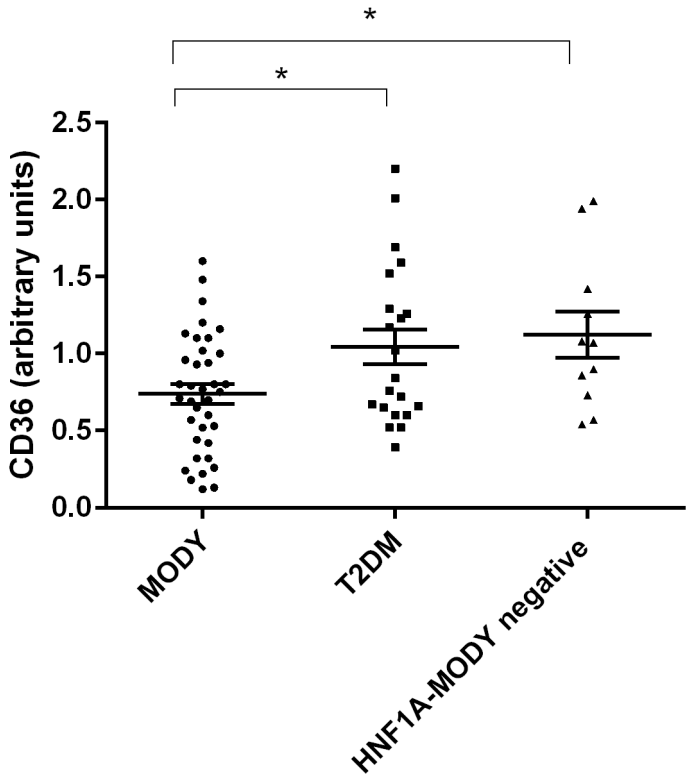

Figure 5-2:sCD36 (arbitrary units) in HNF1A-MODY (black circle),T2DM (black square) and HNF1A-MODY negative normoglycaemic control (black triangle).*=p<0.05

Levels of sCD36 in HNF1A-MODY did not correlate with age, duration of diabetes or glycaemic control as determined using HbA_{1c}. Similarly there was no correlation noted with insulin sensitivity as determined using OGIS or hsCRP. There was a positive correlation noted with ALT (rho=0.37, p=0.04).

The expression of sCD36 is known to be influenced by both aspirin and statin usage (Davi, Catalano et al. 1990, Santilli, Davi et al. 2006, Chmielewski, Bragfors-Helin et al. 2010). It is worth noting that there was significantly less statin (21.6% vs. 61.9%, p=0.001) and aspirin therapy (30% vs. 60.8%, p=0.01) prescribed in the HNF1A-MODY group compared to the T2DM group.

5.5 Discussion:

5.5.1 HNF1A-MODY participants lack features of the metabolic syndrome;

As previously reported, in the current study we demonstrate that participants with HNF1A-MODY have a better metabolic profile compared to BMI-matched T2DM controls (Owen, Shepherd et al. 2002). They have significantly lower triglyceride levels, higher HDL levels with less hypertension.

5.5.2 CD36 is significantly reduced in HNF1A-MODY participants;

In keeping with the absence of the metabolic syndrome features, sCD36 levels are significantly lower in the HNF1A-MODY cohort when compared to the normoglycaemic HNF1A-MODY negative family control group and the T2DM group. CD36 is highly regulated by insulin resistance and diabetes. Insulin resistance *per se* causes an up regulation of CD36 expression on the surface of monocytes. The concentration of CD36 is increased up to five fold in the plasma of obese individuals with diabetes than in lean healthy controls (Handberg, Levin et al. 2006). A further study noted a bimodal distribution for sCD36 whereby an elevated or indeed a very low level was associated with a higher risk of cardiovascular disease (Handberg, Hojlund et al. 2012). A recent publication reported an increase in sCD36 levels in young obese when compared to healthy controls suggesting it may be a useful marker in early atherosclerotic disease (Ramos-Arellano, Munoz-Valle et al. 2014). In addition, a positive relationship was also noted between elevated sCD36 and carotid intima medial thickness in a healthy group (Handberg, Hojlund et al. 2012). Echocardiographic parameters reveal a potential association between sCD36 expression and evidence of impaired left ventricular diastolic function (Krzystolik, Dziedziejko et al. 2015). Overall elevated sCD36 has the potential to be an important marker of atherosclerosis.

In the current study, sCD36 is low in the HNF1A-MODY population when compared to family controls and T2DM participants. We can speculate that HNF1A may play a role in the regulation of sCD36; however, further study with a larger population size is required to confirm an association.

Our current study, in keeping with previous findings, has demonstrated that individuals with HNF1A-MODY have significantly lower levels of hsCRP when compared to individuals with T2DM (McDonald, Shields et al. 2011). There is a plausible biological reason for this as the promoter of the CRP gene has 2 binding sites for hepatocyte nuclear factor 1A (McDonald, Shields et al. 2011). Elevated levels of high sensitivity C-reactive protein (hsCRP) has been shown to be associated with increased cardiovascular risk.

We did not find a significant correlation between sCD36 and insulin sensitivity in the participants with HNF1A-MODY when determined using OGIS. OGIS has been shown to be analogous to the assessment of insulin sensitivity using the hyperinsulinaemic euglycaemic clamp technique and is more practical for use in a clinical setting (Mari, Pacini et al. 2001). This is the first study to look at sCD36 in HNF1A-MODY. As HNF1A-MODY is rare in comparison to T2DM, the sample size studied maybe affecting correlation significance.

We have demonstrated a positive correlation between sCD36 and ALT. Elevated aminotransferases are surrogate markers for liver fat content. In apparently healthy cohorts an elevated ALT is associated with hepatic insulin resistance (Bonnet, Ducluzeau et al. 2011).

In the HNF1A-MODY group studied there was no personal history of myocardial infarction or ischaemic stroke. The 3 subjects who had peripheral vascular disease were all smokers.

In this current study, the T2DM population had lower sCD36 levels than previously reported, however this group was selected to be BMI-matched with the lean HNF1A-MODY population and therefore not comparable to previously studied patients. In addition, the majority of the T2DM group was on statin and aspirin therapy; both of which are known to reduce CD36 levels (Chmielewski, Bragfors-Helin et al. 2010) therefore it is likely that the levels noted in this study for the T2DM group are lower than expected.

5.5.3 CD36 is a cellular receptor for HDL

We propose a potential association between a low sCD36 and the favourable HDL profile noted in HNF1A-MODY. HDL plays an important role in cholesterol homeostasis removing cholesterol from peripheral tissues such as vessel walls. A mouse model with a targeted mutation in the CD36 gene was noted to have a significant increase in HDL cholesterol compared to wild type littermates (Febbraio, Abumrad et al. 1999, Yue, Chen et al. 2010). A recent study also proposed a deficiency of CD36 promoting HDL formation. A lack of CD36 may be associated with an increase in hepatic cholesterol and phospholipid efflux and stimulated hepatic secretion of apolipoproteins. These changes may represent a mechanism that mediates an increase in plasma HDL cholesterol (Yue, Chen et al. 2010). Brundert et al also concluded that CD36 mediates uptake of HDL by tissues particularly by the liver and the adrenal glands (Brundert, Heeren et al. 2011) and that through these two mechanisms; increased HDL biosynthesis and reduced HDL catabolism an increase in the steady state plasma HDL cholesterol is achieved in the CD36 deficient mice.

5.6 Conclusion

In conclusion, HNF1A-MODY carriers have significantly lower levels of sCD36, a marker which is known to cluster with insulin resistance and atherosclerotic plaque development. In accordance with previous studies we have demonstrated that HNF1A-MODY carriers have lower levels of hsCRP when compared to subjects with T2DM. This is the first study to measure sCD36 in the HNF1A-MODY population. We have also provided a possible relationship between low sCD36 levels and the high HDL levels noted in these patients which perhaps is conferring a protective mechanism in HNF1A-MODY carriers against macrovascular disease. The marker sCD36 warrants further investigation in a larger population of HNF1A-MODY carriers and control subjects.

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Chapter 6

Successful maintenance on sulphonylurea therapy and low diabetes complications rate in a HNF1A-MODY cohort.

The contents of this chapter are published as: "*Successful maintenance on sulphonylurea therapy and low diabetes complications rate in a HNF1A-MODY cohort",* Bacon S, Kyithar MP, Ellard S, Byrne MM. Diabet. Medicine. 2015.PMID 26479152

6 Chapter 6: Successful maintenance on sulphonylurea therapy and low diabetes complications rate in a HNF1A-MODY cohort.

6.1 Abstract:

Introduction:

HNF1A gene mutations are the most common cause of MODY in the UK. Persons with HNF1A-MODY display sensitivity to sulphonylurea therapy; however the long term efficacy is not established. There is limited literature as to the prevalence of micro- and macrovascular complications in this unique cohort. The aim of this study was to determine the natural progression and clinical management of HNF1A-MODY diabetes in a dedicated MODY clinic.

Methods:

Sixty participants with HNF1A-MODY and a cohort of 60 BMI-, age-, ethnicity- and diabetes duration- matched participants with Type 1 DM participated in the study. All participants were phenotyped in detail. Clinical follow up of the HNF1A-MODY cohort occurred on a bi-annual basis.

Results:

Following a genetic diagnosis of MODY the majority of the cohort treated with sulphonylurea therapy remained insulin independent at 84 month follow up (80%). The HbA_{1c} in the HNF1A-MODY group treated with sulphonylurea therapy alone improved significantly over the study period (from 49mmol/mol (44-63)/6.6% (6.2-7.9) to 41mmol/mol (31-50)/5.9% (5-6.7), p=0.003). The rate of retinopathy was significantly lower than that noted in the Type 1 DM group (13.6% vs. 50%, p=0.0001).There was also a lower rate of microalbuminuria and cardiovascular disease in the HNF1A-MODY group compared to the Type 1 DM group.

Conclusions:

This study demonstrates that the majority of participants with HNF1A-MODY can be maintained successfully on sulphonylurea therapy with good glycaemic control.

We note a significantly lower rate of micro-and macrovascular complications than that previously reported. The use of appropriate therapy at early stages of the disorder may decrease the incidence of complications.

6.2 Introduction:

It is established that HNF1A-MODY results in diabetes caused by a ß-cell defect with a progressive deterioration in glucose tolerance and a gradual increase in treatment requirements (Hattersley 1998, Pontoglio, Sreenan et al. 1998). Sulphonylureas have been shown to successfully bypass the major ß-cell defects that result in HNF1A-MODY (Pearson, Starkey et al. 2003). The response to sulphonylureas in individuals with HNF1A-MODY has been investigated in a number of small studies (Fajans and Brown 1993, Hansen, Eiberg et al. 1997, Sovik, Njolstad et al. 1998, Pearson, Liddell et al. 2000, Shepherd, Pearson et al. 2003, Hattersley, Bruining et al. 2009, Becker, Galler et al. 2014). The specific therapeutic response to sulphonylureas in HNF1A-MODY was confirmed by a double-blind randomised clinical study comparing the efficacy of gliclazide and metformin in HNF1A-MODY and T2DM (Pearson, Starkey et al. 2003). Likewise a previous study conducted over a 39 month period reported that the majority of participants (71%) who were transferred to sulphonylureas were able to remain off insulin without any deterioration in glycaemic control. The authors noted that the participants who recommenced insulin were those who started sulphonylurea treatment at a later stage in their disease (Shepherd, Shields et al. 2009). There is limited literature available as to both the micro-and macrovascular complications associated with HNF1A-MODY (Velho, Vaxillaire et al. 1996, Isomaa, Henricsson et al. 1998, Steele, Shields et al. 2010).

The MODY cohort in the Mater Misericordiae University Hospital is unique to Ireland in that potential mutation carriers are not only screened by the centre but they also attend a dedicated MODY clinic facilitating the close monitoring and management of both micro-and macrovascular complications over time. The clinic includes at least 3 generations in the majority of families screened and is

therefore in a position to observe the natural progression of the disorder, albeit in a dedicated tertiary referral centre.

A principal aim of the study was to determine the success of sulphonylurea therapy in this group. A further aim was to assess the prevalence and severity of micro-and macrovascular complications in this cohort over an extended period compared to a cohort of participants with Type 1 DM.

6.3 Methods:

6.3.1 Study Population:

A total of 60 individuals from 20 families with a confirmed genetic diagnosis of a *HNF1A* mutation identified as part of the MODY screening study in the Mater Hospital participated. A cohort of 60 participants with T1DM was recruited as detailed in section 2.1.8. The T1DM cohort was Caucasian and matched for BMI, duration of diabetes, ethnicity and age to the HNF1A-MODY cohort.

The details on phenotyping of the cohort are as per section 2.1.5.

6.3.2 Clinical follow up of HNF1A-MODY participants

Individuals with a diagnosis of HNF1A-MODY attend our dedicated MODY clinic on a bi-annual basis. A clinical history detailing symptoms and medication usage was performed at each visit. For the purpose of this study, a severe hypoglycaemic episode is defined as one requiring third party assistance. Suboptimal glycaemic control is defined as a HbA_{1c} of >53mmol/mol/ >7% as per ADA Standards of Medical Care 2014. Retinopathy is assessed using bilateral digital images on an annual basis (Zeiss Visucam Pro NM).

6.3.3 Statistical analysis

Statistical analyses are performed using Prism/MatLab and its statistical toolbox (The MathWorks, Inc. Natick, USA). Non-parametric data are given as median and interquartile range (IQR) and are compared by Mann-Whitney U test and Spearman correlation analysis. Categorical data such as medication usage was compared using Fisher's exact test. Hypotheses tests were considered statistically significant if $p<0.05$.

6.4 Results:

6.4.1 Genotyping and phenotyping of the cohort.

The HNF1A mutations included 13 different mutations, 3 of which were novel (Table 6.1).Novel mutations were deemed to be pathological on the finding of co-segregation with diabetes in the index case. The clinical profiles and complications of HNF1A-MODY affected individuals and the cohort of participants with Type 1 DM are contained within Table 6.2.

Location in HNF1A	cDNA level	Protein level	Protein effect	Position relative to functional domains	No of patients	No of families	Reference
Exon 1	c.50T>A	p.Leu17His	Missense	Dimerization domain	2	1	Kyithar et al Diabetes Metab. 2011 Dec;37(6):512-9
Exon 2	c.411_412del	p.Gly138fs	Frameshift	DNA-binding domain	1	1	Novel
Exon2	c.476G>A	p.Arg159Gln	Missense	DNA-binding domain/Nuclear Localisation Signal	1	1	Vaxillaire et al Hum Mol Genet. 1997 Apr;6(4):583-6.
Exon 3	c.599G>A	p.Arg200Gln	Missense	DNA-binding domain/Nuclear Localisation Signal	2	1	Hattersley Diabet Med. 1998 Jan;15(1):15-24.
Exon 3	c.620G>A	p.Gly207Asp	Missense	DNA-binding domain	2	1	Ellard & Colclough Hum Mutat. 2006 Sep;27(9):854-69.
Exon 3	c.690_691del	p.Glu230fs	Frameshift	DNA-binding domain	4	1	Ellard & Colclough Hum Mutat. 2006 Sep;27(9):854-69.
Exon 4	c.872dup	p.Pro291fs	Frameshift	Transactivation domain	23	6	Yamagata et al Nature. 1996 Dec 5;384(6608):455-8.
Exon 5	c.1004del	p.Ser335*	Nonsense	Transactivation domain	1	1	Awa et al Eur J Endocrinol. 2011 Apr;164(4):513-20
Exon 5	c.363_379del	p.Val351fs	Frameshift	Transactivation domain	1	1	Novel
Exon 5	c.1053del	p.Ser352fs	Frameshift	Transactivation domain	11	2	Ellard & Colclough Hum Mutat. 2006 Sep;27(9):854-69.
Exon 6	c.1135C>A	p.Pro379Thr	Missense	Transactivation domain	4	1	Bellanné-Chantelot C Diabetes. 2008 Feb;57(2):503-8
Exon 6	c.1276_1277insAGGT	p.Phe426*	Nonsense	Transactivation domain	2	1	Kyithar et al Diabetes Metab. 2011 Dec;37(6):512-9
Intron 7	c.1502-6G>A	Skipping of exon 7	Aberrant Splicing	Transactivation domain	3	1	Xu et al Diabetologia. 2002 May;45(5):744-6
Exon 8	c.1504del	p.Leu502fs	Frameshift	Transactivation domain	3	1	Novel

Table 6-1: Mutations in the HNF1A genes in Irish MODY subjects. Mutations described according to HGVS nomenclature guidelines using reference sequence NM_000545.5 for *HNF1A*.

	HNF1A-MODY [median [IQR]] N=59	**T1DM [median [IQR]] N=60**	**P value**
Sex (M:F)	24:35	30:30	na
Smoker	14	18	0.6
Age (yrs.)	35 (21-49)	36 (23-48.5)	0.8
Duration of Diabetes (yrs.)	13 (9-30)	19 (3.5-28)	0.2
BMI (kg/m^2)	24.1(21-26)	25.3 (22-28)	0.08
HbA_{1c} (mmol/mol)/%	43 (31-53)/ 6.1 (5-7)	64 (57-76)/ 8 (7.4-9.1)	**<0.0001**
SBP(mmHg)	120 (112-131)	124(118-131)	0.2
DBP(mmHg)	70 (65-80)	78 (70-82)	**0.005**
Total Cholesterol (mmol/l)	4.4 (3.7-4.9)	4.3 (3.8-4.8)	0.6
Triglycerides (mmol/l)	0.8 (0.6-1.1)	0.8 (0.6-1.2)	0.5
LDL (mmol/l)	2.3 (2-3)	2.3 (2-2.8)	0.6
HDL (mmol/l)	1.3 (1.1-1.7)	1.4 (1.1-1.6)	0.4
Fasting C-peptide (pmol)	495 (345-594)	<66	Na
Aspirin (%)	25.4	32.8	0.4

Anti-hypertensive (%)	15	27.8	0.08
Statin (%)	20.3	40.9	**0.02**
ICA/GAD positivity (%)	1 (1.6)	60 (100)	**<0.0001**
Retinopathy n (%)	8 (13.6)	30 (50)	**<0.0001**
Proliferative Retinopathy n (%)	2 (3.3)	10 (16)	**0.01**
Microalbuminuria n (%)	3 (5)	5 (8.3)	0.2
Nephropathy n (%)	3 (5)	4 (6)	0.4
Coronary Heart Disease n (%)	4 (6.7)	5 (8.3)	0.4
Peripheral Vascular Disease n (%)	3 (5%)	4 (6.6%)	0.3

Table 6-2: Clinical Characteristics and complications of participants with HNF1A-MODY and T1DM (na=not applicable)

6.4.2 Transfer from insulin to sulphonylurea therapy.

Following a molecular diagnosis of HNF1A-MODY, 13/15 (86.6%) of insulin treated patients were switched to sulphonylurea therapy (see Figure 6.1). In 2 participants a transfer from insulin to sulphonylurea was not attempted due to patient preference and chronic renal impairment. One HNF1A-MODY mutation carrier had high GAD titres with undetectable C-peptide consistent with co-existing type 1 DM and was removed from the analyses. The median sulphonylurea dose utilised in the insulin transfer group was Gliclazide 120 mg (40-320) per day.

At clinical follow up (84 months), two of the 13 participants that switched to sulphonylurea therapy required additional basal insulin (median total daily dose: 8

units (3-12)) and one patient electively returned to their multi-daily injections (MDI) insulin regimen. The group requiring the additional basal insulin were older; (52 yrs. (47-56) vs. 27 yrs. (21-46), p=0.006) and had a longer duration of diabetes; (25 yrs. (21-30) vs. 8 yrs. (1.2-16), p=0.001). than the group not requiring additional basal insulin. Those that required additional therapy to sulphonylurea gained an average of 12kg over the duration of the study period whilst those who were successfully maintained on sulphonylurea therapy actually lost an average of 2.7 kg over the same study period.

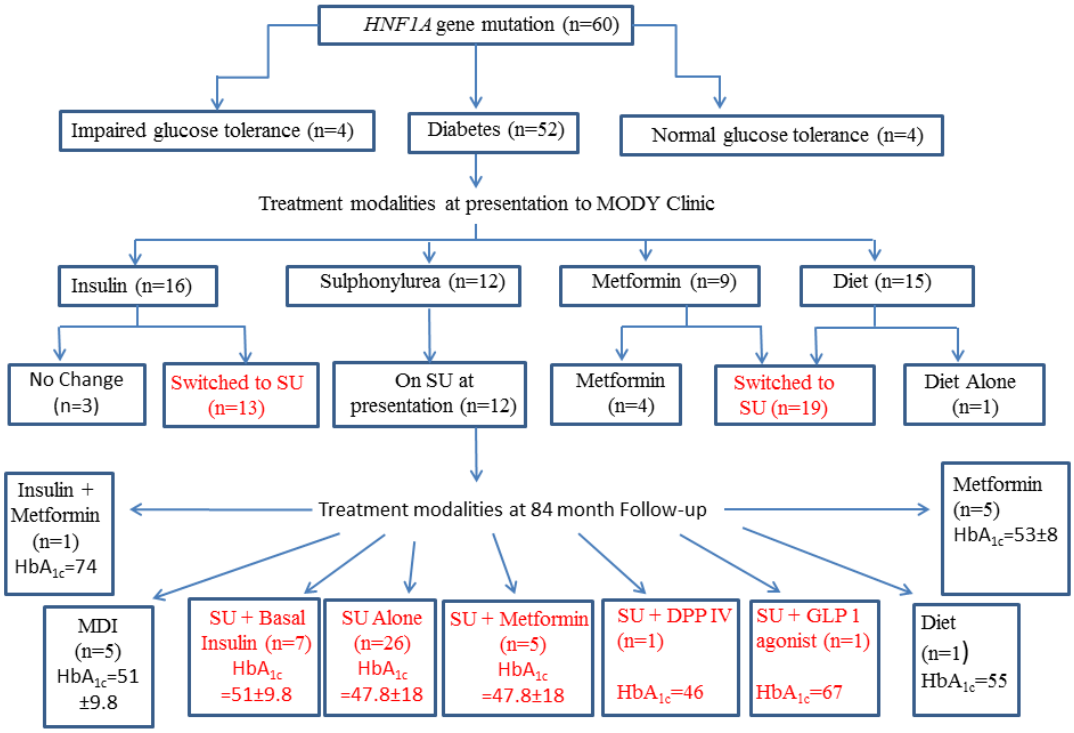

Figure 6-1: Summary of treatment change following genetic diagnosis of HNF1A-MODY

Figure 6.1 is a schematic representation of the degree of glucose intolerance detected in the HNF1A-MODY cohort with their corresponding treatment modality. Of the 60 HNF1A-MODY participants, seven were newly diagnosed with diabetes, four participants were diagnosed with impaired glucose tolerance and four had normal glucose tolerance. We demonstrate the treatment modalities when participants initially presented (prior to HNF1A-MODY diagnosis) and the subsequent change in therapeutic regimen following HNF1A-MODY status being confirmed.

SU=sulphonylurea therapy. MDI=multiple daily injections.GLP-1=glucagon like peptide. DPP-IV=Dipeptidyl peptidase

6.4.3 Chronic sulphonylurea usage in the HNF1A-MODY group.

There were 23.5 % (12/51) of participants with HNF1A-MODY who were receiving sulphonylurea therapy for a mean duration of 14.5±10 years prior to a genetic diagnosis being confirmed. At follow up 5 participants from this cohort were utilizing basal insulin. There was no significant difference in the group requiring additional basal insulin with regards to age; (58yrs. (50-62.8) vs. 49yrs. (37-64), p=0.8) or duration of diabetes; (27.5 yrs. (22-37) vs.27 yrs.(22-32) , p=0.3).However, they did have a higher HbA_{1c} value at clinical follow up; (71.5mmol/mol (57-74)/8.6% (7.4-8.9) vs. 46.5mmol/mol (42-56)/6.4% (6-7.3), p=0.05). The median dose of Gliclazide in this cohort was 80mg (40-80) at initial diagnosis and remained stable over the study duration.

6.4.4 Is sulphonylurea therapy maintained?

A total of 32 participants with HNF1A MODY were not on sulphonylurea treatment at the time of their genetic diagnosis (13 on insulin, 5 on Metformin and 14 on diet) and a change in their treatment regimen to sulphonylurea only was made. A further 12 were already treated with sulphonylurea at the time of their genetic diagnosis. At follow up, 26 of these 44 participants (59.1%) were maintained on sulphonylurea treatment alone (Figure 6.2). The average duration of hyperglycaemia at study follow up in this cohort was 16.5±11.2 years. The additional and/or alternative agents utilised to maintain optimal glycaemic control and their clinical indication are listed in Table 6.3. Of the 44 patients on sulphonylurea therapy, just 8 participants (18%) required an additional agent to sulphonylurea therapy for suboptimal glycaemic control alone over the study period. Those, (n=26) who were successfully treated with sulphonylurea mono therapy had a significant improvement in HbA_{1c} over a duration of 84 months; (49mmol/mol (44-63)/6.6% (6.2-7.9) vs. 41mmol/mol (31-50)/5.9% (5-6.7), p=0.003) (see Figure 3) .The median dose of Gliclazide required to maintain optimal control in this group was 80mg (40-80) per day. Those that required an additional agent had a longer duration of diabetes (20 yrs. (12-28) vs. 9 yrs. (8-21), p=0.01) and had a higher HbA_{1c} at initial presentation to the MODY clinic (60mmol/mol (50-73)/7.6% (6.7-8.8) vs. 49mmol/mol (44-63)/6.6% (6.2-7.0),

p=0.04) when compared to the group successfully maintained on sulphonylureas. In addition, the cohort that required an additional agent gained an average of 7kg weight during study follow up whilst those maintained on sulphonylurea alone only gained an average of 1.6 kg . Table 6.3 contains the clinical characteristics of the HNF1A-MODY cohort who were always on sulphonylurea therapy alone and those who required an additional or alternative agent to maintain euglycaemia.

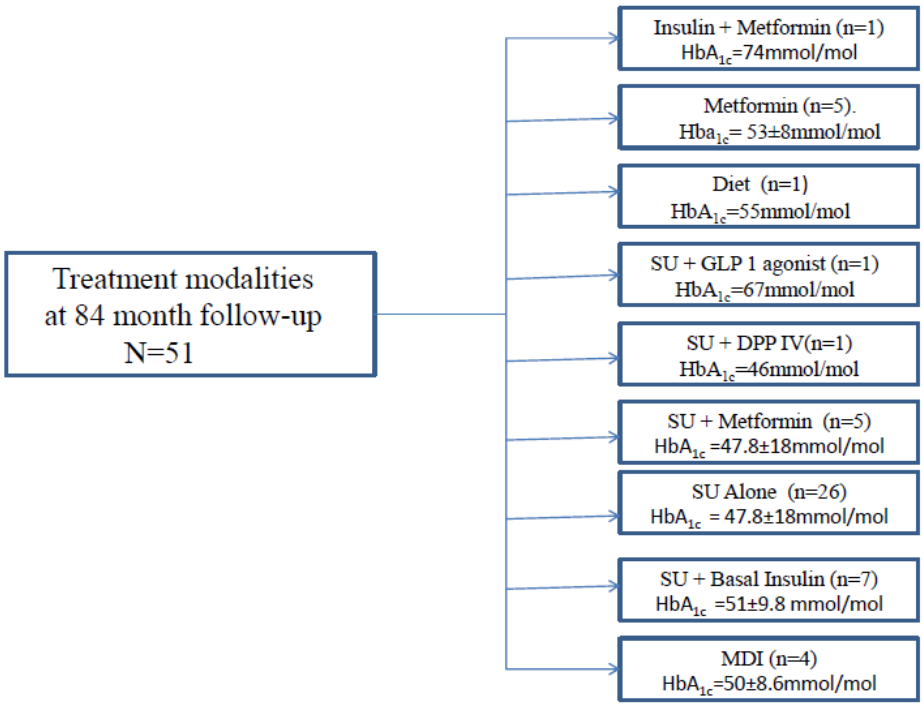

Figure 6-2: Therapeutic regimens at end of study follow up period.

Figure 6.2 contains details of the therapeutic regimens after a median of 84 months following confirmatory genetic diagnosis of HNF1A-MODY and the corresponding current HbA_{1c} value.

Medication	Indication for alternative agent to sulphonylurea
Sulphonylurea + Basal insulin	Suboptimal glycaemic control; n=5 Patient preference ;n=2
Sulphonylurea +Metformin	Weight gain; n=3 Suboptimal glycaemic control; n=2
Sulphonylurea + GLP-1 agonist	Weight gain
Sulphonylurea + DPP 4 inhibitor	Suboptimal glycaemic control
Insulin + metformin	Unable to tolerate sulphonylurea and weight gain
Diet alone	Good glycaemic control
Metformin only	Pre-pregnancy; n=1 Weight gain; n=4
Insulin (Multiple Daily Injections)	Pre-pregnancy; n=1 Contraindication to SU therapy (renal impairment);n=1 Patient preference; n=2 High GAD titres; n=1

Table 6-3: Indication for alternative or additional agent to SU therapy at median 84 month follow up.

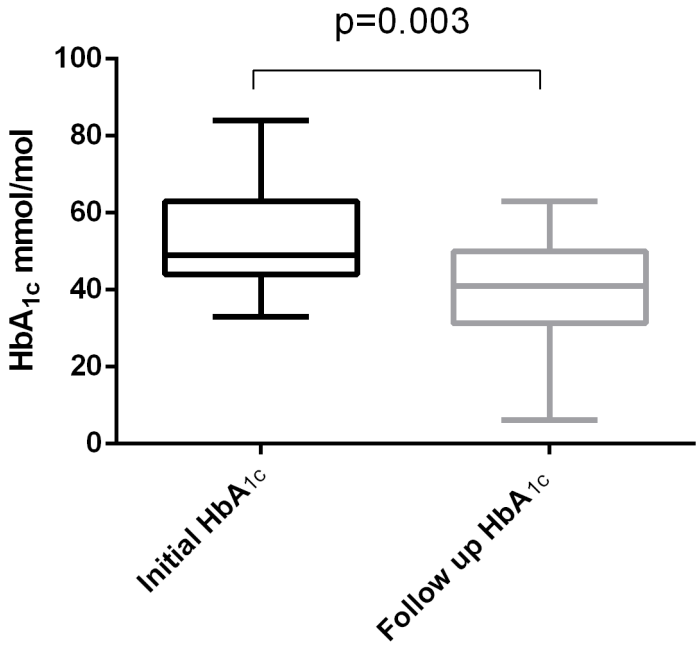

Figure 6-3: HbA_{1c} change with sulphonylurea usage. Those, (n=26) who were successfully treated with sulphonylurea mono therapy had a significant improvement in HbA_{1c} over a duration of 84 months; (49mmol/mol (44-63)/6.6% (6.2-7.9) vs. 41mmol/mol (31-50)/5.9% (5-6.7), p=0.003)

	Always on SU therapy alone (n=19)	Initial additional /alternative agent to SU therapy (n=30)	P-value
Age (yrs.)	51 (28.8-60)	38 (20-52)	0.09
HbA_{1c} (mmol/mol/%) @initial attendance	51.5 (45-68)/	56 (44-64)/	0.9
HbA_{1c} (mmol/mol/%) @follow up	45 (37-54)/	45 (39-59)	0.7
Duration of diabetes (yrs.)	10.5 (6.5-26.2)	17 (8-27)	0.4
Complication rate (any complication) %	10.5%	30%	0.1

Table 6-4: Clinical characteristics of the group always on SU therapy alone compared to the group who were initially on an additional or alternative agent to SU therapy . SU= sulphonylurea. Categorical data was compared using Fisher's exact test.

6.4.5 Complications of diabetes:

There were no signs of retinopathy detected in 86.4% of HNF1A-MODY participants (Table 6.2). The retinopathy detected in the HNF1A-MODY group was almost exclusively background retinopathy (70%). The overall rate of retinopathy was significantly lower in the HNF1A-MODY cohort than in the Type 1 DM group (13.6%

vs. 50%, p=0.0001). We also observed a significantly lower occurrence of proliferative retinopathy in the HNF1A-MODY group when compared to the Type 1 DM group (3.3% vs. 16%, p=0.01). Further analysis revealed that duration of diabetes significantly increased the odds of developing retinopathy in both the HNF1A-MODY (OR=5.85%, p=0.03) and the Type 1 DM groups (OR=7.33%, p=0.001).

Rates of persistent microalbuminuria were low in both the HNF1A-MODY and the Type 1 DM groups (5% vs. 8.3%, p=0.2). There was correspondingly a low incidence of overt nephropathy in both the HNF1A-MODY and Type 1 DM group (5% vs. 6%, p=0.4).

There was no significant difference in the rate of CVD detected in the HNF1A-MODY group than in the Type 1 DM group (6.6% vs. 8.3%, p=0.46). In the HNF1A-MODY group, there were four patients with CVD; two had multi-vessel disease and required coronary artery bypass grafting. PVD was evident in three of the affected HNF1A-MODY participants. Clinically, neuropathy was diagnosed in 3.3% of HNF1A-MODY participants. This low occurrence rate was similar to that noted in the T1DM cohort (6%).

When the HNF1A-MODY and T1DM groups with complications were compared, the HNF1A-MODY group with complications were significantly older than the T1DM group (44 yrs. (21-55) vs. 40 yrs. (31-51), p=0.03). There was no significant difference between the groups for duration of diabetes (25 yrs. (2.7-21) vs. 23 yrs. (18.5-33), p=0.5) or HbA_{1c} (61mmol/mol (44-64)/ vs. 65.5mmol/mol (58-78), p=0.08). Interestingly, hsCRP was significantly higher in the HNF1A-MODY group with complications (0.28 vs. 0.17, p=0.05) suggesting a higher burden of inflammation in these participants. The hsCRP was, as previously described, lower in the HNF1A-MODY than it was in the Type 1 DM cohort (0.22 (0.17-0.36) vs. 1.1 (0.5-2), p=0.0003) (Owen, Thanabalasingham et al. 2010).

6.5 Discussion:

6.5.1 The successful usage of sulphonylureas in the HNF1A-MODY cohort.

In the current study, the significant majority (80%) of the total HNF1A-MODY cohort treated with sulphonylurea therapy remained insulin independent. There was no significant escalation in sulphonylurea dose required despite the lengthy duration of diabetes. Sulphonylurea therapy was well tolerated amongst the current HNF1A-MODY cohort; specifically there were no episodes of severe hypoglycaemia. Basal insulin was required in six patients. The insulin doses required to achieve optimal glycaemic control in these participants were low with no significant increase required over the duration of the study again indicating the stability of the disease. No participants required the addition of multiple daily injections for suboptimal glycaemic control. The success of sulphonylurea therapy appeared to be dependent on the duration of diabetes, the initial HbA_{1c} at presentation to the MODY clinic and the weight gained by HNF1A-MODY mutation carriers.

In diabetes, there are numerous pathophysiological pathways believed to contribute to the development of progressive ß-cell decline including glucotoxicity (Halban, Polonsky et al. 2014). Chronic sulphonylurea usage in HNF1A-MODY does not appear to cause ß-cell exhaustion. We suggest that through the successful maintenance of glycaemic control, the early initiation of sulphonylurea may delay the progression of the disorder and the development of complications.

6.5.2 A decrease in the rate of micro and macrovascular complications amongst HNF1A-MODY mutation carriers.

We report a significantly lower rate of both total retinopathy (13.6%) and proliferative retinopathy (3.3%) in the HNF1A-MODY cohort compared to the Type 1 DM population studied. The higher complication rate in the Type 1 DM group may be explained by suboptimal glycaemic control alone (DCCT, NEJM, 1993), however, the HbA_{1c} in the HNF1A-MODY cohort with complications was not significantly higher than that in the HNF1A-MODY cohort without complications .

Family members with HNF1A mutations are reported to die at a younger age than familial control subjects secondary to cardiovascular disease (Steele, Shields et al 2008).Previous studies have reported rates of diabetic retinopathy of approximately 47% in their HNF1A-MODY cohorts, with proliferative retinopathy ranging from 13-21% (Velho, Vaxillaire et al. 1996, Isomaa, Henricsson et al. 1998, Skupien, Gorczynska-Kosiorz et al. 2008). The study by Skupien et al. reported a 47.7% rate of diabetic retinopathy and a 25% rate of diabetic nephropathy (Skupien, Gorczynska-Kosiorz et al. 2008). This particular cohort were younger in age when compared to our cohort (33.7±14.1yrs), the average HbA_{1c} was 58 mmol/mol/7.5% with the majority of the cohort on insulin therapy (52.3%).

We report a 6.6% incidence of CVD amongst the Irish HNF1A-MODY cohort. Steele et al reported a higher rate of death from a cardiovascular related illness in HNF1A-MODY carriers when compared to non-affected family members (Steele, Shields et al. 2010) .CVD was documented in 16% of the Finnish (Isomaa, Henricsson et al. 1998) and 9.1% in the Polish HNF1A-MODY cohort (Skupien, Gorczynska-Kosiorz et al. 2008). Our research group, in accordance with the findings of multiple research groups have observed a favourable metabolic profile synonymous with HNF1A-MODY (Owen, Shepherd et al. 2002, Schober, Rami et al. 2009, Kyithar, Bacon et al. 2011, Besser, Jones et al. 2012). In the current study, HNF1A-MODY participants who did have CVD had a familial history on both the maternal and paternal sides of the family, in addition to being smokers. Therefore, development of atherosclerosis in these individuals may be under the influence of alternative cardiovascular risk factors. In addition, the diagnosis of HNF1A-MODY was made in these carriers approximately 30 years after initial diagnosis with diabetes. Inappropriate treatment for a prolonged period may also have resulted in suboptimal glycaemic control and hence exposed them to a greater degree of atherosclerotic burden.

We can speculate as to the reasoning for the lower detected rate of micro vascular complications in our HNF1A-MODY cohort when compared to the findings of other European groups. Firstly, two of the studies were conducted in the era prior to extensive statin therapy usage. Secondly, our HNF1A-MODY cohort had a lower rate of hypertension and better glycaemic control when compared to its' predecessor studies. A significantly higher proportion of the participants in the

older studies were treated with dietary therapy alone in comparison to the HNF1A-MODY cohort under current observation. It is noteworthy that there could be a potential bias introduced to the findings as the participants with HNF1A-MODY may represent an extremely motivated cohort. It is likely, however, that the clinical management of this unique cohort in a centre dedicated to the screening and management of MODY have influenced the good outcomes that we have observed in the current study.

6.6 Conclusion:

In conclusion, we report the successful maintenance of participants with HNF1A-MODY on sulphonylurea therapy in the long term. We also report a significantly lower incidence of microvascular complications amongst the HNF1A-MODY study population compared to that previously reported. The early recognition of HNF1A-MODY through clinical awareness and familial screening appears to significantly influence the incidence of complications amongst this unique cohort. The use of appropriate therapy in the early stages of the disease and maintenance of optimal glycaemic control is essential in the prevention of diabetes complications. The study further emphasises the importance of genetic testing for monogenic forms of diabetes to guide personalised treatment

6.7 References

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Chapter 7

MicroRNA-224 is readily detectable in urine of individuals with diabetes mellitus and is a potential indicator of beta cell demise.

The contents of this chapter are published as "MicroRNA-224 is readily detectable in urine of individuals with diabetes mellitus and is a potential indicator of beta cell demise". Siobhán Bacon, Britta Engelbrecht, Jasmin Schmid, Shona Pfeiffer, Ross Gallagher, Ailbhe Mc Carthy, Marie Burke, Caoimhín G Concannon, Jochen H.M.Prehn, Maria M Byrne. Genes . 2015 Jun; 6(2): 399-416. PMID:26110317

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7 Chapter 7: Introduction to biomarkers in MODY

A biomarker refers to a measurable indicator of a biological condition. In medical terms, a biomarker is typically a substance that assists in identifying a disease state. Currently, for a definitive diagnosis of MODY, molecular genetic analysis is required which remains an expensive process. To prioritize individuals who warrant genetic screening, clinical guidelines have been formulated. However, these guidelines will only assist in detecting approximately 50% of MODY mutation carriers (Ellard, Bellanne-Chantelot et al. 2008). Of late, the use of next generation sequencing (NGS) has enabled the rapid detection of a number of genes simultaneously rather than in the traditional sequence of screening. NGS will confirm an increased number of monogenic diabetes cases; however, it is still unfeasible to many. (Ellard, Lango Allen et al. 2013, Alkorta-Aranburu, Carmody et al. 2014, Szopa, Ludwig-Galezowska et al. 2015)

To this end, much research has focused on biomarker discovery as an adjunct to clinical guidelines. Biomarker research has been largely based on the findings from *Hnf1a* knock-out mouse models. *HNF1A* encodes transcription factors involved in the regulation of multiple genes. One of the first detected biomarkers was glycosuria in the presence of normoglycaemia in HNF1A-MODY mutation carriers (Menzel, Kaisaki et al. 1998). The threshold value for glucose appearing in the urine is significantly lower in HNF1A-MODY carriers than patients with T1DM. This phenomenon is believed to occur due to disruption of the renal sodium glucose co-transporters (SGLT 1 and 2). Another biomarker investigated was 1-5 anhydroglucitol (1-5 AG) which is a metabolically inactive monosaccharide with near complete renal reabsorption at a specific fructose-mannose active transporter (Skupien, Gorczynska-Kosiorz et al. 2008, Pal, Farmer et al. 2010). Glucose competes with 1-5 AG at this renal transporter. Therefore, in the presence of glycosuria, 1-5 AG is detected at a lower serum level in HNF1A-MODY

carriers. *Hnf1a* -/- mice have defective bile acid transport, increased bile synthesis and increased liver cholesterol synthesis (Shih, Bussen et al. 2001). Likewise, a specific lipid profile is synonymous with human mutation carriers. HNF1A-MODY is associated with a high HDL and a lower triglyceride level to that of individuals with T2DM (McDonald, McEneny et al. 2012). Apolipoprotein M (Apo M) is a 25 kDa apolipoprotein associated with HDL that is also regulated by *HNF1A* ((Skupien, Kepka et al. 2007, Mughal, Park et al. 2013). Apo M levels have been studied as a potential biomarker for differentiating HNF1A-MODY from the more common diabetes types with conflicting results.

GWAS revealed common variants mapping near the *HNF1A* gene on chromosome 12 are associated with alterations in hsCRP (Reiner, Barber et al. 2008). It was found that a cut off for hsCRP of <0.5g/l could differentiate HNF1A-MODY from T1DM, T2DM and normal controls (Owen, Thanabalasingham et al. 2010). Detectable C-peptide beyond the typical honeymoon period of T1DM is a clinical characteristic of HNF1A-MODY; therefore urinary C-peptide has been investigated as a potential outpatient screening tool for differentiating long standing T1DM patients from HNF1A-MODY mutation carriers (Besser, Jones et al. 2012, Besser, Shields et al. 2013).An altered glycan profile (Thanabalasingham, Huffman et al. 2013), generalised aminoaciduria (Bingham, Ellard et al. 2001), cystatin C (Nowak, Szopa et al. 2013), ghrelin (Nowak, Hohendorff et al. 2015) and complement 5 and 8 (Karlsson, Shaat et al. 2008) have also been studied with varying degrees of success.

Our research group has previously identified a pancreatic marker; *PSP/reg* (pancreatic stone protein/regenerating protein) to be elevated in HNF1A-MODY carriers when compared to mutation negative family controls (Bonner, Bacon et al. 2010). *PSP/reg* is a protein associated with islet cell regeneration and diabetogenesis. We provided biological evidence both in insulinoma cell lines and transgenic mouse models of *HNF1A* that ß-cells undergoing apoptosis induce the expression of *PSP/reg* in neighbouring cells. A reduction in functional ß-cell mass is a known feature of HNF1A-MODY (Vesterhus, Haldorsen et al. 2008). It is now widely accepted that ß-cell mass is a dynamic body involving both apoptosis and regeneration. However, direct visualization of pancreatic islets and ß-cells is not possible in humans. Therefore, there is a demand for molecular markers to

monitor ß-cell mass and function. We presented findings that *PSP/reg* plays a role in ß-cell mass regulation and can be easily measured. A follow on study performed in a larger group of HNF1A-MODY mutation carriers and GCK-MODY carriers confirmed the preliminary findings with higher expression of *PSP/reg* in HNF1A-MODY carriers but not in *GCK* participants (Bacon, Kyithar et al. 2012). We also studied a combination of biomarkers; *PSP/reg* and hsCRP to differentiate HNF1A from HNF4A-MODY (Kyithar, Bonner et al. 2013). These are difficult entities to distinguish between as they act in a synergistic fashion and result in a clinically similar phenotype. Inducible repression of *HNF1A* and *HNF4A* function in INS-1 cells suggested that *PSP/reg* induction required *HNF4A*, but not *HNF1A*. In contrast, *crp* gene expression was significantly reduced by repression of *HNF1A*, but not *HNF4A* function. *PSP/reg* and hsCRP levels were significantly lower in the HNF4A-MODY cohort when compared to HNF1A-MODY cohort. Parallel measurements of serum *PSP/reg* and hsCRP levels were able to discriminate HNF1A- from HNF4A-MODY subjects.

However, to date, no biomarker has translated into routine clinical practice.

The discovery of microRNAs (miRNA) and their distinct expression in multiple disease states offered the opportunity to explore their role in diabetes, specifically in MODY. This chapter is dedicated to miRNA research that has been conducted over the duration of the presented thesis. Initially, the differential expression of miRNAs in serum was investigated and the follow on project, which is the main subject matter of this chapter utilized urine. Subsequent to the publication of this manuscript the role of miRNA in MODY has been further explored. Fendler et al. have described the differential expression of serum miR-24, -27b, -223 and -199a in participants with HNF1B-MODY (Fendler, Madzio et al. 2016).

MicroRNA-224 is readily detectable in urine of individuals with diabetes mellitus and is a potential indicator of beta cell demise.

7.1 Abstract:

Introduction:

MicroRNA (miRNA) are a class of non-coding, 19–25 nucleotide RNA critical for network-level regulation of gene expression. miRNA serve as paracrine signalling molecules. Using an unbiased array approach, we previously identified elevated levels of miR-224 and miR-103 to be associated with a monogenic form of diabetes; HNF1A-MODY. miR-224 is a novel miRNA in the field of diabetes.

Aims:

We sought to explore the role of miR-224 as a potential biomarker in diabetes, and whether such diabetes-associated-miRNA can also be detected in the urine of patients.

Results:

Absolute levels of miR-224 and miR-103 were determined in the urine of *n* = 144 individuals including carriers of a *HNF1A* mutation, participants with T1DM, T2DM and normal controls. Expression levels were correlated with clinical and biochemical parameters. miR-224 was significantly elevated in the urine of carriers of a *HNF1A* mutation and participants with T1DM. miR-103 was highly expressed in urine across all diabetes cohorts when compared to controls. For both miR-224 and-103, we found a significant correlation between serum and urine levels ($p < 0.01$).

Conclusion:

We demonstrate that miRNA can be readily detected in the urine independent of clinical indices of renal dysfunction. We surmise that the differential expression

levels of miR-224 in both HNF1A-MODY mutation carriers and T1DM may be an attempt to compensate for ß-cell demise.

7.2 Introduction

miRNA have emerged as potent regulators of glucose homeostasis. miRNA are 19–25 nucleotide non-coding RNA molecules which suppress gene expression via imperfect base pairing to the 3' untranslated region of target mRNAs, leading to repression of protein production or mRNA degradation. miRNA have been shown to play a crucial role in pancreatic development (Poy, Eliasson et al. 2004, Poy, Hausser et al. 2009), insulin secretion (Poy, Hausser et al. 2009, Keller, Clark et al. 2012, Latreille, Hausser et al. 2014) and insulin resistance (Trajkovski, Hausser et al. 2011). In addition to being potential therapeutic targets, miRNA are also considered as future diagnostics and biomarkers for disease progression and therapeutic responses. They are differentially expressed in tissues affected by diabetes when compared to normal controls, and have been detected in serum, plasma and whole blood in animal models and human subjects with diabetes (Bhatt, Mi et al. 2011, Feng, Chen et al. 2011, Greco, Fasanaro et al. 2012).

Unique miRNA profiles have been identified in the serum of participants with diabetes, including both paediatric and adult cohorts (Ortega, Moreno-Navarrete et al. 2010, Zampetaki, Kiechl et al. 2010, Nielsen, Wang et al. 2012, Pescador, Perez-Barba et al. 2013, Osipova, Fischer et al. 2014, Yang, Chen et al. 2014, Higuchi, Nakatsuka et al. 2015). miRNA have also been identified as biomarkers for micro- and macrovascular complications with the potential to be of clinical utility in diabetes care (Kato, Zhang et al. 2007, Xiao, Luo et al. 2007, Baldeon, Weigelt et al. 2014, Zhou, Lv et al. 2014). The role of circulating miRNA in diabetes has been studied almost exclusively using the serum of human subjects. However, microRNA detection in various more accessible body fluids has attracted recent attention (Weber, Baxter et al. 2010).

HNF1A-maturity-onset diabetes of the young (HNF1A-MODY) is the most common monogenic form of diabetes resulting from mutations in the gene encoding the pancreatic transcription factor hepatocyte nuclear factor 1α (*HNF1A*) (Frayling,

Bulamn et al. 1997). Our research group recently demonstrated that induced suppression of endogenous *HNF1A* function in INS-1 cells, a cellular model of HNF1A-MODY (Wang, Maechler et al. 1998, Servitja, Pignatelli et al. 2009, Bonner, Bacon et al. 2010), increased the levels of two specific miRNA; miR-224 and miR-103 (Bonner, Nyhan et al. 2013). Using absolute quantitative PCR analysis, we demonstrated that miR-224 and miR-103 levels were significantly elevated in the serum of HNF1A-MODY mutation carriers when compared to controls (Bonner, Nyhan et al. 2013). miR-103 has been previously shown to play a key role in insulin resistance and glucose homeostasis. The overexpression of miR-103 had been shown to result in impaired insulin sensitivity (Trajkovski, Hausser et al. 2011).

miRNA can be secreted from cells and accumulate in microvesicles, either bound to Ago2 protein, or as free miRNA in extracellular fluids (Fevrier and Raposo 2004, Hunter, Ismail et al. 2008, Arroyo, Chevillet et al. 2011). Of note, *HNF1A* is expressed not only in the pancreas but also the liver, digestive tract and in particular the kidney (Kritis, Argyrokastritis et al. 1996). Urine has proven to be a stable biofluid (Mall, Rocke et al. 2013) which can be acquired non-invasively and is not subject to collection difficulties such as the haemolysis associated with blood draw.

The aim of this current study was to obtain proof-of-concept that miR-224 and miR-103 are detectable in the urine of HNF1A-MODY mutation carriers, and to determine whether these diabetes-associated miRNA are also elevated in the urine of patients with T1DM and T2DM. We also sought to correlate urinary levels of miRNA with serum levels, and with clinically relevant indices of renal disease.

7.3 Methods:

7.3.1 Study population:

In this study I included 38 genetically confirmed HNF1A-MODY mutations. The specific mutations included G207D, P291finsC, S352fsdelG, F426X, P379T, E230fsdelGA, p.L502fs, p.Leu17His, p.R159Q, R200Q, S335X and V351fsdelG. As control groups, I recruited n=44 participants with T1DM, n=36 participants with T2DM and n=26 normal controls. The cohorts were selected and phenotyped as detailed in sections 2.1.8, 2.1.9 and 2.1.10. In addition to basic phenotyping described in section 2.1.5 and 2.1.6, urinary osmolality was also measured using the Semi-micro Osmometer K-7400 (Knauer, Berlin, Germany).

7.3.2 RNA Isolation from Human Urine

A urinary sample was collected in a fasting state and collected in a specimen jar (20 mL volume). A mid-stream clean catch void was advised. Immediately after collection, a 1 mL aliquot of urine was drawn from the specimen and immediately frozen. For miRNA analysis, 100 µL of the urine specimen was utilized. Following centrifugation at 3000× g for 10 min at 4 °C, total RNA containing small RNA was extracted from 100 µL urine using the miRNeasy Serum/Plasma kit (Qiagen, Hilden, Germany). In brief, 700 µL of QIAzol reagent was added to 100 µL of urine sample and mixed well. To allow for normalization of sample-to-sample variation in RNA isolation, 25 fmol synthetic Caenorhabditis elegans miRNA cel-miR-39, (Sigma-Aldrich, Wicklow, Ireland) was added to each sample after 5 min incubation in QIAzol. Samples were mixed followed by adding 100 µL of chloroform. After mixing vigorously for 15 s, samples were centrifugated at 12,000× g for 15 min at 4 °C. The upper aqueous phase was carefully transferred avoiding the interphase to a new tube, and 700 µL of ethanol was added and mixed. At that point, the manufacturer's protocol was followed, with the entire aqueous phase from each sample loaded onto a single affinity column. Total RNA was eluted by adding 16 µL of RNase-free water to the membrane of the spin column and incubating for 1 min before centrifugation at 15,000× g for 1 min at room temperature and was stored at −80 °C. For the direct comparison of urine

and serum miRNA levels, 200 µL urine or serum were isolated and quantified as described in Bonner et al. (Bonner, Nyhan et al. 2013)]

7.3.3 Reverse Transcription and Quantitative Real-Time-PCR of Mature miRNA

Reverse transcription (RT) reactions are performed using the TaqMan miRNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA) and mature miRNA-specific stem-loop primers [hsa-miR-224, AssayID_002099 (MI0000301), hsa-miR-103, AssayID_000439 (MI0000109)] according to the manufacturer's instructions. Because the concentration of total RNA in urine has been below the limit of accurate quantitation by spectrophotometry, a fixed volume of 3 µL total RNA was used for cDNA preparation, as previously described (Mitchell, Parkin et al. 2008)]. For generation of standard curves, synthetic single-stranded RNA oligonucleotides corresponding to the mature miRNA sequences were purchased from Sigma-Aldrich. A serial of 10-fold dilutions of each oligonucleotide were made in water and run in parallel with the urine samples. Quantitative real-time PCR (qPCR) was performed in 96-well plates by use of the AIB 7500 instrument (Applied Biosystems, Foster City, CA, USA). The qPCR reaction mixture (20 µL reaction volume) includes 1.33 µL product from RT reaction, 1 µL TaqMan MicroRNA Assay (Life Technologies, Carlsbad, CA, USA), 10 µL of TaqMan 2× Universal PCR Master Mix (no AmpErase) (Life Technologies, Carlsbad, CA, USA) and 7.67 µL nuclease-free water. Amplification was performed under following conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The cycle threshold (C_t) values were calculated with the SDS 2.1 software (Applied Biosystems, Foster City, CA, USA), with the automatic setting for automatic assigning of baseline and threshold for C_t determination. Each patient sample, sample with spike-in cel-miR-39 and dilutions of synthetic miRNA for generation of standard curve were run in triplicates. C_t measurements with high standard deviation between triplicates were considered outliers and excluded from further analysis.

7.3.4 Absolute Quantification of miRNA and Data Normalization against Spiked-in Synthetic C. elegans miRNA Control

Standard curves for each miRNA of interest were generated by plotting C_t values of the dilutions of synthetic miRNA vs. number of copies per reaction followed by exponential fitting. For normalisation of thus quantified miR-103 and miR-224 levels, a median normalization procedure with spike-in cel-miR-39 was performed as previously described (Mitchell, Parkin et al. 2008)]. In brief, a normalisation factor for each sample was defined as ΔC_t of mean cel-miR-39 run in triplicate to the median of all cel-miR-39 that were run in parallel. Quantifications of miRNA in patient samples were then normalised by dividing by $2^{\Delta Ct}$.

7.3.5 Statistical Analysis

Statistical analyses were performed using MatLab and its statistical toolbox (The MathWorks, Inc., Natick, MA, USA). The individual miRNA copy number was calculated based on 1.7 µL of urine. Areas under the curves (AUCs) for insulin, glucose and C-peptide were calculated using the trapezoidal rule. Data are given as median and interquartile range (IQR) and were compared by Mann-Whitney U test and Spearman correlation analysis. Repeated samples of urine were analyzed using Wilcoxon signed rank test for paired data. Categorical data such as medication usage was compared using Fisher's exact test. The potential of miRNA to discriminate between normal controls and participants of the T1DM, T2DM or HNF1A-MODY mutation carrier groups or a pooled group thereof were assessed by receiver operating characteristic (ROC) analysis. In the ROC analysis, each level of miRNA is tested as a potential cut-off to distinguish between two groups. The resulting sensitivity and specificity is presented as a curve where an AUC = 1 would indicate perfect discrimination. An association between miRNA and renal indices was investigated by linear regression analysis and Spearman correlation. Hypotheses tests were considered statistically significant if $p < 0.05$.

7.4 Results

7.4.1 Clinical characteristics of all groups

A total of 144 individuals participated in the study. The clinical characteristics of the HNF1A-MODY mutation carriers (*n* = 38), T1DM (*n* = 44), T2DM (*n* = 36) and controls (*n* = 26) are contained in Table 1. The HNF1A-MODY carriers, T1DM and control groups were matched for age (41.5 years (21–52), 37 years (23–49.5) and 38 years (30–44), respectively, p = n.s.) and BMI (24.1 kg/m^2 (21.8–25), 25 kg/m^2 (22–27) and 23.4 kg/m^2 (22–27), respectively, p = n.s.). In terms of metabolic parameters, the HNF1A-MODY carriers, T1DM and control groups were comparable for systolic blood pressure (SBP), diastolic blood pressure (DBP), high density lipoprotein (HDL) cholesterol, total cholesterol, low density lipoprotein (LDL) cholesterol and triglyceride level. As expected, the T2DM group had a higher DBP (median (IQR) 80 mmHg (73–85)) and triglyceride level (1.24 mmol/L (0.8–2.68)) than all the remaining groups ($p < 0.05$). There was a significantly lower use of secondary preventative medication amongst both the HNF1A-MODY mutation carriers and T1DM group when compared to the T2DM group. Specifically, in the HNF1A-MODY mutation carriers there was lower use of aspirin (31.5% *vs.* 69.4%, $p \leq 0.05$), anti-hypertensive agents (15.7% *vs.*58.3% $p \leq 0.05$) and lipid lowering medications in particular statin therapy (69.4% *vs.* 23.6%, $p \leq 0.05$) when compared to the T2DM group. Likewise, in the T1DM group there was a lower use of aspirin (36.3% *vs.* 69.4%, $p \leq 0.05$) and anti-hypertensive agents (34% *vs.* 58.3% $p \leq 0.05$) when compared to the T2DM group.

As expected, the T1DM group had the highest HbA_{1c} when compared to all remaining groups (63 mmol/mol (8%) (55–75) / (7.2–9); $p < 0.05$) with an undetectable C-peptide level (<66 mmol/L). The T2DM group had a lower OGIS level than the HNF1A-MODY mutation carriers (295 mL/min/m^2 (223–344) *vs.* 355 mL/min/m^2 (296–410) $p < 0.05$) in keeping with the presence of insulin resistance.

Parameter	Normal Controls	T1DM	T2DM	HNF1A-MODY	Statistical Analysis *p* Value (* Bonferroni Corrected)					
Group	0	1	2	3	0 *vs.* 1	0 *vs.* 2	0 *vs.* 3	1 *vs.* 2	1 *vs.* 3	2 *vs.* 3
N	26	44	36	38						
Duration of diabetes (years)	n.a.	19 (4–28)	3 (1–8)	8 (2–24.25)	n.a.	n.a.	n.a.	0.0009 (*)	n.s.	n.s.
HbA_{1c} (mmol/mol)/ (%)	32.5 (31–35)/ 9.2 (9.19.4)	63 (55–75)/ 8 (7.2–9)	53 (49–65)/ 7 (6.6–8.1)	54 (44–63)/ 7.1 (6.2–7.9)	<0.0001 (*)	<0.0001 (*)	<0.0001 (*)	0.004	0.001	n.s.
SBP (mmHg)	121 (113–128)	125 (119.5–130)	135 (130–149.5)	120.5 (113.5–129)	n.s.	<0.0001 (*)	n.s.	0.0003 (*)	n.s.	0.0001 (*)
DBP (mmHg)	73 (69–79)	76.5 (69–80)	80 (74–85)	71 (66.5–77)	n.s.	0.0156	n.s.	0.0441	n.s.	0.0002 (*)
T.Cholesterol (mmol/L)	4.8 (4.13–5.5)	4.25 (3.8–4.7)	4 (3.4–4.68)	4.35 (3.7–5.1)	0.0465	0.0079	n.s.	n.s.	n.s.	n.s.

Creatinine (mmol/L)	79 (71.25–95)	70 (65.5–83)	76 (70–85.75)	68 (58–76.25)	0.0282 (n.s.)	n.s.	0.0014 (n.s.)	n.s.	n.s.	0.0136 (n.s.)
ACR (g/mol)	0 (0–0.8)	0.5 (0–1.33)	0.9 (0.5–2.7)	0.6 (0.4–0.96)	n.s.	0.0001 (*)	0.0130 (n.s.)	0.0101 (n.s.)	n.s.	0.04 (n.s.)
GFR (mL/min/1.73 m^2)	74 (70–83)	89 (78–113)	85.5 (68–103)	88.5 (75–112)	0.0005 (*)	n.s.	0.0024 (n.s.)	n.s.	n.s.	n.s.
Fasting C-Peptide (pmol/L)	367 (236.5–581)	<66	924 (775–1295)	533 (365–681)	n.a.	0.0009 (*)	n.s.	<0.0001 (*)	n.a.	0.0001 (*)

Table 7-1: Clinical Characteristics of the normal controls, T1DM, T2DM and HNF1A-MODY cohorts. SBP/DBP=systolic diastolic / blood pressure, ACR=albumin creatinine ratio, GFR=glomerular filtration rate. n/a=not applicable, n.s. =not significant

7.4.2 miR-224 is Detectable in Urine and is Highly Expressed in HNF1A-MODY Mutation Carriers and Participants with T1DM

In our initial report, we identified a strong association between elevated serum levels of miR-224 and the presence of a HNF1A-MODY mutation (Bonner, Nyhan et al. 2013). To test whether miR-224 was also detectable in the urine of participants with diabetes, RNA from human urine samples was isolated and hsa-miR-224 expression was quantitated via TaqMan qPCR assays. Samples were measured in triplicate using synthetic miR-224 single-stranded RNA Oligonucleotides as standards to obtain absolute miRNA copy numbers that were furthermore normalized against spiked-in synthetic *C. elegans* miRNA control. miR-224 levels were detected at a significantly higher level in the urine of HNF1A-MODY mutation carriers when compared to the control group (2790 × 10^3 copies per 1.7 µL (1430–7990 × 10^3) *vs.* 1180 × 10^3 copies per 1.7 µL (575–2030 × 10^3), $p < 0.05$) (Figure 7.1). In a subgroup of patients, a repeat urine sample was attained during clinical follow up (median follow up; 16 months (4–36 months)) and analyzed to determine repeat levels of miR-224. On repeat analysis, there was no significant difference in expression levels for miR-224 ((initial sample); 660 × 10^3 copies per 1.7 µL *vs.* (subsequent sample); 710 × 10^3 copies per 1.7 µL, $p = 0.5$), again emphasizing the robust nature of miR-224. In addition, we compared HNF1A-MODY mutation carriers with the highest levels of miR-224 to those having a "low to normal" level for all clinical parameters including age, duration, BMI and HbA_{1c}. Findings demonstrate that participants with the highest miR-224 values have measurements within the same range as the "low to normal" group for the clinical parameters studied.

We also determined urine levels of miR-224 in individuals with T1DM and T2DM (Figure 7.1). miR-224 was significantly elevated in the urine of individuals with T1DM when compared to controls (2380 × 10^3 copies per 1.7 µL (1120–7970 × 10^3) *vs.* 1180 × 10^3 copies per 1.7 µL (575–2030 × 10^3), $p < 0.05$), but there was no significant difference noted in miR-224 levels in the T1DM population studied when compared to the HNF1A-MODY group (2380 × 10^3 copies per 1.7 µL (1120–7970 × 10^3) *vs.* 2790 × 10^3 copies per 1.7 µL (1430–7990 × 10^3), $p = 0.8$). miR-224 expression levels were significantly higher in both the HNF1A-MODY mutation carriers and T1DM groups when compared to the T2DM group (HNF1A-MODY mutation carriers: 2790 × 10^3 copies per 1.7 µL (1430–7990 × 10^3) *vs.* 1520 × 10^3 copies per 1.7 µL (630–3960 × 10^3), $p< 0.05$) and (T1DM: 2380 × 10^3 copies per 1.7 µL (1120–7970 × 10^3) *vs.* 1520 × 10^3 copies per 1.7 µL (630–3960 × 10^3), $p < 0.05$).

The ROC analysis for miR-224 in the HNF1A-MODY mutation carriers, T1DM, T2DM and normal control groups are presented in Figure 7.2. miR-224 can reasonably differentiate HNF1A-MODY from normal controls (AUC = 0.74) as well as T1DM from normal controls (AUC = 0.76). Furthermore, ROC analysis of pooled groups with diabetes (T1DM, T2DM and HNF1A-MODY mutation carriers) indicates that miR-224 can distinguish between normal controls and diseased patients (AUC = 0.72).

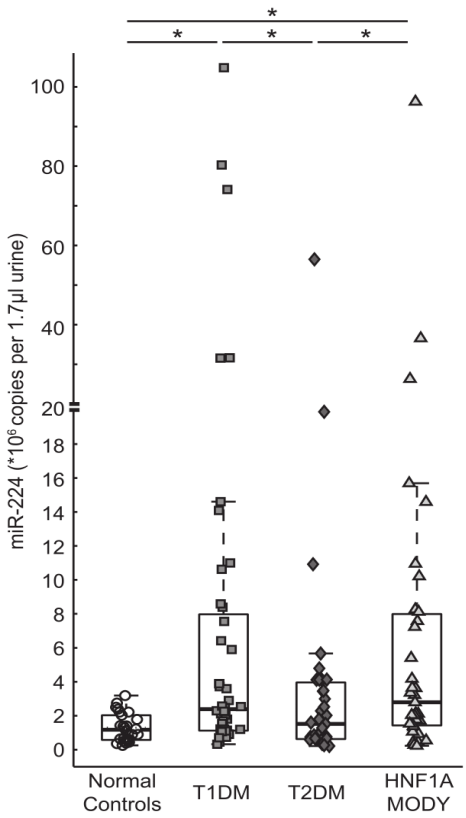

Figure 7-1: miR-224 levels detected in urine. Copies of miRNA per reaction were determined by quantitative qPCR in urine samples of T1DM (*n* = 44), T2DM (*n* = 36), HNF1A-MODY (*n* = 38) and normal control (*n* = 26) subjects. Box plots depict median and inter quartile range. The HNF1A-MODY mutation carriers had the highest median value of miR-224 and was significantly different from normal controls showing the lowest median value. In addition, the T1DM group had a higher median value than normal controls. miR-224 expression levels in the T2DM group were significantly lower than in T1DM group and the HNF1A-MODY mutation carriers but were similar to normal controls. (* significant differences by Mann-Whitney test).

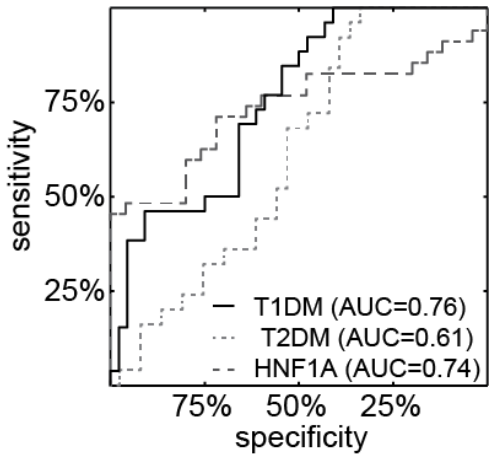

Figure 7-2: ROC analysis of miR-224. Levels of miR-224 in urine differentiated the normal control group from the T1DM, HNF1A-MODY mutation carrier and T2DM groups, respectively.

7.4.3 miR-103 is Detectable in Urine and is Highly Expressed in Patients with Diabetes

We also found strongly elevated levels of miR-103 in the urine of HNF1A-MODY mutation carriers when compared to the control group (median (IQR) 2020 × 103 copies per 1.7 µL (622–5840 × 103) vs. 559 × 103 copies per 1.7 µL (385–997 × 103) $p < 0.05$) (Figure 7.3). Similar to miR-224, we found no significant difference on subsequent measurement for miR-103 ((initial sample) ;390 × 103 copies per 1.7 µL vs. (subsequent sample); 260 × 103 copies per 1.7 µL, $p = 0.6$).We next determined urine levels of miR-103 in individuals with T1DM and T2DM. There were significantly higher levels of miR-103 in the urine of individuals with T1DM and T2DM when compared to controls (T1DM: 1800 × 103 copies per 1.7 µL (698–453 × 103) vs. 559 × 103 copies per 1.7 µL (385–997 × 103), $p < 0.05$) and (T2DM: 1300 × 103 copies per 1.7 µL (478–309 × 103) vs. 559 copies per 1.7 µL (385–997 × 103), $p < 0.05$), respectively. There was no difference noted in miR-103 expression levels between all diabetes cohorts studied (T1DM, T2DM and HNF1A-MODY mutation carriers) (Figure 3).

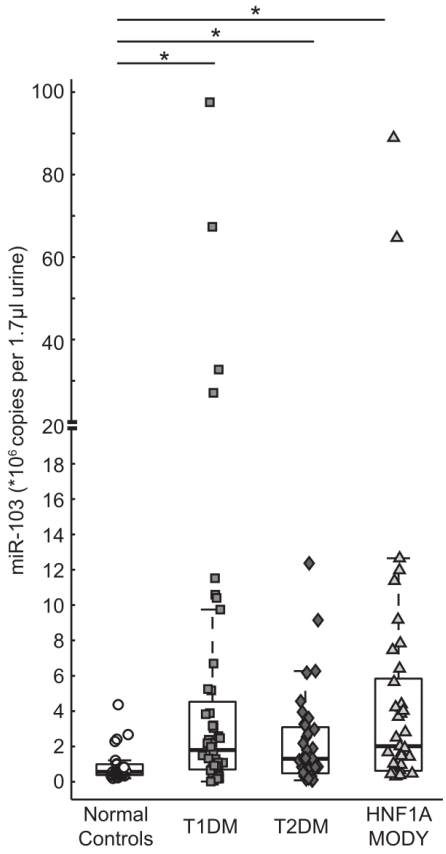

Figure 7-3: miR-103 levels detected in urine. Copies of miRNA per reaction were determined by quantitative qPCR in urine samples of T1DM (*n* = 44), T2DM (*n* = 36), HNF1A-MODY mutation carriers (*n* = 38) and normal control (*n* = 26) subjects. Box plots depict median and inter quartile range. Levels of miR-103 were statistically higher in all disease groups compared to normal controls where HNF1A-MODY mutation carriers showed the highest expression values (* significant differences by Mann-Whitney test).

7.4.4 Correlation of Urine miR-224 and miR-103 Levels with Clinical and Biochemical Characteristics

We next performed Spearman correlation analyses to identify any correlation of urine miRNA levels with clinical and biochemical parameters. Since any published work available to date on urinary miRNA has been performed in the setting of renal

pathology, we analyzed any potential correlation of miR-224 and miR-103 levels with indices of renal disease. The median eGFR was >60 mL/min/1.73 m^2 in all groups signifying the absence of overt renal disease. Likewise, the median ACR values in all patients studied did not reveal microalbuminuria or overt albuminuria. The miR-224 and miR-103 levels in HNF1A-MODY mutation carriers, T1DM or T2DM did not correlate with the renal indices; ACR or eGFR (Figure 7.4). In addition, there was no correlation detected between miR-224 and miR-103 and urinary osmolality (miR-224; $\rho = 0.04$, $p = 0.6$ and miR-103; $\rho = -0.002$, $p = 0.9$). Serum and urine miRNA analysis performed in matched cohort samples, using identical miRNA quantification methods revealed a significant correlation between serum and urine miRNA levels (miR-224: $\rho = 0.55$, $p \leq 0.01$ and miR-103: $\rho = 0.38$, $p = 0.01$). Figure 7.5 demonstrates the statistically significant association between serum and urine for miR-224 in a cohort of HNF1A-MODY mutation carriers and normal controls.

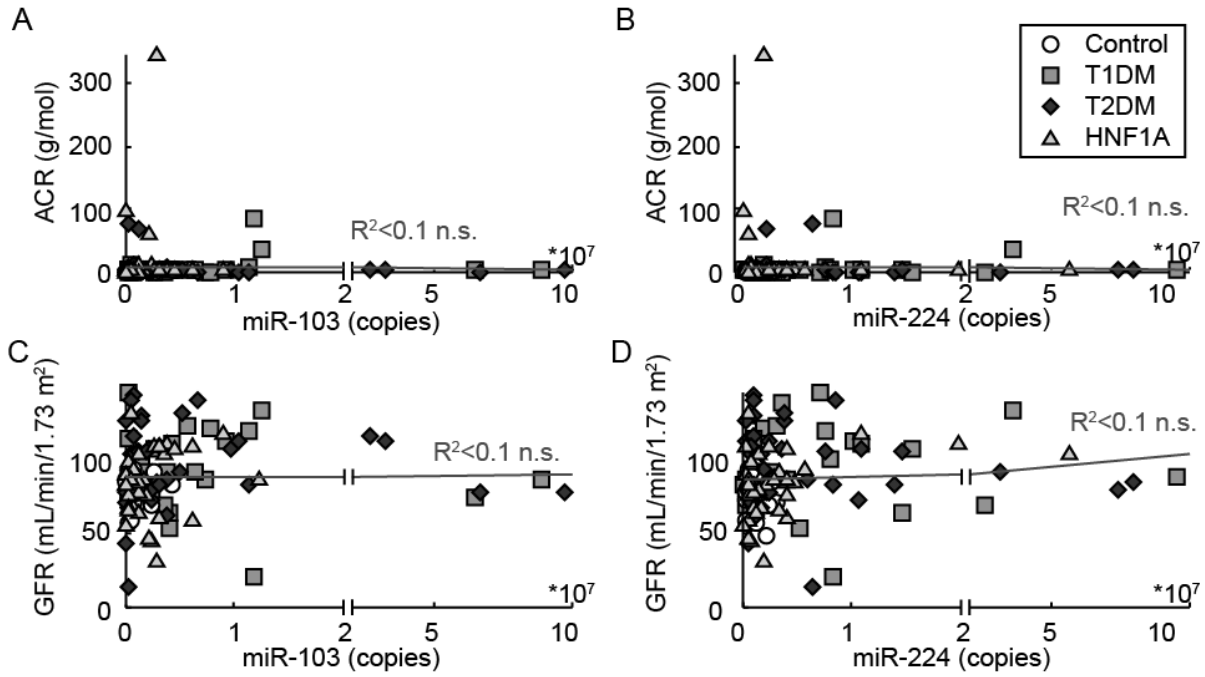

Figure 7-4: Correlation of miR-224 and miR-103 with renal indices; eGFR/ACR. Linear regression analysis indicates no statistically significant association of miR-224/-103 with GFR/ACR in the HNF1A-MODY mutation carrier, T1DM, T2DM and normal control groups; n.s. not significant.

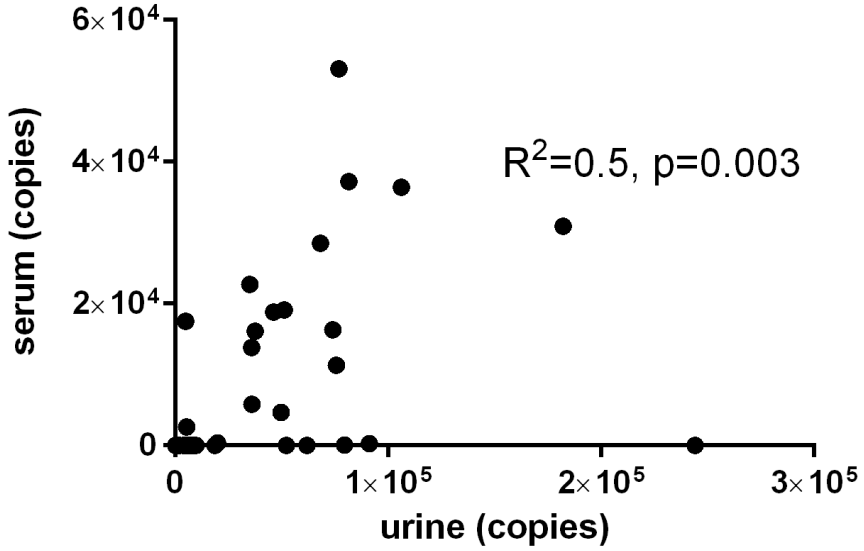

Figure 7-5: Correlation of miR-224 in the serum and urine of a matched cohort of HNF1A-MODY mutation carriers (*n* = 30) and normal controls (*n* = 8). Spearman correlation analyses demonstrated a significant correlation between serum and urine for miR-224 (R^2 = 0.5, p < 0.003).

7.5 Discussion

miRNA represent an important mechanism in the regulation of gene expression and the potential to manipulate this expression has significant implications in the management of disease states including diabetes. In this study, we report, for the first time, detection of circulating diabetes-associated miR-224 and miR-103 in the urine of patients, notably in the absence of renal pathology. This is also the first study to detect miRNA in the urine of a large cohort of HNF1A-MODY mutation carriers. HNF1A-MODY is a monogenic form of diabetes and therefore an ideal model for the study of the ß-cell. HNF1A-MODY is primarily a disorder resulting in ß-cell dysfunction, with mutation carriers lacking features of the metabolic syndrome such as insulin resistance, as opposed to the multifactorial aetiology synonymous with T2DM. We chose to study miR-224 and miR-103 in particular based on preliminary work performed in our laboratory whereby the endogenous expression

of *HNF1A* in INS-1 cell lines resulted in the elevated expression of both miR-224 and miR-103 (Bonner, Nyhan et al. 2013).

Of interest, we have found that miR-224 is highly expressed in the urine of patients with T1DM and HNF1A-MODY mutation carriers when compared to both the T2DM and the normal control cohorts. miR-224 is a novel miRNA in the field of diabetes. To date, it has been shown that miR-224 is aberrantly expressed in a wide variety of malignancies including prostatic, hepatocellular, renal cell and colorectal. It is known to promote cell migration, proliferation, and invasion and has been shown to target TGF-ß signaling via the Smad 4 pathway (Mees, Mardin et al. 2009, Boguslawska, Wojcicka et al. 2011, Zhang, Zhou et al. 2013, Li, Ding et al. 2014). The elevated expression of miR-224 in both HNF1A-MODY mutation carriers and T1DM is not surprising given that both share certain traits. ß-cell failure and ultimately a reduction in ß-cell mass are noted in both HNF1A-MODY mutation carriers and T1DM (Kloppel, Drenck et al. 1984, Vesterhus, Haldorsen et al. 2008). In contrast to T2DM, which is a *relative* insulin deficient state with the pathology attributed largely to insulin resistance, it is a defect in insulin secretion that is the principal pathology of both HNF1A-MODY mutation carriers and T1DM (Fajans, Bell et al. 2001). This defect predates the clinical manifestation of diabetes as demonstrated by research in the carriers of HNF1A-MODY who were normoglycaemic and likewise in islet antibody positive relatives of those with T1DM (Byrne, Sturis et al. 1996, Siewko, Poplawska-Kita et al. 2014). We can hypothesize that the altered miR-224 expression profile may be a contributor to the ß-cell dysfunction noted in both HNF1A-MODY mutation carriers and T1DM. We can speculate that miR-224, as a marker of ß-cell demise, has the potential to be an important clinical development given the current inability to perform pancreatic biopsies in humans and lack of ß-cell imaging techniques. There are currently no available biomarkers of ß-cell demise.

miR-224 was significantly elevated in the urine of HNF1A-MODY mutation carriers when compared to the T2DM cohort studied. We therefore propose that the clinical utility of miR-224 may be further expanded as an additional screening tool to decipher who should be screened for HNF1A-MODY genetic testing. This is of clinical relevance, given that it is not feasible to perform genetic testing on all potential mutation carriers, and the greatest clinical challenge is in the discrimination of potential HNF1A-MODY mutation carriers from lean patients with T2DM.

In the current study, miR-103 was highly expressed in the urine of all participants with diabetes when compared to the normal control cohort. To our knowledge, this is the first description of miR-103 in the urine of patients with diabetes. A recently published meta-analysis ranked miR-103 as one of the most common, consistently deregulated miRNA in diabetes across multiple tissue types including pancreatic, adipose and serum (Zhu and Leung 2015). Our novel finding of such a well-established diabetes-associated marker in urine of patients with both mono- and polygenic forms of diabetes lends credence to the validity of the methodology employed to detect miRNA in the current study. Whilst the pathway regulated by miR-224 is not fully elucidated, the principal targets of miR-103 have been investigated extensively. Caveolin 1 is a known target of miR-103; *in vitro* studies have previously demonstrated that caveolin 1 interacts with the insulin receptor improving insulin mediated phosphorylation of IRS-1 (Trajkovski, Hausser et al. 2011). *Cav-1* deficient mice develop post-prandial hyperglycaemia and insulin resistance. It may be that the increased miR-103 expression in diabetes is an attempt to surmount the lack of insulin with enhanced insulin signaling.

In the current study, we can only speculate as to the origin of miR-224 and miR-103 in the urine of individuals with diabetes. The miRNA detected in the urine may have originated from ß-cells or indeed from tissues affected by diabetes such as liver, muscle, or kidneys. It is also possible that miRNA are actively secreted by the kidneys. A likely explanation is that miRNA derive in urine (at lower quantities than in serum) from the blood through the process of glomerular filtration. The approximate molecular weight of miRNA is 6.2–7.2 kDa (Blondal, Jensby Nielsen, 2013). The threshold of the glomerular filtration barrier is approximately 60 kDa. Therefore, a substantial portion of circulating miR-224 and miR-103 could be ultra-filtrated through the kidneys.

The utilization of urine as a biofluid has multiple advantages in the clinical setting. Urine sampling is non-invasive and it is collected at every diabetes and nephrology clinic. Urine is not subject to hemolysis. This of utmost importance, as blood contamination and hemolysis are the two most common artifacts encountered in miRNA detection in fluids acquired by invasive means (serum/plasma/CSF) (Weber, Baxter et al. 2010, Kirschner, Kao et al. 2011, Blondal, Jensby Nielsen et al. 2013). A publication by Mall *et al*. showed that urinary miRNA remain stable under harsh conditions including freeze/thaw cycles and prolonged storage at room temperature. Although there may be some degradation over time, lower levels have been shown to remain detectable (Mall,

Rocke et al. 2013). We have performed repeat urinary miR-224 and miR-103 measurements on a subset of participants. The repeat samples were drawn on separate days in a non-fasting state. There was no significant difference noted between both measurements. These particular urinary miRNA appear to be robust and independent of dietary restriction which is important in a clinical setting.

A limitation of the current investigation is that we cannot provide a definitive causation for the differential expression of urinary miR-224 between the different forms of diabetes. However, reassuringly, we have demonstrated that the elevated expression of miR-224 and miR-103 levels is a common phenotype *in vitro*, in serum and now in the urine of HNF1A-MODY mutation carriers. As aforementioned, HNF1A-MODY represents an ideal ß-cell model. The establishment of gene manipulated *in vitro* and rodent models may facilitate the functional analyses of such miRNA and promote further translational research for the diagnosis and treatment of diabetes. A further strength of this study is the inclusion of both mono- and polygenic diabetes forms.

7.6 Conclusions

In conclusion, we here provide the first proof-of-concept study that miRNA can be readily detected in the urine of participants with diabetes. miR-224 is a novel diabetes-associated miRNA which is highly expressed in the urine of HNF1A-MODY mutation carriers and a T1DM cohort. We surmise that the differential expression levels of miR-224 in both insulin deficient states may be an attempt to compensate for ß-cell demise. A further novel finding of the current study is the detection of the well-established diabetes-associated miR-103 in urine. Finally, our study highlights the benefits in the use of urine as a biofluid for miRNA detection in the clinical setting.

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Chapter 8

Concluding Discussion

8 Chapter 8: Concluding Discussion

In the most recent decades our understanding of the pathophysiology of the condition coined "MODY" has improved significantly. However, despite an increasingly refined knowledge of the condition the majority of individuals with MODY remain undiagnosed. The estimated prevalence is 70-110 per million of population. MODY, given that it is inherited in an autosomal fashion and has a 95% penetrance rate is an extremely important diagnosis to make for a pedigree. The MODY clinic at the Mater Hospital was established a decade ago to promote awareness, reduce complication rate and ultimately to improve patient care amongst MODY carriers. This candidature is a compilation of work gleaned from the study of this unique cohort.

8.1 Main Findings

The principal findings of are as follows;

Firstly, there is a paucity of data available on the management of MODY in pregnancy. The presence of a HNF1A-MODY mutation in the mother is not believed to influence birth weight in the offspring. It is also believed that GCK-MODY is a stable, mildly progressive form of diabetes not necessitating treatment outside of pregnancy. However, controversy exists as to the most appropriate management of pregnancies complicated by GCK-MODY. The prevailing school of thought would be to withhold insulin treatment pending fetal growth sizing at 27 weeks gestation. If at this juncture the abdominal circumference is greater than the 75th centile, insulin treatment should be instigated. However, I would argue that insulin initiated at this late stage may not prevent macrosomia in the offspring. To provide clinical clarification, I performed a retrospective chart review on pregnancies complicated by MODY in the three tertiary referral maternity centres in Dublin. For the first time, glycaemic variability using HBGM profiles were analyzed. The findings of this pertinent study were as follows; firstly the current conventional management of HNF1A-MODY in pregnancy as per pre-gestational

diabetes protocols is appropriate. However, in GCK-MODY pregnancies we demonstrated significant glycaemic variability particularly in the first trimester. I highlight the difficulty in achieving reasonable glycaemic control in this population even with insulin therapy. I report a higher miscarriage rate in GCK – MODY pregnancies than that of the background population. There was also an increased macrosomic rate in GCK N/N group not treated with insulin and consequently an increased rate of neonatal co morbidities. Overall, I reiterate the importance of pre-conception diagnosis and planning of pregnancies complicated by GCK-MODY.

HNF4A-MODY accounts for approximately 10% of all MODY cases (Kyithar, Bacon et al. 2011). Despite its rarity relative to GCK and HNF1A-MODY it is an important entity to recognise. Congruent clinical characteristics exist between HNF4A and HNF1A-MODY largely with the exception of macrosomia and hyperinsulinemic hypoglycaemia. Congenital hyperinsulinaemic hypoglycaemia is characterised by hypoglycaemia caused by the unregulated secretion of insulin from pancreatic beta cells. HNF4A-MODY is now a well-recognised cause of congenital hyperinsulinism with the oldest reported case requiring treatment being 12 years of age. Following this phase there appears to be a period of "wellness" prior to the onset of diabetes. My clinical observation of asymptomatic fasting hypoglycaemia in our adult HNF4A-MODY cohort stimulated speculation as to whether hyperinsulinaemic hypoglycaemia persists into adult hood in a proportion of mutation carriers. To investigate this potential phenomenon further I studied serial OGTTs performed on HNF4A-MODY IGT carriers. I also utilised CGMS to appreciate glycaemic variability and in particular to capture episodes of asymptomatic hypoglycaemia. Findings from this study revealed preserved insulin, C-peptide and glucose levels over time in the HNF4A-IGT group. Of clinical significance was the incidence of asymptomatic hypoglycaemia detected using CGMS in the HNF4A-MODY cohort. The incorporation of such information into the clinical management of MODY enables better care for these patients.

Cardiovascular disease is a significant cause of morbidity and mortality in individuals with diabetes. Cardiovascular outcomes in MODY cohorts are relatively unknown. The studies to date were largely conducted in an era prior to stain therapy and the adoption of strict guidelines for blood pressure and glycaemic controls in diabetes. Diabetes in HNF1A-MODY cohorts is typically diagnosed before the age of 25 years; therefore these individuals are exposed to a similar

period of dysglycaemia as that of a T1DM population. As a result one may expect a similar atherosclerotic burden in HNF1A-MODY mutation carriers. However, HNF1A-MODY is synonymous with a favourable metabolic profile, in particular a high HDL level. To this end, I investigated sCD36, a known marker of atherosclerotic burden in a MODY cohort. CD36 expression is increased in the presence of insulin resistance and excess CD36 is thought to initiate atherosclerotic lesions. To our surprise, we found a significantly lower level of sCD36 in the HNF1A-MODY cohort when compared to a T2DM population. I hypothesised that the decreased levels of CD36 may be instrumental in increasing HDL levels in HNF1A-MODY and perhaps confer a protective mechanism against atherosclerotic burden in this population.

There are limited studies pertaining to the natural progression of HNF1A-MODY in individuals over their lifetime. To facilitate accurate counselling and appropriate clinical management, I conducted a retrospective review of the HNF1A-MODY cohort in the Mater University Hospital. The principal findings were as follows; firstly, the significant majority of the MODY cohort treated with sulphonylurea therapy remain insulin independent. The success of sulphonylurea therapy was determined by the duration of diabetes, HbA_{1c} at presentation to the MODY clinic, early initiation of sulphonylurea therapy and the weight gained throughout the study duration. Another important finding from this study was that of a reduced microvascular complication rate in HNF1A-MODY when compared to that reported in the existing literature. I hypothesised that the early recognition of HNF1A-MODY through clinical awareness and familial screening significantly influences the incidence of complications amongst the HNF1A-MODY cohort.

As previously stated, diagnosing MODY can be a clinical challenge given the overlap in characteristics with T1DM and T2DM. The absence of a cheap diagnostic screening tool as an alternative to genetic testing is a principal reason for under diagnosing the condition. We therefore decided to determine if the expression of specific miRNAs could differentiate between MODY and T1DM or T2DM. For the first time, miRNAs were studied in the urine of individuals with HNF1A-MODY. We found miR-224 to be significantly elevated in the urine of individuals with HNF1A-MODY and T1DM when compared to both normal controls and a T2DM cohort.

Pooled data also demonstrated that miR-224 could differentiate between a diseased and normal state. miR-224 is a novel miRNA in the field of diabetes. I also emphasised the clinical utility of miRNAs in urine as attractive biomarkers in the clinical setting. Urine is a readily available and cheap biofluid. In addition, we demonstrated the robust nature of miRNAs given their reproducibility following freeze/thaw cycling and diurnal variability.

8.2 Implications of the thesis

As clinicians, we aim to deliver "personalised medicine", that is the tailoring of treatment to the needs of the individual patient. Overall, I hope I have emphasized the clinical importance of making an accurate diagnosis of MODY in an individual. The advent of next generation sequencing technology will facilitate the simultaneous analysis of a large panel of genes at a cost comparable to Sanger sequencing (Ellard, Lango Allen et al. 2013, Bonnefond, Philippe et al. 2014, Szopa, Ludwig-Galezowska et al. 2015). As a result there will be an increase in the number of individuals diagnosed with a monogenic form of diabetes and an increased requirement to understand these disorders. We have an obligation to our patients to recognise the implications of making a diagnosis of MODY.

The study of the MODY cohort in the Mater hospital has revealed several important findings which will improve our understanding of the condition. For the first time, I utilised HBGM profiles to determine glycaemic variability in pregnancies complicated by GCK-MODY. This revealed that it is difficult to achieve optimal glycaemic control in this population particularly in the first trimester. This is of importance given the controversy that currently abides in relation to insulin treatment in this cohort. The biphasic phenomenon associated with HNF4A-MODY was investigated further utilising CGMS and serial OGTT profiling. I noted a significant incidence of asymptomatic hypoglycaemia in this unique cohort, the clinical implication of which remains unknown. I have noted that a marker of atherosclerotic burden; sCD36 is expressed at significantly lower levels in HNF1A-MODY than those with T2DM. I have reported on a lower incidence of microvascular complications than that reported in the literature to date. These findings will assist clinicians in counselling HNF1A-MODY patients and their family members as to the natural progression of the condition. I have also reported that

the usage of sulphonylurea therapy in HNF1A-MODY is optimal management, with the early initiation of same resulting in insulin independence. This is extremely important not only for improving quality of life but also from an economic prospective. The psychosocial implication of a MODY diagnosis cannot be underestimated. The transfer from insulin to OHAs is of significant benefit to the patient. In relation to diagnosis, we have proposed a role for miR-224 in differentiating MODY from T2DM. The use of a panel of bio-markers complimenting clinical acumen may direct the appropriate patient to next generation sequencing. This use of NGS will indefinitely reduce the interval between clinical suspicion of MODY and confirmatory molecular diagnosis.

8.3 Strengths of the study:

This is the largest cohort of individuals with MODY that exists in Ireland. In addition, since the establishment of the MODY clinic, individuals diagnosed with the condition are followed up clinically at least bi-annually. As a result, much additional information as to the management of the condition has been acquired. Many novel approaches and technologies have been adopted in this study including the usage of CGMS and miRNA measurement. This is also the first study to utilise HBGM profiles to determine glycaemic variability in pregnancies complicated by MODY and to highlight the importance of pre-conception care.

8.4 Future research opportunities:

A prospective study on the development of micro and macrovascular complications, in particular CVD in a larger cohort would be of benefit to strengthen our findings. A longitudinal study on MODY in pregnancy would facilitate the establishment of a protocol for the clinical management of hyperglycaemia throughout the trimesters. Further investigation into the dual phenotype of HNF4A-MODY and the mechanism for prolonged asymptomatic hypoglycaemia in a proportion of adult HNF4A-MODY IGT mutation carriers is warranted.

There is an inherent public perception that diabetes per se is a self-inflicted condition. As advocates for our patients with diabetes we need to raise awareness of the entity MODY and deliver the best possible care.

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Appendices

9

9.1 Appendix 1: Co-Authorship Signature Forms

Appendix 2: Ethical Approval Confirmatory Letters for studies contained within the thesis.

9.2 Appendix 3: MODY referral form (available on MaterNet)

Referral Form

Screening for Maturity Onset Diabetes of the Young.

(MODY)

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9.3 Appendix 4: Published articles