The clinical and genetic characterisation of young onset diabetes

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by

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Memorandum

The work in this thesis is the original work of the author. Experiments were carried out at the Oxford Centre for Diabetes, Endocrinology & Metabolism, University of Oxford, under the supervision of Dr Katharine Owen and Prof Mark McCarthy. Funding was provided by the Higher Education Commission of Pakistan, Oxfordshire Health Services Research Committee and Biomedical Research Council.

Evaluation of apolipoprotein M as a biomarker for HNF1A-MODY (chapter 3) has been published in Diabetic Medicine. The manuscript is provided as an appendix (Mughal, et al., 2013a).

Rebbeka Park performed the measurement of apoM levels (Institute of Molecular Health Sciences, ETH Zurich, Switzerland). Plasma glycan release and HPLC analysis of plasma and membrane glycans was performed by Mislav Novokmet (Glycobiology Laboratory, Genos Ltd, Zagreb, Croatia).

I hereby state that no part of this thesis has been submitted for any other degree at this or any other university.

This thesis is approximately 45,000 words.

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The clinical and genetic characterisation of young-onset diabetes

Saima Amin Mughal, Green Templeton College, DPhil thesis, Trinity term 2014

Abstract

Maturity-onset diabetes of the young (MODY), due to hepatocyte nuclear factor 1 alpha mutations (HNF1A-MODY), is the most common form of monogenic diabetes presenting in young adults. An accurate genetic diagnosis of HNF1A-MODY has therapeutic implications for the patients and their family members. However, the majority of people with HNF1A-MODY are not referred for genetic testing and remain misdiagnosed as type 1 or type 2 diabetes.

As part of measures to address this misdiagnosis, over the last few years there have been efforts to define clinical features and biomarkers that can be used to identify those at high risk of HNF1A-MODY. Secreted hepatic proteins regulated by HNF1A are attractive candidates for diagnostic biomarkers that would be specific for this form of diabetes. Apolipoprotein M (apoM), C-reactive protein (CRP) and plasma glycan profile have all been investigated as biomarkers to improve selection of suspected MODY cases for genetic testing. In my thesis, I have addressed questions about the variation in apoM between different forms of diabetes and assessed the performance of hsCRP and plasma glycan profile to identify HNF1A-MODY in previously uninvestigated individuals with young-onset diabetes and in a non-European population. Additionally because CRP and plasma glycans are both important components of an acute inflammatory response, I examined the effect of haploinsufficiency of *HNF1A* in a standardised model of inflammation.

When investigating apoM, I showed that serum apoM levels are lower in HNF1A-MODY than controls, and have demonstrated for the first time that serum apoM provides good discrimination between HNF1A-MODY and type 1 diabetes. CRP and plasma glycan profile both performed well in identifying HNF1A-MODY cases in unselected young adults with diabetes. The results also suggested that both biomarkers have value for assessing the functional impact of novel *HNF1A* variants. I went on to examine the use of a low CRP for selecting those at risk of HNF1A-MODY in South Asian subjects with young-onset diabetes. This study suggests that the overall population prevalence of HNF1A-MODY is similar in South Asians to Europeans, but that MODY represents a lower proportion of those with diabetes (due to the higher prevalence of type 2 diabetes in South Asians). The specific selection strategy employed in this study was not successful in identifying subjects at high risk of HNF1A-MODY (only 3% of those sequenced had mutations), suggesting that additional clinical and biochemical features will be required in addition to CRP to distinguish South Asians at high risk of HNF1A-MODY.

Lastly, using endotoxaemia as a standardised model of acute inflammation for the first time in HNF1A-MODY, I have shown that despite low baseline levels, subjects with HNF1A-MODY had peak stimulated CRP levels comparable to non-diabetic controls. An attenuated cytokine response was observed in HNF1A-MODY, which requires further investigation. This is also the first report of inflammation-associated changes in plasma and white cell membrane glycan profile in diabetes.

This research work adds substantially to current understanding of performance of HNF1A-MODY biomarkers, a critical step before their clinical translation. The work presented also provides novel insights into the regulation of the acute inflammatory response in HNF1A-MODY.

List of common abbreviations

ADA	American Diabetes Association
ApoM	Apolipoprotein M
BMI	Body mass index
DG9-glycan index	Desialylated glycan 9 index
ESR	Erythrocyte sedimentation rate
GCK	Glucokinase
GWAS	Genome-wide association study
HDL	High density lipoprotein
HNF1A	Hepatocyte nuclear factor 1 alpha
HNF4A	Hepatocyte nuclear factor 4 alpha
HPLC	High performance liquid chromatography
HsCRP	High-sensitivity C-reactive protein
IL-6	Interleukin 6
IL-1ra	Interleukin 1 receptor antagonist
TNFα	Tumour necrosis factor α
LADA	Latent autoimmune diabetes of adults
LBP	Lipopolysaccharide binding protein
LPS	Lipopolysaccharide
LOLIPOP	London life Sciences prospective population study
MAF	Minor allele frequency
MODY	Maturity-onset diabetes of the young

ROC	Receiver operating characteristic
OXBB	Oxford Biobank
WBC	White blood cells
WHO	World Health Organisation
YDX	Young diabetes in Oxford study

List of tables

List of figures

Contents

Chapter 1

General Introduction

1.1 Diabetes: a heterogeneous disorder

Diabetes is one of the oldest diseases known to mankind, with earliest descriptions dating back to 1550 B.C. It was described as an illness of “too great emptying of the urine” (Ebers Papyrus; ancient Egyptian medical manuscript). It is a heterogeneous disease encompassing many subtypes all resulting in the same end point of chronic hyperglycaemia (Tuomi, et al., 2014). Physicians recognised this heterogeneity of diabetes as early as the fifth century. They described two forms of diabetes: one in older obese individuals and the other in thin individuals with a short life span (Sushruta Samhita and Charaka Samhita, early texts of Indian traditional medicine). Himsworth provided the first insight into the aetiological difference between the two most common forms of diabetes, today known as type 1 and type 2 diabetes, in 1936. He discriminated the two forms of diabetes as the “insulin insensitive patients”, who tend to be older and obese, and “insulin sensitive patients”, who are generally young and lean (Himsworth, 1936).

The first formal diagnostic classification of diabetes was published in 1980 (1980) and modified in 1985 (1985a). Even at the time this initial classification was developed, the understanding of pathophysiology of diabetes was still in its very early stages. This classification was based on the need for insulin and included two major classes of diabetes: Insulin Dependent Diabetes Mellitus (IDDM) and Non-Insulin Dependent Diabetes Mellitus (NIDDM). In addition, it included Gestational Diabetes Mellitus (GDM) as well as other, such as diabetes due to hormonal or pancreatic aetiologies. Clinicians at that time found this classification as too restrictive and imprecise (1985b; Hother-Nielsen, et al., 1988). This

classification was suboptimal, as patients were often classified based on the treatment they were receiving rather than underlying disease pathology. Similarly, it did not take into account the clinical stage of hyperglycaemia. Difficulties in classification occurred both at diagnosis and years after insulin treatment because of an atypical presentation or due to an increase in insulin requirement when the hyperglycaemia progressed in otherwise non-ketosis-prone middle-aged diabetes patients (1985b).

Within the last few decades, a large improvement in understanding of diabetes pathogenesis, subtypes and their optimal treatment has taken place consequently leading to a change in diabetes classification (1997; Alberti and Zimmet, 1998). It was realised that an aetiology-based classification offers better prospects of optimal treatment, good glycaemic control and decreasing diabetes related complications.

The diabetes classification was revisited in 1997 and 1998 by the American Diabetes Association (ADA) and World Health Organisation (WHO) (1997; Alberti and Zimmet, 1998). Updated guidelines incorporate both clinical stages of hyperglycaemia and the aetiological subtypes (American Diabetes, 2013) **(Table 1.1)**.

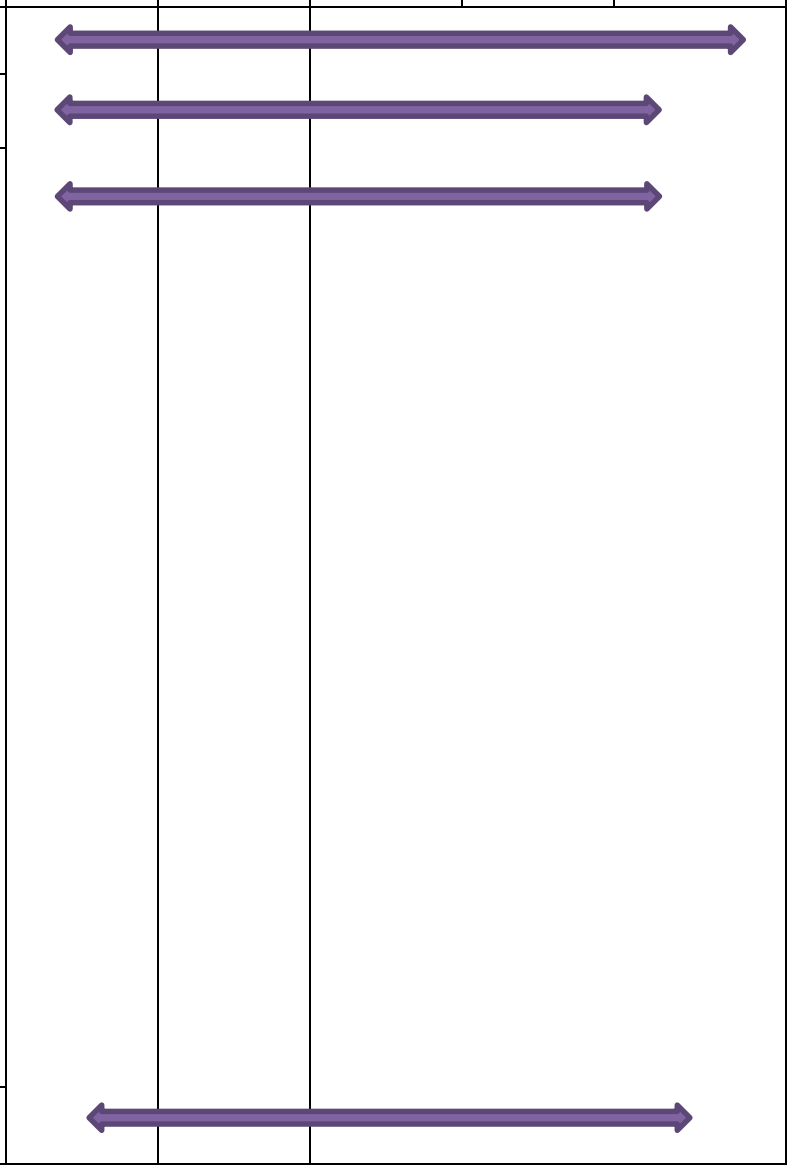
Types/Stages	Normoglycaemia	Hyperglycaemia			
		Impaired glucose tolerance	Diabetes Mellitus		
			Not insulin requiring	Insulin requiring for control	Insulin requiring for survival
Type 1 diabetes					
Type 2 diabetes					
Other specific types **A. Genetic defects of β-cell function** 1. HNF1A-MODY 2. GCK-MODY 3. HNF4A-MODY 4. Mitochondrial diabetes 5. Others **B. Genetic defects in insulin action** 1. Type A insulin resistance 2. Rabson-Mendenhall syndrome 3. Lipoatrophic diabetes 4. Others **C. Diseases of the exocrine pancreas** **D. Endocrinopathies** **E. Drug or chemical induced** **F. Infections** **G. Uncommon forms of immune-mediated diabetes** **H. Other genetic syndromes associated with diabetes**					
Gestational diabetes					

Table 1.1: Classification of diabetes mellitus (Adapted from American Diabetes Association "Diagnosis and Classification of Diabetes" 2013)

MODY = maturity onset diabetes of the young, *HNF1A* = hepatocyte nuclear factor 1 alpha, *HNF4A* = hepatocyte nuclear factor 4 alpha and *GCK* = glucokinase

The current guidelines recommend diabetes classification into four main categories:

- type 1 diabetes (due to β-cell destruction)
- type 2 diabetes (due to insulin resistance and β-cell dysfunction)
- gestational diabetes and
- other specific types

The "other specific types" include different forms of monogenic diabetes (genetic defects of β-cell function or insulin action), diabetes due to endocrinopathies, drugs and infections.

1.2 Young adult-onset diabetes

Despite an improved understanding of diabetes pathogenesis and better classification criteria, the assignment of an appropriate diagnosis of diabetes subtype is still challenging in a significant number of patients, particularly those presenting in young adult life (diagnosed 18-45 years). This is due to the broad range of diabetes differential diagnoses and substantial overlap in the clinical features of diabetes subtypes presenting in this age range (Tuomi, et al., 2014) (**Table 1.2**). Along with classical type 1 diabetes, a related, gradually progressive form of autoimmune diabetes known as latent autoimmune diabetes of adults (LADA) is also diagnosed in young adults. The clinical characteristics of LADA often do not fit the classic profile of either type 1 or type 2 diabetes, but lie somewhere in the middle.

Features	Type 1 diabetes	LADA	Young-onset type 2 diabetes	MODY	
				HNF1A/HNF4A MODY	GCK-MODY
Typical age of onset	6 months-young adulthood	Usually older than 35 years	Adolescence and young adulthood-45 years	10-45 years	From birth
Pathogenesis	Autoimmune	Autoimmune	Insulin resistance, β-cell dysfunction	β-cell dysfunction	Defect in β-cell glucose sensing
Diabetic ketoacidosis	Common	Rare	Rare	Rare	Rare
First line Treatment	Insulin	Diet and OAD agents initially, require insulin earlier than type 2 diabetes	Diet, OAD agents, may require insulin in later years	Low dose Sulphonylureas	Diet alone
Obesity	Unusual	Not usual (Usually lower BMI than type 2 diabetes)	Usual	Not usual	Not usual
Affected parent	0-1	0-1	1-2	Classically 1 parent	Usually one parent with fasting
C peptide	Undetectable	Low/Normal	Normal/high	Low/normal	Normal
Presence of β-cell antibodies	Very common (>90%)	Common	Rare	Rare	Rare
Features of insulin resistance	Unusual	Unusual	Typical	Unusual	Unusual

Table 1.2: Comparison of the characteristics of type 1 diabetes, LADA, young-onset type 2 diabetes and the common subtypes of maturity onset diabetes of the young (MODY)

OAD = oral antidiabetic, BMI= Body Mass Index

Like type 1 diabetes, LADA individuals are typically lean and positive for at least one of the four islet autoantibodies (Tuomi, et al., 2014). However, due to the late age of presentation (as compared with type 1 diabetes) and non-insulin-dependence early in course of disease, LADA patients are often initially misdiagnosed as type 2 diabetes. Similarly, type 2 diabetes, once considered as a disease of middle-aged and older people, is now increasingly being diagnosed in children and young adults (Alberti, et al., 2004; Pinhas-Hamiel and Zeitler, 2005; Wilmot, et al., 2010). Different monogenic forms of diabetes, of which Maturity-Onset Diabetes of the Young (MODY) is the most common, can also present in young adult life.

Establishing the correct aetiology is important, as the first line of treatment is not the same for the different types of diabetes (**Table 1.2**). An excellent example of aetiology-based treatment is HNF1A-MODY, where a confirmed molecular diagnosis guides the optimal treatment (Pearson, et al., 2003). For instance, patients having MODY due to mutations in *HNF1A* are extremely sensitive to low-dose sulphonylureas. On the other hand, MODY due to *GCK* mutations does not require any pharmacological treatment (Gill Carey, et al., 2007).

A correct aetiological diagnosis also informs the clinical course of diabetes. For example, with a correct diagnosis, patients with LADA can be made aware that, unlike type 2 diabetes (more common to occur at their age), they might need insulin early in the course of the disease.

1.2.1 Monogenic diabetes

Monogenic diabetes is a heterogeneous group of diseases that are caused by mutations in 29 genes identified so far (Ellard, et al., 2013). **Table 1.3** summarizes causal genes and clinical features of various monogenic forms of diabetes.

1.2.1.1 Maturity-Onset Diabetes of the Young (MODY)

MODY is a group of monogenic disorders characterised by autosomal dominant inheritance, young-onset diabetes, insulin independence (although insulin may be required for better glycaemic control) and absence of β cell autoimmunity and signs of insulin resistance (Owen, 2013). The minimum population prevalence of MODY in the UK (based on MODY case referral to the UK genetic testing centre) is estimated to be approximately 68-108 cases per million (Shields, et al., 2010). Mutations in at least 13 genes have been reported to cause MODY-like phenotype (Ellard, et al., 2013). The most common forms seen in clinical practice are due to heterozygous mutations in the gene encoding the glycolytic enzyme glucokinase (*GCK)* and genes encoding the transcription factors hepatocyte nuclear factor-1α (*HNF1A*), hepatocyte nuclear factor-4α (*HNF4A*) and hepatocyte nuclear factor-1β (*HNF1B*) (Shields, et al., 2010). Other forms of MODY occur due to mutations in the genes listed in **Table 1.3**; however, they are much rarer.

Gene mutated	Protein affected	Mutation frequency (%)	Type of monogenic diabetes	Clinical features	Ref
GCK	Glucokinase (enzyme that catalyses first step of glycolysis leading to defect in β-cell glucose sensing)	32% [∞]	MODY, PNDM	Onset age is from birth to entire life time, persistent mild fasting hyperglycaemia (5.5–8 mmol/l), do not suffer from microvascular complications, treated with diet alone	Froguel, et al. (1992)
HNF1A	Hepatocyte nuclear factor 1 alpha transcription factor	52%[∞]	MODY	Onset age is 10-45 years, 1st line of treatment are low dose sulphonylureas, associated with low renal threshold for glucose	Yamagata, et al. (1996b)
HNF4A	Hepatocyte nuclear factor 4 alpha transcription factor	10%[∞]	MODY	Similar to HNF1A-MODY except low renal threshold for glucose. Associated with macrosomia and neonatal hypoglycaemia caused by foetal hyperinsulinemia	Yamagata, et al. (1996a)
HNF1B	Hepatocyte nuclear factor 1 beta transcription factor	6% [∞]	MODY	Onset age is 10-45 years, treated with diet, OAD agents or insulin depending on severity, associated with renal abnormalities and genital malformations	Nakajima, et al. (1996)
NEUROD1	Neurogenic differentiation factor 1	Rare	MODY, PNDM	PNDM associated with severe cerebellar hypoplasia, sensorineural deafness and visual impairment	Malecki, et al. (1999); Rubio-Cabezas, et al. (2010)
MTTL1 (A3243G mutation in the mitochondrial DNA)	Mitochondrially encoded tRNA leucine 1 (UUA)	Rare	Mitochondrial diabetes	Associated with progressive sensory neural hearing loss, known as a syndrome of Maternally Inherited Diabetes and Deafness (MIDD), majority of patients become insulin dependent few years after diagnosis	Ballinger, et al. (1992)
INSR	Insulin receptor	Rare	Severe IR	Severe IR due to primary insulin signalling defects. Treated with insulin sensitisers, high doses of insulin and leptin	Kahn, et al. (1976); Krook, et al. (1993)
LMNA, PPARG	Lamin A and C (nuclear lamina structural proteins), Peroxisome proliferator-activated receptor	Rare	Severe IR	Severe IR secondary to adipose tissue abnormalities. Treatment as above	Hegele, et al. (2002); Shackleton, et al. (2000)
PDX1 or *IPF1*	Pancreatic duodenal homeobox-1	Rare	MODY, PNDM	Pancreatic agenesis	Cockburn, et al. (2004)
KLF11	Krüppel-like factor 11	Rare	MODY	No other associated features	Neve, et al. (2005)

CEL	Carboxyl ester lipase	Rare	MODY	MODY with exocrine dysfunction	Raeder, et al. (2006)
KCNJ11	Inwardly-rectifying potassium channel pore forming subunit	Rare	MODY, PNDM	1st line of treatment are Sulphonylureas. PNDM associated with Developmental delay, Epilepsy, Neonatal Diabetes (DEND syndrome)	Gloyn, et al. (2004)
ABCC8	Sulphonylurea receptor 1 subunit of potassium channel	Rare	PNDM TNDM MODY	1st line of treatment are Sulphonylureas, associated with DEND syndrome	Babenko, et al. (2006)
WFS1	Wolframin membrane glycoprotein	Rare	PNDM	Associated with Diabetes Insipidus, Diabetes Mellitus, Optic Atrophy and Deafness (DIDMOAD syndrome)	Strom, et al. (1998)
PAX4	Paired box 4	Rare	MODY	No other associated features	Plengvidhya, et al. (2007)
INS	Preproinsulin, Insulin	Rare	MODY, PNDM	Usually associated with neonatal diabetes	Stoy, et al. (2007)
BLK	B lymphoid tissue kinase	-	-	Initially reported as causal for MODY in 3 families but could not be not confirmed in other cohorts with autosomal dominant diabetes	Bonnefond, et al. (2013); Borowiec, et al. (2009)

Table 1.3: Causal genes and clinical features of various monogenic forms of diabetes

‰= percentage of UK MODY cases, Rare = reported in few families worldwide, MODY = maturity-onset diabetes of the young, PNDM = permanent neonatal diabetes mellitus, TNDM = transient neonatal diabetes mellitus, OAD = oral antidiabetic and IR = Insulin resistance

A correct diagnosis of MODY has significant clinical implications for the patient. Low dose sulphonylureas are the first-line treatment for patients with HNF1A-MODY and HNF4A-MODY (Pearson, et al., 2003), while no treatment is required for GCK-MODY (Carey, et al., 2007). This is different from both type 1 and type 2 diabetes where insulin and metformin, respectively, are the treatments of choice. A correct diagnosis also makes it easier to predict the course of the hyperglycaemia and allows genetic testing of family members who already have diabetes or who are at risk of having inherited the mutation.

The focus of my DPhil research is HNF1A-MODY. The remaining part of this chapter will discuss HNF1A-MODY, challenges associated in identifying MODY cases, current state of knowledge on HNF1A-MODY biomarkers and necessary steps needed before application of biomarkers in clinical practice.

1.3 HNF1A-MODY (formerly known as MODY 3)

HNF1A, previously known as the gene encoding liver specific transcription factor, was implicated as the MODY 3 causing gene in 1996. A reverse genetics linkage analysis initially mapped the MODY 3 causal gene to a 7 centimorgan interval on chromosome 12 (Vaxillaire, et al., 1995). The site was then fine-mapped to 12q24.2, containing *HNF1A* by Yamagata *et al* (Yamagata, et al., 1996a). *HNF1A* consists of 10 exons that span ~23 kilo-base pairs and encodes for a 631-amino acid protein (HNF1A transcription factor), expressed in the liver, kidney, pancreas and intestine.

To date, a total of 414 different *HNF1A* mutations in 1247 families have been

reported in association with MODY (Colclough, et al., 2013). Heterozygous mutations of *HNF1A* are the most common form of MODY, accounting for ~50% of UK MODY cases (Shields et al., 2010).

1.3.1 The pancreatic phenotype of HNF1A-MODY

Patients with HNF1A-MODY have a progressive decrease in insulin secretion. Diabetes usually presents itself in the second to fourth decade and requires pharmacological treatment. A randomised-controlled trial confirmed anecdotal suggestions that patients with HNF1A-MODY are exquisitely sensitive to low dose sulphonylureas (Pearson, et al., 2003) (**Figure 1.1**). Sulphonylureas are thus recommended as first-line treatment, maintaining good glycaemic control for a number of years although, eventually, insulin treatment may be needed (Pearson et al., 2003). Patients with HNF1A-MODY can develop severe diabetes-related complications and require regular medical follow up comparable to that employed with type 1 and type 2 diabetes.

In contrast to the human phenotype, *Hnf1a* heterozygous (*Hnf1a*+/-) knockout mice do not develop diabetes and are phenotypically indistinguishable from wild type mice (Pontoglio, et al., 1998). However, *Hnf1a* homozygous (*Hnf1a*-/-) knockout mice have reduced expression of a large number of islet genes (Servitja, et al., 2009; Shih, et al., 2001).

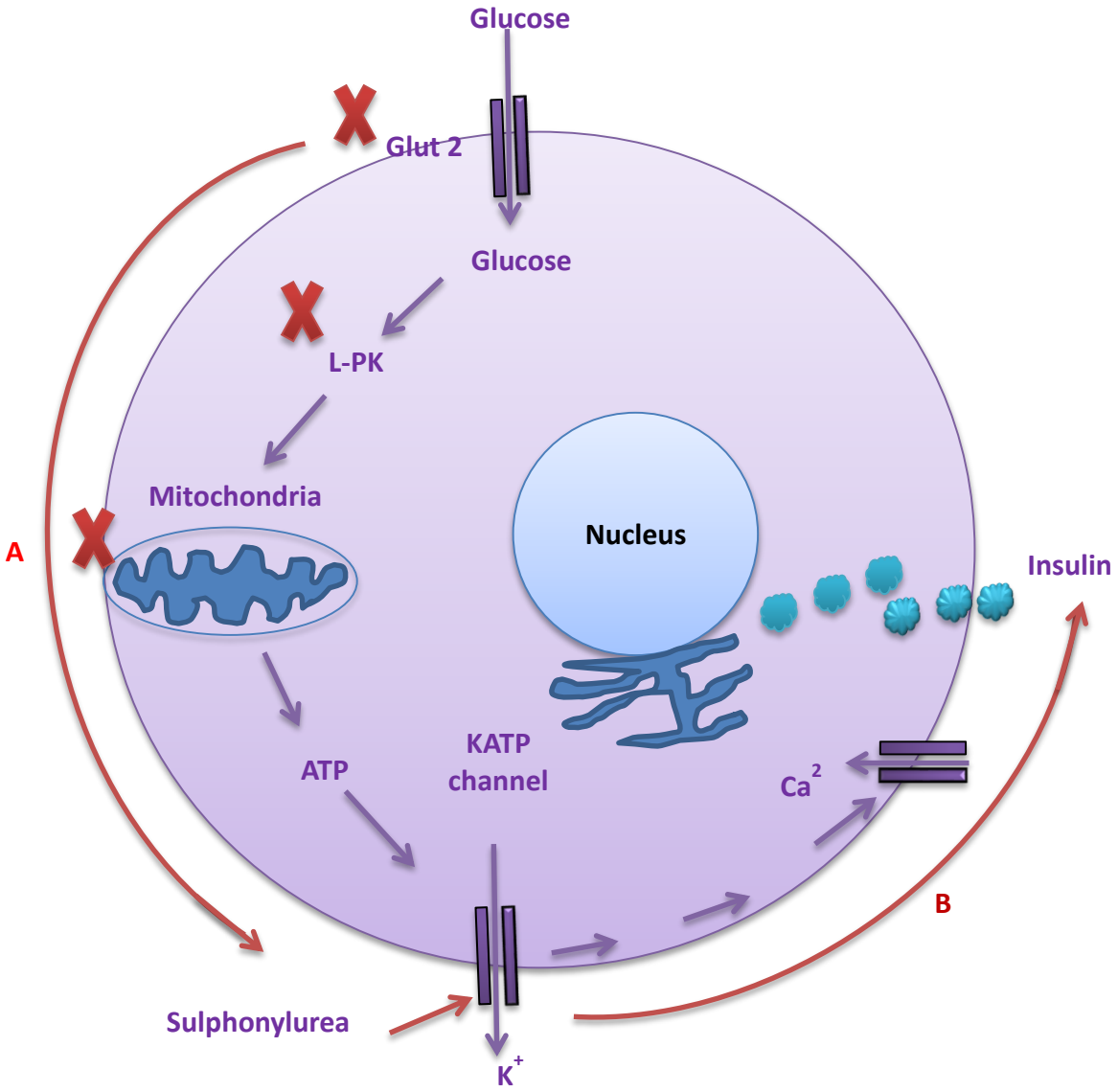


Figure 1.1: Figure illustrating steps of glucose-induced insulin secretion pathway affected by *HNF1A* mutation and bypassing of these steps by sulphonylureas

A = pre-KATP channel, B = post-KATP channel, L-PK = L-type pyruvate kinase. The three red cross represent steps of glucose metabolism pathway (pathway A) regulated by HNF1A and hence affected in HNF1A-MODY. The suggested mechanism for HNF1A-MODY sensitivity to sulphonylureas is that sulphonylureas bypass sites of action of HNF1A (pathway A) and stimulate insulin secretion by closing the KATP channel. (Adapted from Pearson e*t al* 2003)

Some of the key genes downregulated in *Hnf1a* homozygous mutant mice are those involved in glucose stimulated insulin secretion such as genes encoding glucose transporter 2 (*Glut2*), liver pyruvate kinase (*L-pk*) and tricarboxylic acid cycle enzymes (Servitja, et al., 2009) (Figure 1.1). *Hnf1a* homozygous mutant mice show impaired β-cell growth, deranged glucose metabolism, decreased ATP production and insulin release.

1.3.2 The hepatic phenotype of HNF1A-MODY

HNF1A is also expressed in liver where it regulates diverse hepatic functions. HNF1A regulates genes responsible for bile acid and cholesterol homeostasis, detoxification and glucose, lipid and amino acid metabolism (Servitja, et al., 2009). HNF1A also regulates various hepatic acute phase proteins (such as C-reactive protein [CRP], fibrinogen, complement components and apolipoprotein M [apoM]) (Armendariz and Krauss, 2009) .

Patients with HNF1A-MODY show a clinically heterogeneous hepatic phenotype. They have been shown to have low basal levels of CRP, complement 5, 8 and apoM (discussed in detail in section 1.5). The clinical significance of low basal levels of these proteins in HNF1A-MODY is hitherto unknown. Patients with HNF1A-MODY have a normal lipid profile. They have lower fasting plasma triglyceride levels than those having type 2 diabetes (Owen, et al., 2002) and HDL levels similar to non-diabetic individuals (McDonald, et al., 2012). There are also isolated reports of association of HNF1A-MODY with hepatic adenomatosis (Reznik, et al., 2004) (Bluteau, et al., 2002; Nakamura, et al., 2012). Biallelic somatic inactivation of *HNF1A* is a known cause of hepatic adenomas (Bluteau,

et al., 2002). Co-occurrence of hepatic adenomas with HNF1A-MODY is believed to be due to a somatic event in the presence of a germline *HNF1A* mutation (Bluteau, et al., 2002).

Hnf1a homozygous mutant mice show a marked hepatic phenotype with hepatomegaly, progressive liver damage, elevated plasma bile acids, hypercholesterolaemia and hyperphenylalaninemia (Pontoglio, et al., 1996; Shih, et al., 2001).

1.3.3 The renal phenotype of HNF1A-MODY

Patients with HNF1A-MODY have a low renal threshold for glucose with glycosuria inappropriate for the blood glucose levels (Menzel, et al., 1998). They often have glycosuria following a carbohydrate load, before the clinical features of diabetes become apparent (Menzel, et al., 1998). Experiments on *Hnf1a* homozygous mutant mice suggest that this is probably due to reduced expression of the high affinity low capacity sodium-glucose transporter-2 (*Sglt2*) in the proximal renal tubule (Pontoglio, et al., 2000).

1.3.4 Heterogeneity in clinical phenotype of HNF1A-MODY

There is marked heterogeneity in clinical phenotype of HNF1A-MODY. This heterogeneity is demonstrated by variation in the age of onset of diabetes, severity of symptoms at presentation and disease progression. Incomplete penetrance of *HNF1A* variants is one of the several possible explanations for these observations (Cooper, et al., 2013). There is increasing evidence that suggests a complex relationship exists between *HNF1A* variation and clinical

phenotype. For example:

- variants in *HNF1A* exons 8-10 have been shown to have reduced penetrance and a less severe phenotype with diabetes diagnosed at a later age compared with variants in exons 1-7 (Bellanne-Chantelot, et al., 2008; Harries, et al., 2006). This is likely to be because exons 1-7 encode for all three HNF1A isoforms (isoforms A, B and C), while exons 8-10 only encode for isoform A. These isoforms differ in expression patterns, with isoform A expressed more in fetal pancreas and adult kidney and liver, and isoforms B and C more so in adult pancreas, hence explaining the less severe phenotype of variants in exon 8-10.

- variants leading to HNF1A-MODY phenotypes in some family members have been observed to be non-penetrant in other members of the same family (euglycaemic by age of 87 and 46) (Miedzybrodzka, et al., 1999). Recently Flannick *et al* reported presence of rare conserved protein damaging variants in *HNF1A* (as well as other MODY genes) in the asymptomatic general population (Flannick, et al., 2013).

In addition, there are *HNF1A* variants that do not lead to a MODY phenotype but are associated with an increased risk of type 2 diabetes (Triggs-Raine, et al., 2002; Voight, et al., 2010; Weedon, et al., 2005). These observations suggest that there is a continuous spectrum of *HNF1A* variants ranging from neutral common variants through functional variants increasing susceptibility to type 2 diabetes, to low to highly penetrant MODY causing variants.

1.4 Diagnosing MODY: challenges and current approaches

Despite the advantages of a correct diagnosis, the majority of MODY cases remain undiagnosed or misdiagnosed as type 1 or type 2 diabetes. A report from the UK MODY diagnostic centre estimated that the minimum population prevalence of MODY in UK is 68-108 cases per million. These figures are based on data from two places [Exeter (12 confirmed MODY cases from a population of 111,076 amounting to 6,351 in total or 108 cases/million) and Edinburgh/Lothians (53 MODY cases from a population of 778,367 amounting to 4,003 in total or 68 cases/million)]. The authors calculated that with confirmed genetic diagnosis of only 1,177 UK MODY patients, more than 81% of MODY cases in the UK remain unidentified and are unlikely to be receiving appropriate treatment (Shields, et al., 2010). These figures are comparable with a population-based cross-sectional study in Oxfordshire (UK) that reported minimum population prevalence of 84 cases per million of HNF1A-MODY (Kropff, et al., 2011). The authors estimated that 90% of HNF1A-MODY cases in UK remained misdiagnosed.

Possible reasons for the missed diagnosis of MODY include overlap in clinical features with the more common forms of diabetes, the high cost of genetic testing (~£350 per gene) and lack of physician awareness. The current diagnostic guidelines for MODY emphasise that genetic testing should be offered to individuals who have diagnosis of diabetes at a young age (<25 years), family history of diabetes (at least 2 consecutive generations) and evidence of endogenous insulin secretion (Ellard, et al., 2008). However, it is clear that most individuals meeting these criteria are not referred for diagnostic genetic testing,

and in addition, ~50% of subsequently proven MODY cases do not match these criteria (Bellanne-Chantelot, et al., 2011; Shields, et al., 2010).

1.4.1 Translation of scientific findings into biomarker development

Non-genetic biomarkers of MODY could improve identification of cases and help prioritise patients for molecular diagnostic testing. In this context, biomarker discovery has been an area of major interest in the last decade with a particular focus on HNF1A-MODY. Different approaches using knockout mouse models, human studies and bioinformatics have revealed the regulatory functions of HNF1A and have identified a number of candidate molecules regulated by HNF1A in pancreas, liver and kidney. As all kinds of diabetes have an underlying β-cell defect (or β-cell loss in autoimmune diabetes), a biomarker utilising the specific extra-pancreatic features of HNF1A would likely be a better tool to differentiate HNF1A-MODY from other forms of diabetes.

1.4.1 (a) What is a biomarker?

Biomarkers are adjunct tools that help clinicians determine the risk, screen or diagnose a specific disease. Biomarkers can also be used to monitor disease progress or to measure the therapeutic response. Accordingly biomarkers can be categorised as risk factors, screening, diagnostic or prognostic biomarkers (Vasan, 2006). This thesis will focus on diagnostic biomarkers for MODY and issues related to their clinical use.

1.4.1 (b) Measures of Biomarker performance

A clinically useful biomarker is one that demonstrates a high sensitivity and

specificity for the disease in question, is less expensive, less invasive and broadly available (than the gold standard), is not operator or assay dependent and is consistent across ethnic groups (Vasan, 2006). Results of diagnostic biomarkers can be compared with a gold standard test, which in the case of MODY, is identification of a pathogenic mutation (through genetic sequencing and testing for co-segregation of variant with MODY phenotype in the family). The discriminative potential can be assessed using various measures of diagnostic accuracy such as sensitivity, specificity, predictive values and likelihood ratios (**Table 1.4**) (Altman and Bland, 1994a; Altman and Bland, 1994b; McGee, 2002; Vasan, 2006) (Soreide, 2009). For tests that yield a continuous outcome, an optimum threshold or cut-off value is used for discriminating diseased individuals from healthy ones. This optimum cut-off value, as well as the discriminant potential of a biomarker, can be determined by receiver operating characteristic (ROC) curve analysis (**Figure 1.2**). ROC curve analysis was initially developed by British radar engineers in World War II to differentiate between enemy planes and background noise. It was introduced for use in medicine in late 1940's and since then has been used in a large number of studies evaluating biomarkers (Lusted, 1971; Zou, et al., 2007).

A ROC curve is a graphical display of sensitivity vs. 1-specificity for every possible cut-off point of a test result.

Sensitivity	The ability of the test to correctly identify those who have the disease i.e. the proportion of patients with disease who test positive
Specificity	The ability of the test to correctly identify those who do not have the disease i.e. the proportion of patients without disease who test negative
Predictive value	The probability that the test will give the correct diagnosis; predictive values are affected by the prevalence of the disease in given population
	Positive predictive value: the probability of having the disease if a person tests positive
	Negative predictive value: the probability of not having the disease if a person tests negative
Likelihood ratio	The probability of having a certain test result in patients with disease compared with those without the disease. Likelihood ratios are not affected by the prevalence of the disease in given population
	Positive Likelihood ratio: the ratio of the probability that a positive result will occur in subjects with the disease to the probability that the same result will occur in subjects without the disease
	Negative Likelihood ratio: the ratio of the probability that a negative result will occur in subjects with the disease to the probability that the same result will occur in subjects without the disease

Table 1.4: Measures of biomarker performance

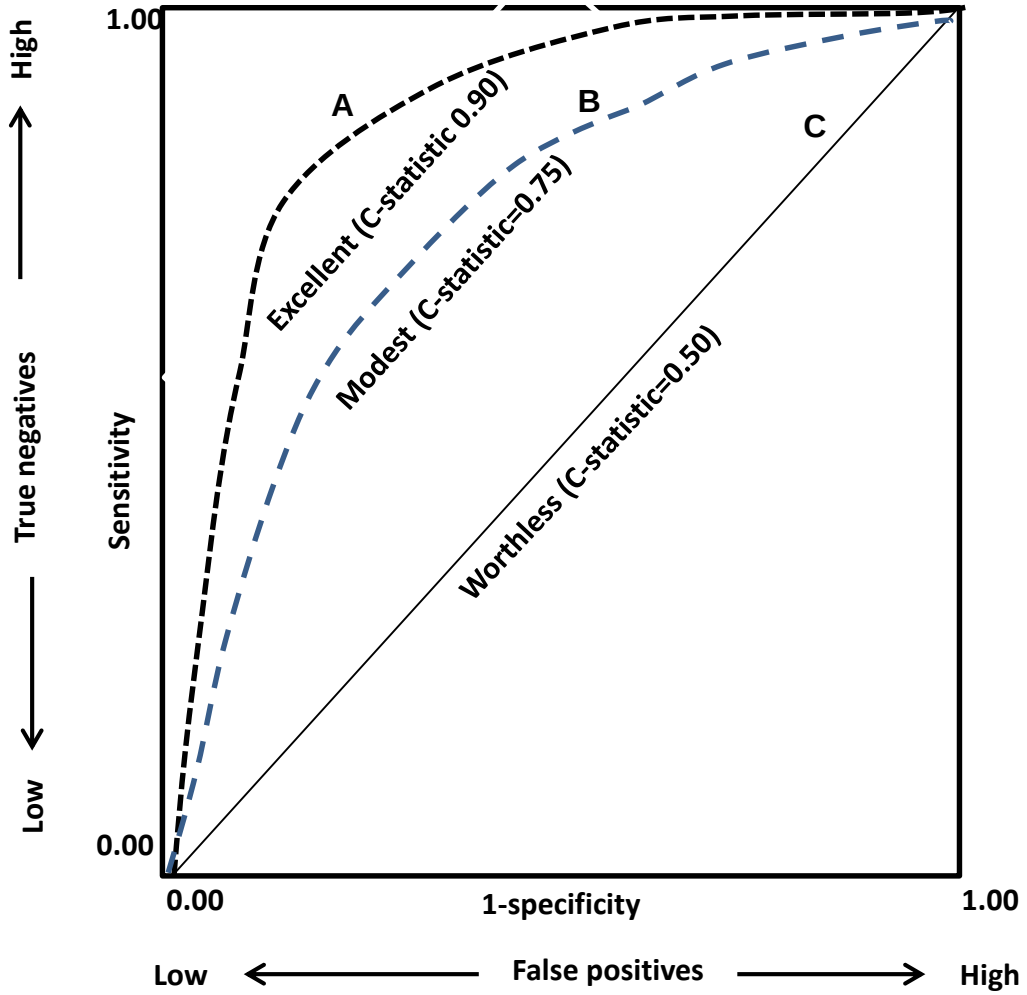


Figure 1.2: Receiver operating characteristic (ROC) curve

The graph above shows three hypothetical ROC curves with different degrees of discrimination. The closer a curve follows the upper left hand corner the more clinically useful the test is.

The area under the ROC curve (also known as the C-statistic) is a summary measure of the discriminatory potential of the diagnostic test. The C-statistic can range from 0.5 (which means the diagnostic test is as good as a random guess in discriminating cases with and without disease) to 1 (perfect discrimination).

The point on the ROC curve that combines the optimal sensitivity and specificity determines the cut-off point for clinical use. There is usually a trade-off between high sensitivity (to detect most affected cases) and high specificity (to exclude most non-cases). For diseases such as breast cancer, where misclassification results in serious consequences, a higher sensitivity would be preferred over specificity. For a disease such as MODY, with relatively low prevalence, a higher specificity may be set to prevent investigation of a large number of individuals with other forms of diabetes. However, this will be at the expense of missing a proportion of MODY cases.

1.4.1 (c) Typical phases of biomarker development and associated challenges

Figure 1.3 illustrates the various stages of biomarker development, starting from the discovery of a biomarker to its clinical application (Pepe, et al., 2001; Vasan, 2006). Biomarkers can be identified through one of various technologies (e.g imaging, phenotype studies, animal studies or one of the 'omics' tools: genomics, proteomics or metabolomics). They are then evaluated in pilot case-control studies (including cases with established diagnoses) (Vasan, 2006).

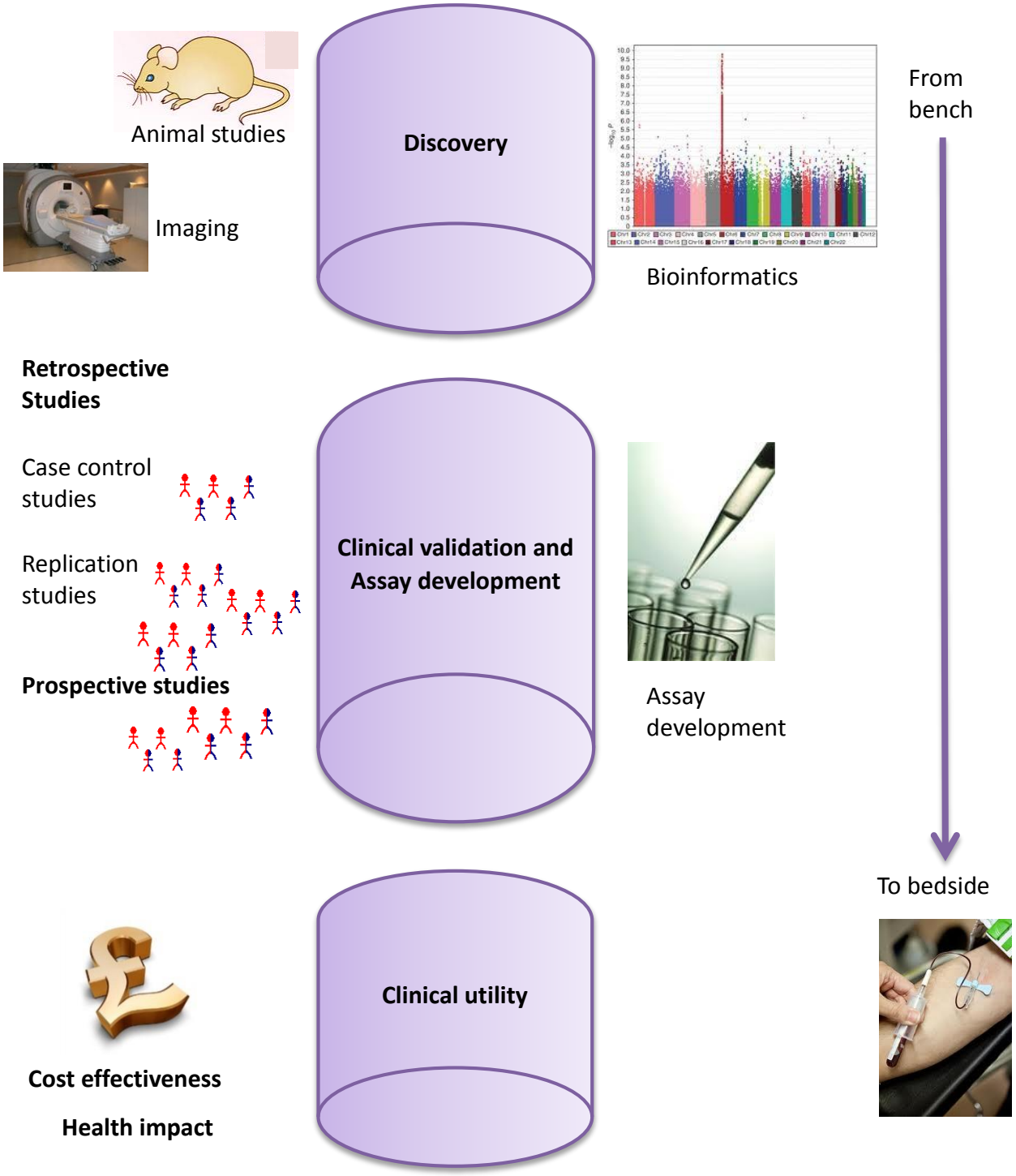


Figure 1.3: Phases of Biomarker Development

Pilot case control studies are feasible for initial testing of the biomarkers as they require fewer resources and do not need patient follow up. However, they are prone to bias and often the results are either not reproducible or not transferrable to different clinical settings. Various sources of variation or bias that can affect the results of studies evaluating biomarkers are listed in **Table 1.5** (Lijmer, et al., 1999; Rutjes, et al., 2006; Whiting, et al., 2004).

If the diagnostic biomarker shows good discrimination of those with and without the disease, the results are then typically replicated in large-scale case control studies. Often, these large-scale follow up studies fail to replicate the initial findings due to presence of sources of bias and variation in pilot studies or sometimes in their own design (**Table 1.5**). If, however, the results are reproducible, biomarkers are then evaluated in prospective studies. In prospective studies, the disease status of the patient is unknown at the time of recruitment and patients are selected based on their symptoms. In prospective studies, all subjects undergo testing for the biomarker and the gold standard reference test. Prospective studies are less prone to selection bias as compared with case control studies. However they are expensive to conduct particularly when the disease being detected has a low prevalence and a large sample size is required (Obuchowski and Zhou, 2002). The final phase of biomarker development includes comparison of using the biomarker to already existing clinical criteria, determining cost effectiveness and impact on disease management and quality of life of the patient. Where more than single biomarkers are available for a particular disease, it is also important to determine the incremental value of new biomarkers.

Source	Description of subsequent Bias
Selection of participants	
Demographic features	Diagnostic test may perform differently in other ethnic populations, across gender, BMI range or age groups. Therefore, demographic features may lead to variation in biomarker performance.
Disease severity	Spectrum bias (better known as spectrum effect) results if cases and controls lying on extremes of disease spectrum are included and patients with mild difficult to diagnose disease are omitted. This leads to overestimation in biomarker performance.
Disease prevalence	Prevalence of the disease in question may vary according to clinical setting and lead to variation in diagnostic test performance. For a disease with low prevalence, the number of false positives will outnumber the true positives, no matter how sensitive the test is.
Verification procedure	
Differential verification	If part of diagnostic test results are confirmed with an alternative reference standard. Difference in sensitivity of the reference standards used may affect the diagnostic test performance.
Partial verification	Only subset of diagnostic test results verified with reference standard. May lead to incorrect labelling of participants not verified by reference standard (e.g. true positive might be false positive) and hence affect the diagnostic test performance.
Interpretation of test results	
Clinical review bias	Knowledge of patient characteristics may affect interpretation of diagnostic test result.
Observer variability	If results of diagnostic test are operator or assay dependent this may lead to variation in diagnostic test performance.
Double/Single/Non-blinded reading	Interpretation of diagnostic test performance is influenced by knowledge of the result of standard reference.
Analysis	
Choice of threshold value	Overfitting due to selection of a threshold value that maximises the sensitivity and specificity in a particular set of sample. The performance of this cut-off may not be reproducible in a follow up study.
Non-interpretable test results reported	Non-interpretable test results or outliers may be omitted and not reported. This may lead to biased assessment of diagnostic test performance.

Table 1.5: Description of sources of variation or bias in diagnostic studies (Adapted from (Lijmer et al., 1999; Rutjes et al., 2006; Whiting et al., 2004))

1.5 Biomarkers for HNF1A-MODY

A molecular diagnosis of MODY (such as HNF1A, HNF4A or GCK-MODY) requires expensive genetic testing (£350 per gene). To prioritise patients for genetic testing and reduce unnecessary referrals, biomarker discovery has been an area of major interest in the last decade with particular focus on HNF1A-MODY. Several approaches have been used for identifying potential biomarkers. These include knockout mouse models, human studies and bioinformatics.

1.5.1 Candidate biomarkers for HNF1A-MODY identified from *Hnf1a* homozygous knockout mouse models

Several candidate biomarkers identified from *Hnf1a* homozygous mutant mice have been examined in different human studies (discussed in detail below). The common hypothesis of these studies was that if changes similar to those seen in mice could be observed in HNF1A-MODY patients, these could then serve as potential biomarkers.

1.5.1 (a) Urinary amino acids

The *Hnf1a* homozygous knockout mouse has a striking phenotype of renal Fanconi syndrome with polyuria, glycosuria and increased renal fractional excretion of amino acids (Pontoglio, et al., 1996). The severe renal phenotype of *Hnf1a* knockout mice led to the hypothesis that aminoaciduria would be seen in human *HNF1A* mutation carriers. To test this, the urine levels of 16 amino acids were analysed in patients with HNF1A-MODY (n=50), type 1 diabetes (n=25), type 2 diabetes (n=25) and patients with coexisting diabetes and chronic renal

failure (n=10). This study found that generalised aminoaciduria was not specific to HNF1A-MODY and was a common feature of all diabetes groups due to glycosuria (Bingham, et al., 2001).

The above results were independently confirmed in a recent study, which compared the metabolomic urine profiles of subjects with HNF1A-MODY (n=14), GCK-MODY (n=17), young-onset type 2 diabetes (n=14) and non-diabetic individuals (n=34) (Gloyn, et al., 2012). The authors hypothesised that, due to different genetic aetiology and metabolic pathways affected, HNF1A-MODY cases would have a distinct urinary metabolic profile as compared with those having GCK-MODY and type 2 diabetes. Urine samples were analysed using liquid chromatography mass spectrometry and ^{1}H-nuclear magnetic resonance spectroscopy (NMR). Examination of NMR acquired data revealed significant difference in valine and glycine levels in subjects with HNF1A-MODY compared with type 2 diabetes. Direct quantification of these amino acids was undertaken to confirm the findings of the NMR data. The urine samples were matched for urinary glucose to control for the effect of glycosuria. No difference in the levels of urinary amino acids was observed among the diabetes subtypes when the subjects were matched for urine glucose. This confirms the previous report that any difference in urinary amino acid profile between the diabetes subtypes was driven by glycosuria.

1.5.1 (b) Serum amino acids

Hnf1a knockout mice also exhibit alteration in serum levels of amino acids, in particular demonstrating raised levels of phenylalanine (as phenylalanine

hydroxylase gene expression is regulated by Hnf1a). Serum amino acids in HNF1A-MODY patients were compared with healthy controls (n=20 in both groups) (Stride, et al., 2004). However, the specific changes seen in serum amino acids of mouse models were not observed in subjects with HNF1A-MODY.

1.5.1 (c) Complement 5 (C5), complement 8 (C8) and transthyretin (TTR)

Both HNF1A and HNF4A regulate the genes encoding complement 5 (C5), complement 8 (C8) and transthyretin (TTR). *Hnf1a* knockout mice fail to express C5 and C8. C5, C8 and TTR were evaluated as potential biomarkers for MODY in a study including subjects having HNF1A-MODY (n=29), HNF4A-MODY (n=13), type 2 diabetes (n=14) and healthy controls (n=20) (Karlsson, et al., 2008). Although sensitivity to distinguish HNF1A/HNF4A MODY from type 2 diabetes was quite good (60-90%), these candidate biomarkers had extremely poor specificity (2-10%).

1.5.1 (d) Apolipoprotein M (apoM)

Apolipoprotein M (apoM), a ~25 kDa apolipoprotein, was discovered by Xu and Dahlback in 1999 (Xu and Dahlback, 1999). ApoM is found in all major lipoprotein classes but is mainly associated with high density lipoprotein (HDL) cholesterol (Dahlback and Nielsen, 2009). It is important for reverse cholesterol transport and anti-oxidant functions of HDL-cholesterol (Dahlback, et al., 2008; Wolfrum, et al., 2005).

ApoM is regulated by a variety of transcription factors (Richter, et al., 2003; Venteclef, et al., 2008; Wolfrum, et al., 2008; Zhang, et al., 2008), one of which

is HNF1A. ApoM was suggested as a candidate biomarker for HNF1A-MODY following the observation of reduced *apoM* gene expression in *Hnf1a* homozygous knockout mice (Richter, et al., 2003). However, subsequent human studies examining apoM concentrations in HNF1A-MODY have yielded conflicting results (Cervin, et al., 2010; Skupien, et al., 2007). **Table 1.6** describes the study subjects, techniques used and results of the previous studies.

Due to inconsistency between the results of the previous studies, the role of apoM as HNF1A-MODY biomarker is unclear. Chapter 3 aims to re-examine the use of apoM as a biomarker for HNF1A-MODY.

1.5.2 Candidate biomarkers for HNF1A-MODY identified from human studies

1.5.2 (a) 1,5 anhydroglucitol

1,5 anhydroglucitol (1,5 AG) is a dietary monosaccharide with a structure similar to glucose. Usually 1,5 AG is re-absorbed in the kidney by a AG/fructose/mannose common transport system. However, in the presence of hyperglycaemia, glucose can compete with 1,5 AG for reabsorption at this monosaccharide common transport system leading to lowered plasma concentration of 1,5 AG. Given the low renal threshold and resulting glycosuria seen in HNF1A-MODY, Skupien *et al* hypothesised that this would present a stronger competition for 1,5 AG leading to its increased urinary loss.

	Subjects	Technique used	Results	Mean (SD) apoM concentration in healthy controls (µmol/L)
Richter *et al.* 2003	9 HNF1A-MODY 9 HNF4A-MODY 9 Healthy controls	Western blotting	Serum apoM concentration was significantly lower in subjects with HNF1A-MODY as compared with normal controls.	4 (SD not reported)
Skupien *et al.* 2008	48 HNF1A-MODY 55 Type 2 diabetes subjects 19 Healthy controls	Dot blot technique	No significant difference in apoM concentration of HNF1A-MODY subjects as compared with non-diabetic controls and those with type 2 diabetes.	0.56 (0.08)
Cervin *et al.* 2010	**Family study:** 71 carriers of *HNF1A* mutation (53 diagnosed with diabetes) 75 family controls (5 diagnosed with diabetes)	ELISA	Family study: Serum apoM concentration lower in female carriers of *HNF1A* mutation as compared with family controls	0.95 (0.21)
	Case/Control study: 24 HNF1A-MODY 11 HNF4A-MODY 18 Type 2 diabetes subjects 19 Healthy controls	ELISA	Case/Control study: No significant differences in apoM concentration between HNF1A-MODY patients and type 2 diabetes subjects.	0.97 (0.19)

Table 1.6: Studies evaluating apolipoprotein M as biomarker for HNF1A-MODY

An increased urinary loss would result in lower plasma levels and thus 1,5 AG could serve as a biomarker for HNF1A-MODY. It was found that plasma levels of 1,5 AG in HNF1A-MODY patients (n=33) were 50% lower (p=0.003) than type 2 diabetes subjects (n=43) with matched glycaemic control (Skupien, et al., 2008). A later study evaluated these results in a larger sample set with wider range of diabetes subtype again noting that the difference in plasma levels of 1,5 AG between HNF1A-MODY (n=23) and type 2 diabetes subjects (n=206) was evident only after adjustment for HbA1c (Pal, et al., 2010). The receiver operating characteristic (ROC) curve-derived C-statistic was 0.60 (a C-statistic of > 0.80 is considered a good discrimination) for HNF1A-MODY versus type 2 diabetes. An interesting finding in this study was that levels of 1,5 AG provided good discrimination between GCK-MODY (n=23) and HNF1A-MODY (C-statistic of 0.86). It was proposed that 1,5 AG could be used as a practical alternative to the oral glucose tolerance test which is sometimes used to discriminate between GCK-MODY and HNF1A-MODY (Pal, et al., 2010).

1.5.2 (b) Urine glucose

Urinary glucose was directly measured in the study, described in section 1.5.1 (a), investigating the metabolomic urine profile of HNF1A-MODY, GCK-MODY and young onset type 2 diabetes (Gloyn, et al., 2012). Urine glucose was highest in the HNF1A-MODY subjects and lowest in the GCK-MODY cases on both liquid chromatography mass spectrometry and direct urinary glucose measurement. Urine glucose derived parameters (urinary glucose/creatinine, plasma glucose to urinary glucose/creatinine ratio) were found to be significantly different across the diabetes subtypes. However, there was a huge variation in urine glucose levels,

and the C-statistic for urine glucose derived measures was <0.60, indicating that parameters based on urine glucose will not be very useful clinical discriminators of HNF1A-MODY from other diabetes subtypes.

1.5.2 (c) Lipid profile

Type 2 diabetes is characterised by diabetic dyslipidaemia, which includes elevated plasma triglyceride (TG) levels and low levels of HDL. Previous studies investigating the phenotypic characteristics of HNF1A-MODY have shown that fasting triglyceride levels are lower in patients with HNF1A-MODY as compared with patients with young-onset type 2 diabetes (Owen, et al., 2002). Moreover, patients with HNF1A-MODY have HDL levels similar to non-diabetic individuals. HDL was investigated as a candidate biomarker for discriminating HNF1A–MODY and type 2 diabetes in a small study (n=14 in both groups) (McDonald, et al., 2012). HDL was found to be significantly lower in patients with type 2 diabetes as compared with those with HNF1A-MODY with a C-statistic of 0.76 indicating modest discrimination. However, the difference in HDL between diabetes subtypes was lost when adjusted for covariates, such as age of diagnosis and BMI, suggesting that HDL does not add much discrimination above that available from clinical features.

1.5.2 (d) C-peptide

C-peptide is co-secreted with insulin from β-cells and measurable levels indicate residual β-cell function. In type 1 diabetes, due to autoimmune destruction of β-cells, C-peptide levels gradually decline. However, in type 1 diabetes, C-peptide levels can be detected during the “honeymoon period”, a period of partial

remission in type 1 diabetes due to small amounts of insulin production from remaining β-cells. Patients with MODY retain some endogenous β-cell function. Two independent studies have shown serum C-peptide and urinary C-peptide to creatinine ratio (UCPCR) are useful biomarkers to discriminate HNF1A-MODY from type 1 diabetes (Besser, et al., 2011; Thanabalasingham, et al., 2012b). In the study by Besser *et al,* including subjects with long standing diabetes, it was found that UCPCR had a C-statistic of 0.98 for differentiating HNF1A-MODY (n=54) from type 1 diabetes (n=69). In the second study, C-peptide was used to select suspected MODY cases for genetic sequencing (Thanabalasingham, et al., 2012b). It was observed that 10% of clinically labelled, post-honeymoon, type 1 diabetes subjects with residual β-cell function (C-peptide >0.2 nmol/l) had HNF1A-MODY. However, C-peptide would be less useful in discriminating MODY from type 1 diabetes during the honeymoon period. This is a disadvantage of the use of C-peptide, as identifying MODY close to diagnosis of diabetes is highly desirable to prevent patients from long periods of inappropriate insulin treatment.

1.5.2 (e) Islet-auto antibodies

Type 1 diabetes is characterised by the presence of pancreatic islet autoantibodies including glutamic acid decarboxylase (GAD) and islet cells (IA-2). Eighty-five to ninety percent of individuals with type 1 diabetes have presence of one or more pancreatic islet autoantibodies at the time of diagnosis (American Diabetes, 2013). Unlike type 1 diabetes, MODY is not an autoimmune disease and patients with MODY generally do not have pancreatic islet autoantibodies. Current diagnostic guidelines for MODY suggest genetic testing of those who are

antibody negative (Ellard, et al., 2008). Data are mixed however: a study by the UK diagnostic testing centre for MODY reported < 1% prevalence of GAD and IA-2 antibodies (McDonald, et al., 2011a), while another UK-based study reported prevalence of 21% in MODY patients (Thanabalasingham, et al., 2012b). In a registry-based German paediatric cohort, Schober *et al* reported presence of pancreatic islet autoantibodies in 17% of patients with confirmed MODY mutations (Schober, et al., 2009). In a Swedish study, GAD antibodies were detected in 4.8% of MODY patients (Lehto, et al., 1999). Another recently published study by Czech researchers reported 25% of the investigated MODY cohort (7/28) to be positive for GAD or IA-2 autoantibodies (Urbanova, et al., 2014).

Data from these different studies needs to be interpreted with caution. For instance the difference in prevalence of autoantibodies in MODY patients among the two UK-based studies could be explained by the different cut-offs used for GAD antibodies. McDonald *et al* reporting <1% prevalence used a cut-off of 64 WHO units/ml, as compared with Thanabalsingham *et al* reporting 21% prevalence using a cut-off of 14 WHO units/ml. This shows that the difference in antibody prevalence between the two UK studies seems likely to be due to the different cut-offs used. McDonald *et al* also reported that 36% of MODY patients included might have undergone pre-screening for islet autoantibodies (before referral to the UK diagnostic testing centre), and this could have contributed to the low prevalence in their study.

The variability in the results of the studies above mentioned suggests that, in the case of strong clinical suspicion of monogenic diabetes, presence of islet

autoantibodies should not preclude genetic testing.

1.5.3 Candidate biomarkers for HNF1A-MODY identified from Genome wide association studies

Genome wide association studies (GWAS) found evidence of association between common variants near *HNF1A* and plasma C-reactive protein (CRP) concentration (Reiner, et al., 2008; Ridker, et al., 2008) and plasma protein fucosylation (Lauc, et al., 2010), both of which were then investigated as HNF1A-MODY biomarkers. These are discussed in detail below.

1.5.3 (a) High-sensitivity C-reactive protein (hsCRP)

CRP is an acute phase reactant produced mainly in the liver and to a small extent in mononuclear cells, adipose tissue, kidneys and gut (Anty, et al., 2006; Haider, et al., 2006; Peyrin-Biroulet, et al., 2012). The exact in vivo role of CRP has not yet been determined; however, it has been shown that CRP can activate the complement system, opsonise pathogens and promote their phagocytic clearance (Wolbink, et al., 1996). Its serum levels rise dramatically (more than 1000 fold) during an acute inflammation. It has been used in clinical practice as a diagnostic and prognostic marker of inflammation for decades (Gabay, 1999; Pepys and Hirschfield, 2003).

Serum CRP levels are influenced by genetic and non-genetic factors. Non-genetic factors include body mass index, smoking, hormone replacement therapy and alcohol intake. Twin studies have indicated that genetic factors account for 50% of the inter-individual variability in CRP levels (de Maat, et al., 2004). So far, GWAS have revealed 18 loci explaining 5% of heritability in CRP levels

(Dehghan, et al., 2011). These include variants near *CRP*, interleukin 6 (*IL6)*, leptin receptor (*LEPR)* and *HNF1A (Dehghan, et al., 2011).* The GWAS findings are supported by functional evidence. For example, it has been shown that the *CRP* promoter contains binding sites for HNF1A (Toniatti, et al., 1990a) and that *CRP* expression is down-regulated in *Hnf1a* knockout mice (Shih, et al., 2001). HNF1A is part of the transcriptional complex involved in interleukin-6 stimulated CRP production during acute inflammation (**Figure 1.4**) (Nishikawa, et al., 2008).

In a large number of epidemiological studies, elevated CRP levels have been shown to be consistently associated with risk of cardiovascular disease (Danesh, et al., 1998; Ridker, et al., 1998). It is not clear whether CRP is a mediator or a marker of cardiovascular events. The possible pathogenic role of CRP is suggested by studies showing presence of CRP in atherosclerotic plaque and CRP-mediated low density lipoprotein uptake by macrophages (Torzewski, et al., 1998; Zwaka, et al., 2001). The causal role of CRP in cardiovascular disease was explored by two large-scale studies using Mandelian randomisation technique (Wensley, et al., 2011; Zacho, et al., 2008). These studies used *CRP* variants as proxies to investigate whether genetic variants related to changes in CRP concentration were associated with altered risk of cardiovascular events.

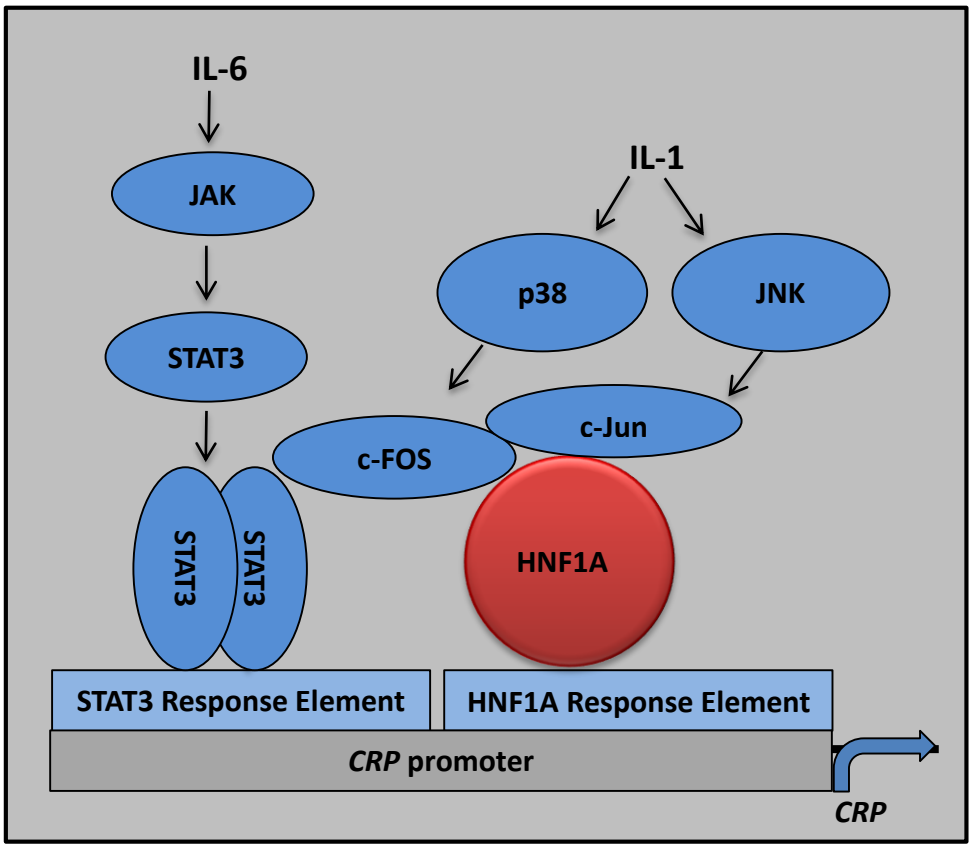


Figure 1.4: Regulation of human *CRP by* cytokines and transcription factors
Transcriptional complex formation of c-Fos, STAT3 and HNF1A is required for cytokine driven *CRP* expression. **Kinases:** Janus Kinase (JAK) and c-JUN N terminal kinase (JNK) transduction of cytokine mediated signals **Transcription factors:** Signal transducer and activator of transcription 3 (STAT3), c-JUN and c-FOS (together known as Activator Protein-1 transcription factor), Hepatocyte nuclear factor 1 alpha (HNF1A). (Adapted from Nishikawa *et al* Journal of Immunology 2008)

Neither study found significant association between *CRP* variants and cardiovascular risk. Further investigation with randomised controlled trials of CRP lowering therapy may refine the effect of CRP levels on cardiovascular outcomes.

Following on from the GWAS findings, it was hypothesised by our group that if common variation near *HNF1A* is associated with modest differences in CRP levels, then loss of function mutations in *HNF1A* (as in HNF1A-MODY) would lead to more marked effects on CRP levels. If so, CRP could be used as a biomarker. It was shown in an initial pilot study, including HNF1A-MODY (n=31), type 1 diabetes (n=316), type 2 diabetes (n=240) and GCK-MODY (n=24), that baseline hsCRP levels were significantly lower in HNF1A-MODY patients as compared with other study groups (Owen, et al., 2010). The largest difference was observed between HNF1A-MODY and subjects with type 2 diabetes ($P<1\times10^{-6}$), with a sensitivity of 71%, specificity of 77% and ROC curve-derived C-statistic of 0.80 for distinguishing HNF1A-MODY from type 2 diabetes. This initial finding was then replicated in a large-scale study including subjects with HNF1A-MODY (n=457) and type 2 diabetes (n=582) from seven European centres. This study confirmed the results of the pilot study and reported a C-statistic ranging from 0.79-0.97 (for the seven European centres) for discrimination of HNF1A-MODY from young-onset type 2 diabetes (**Figure 1.5**). Given the good discriminative capacity and common use in clinical practice as a clinical and prognostic indicator of inflammation, hsCRP could serve as a promising biomarker for prioritisation of patients with young-onset diabetes for molecular diagnostic testing.

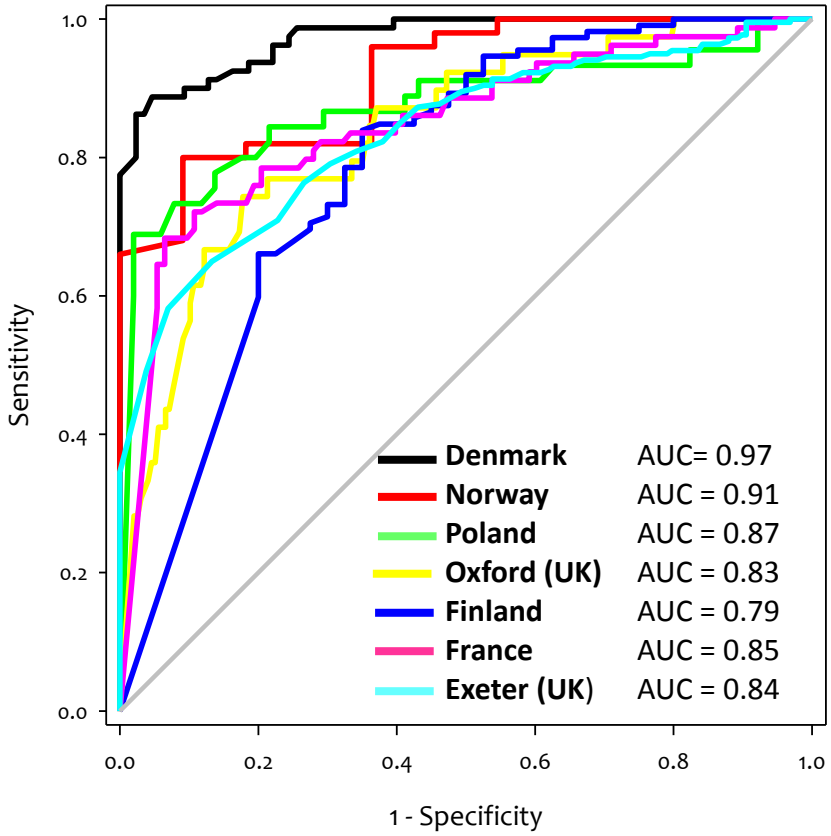


Figure 1.5: ROC curve illustrating the discriminative capacity of hsCRP to distinguish between subjects with HNF1A-MODY and type 2 diabetes across 7 European centers.
Figure created using data from two large scale studies evaluating CRP as HNF1A-MODY biomarker (Thanabalasingham *et al* 2011 and McDonald et al 2011).

1.5.3 (b) DG9-glycan index as a biomarker for HNF1A-MODY

Glycosylation is a posttranslational modification of proteins. It is an enzyme mediated process in which oligosaccharide side chains (glycans) are covalently attached to asparagine (N-linked) or serine, threonine, hydroxylysine or hydroxyproline residues (O-linked) (Ohtsubo and Marth, 2006). It is important for diverse biological processes such as cell-cell interaction, intra and extracellular signalling and endocytosis.

N-glycans have a common core structure composed of mannose and N-acetylglucosamine residues. N-glycans are divided into three types: oligomannose, containing mannose residues added to the core; complex (branched structures), containing branches or antennae added to core and hybrid glycans containing mannose residues and one or two branches. After synthesis in the endoplasmic reticulum, glycans undergo further modifications in the Golgi apparatus such as addition of a fucose group to the core (core fucosylation) or to the antennae (antennary fucosylation).

A recent GWAS drew attention to another functional role of HNF1A. It was shown that HNF1A affects N-glycan levels in the plasma and is a master regulator of plasma protein fucosylation (Lauc, et al., 2010). It was found that HNF1A regulates expression of fucose synthesis genes as well as genes encoding enzymes necessary for core and antennary fucosylation. HNF1A increased antennary fucosylation and repressed genes involved in core fucosylation.

Following on from the GWAS finding, it was shown by our group that there are marked differences in plasma glycan profiles in HNF1A-MODY as compared with other diabetes subtypes (Thanabalasingham, et al., 2011). In a pilot comparison, plasma glycan profiles of subjects having HNF1A-MODY (n=33) and type 2 diabetes (n=41) were compared (Thanabalasingham, et al., 2013). Consistent with GWAS findings, patients with HNF1A-MODY had an increase in the proportion of glycans without antennary fucose. These findings were then validated in a larger number of subjects including those with HNF1A-MODY (n = 188), type 1 diabetes (n = 98) and type 2 diabetes (n = 167) (Thanabalasingham et al., 2013). The DG9-glycan index (a ratio of triantennary glycans with and without antennary fucose) was found to provide excellent discrimination of HNF1A-MODY from both type 1 and type 2 diabetes (C-statistic of 0.94 and 0.90 respectively) (**Figure 1.6**).

1.5.4 Limitations of studies investigating hsCRP and DG-9 glycan index as HNF1A-MODY biomarkers

The studies evaluating hsCRP and DG9-glycan index as HNF1A-MODY biomarkers had certain limitations. Firstly, these were cross-sectional studies including subjects having well-established diagnoses of a specific diabetes subtype. The MODY subjects included in these studies had undergone genetic sequencing and had a confirmed mutation in *HNF1A, HNF4A or GCK*. Subjects having type 1 diabetes had been on permanent insulin treatment since diagnosis with additional evidence of severe β-cell dysfunction (C-peptide≤0.09 nmol/l or HOMA %B < 10%), positive GAD antibodies (>14 WHO units/ml) or both. Subjects having type 2 diabetes had no requirement for permanent insulin within

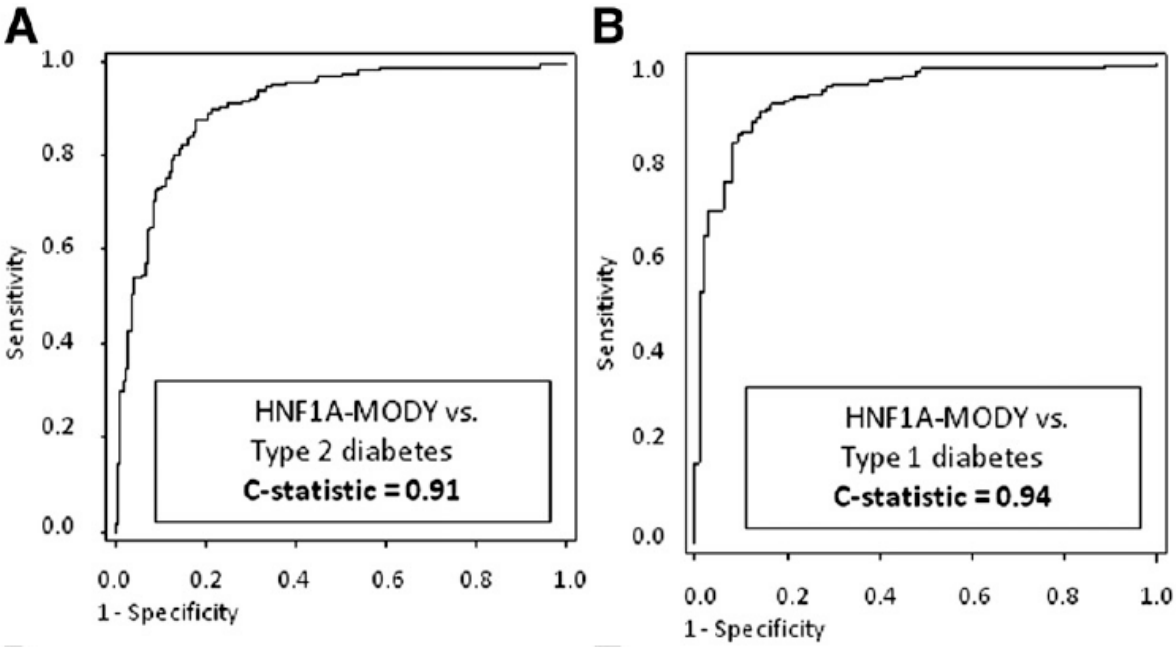


Figure 1.6: ROC curve illustrating the discriminative capacity of DG9-glycan index to distinguish between subjects with HNF1A-MODY and those with type 2 diabetes (A) and type 1 diabetes (B) (Taken with permission from Thanabalasingham *et al* Diabetes 2012)

six to twelve months of diagnosis and negative islet cell or GAD antibodies. Subjects in type 1 and type 2 diabetes groups either did not meet clinical criteria for MODY diagnostic testing or had been tested and were negative for mutations in *HNF1A, HNF4A or GCK*.

Evidence suggests that such a restricted sampling of the study groups (inclusion of those with well-established diagnoses of HNF1A-MODY, type 2 and type 1 diabetes) can result in an overestimation of measures of diagnostic accuracy (Lachs, et al., 1992; Mulherin and Miller, 2002; Whiting, et al., 2004). This phenomenon is known as the “spectrum effect” and is due to the selection of study groups lying on extremes of the spectrum. The spectrum effect limits applicability of the study results to the clinical setting where a clinician faces a greater degree of diagnostic uncertainty and patients that do not always fall neatly into specific category of diabetes subtype. Before the results of studies evaluating hsCRP and DG-9 glycan index can be extended to the clinical setting, it is important to assess the performance of these biomarkers in unselected datasets of young-onset diabetes patients. This will be investigated in chapter 4.

Secondly, the assessment of hsCRP as a biomarker for HNF1A-MODY was carried out in studies featuring subjects mainly of European ancestry (McDonald, et al., 2011b; Owen, et al., 2011; Owen, et al., 2010). It is not known how hsCRP would perform in identifying HNF1A-MODY patients in other ethnic populations. Type 2 diabetes is common in young South Asians, presenting at a lower BMI than in North Europeans, making the differentiation from MODY challenging (1994). MODY occurs in UK Asian families; however there is lower referral rate

for genetic testing (Porter, et al., 2006). In the UK MODY database (a record of all referrals for genetic testing for diabetes since 1996), only 8 out of 1369 (0.5%) UK index case referrals for MODY testing were of South Asian origin (Porter, et al., 2006). These numbers suggest a low referral for genetic testing given that South Asians comprise 4% of UK population and are 4 times more likely to have diabetes.

Population studies have suggested that CRP levels differ among adults from different ethnic backgrounds (Chambers, et al., 2001; Dalan, et al., 2010). For instance in the study by Chambers *et al*, CRP levels were found to be 17% higher in Indian Asians as compared with Europeans (Chambers, et al., 2001). The authors found that the difference in CRP levels between the two ethnic groups disappeared when adjusted for waist-hip ratio or insulin resistance score. Given the relatively higher hsCRP levels in South Asians, the difference in CRP might be greater than that seen in Europeans, allowing better discrimination between the two forms of diabetes. The use of hsCRP as HNF1A-MODY biomarker in South Asians will be investigated in chapter 5.

Table 1.7 summarizes all the biomarkers for HNF1A-MODY investigated to date, the types of diabetes they distinguish, which phase of development they have reached and the cost and availability of the assays.

Biomarkers	HNF1A-MODY vs. T1D	HNF1A-MODY vs. T2D	HNF1A vs. HNF4A-MODY	HNF1A vs. GCK-MODY	Replication in large scale case-control studies	Replication in prospective cohorts	Approx Cost	Available
Urinary amino acids	X	X	-	-	-	-	£75	✓
Serum amino acids	-	-	-	-	-	-	£75	✓
C5, C8 and TTR	-	X	X	-	-	-	unknown	?
ApoM	-	X	X	-	-	-	unknown	Mainly research
1, 5 AG	-	X	-	✓	-	-	£20	USA/Japan Not widely used in UK
Islet autoantibodies	✓	-	-	-	-	-	£15	✓
C-peptide	✓	X	X	X	-	-	£10	✓
HDL	-	¥	-	-	-	-	£2	✓
hsCRP	X	✓	✓	X	✓	-	£2	✓
DG9-glycan index	✓	✓	-	-	✓	-	£30	Mainly research*

Table 1.7 Biomarkers of MODY subtypes investigated to date and their differential diagnosis potential

✓= good discrimination (C-statistic > 0.80 or clinically proven useful discrimination) between subtypes of diabetes, ¥ = modest discrimination (C-statistic =0.76), X = no clinically relevant, reproducible discrimination demonstrated, * = On-going assay development, **-** = no relevant investigation carried out. None of the biomarkers has been investigated for cost-effectiveness and for impact on the quality of life of the patient. References: (Bingham, et al., 2001; Gloyn, et al., 2012; Karlsson, et al., 2008; McDonald, et al., 2011a; McDonald, et al., 2012; Nowak, et al., 2013; Pal, et al., 2010; Richter, et al., 2003; Stride, et al., 2004; Thanabalasingham, et al., 2013; Thanabalasingham, et al., 2011)

1.6 Derangement of acute inflammatory response in HNF1A-MODY

HNF1A is transcriptional regulator of a number of inflammatory response proteins, of which CRP has been shown to be lower in HNF1A-MODY (Armendariz and Krauss, 2009). HNF1A regulates fucosylation, important for cell adhesion and leukocyte migration into the inflamed tissues during acute inflammation (Lauc, et al., 2010). Together, these findings suggest that *HNF1A* haploinsufficiency could result in derangement of the acute inflammatory response in subjects with HNF1A-MODY with subsequent clinical implications. A significant amount of my DPhil research focusses on investigation of acute inflammatory response in HNF1A-MODY. The next few sections describe the dynamics of a normal acute inflammatory response, important mediators of response regulated by HNF1A and how a deranged acute inflammatory response could have significant clinical implications for the HNF1A-MODY subjects.

1.6.1 The Acute Inflammatory Response

The acute inflammatory response is the normal response of the body to infection, physical or chemical injury, and is pivotal in removing the inflammatory stimuli and promoting tissue repair (Ward and Lentsch, 1999). A successful inflammatory response comprises a fine balance of pro- and anti-inflammatory mediators, as an uncontrolled systemic inflammatory response can lead to lethal effects such as toxic shock, organ failure and death (Ward and Lentsch, 1999).

1.6.1 (a) Initiation of the acute inflammatory response by Lipopolysaccharide

The inflammatory response is initiated by the recognition of Pathogen-Associated Molecular Patterns (PAMP) on the surface of invading microorganisms by pattern recognition receptors on the host cells (Heumann and Roger, 2002) (**Figure 1.7**). Lipopolysaccharide (LPS; also known as endotoxin) is a well-known PAMP present on the outer membrane of most gram-negative bacteria and can elicit a strong inflammatory response. LPS is usually composed of three parts; Lipid A, core oligosaccharide and a polysaccharide O antigen. Of these, the lipid A region is responsible for much of the toxicity of LPS.

1.6.1 (b) LPS-sensing machinery

The LPS-sensing machinery comprises of circulating lipopolysaccharide binding protein (LBP) and pattern recognition receptors (**Figure 1.7**).

LBP is a hepatocyte secreted acute phase protein. The mean serum concentration of LBP in healthy individuals is 5-20 µg/ml and rises 10-50 fold during inflammatory conditions (Gutsmann, et al., 2001). LBP levels also increase with increasing age and BMI (Gonzalez-Quintela, et al., 2013). LBP has a concentration dependent dual role. A low dose of LBP leads to enhanced inflammatory response and a high dose of LBP leads to inhibition (Zweigner, et al., 2006).

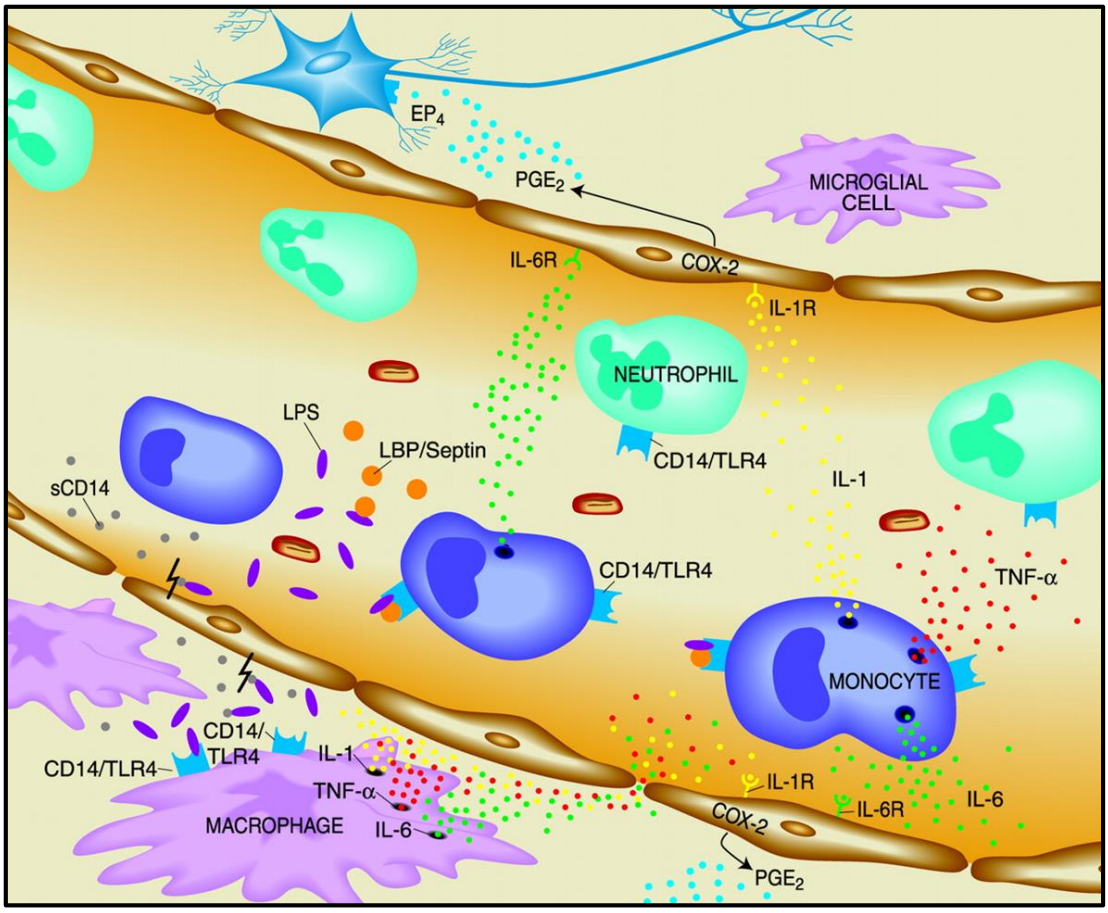


Figure 1.7 Inflammatory response generated by the presence of lipopolysaccharide (LPS). Taken with permission from (Zhang and Rivest, 2003)

COX-2=cyclooxygenase 2, IL-1=interleukin 1, IL-6=interleukin 6, LPS=Lipopolysaccharide, LBP=lipopolysaccharide binding protein, TLR4=toll like receptor 4, TNFα=tumour necrosis factor alpha, PGE2=prostaglandin E2

LPS initiates the inflammatory response by binding with circulating LBP. LBP either presents LPS to the pattern recognition receptors, hence activating them, or neutralises LPS by transferring it to HDL. LPS-specific pattern recognition receptors include CD14 (membrane protein) and toll like receptor 4 (TLR4; signal-transducing integral membrane protein) on the surface of neutrophils and monocytes (**Figure 1.7**). The LPS-LBP complex binds with CD14/TLR-4. This results in dimerization of the receptor and initiation of the intracellular signalling cascade, resulting in the production of pro-inflammatory cytokines (such as TNFα, IL-1, IL-6) (Raetz and Whitfield, 2002). The pro-inflammatory cytokines induce a series of local and systemic responses discussed below.

1.6.1 (c) Selectin and selectin ligand mediated leukocyte adhesion, rolling and migration through endothelium

Inflammatory mediators (such as cytokines, leukotrienes, histamine) stimulate expression of carbohydrate binding transmembrane proteins called selectins on the surface of endothelial cells and leukocytes. Selectins slow down the free flowing leukocytes on the inflamed endothelial wall, a process known as rolling (Barthel, et al., 2007; Kolaczkowska and Kubes, 2013). The selectins bind to oligosaccharide epitopes on specific carrier proteins known as selectin ligands (**Table 1.8**). Selectin-selectin ligand mediated rolling allows leukocytes to come in close contact with endothelial cells and subsequently adhere and transmigrate through the endothelial wall. Selectin-selectin ligand binding is dependent on proper sulphation and fucosylation of oligosaccharide epitopes on the selectin ligands (Maly, et al., 1996; Moore, et al., 1994; Rosen and Bertozzi, 1996; Schottelius, et al., 2003).

Selectin ligands	Expressing cells	Function
P-selectin glycoprotein ligand 1 (PGSL1)	Most leukocytes, chronically inflamed endothelium	Most important inflammatory selectin ligand. Mediates P, L and E selectin dependent rolling
CD44	Leukocytes	E selectin ligand
Glycosylation dependent cell adhesion molecule (GlyCAM-1), CD34	Endothelial cells	L selectin ligand

Table 1.8: Selectin ligands

A defect in fucosylation is the underlying mechanism in leukocyte adhesion deficiency (LAD) type II, an immune disorder characterised by repeated uncontrolled infections. The decrease in fucosylated selectin ligands in LAD type II leads to severe defects in neutrophil adhesion and motility, and impairs their ability to migrate through the vessel wall and reach inflamed tissue.

1.6.1 (d) Acute phase proteins

Pro-inflammatory cytokines also stimulate the production of acute phase proteins by binding to cytokine receptors on the cell surface (mainly hepatocytes) (Ceciliani, et al., 2002). Some of the major acute phase proteins are apolipoproteins serum amyloid A1 and 2, CRP and α 1 acid-glycoprotein. Acute phase proteins play an important role in the acute inflammatory response. For instance, CRP activates the complement system, facilitates phagocytosis and induces the expression of inflammatory cytokines. At high concentration, CRP mediates resolution of the inflammatory response by inhibiting release of superoxide radicals by neutrophils and by stimulating the synthesis of IL-1 receptor antagonist (Ceciliani, et al., 2002). CRP is used as a diagnostic and prognostic indicator of inflammation in clinical practice.

Acute inflammatory response subsides after 1-2 days depending on removal of inflammatory stimuli. Glucocorticoids, anti-inflammatory cytokines (IL-4, IL-10) and receptor antagonists for IL-1 and IL-6 play an important role in resolution of the inflammatory response (Ceciliani, et al., 2002).

1.6.2 Acute inflammatory response and *HNF1A* mutations

Patients with HNF1A-MODY have loss of function *HNF1A* mutations. They have been found to have low baseline levels of CRP levels compared with healthy controls (Owen, et al., 2010). This might have important implications in the clinical management of HNF1A-MODY patients because if CRP does not rise normally during an inflammatory response, it would not be a reliable marker of inflammation in those with HNF1A-MODY. Moreover, low CRP levels during an acute inflammatory response could affect CRP-mediated complement activation and phagocytic clearance of pathogens. Patients that have HNF1A-MODY also show altered plasma glycan profile, and have a lower ratio of fucosylated to unfucosylated plasma glycans as compared with healthy controls (Thanabalasingham, et al., 2013). Various acute phase proteins (alpha 1 acid glycoprotein, CRP, haptoglobin) undergo increase in fucosylated content during systemic inflammatory response conditions, such as sepsis and pancreatitis (Gornik, et al., 2007). Fucosylation is also important for selectin-selectin ligand mediated neutrophil rolling and migration. As HNF1A appears to be a master regulator of plasma protein fucosylation, it can be hypothesised that loss of one functional allele in HNF1A-MODY might lead to disturbances in the plasma glycan profile and white blood cell rolling during an acute inflammatory response. Lastly, *Hnf1a* knockout mouse models have shown that HNF1A regulates LBP expression (a major mediator of LPS stimulated response) (Armendariz and Krauss, 2009; Shih, et al., 2001).

Together, these findings suggest that patients with HNF1A-MODY could have a deranged acute inflammatory response. This will be investigated in chapter 6

using an inflammatory response model.

1.7 Thesis Aims

This introduction has outlined the different types of diabetes that present themselves in young adults and the importance of a correct aetiological diagnosis. Sections 1.3 and 1.5 outlined the transcriptional regulatory role of HNF1A, translation of the findings to date into biomarker development and the challenges remaining for evaluation of these biomarkers before clinical use. Section 1.6 provided evidence suggesting the role of HNF1A in regulating different components of acute inflammatory response.

Following from above, the work presented in this thesis has the following specific aims.

Aim 1: To evaluate the use of apoM as a biomarker for HNF1A-MODY

Human studies examining apoM levels in HNF1A-MODY have yielded conflicting results. I aim to re-examine the use of apoM as a biomarker for HNF1A-MODY by employing a recently described, highly sensitive and specific ELISA. Details of this study are given in chapter 3.

Aim 2: To evaluate the use of hsCRP and DG9-glycan index for differential diagnosis of HNF1A-MODY in young adults

Previous studies investigating hsCRP and DG9-glycan index included large groups of known HNF1A-MODY, type 1 and type 2 diabetes subjects. Due to the strict selection criteria, these studies may have been prone to “spectrum bias” resulting in inflated estimates of measures of biomarker performance. I aim to

evaluate the performance of hsCRP and DG9-glycan index in identifying HNF1A-MODY in a group of subjects previously uninvestigated for MODY, clinically diagnosed as having young-onset non-autoimmune diabetes. Details of this study are given in chapter 4.

Aim 3: To evaluate the use of high sensitivity C-reactive protein for identifying young South Asians with HNF1A-MODY

Previous studies investigating hsCRP included mainly North European subjects and its potential role as a biomarker in other ethnic groups has not yet been investigated. I aim to evaluate the use of low hsCRP to identify South Asian subjects at high risk of having HNF1A-MODY. Details of this study are given in chapter 5.

Aim 4: To investigate the acute inflammatory response in HNF1A-MODY

This study aims to investigate any abnormalities in the acute inflammatory response in HNF1A-MODY by measuring hsCRP, plasma and white blood cell membrane glycans, serum cytokines as well as other parameters of inflammation at baseline and after an acute inflammatory stimulus. Details of this study are given in chapter 6.

Chapter 2

Subjects and Methods

2.1 SUBJECTS

2.1.1 The Young Diabetes in Oxford cohort

The Young Diabetes in Oxford (YDX) cohort includes subjects diagnosed with diabetes ≤45 years of age, ascertained from a survey of GP surgeries and a search of the hospital clinic database in Oxfordshire, U.K (Thanabalasingham, et al., 2012b). After investigations, subjects meeting the criteria listed below were categorized into MODY, type 1 or type 2 diabetes groups.

Type 2 diabetes was defined as: C-peptide positive, no requirement for permanent insulin within 3 months of diagnosis and negative glutamic acid decarboxylase antibodies (GAD; positive level defined as >14 WHO units/ml) (Bingley, et al., 1997). Type 1 diabetes was defined as: permanent insulin treatment since diagnosis with additional evidence of severe beta cell dysfunction (C-peptide≤0.2nmol/L), positive GAD antibodies or both.

MODY was defined as the presence of pathogenic variants in *HNF1A, HNF4A or GCK* confirmed by sequencing in a certified diagnostic centre. The pathogenicity of novel variants was determined by family studies looking for co-segregation of the variant with dysglycaemia, presence of typical MODY phenotype and evidence of evolutionary conservation and impact of the variant on protein function from bioinformatics programs, SIFT and PolyPHEN (Adzhubei, et al., 2010; Kumar, et al., 2009).

Subjects with type 1 and type 2 diabetes either did not meet clinical criteria for MODY diagnostic testing or had been tested and were negative for likely disease causing variants in *HNF1A, HNF4A or GCK*.

The YDX study was approved by the Oxfordshire Research Ethics Committee and all subjects gave their written informed consent. Clinical characteristics of the full data set of type 1, type 2 and MODY subjects in the YDX cohort, after investigation and final categorisation, are provided in **table 2.1**. Subjects with HNF1A-MODY, type 1 and type 2 diabetes from the YDX cohort were used to provide cases for the studies described in chapters 3, 5 and 6. The characteristics of the subsets of subjects used for each specific study are tabulated in the respective chapters.

2.1.2 The Oxford Biobank

The Oxford Biobank (OXBB) is a population-based cohort of healthy men and women (n=6,194, recruited until Dec 2013), aged 30-50 years, living in Oxfordshire and randomly selected from the UK National Health Service population register (http://www.oxfordbiobank.org.uk). All participants underwent detailed physical assessment (including anthropometric measurements and DEXA scan) and laboratory tests (such as hsCRP and lipid profile). The OXBB study was approved by the Oxfordshire Research Ethics Committee and all subjects gave their written informed consent. The clinical and biochemical characteristics of all participants in the OXBB cohort (recruited until Dec 2013) are given in **table 2.2**.

	Type 1 diabetes (n=245)	Type 2 diabetes (n=264)	HNF1A/HNF4A MODY (n=14)	GCK-MODY (n=1)
% Male	53.9	62.9	35.7 #	0.0
Age of diagnosis (years)	25.0 (15.0-32.0)	39.0 (34.0-42.0)	25.5### (20.3-30.7)	19.0
Duration of diabetes (years)	12.5 (8.6-15.7)	11.0 (5.5-22.0)	17.8 (9.0-26.6)	27.0
BMI (kg/m^2)	25.5 (22.6-28.5)	32.6 (28.4-36.7)	27.1## (23.7-30.6)	21.4
% Parental diabetes	25.7	54.2	64.3***	0.0
HbA1c (%)	NA	7.8 (6.9-9.0)	7.7 (6.6-8.7)	7.1
Fasting glucose (mmol/L)	NA	8.3 (6.6-10.5)	7.4 (6.1-8.7)	7.6
Fasting C-peptide (nmol/L)	NA	0.69 (0.42-1.04)	0.49# (0.17-0.81)	0.56
Total cholesterol (mmol/L)	NA	4.3 (3.7-5.1)	4.7 (4.0-5.4)	5.1
HDL cholesterol (mmol/L)	NA	1.1 (0.9 -1.4)	1.3 (1.1-1.5)	1.7
Triglycerides (mmol/L)	NA	1.48 (1.01-2.10)	1.34# (0.49-2.19)	0.59

Table 2.1: Clinical and biochemical characteristics of subjects in the Young Diabetes in Oxford cohort after investigation and final categorisation (section 2.1.1). Data are shown as median (interquartile range) unless otherwise stated. HNF1A/HNF4A-MODY comparisons with type 1 diabetes (* P<0.05, ** P<0.01, *** P<0.001) and type 2 diabetes (# P<0.05, ## P<0.01, ### P<0.001) using Mann-Whitney U test or Chi-squared test for proportions. Parental diabetes=one or both parents has diabetes of any aetiology, NA=Not available.

Characteristics	Subjects (n=6,194)
Males (%)	44.1
Age at recruitment (years)	43 (37-46)
BMI (kg/m^2)	25.2 (22.8-28.4)
Fasting glucose (mmol/L)	5.1 (4.9-5.5)
Total Cholesterol (mmol/L)	5.1 (4.5-5.8)
HDL cholesterol (mmol/L)	1.3 (1.1-1.6)

Table 2.2: Clinical and biochemical characteristics of all subjects in the Oxford Biobank cohort (recruited until Dec 2013) (section 2.1.2). Data are shown as median (interquartile range) unless otherwise stated

Subjects from OXBB were made available for the study described in chapter 3, through collaboration with Professor Fredrik Karpe, Oxford Center for Diabetes, Endocrinology and Metabolism (OCDEM), University of Oxford. The characteristics of the subsets of subjects used from OXBB are tabulated in the respective chapters.

2.1.3 Subjects recruited from Poland

This dataset included subjects having HNF1A-MODY (n=47) and type 2 diabetes (n=70), ascertained from Poland. Subjects from this data set were made available for the study described in chapter 3 through collaboration with Dr Maceij Malecki, Department of Metabolic Diseases, Jagiellonian University, Krakow, Poland.

HNF1A-MODY was defined as the presence of heterozygous loss-of-function *HNF1A* variants with subjects identified through the Polish MODY registry.

Type 2 diabetes subjects were selected from individuals diagnosed with diabetes ≤45 years of age from a cohort collected in the Department of Metabolic Diseases. These subjects did not require permanent insulin within 1 year after the diagnosis and did not meet clinical criteria for MODY or type 1 diabetes. The study was approved by the Bioethical Committee of the Jagiellonian University and all subjects gave informed consent. The clinical and biochemical characteristics of Polish subjects are given in **table 2.3**.

Characteristics	HNF1A-MODY (n=47)	Type 2 diabetes (n=70)	P value
Males (%)	38	71	<0.01
Diabetes Duration (years)	7.0 (2.0-16.5)	10.5 (7.0-15.2)	0.09
Age at Diagnosis (years)	18.0 (16.0-31.2)	40.5 (35.1-44.0)	<0.01
BMI (kg/m^2)	22.8 (19.8-25.1)	30.8 (26.9-34.1)	<0.01
Fasting glucose (mmol/L)	5.8 (5.2-7.8)	6.8 (6.0-7.8)	0.04
HDL cholesterol (mmol/L)	1.2 (1.0-1.6)	1.0 (0.9-1.3)	0.02

Table 2.3: Clinical and biochemical characteristics of Polish subjects (section 2.1.3). Data are shown as median (interquartile range). P values compare HNF1A-MODY with type 2 diabetes and were determined by Mann-Whitney U test.

2.1.4 Subjects from Germany

This dataset comprised North European subjects (n=396) clinically diagnosed and treated as young-onset non-autoimmune diabetes (age of onset < 45 years). Subjects were ascertained from the Diabetes Outpatient Clinics of Ulm University Medical Centre and Bad Mergentheim Diabetes Centre, as well as from three outpatient clinics in Ulm, Frankfurt am Main and Wangen, Germany. All patients were of North European ethnicity. Local ethical approval was obtained and all the patients gave informed consent for the study. Plasma and DNA samples were available for all subjects. Subjects from this dataset were made available through collaboration with Professor Bernhard Boehm, Diabetes centre, Ulm University, Germany. The characteristics of all subjects included in this cohort are provided in **table 2.4.**

As discussed in chapter 1 (section 1.7), one of the aims of my research work is to evaluate the use of high sensitivity C-reactive protein (hsCRP) and plasma glycan profile for identifying HNF1A-MODY in a group of subjects with young-onset diabetes not previously investigated for MODY. This dataset of patients ascertained from Germany met these requirements. Subjects from this dataset were used for the study described in chapter 4. The characteristics of the subjects used are tabulated in chapter 4.

Characteristics	Subjects (n=396)
Males (%)	56
Age at sampling (years)	31 (27-35)
Age at diagnosis (years)	28 (25-32)
BMI (kg/m^2)	27 (23-32)
Islet antibodies % (Present/Absent/Unknown)	0/299/97
Diabetes Treatment % (Diet/Insulin/Oral antidiabetic agents)	1/3/97

Table 2.4: Clinical and biochemical characteristics of German subjects (Section 2.1.4). Data are shown as median (interquartile range).

2.1.5 London Life Sciences Prospective Population

The London Life Sciences Prospective Population (LOLIPOP) is a population-based study of more than 30,000 multi-ethnic men and women recruited from GP surgeries in West London (http://www.lolipopstudy.org/). Detailed health assessment, biochemical tests and DNA samples are available for all the subjects included in this study. The LOLIPOP study was approved by the Ealing and St Mary's Hospitals, North West London Research Ethics Committee and all subjects gave written, informed consent. The clinical and biochemical characteristics and DNA samples for subjects (n=9,448) from the LOLIPOP study were made available, for the study described in chapter 5 through collaboration with Professor Jaspal Kooner and Dr John Chambers (Imperial College London). The clinical and biochemical characteristics of all subjects, for whom data were available, are illustrated in **table 2.5**.

2.1.6 Young Asians with diabetes from Birmingham

This dataset comprised South Asian subjects (n=72) ascertained from Birmingham, clinically labelled and treated as young-onset type 2 diabetes. Plasma and DNA samples were available for these subjects. Detailed clinical and biochemical characteristics were not available for these subjects. Age at onset, age at sampling and diabetes duration of the subjects present in this dataset are given in **table 2.6**. The subjects from this dataset were made available for the study described in chapter 5 through collaboration with Dr Ann Kelly, Centre for Endocrinology, Diabetes and Metabolism, University of Birmingham.

Characteristics	Controls (n=8,196)	Type 2 diabetes (n=1,252)	P value
Age at sampling (years)	42.0 (37.4-46.6)	47.1 (42.8-51.8)	<0.01
BMI (kg/m^2)	26.5 (24.0-29.3)	27.6 (25.2-30.8)	<0.01
Total Cholesterol (mmol/L)	5.2 (4.5-5.9)	4.9 (4.1-5.8)	<0.01
HDL-Cholesterol (mmol/L)	1.2 (1.0-1.4)	1.1 (0.9-1.3)	<0.01
HbA1c (%)	5.5 (5.2-5.8)	7.7 (6.6-9.0)	<0.01
Glucose (mmol/L)	5.0 (4.7-5.3)	9.1 (7.0-10.6)	<0.01

Table 2.5: Clinical and biochemical characteristics of LOLIPOP subjects (section 2.1.5) used for the study described in chapter 5. Data are shown as median (interquartile range). P values compare subjects with and without diabetes and were determined by Mann-Whitney U test.

Characteristics	Type 2 diabetes n=72
Age at sampling (years)	40.0 (32.7-49.0)
Age at onset (years)	29.0 (24.7-31.0)
Diabetes duration (years)	10.5 (4.0-19.0)

Table 2.6: Characteristics of South Asian subjects from Birmingham (section 2.1.6). Data are shown as median (interquartile range).

2.1.7 Other samples

Some of the subjects used in the studies described in chapter 3 and 6 were from other resources. One of the resources was MODY in Oxford (MOX) study. The MOX study includes subjects ascertained from Oxford and diagnosed with HNF1A, HNF4A and GCK-MODY and type 2 diabetes. Another resource was Diabetes Alliance for Research in England (DARE) study. DARE is a UK-wide study, including type 1 and type 2 diabetes subjects >18 years of age. HNF1A-MODY and type 2 diabetes subjects from MOX and DARE respectively, were used for the studies described in chapter 3 and 6. Healthy controls for the study described in chapter 6 were recruited through local advertisement in Oxford and from OCDEM Clinical Research Unit database (a dataset of subjects willing to be contacted again).

2.2 Methods

2.2.1 Genetic Sequencing

Sequencing was carried out using Sanger sequencing (studies described in chapters 3, 4, 5 and 6) or Exome sequencing (study described in chapter 5). Sanger sequencing is a traditional method that comprises of chain termination sequencing method followed by gel electrophoresis. It requires presence of a template strand, four standard deoxynucleotides, four fluorescently labelled dideoxynucleotides, primer and DNA polymerase. DNA polymerase adds complimentary standard nucleotides to the template strand and the chain is terminated when a dideoxynucleotide is inserted. As a result, strands of variable length are produced. The strands are then subjected to electrophoresis and sequence is determined from the colour of fluorescently labelled dideoxynucleotides. Sanger sequencing has a low throughput and is used for small-scale sequencing. Exome sequencing is one of the newer "next generation sequencing methods" that allows parallel sequencing of millions of DNA strands, hence has a high-throughput and can be used for sequencing at a large scale.

Sanger sequencing for *HNF1A*, *HNF4A* and *GCK* was performed by Meg Mashbat and Kevin Colclough (through collaboration with Professor Sian Ellard), in the CPA-accredited Molecular Genetics Laboratory at the Royal Devon and Exeter National Health Service (NHS) Foundation Trust. Semiautomated unidirectional sequencing of *HNF1A* exons 1–10, *HNF4A* promoter P2, exons 1a and 2–10 and *GCK* promoter and exons 1–10 was performed on an ABI 3730 capillary sequencer (Carlsbad, CA) and analyzed using Mutation Surveyor v3.24

(SoftGenetics, State College, Pennsylvania). This method has 99% sensitivity to detect heterozygous base substitutions (Ellard, et al., 2009).

Exome data were generated using Agilent Truseq capture reagents, and individually-barcoded samples sequenced on Illumina HiSeq2000 instruments. Eighty two fold mean coverage and an estimated genotyping rate of 99.2% was achieved across the coding sequence of 18,281 genes sequenced as part of T2D-GENES project.

2.2.1.1 Assessment of pathogenicity of *HNF1A* variants

HNF1A variants were categorized as likely disease causing or benign using conventional criteria listed below:

- previously published reports of the association of the variants with a MODY phenotype
- co-segregation of the variant with a MODY phenotype when relatives were available
- presence of the variants resulting in a premature stop codon
- variants likely to result in exon skipping (variants at canonical splice sites)
- rare, conserved missense variants with likely damaging consequence on protein function

Novel variants were defined as those not previously reported, absent from the Exome Variant Server (EVS) and the 1000 Genomes Project (www.1000genomes.org, http://evs.gs.washington.edu/EVS) (EVS Release Version: v.0.0.22. (Oct. 17, 2013); Genomes Project, et al., 2010).

In silico analysis was performed to predict the effect of the variants using following bioinformatics software:

- Sorting Intolerant From Tolerant (SIFT)

  SIFT is a web-based tool that predicts whether an amino acid substitution affects protein function based on sequence homology and the physical properties of amino acids (Kumar, et al., 2009).

- MutationTaster

  MutationTaster is also a web-based tool that integrates information from different biomedical databases (listed in **table 2.7**) and analyses variants for evolutionary conservation, splice-site changes, loss of protein features and changes that might affect the amount of mRNA (Schwarz, et al., 2010). The test results are then evaluated by a Bayes classifier.

- CONsensus DELeteriousness score of missense single nucleotide variants (CONDEL) (Gonzalez-Perez and Lopez-Bigas, 2011)

- Alamut (http://www.interactive-biosoftware.com/alamut.html)

  CONDEL and Alamut are consensus prediction software that integrate genetic information from the data sources listed in **table 2.7.**

  CONDEL predicts functional impact of missense variants only, classifying them as either Deleterious (pathogenic) or Neutral (benign). The classification is based on weighted average of the normalized scores of the individual methods listed in **Table 2.7**. Alamut integrates information from a larger number of data sources compared with CONDEL. Alamut

Bioinformatic tools and data sources	Description	Used by
UCSC genome browser	Genome database used for genomic sequence, transcript information and evolutionary conservation scores	Alamut
Ensembl	Genome database used for SNP data and protein information	CONDEL, Alamut, MT
dbSNP	Core variation database used for SNP information	Alamut, MT
InterPro	Protein database	Alamut
NCBI RefSeq database	Reference sequence database used for transcript information	Alamut
SwissProt	Protein database used for missense variant information	Alamut, MT
MEDLINE	Bibliographic database	Alamut
NCBI browser	Used for genome browsing	Alamut, MT
SIFT	Web-based tool that uses sequence homology from MSA to predict if amino acid substitutions would be tolerated or damaging	CONDEL, Alamut
POLYPHEN-2	Uses sequence and structure-based information to predict the effect of variants using a Bayesian approach	CONDEL, Alamut
Align GVGD	Uses a MSA to characterise the biochemical properties of the observed amino acids at each position	Alamut
Mutation assessor	Uses MSA to reflect functional specificity and generates conservation scores to represent the functional impact of a missense variant of a mutation	CONDEL
Mutation taster	Web based Bayes classifier trained with known polymorphisms and disease mutations	Alamut
Splice Site Finder	Splice site prediction program	Alamut
NNSplice	Splice site prediction program	Alamut, MT
MaxEntScan	Splice site prediction program	Alamut
GeneSplicer	Splice site prediction program	Alamut

Table 2.7 Brief description of bioinformatic tools and data sources used by Alamut, CONDEL and Mutation taster. SNP=Single Nucleotide Polymorphism, MSA=Multiple Sequence Alignment

provides detailed information on nucleotide and amino acid conservation, providing PhyloP score (method to determine the grade of conservation of a given nucleotide) and Grantham score (chemical distance that measures the difference between two amino acids, taking into account their volume, polarity and composition of side chain).

The programs used for prediction of the functional effect of the variants, have individual strengths and limitations (Davies, et al., 2012; Flanagan, et al., 2010; Xue, et al., 2012). These tools have different attributes. Some use sequence alignment and machine learning algorithms, others take into account protein structure or some are consensus software that integrate information from multiple sources. The prediction of the pathogenicity of a specific variant can differ from one program to another. For example, a change in the evolutionary tree (number or type of orthologues) used for sequence alignment resulting in an increase or decrease in the diversity, can change the prediction of the variant from disease causing to neutral. To improve prediction of the pathogenic variants and to reduce the likelihood of a false positive or negative test, a combination of prediction algorithms listed above was used and the most consistent result among them were selected. Any overall conclusion on the functional impact of the variants identified was based on evidence from the clinical characteristics, family history where available and predictions of more than one bioinformatics programs.

Access to family members was not possible for most of the novel *HNF1A* variants identified in the studies described in chapters 4 and 5.

2.2.2 Biochemical and haematological analysis

2.2.2.1 Fasting glucose, HDL-cholesterol and hsCRP

Fasting glucose, HDL-Cholesterol and hsCRP were measured through collaboration with Dr Tim James at the biochemistry laboratory, John Radcliffe Hospital, Oxford.

Fasting glucose and HDL-cholesterol were measured using Glucose Hexokinase II method, and the elimination/catalase method, respectively, on an ADVIA 2400 analyser (Siemens Healthcare Diagnostics, Frimley, U.K.).

HsCRP was measured using a wide-range latex-enhanced immunoturbidometric assay on an ADVIA 2400 analyser (Siemens Healthcare Diagnostics, Frimley, U.K.). This assay has a quoted method linearity of 0.03–160 mg/L and estimate of imprecision, expressed as percentage CV of 10% at 0.05 mg/L and <1% at 23.5 mg/L.

2.2.2.2 ApoM assay

Serum apoM was measured using a sandwich ELISA provided by Roche (Karuna, et al., 2011). As discussed in chapter 1 (section 1.5.1), apoM was previously investigated as a HNF1A-MODY biomarker by three separate studies using different methods for apoM analysis (Cervin, et al., 2010; Richter, et al., 2003; Skupien, et al., 2007). The sandwich ELISA, the technique used in the current study, is a more sensitive and quantitative method for protein quantification compared with western blot and dot blot, methods used by Richter *et al* and Skupien *et al*. Moreover, based on in-house measurement of average values from healthy controls, it is estimated that the current ELISA is 1.5 fold

more sensitive than the ELISA used by Cervin *et al*.

Measurement of serum apoM levels was carried out by Rebekka Park (through collaboration with Professor Markus Stoffel) at the Institute of Molecular Health Sciences, ETH Zurich, Switzerland, using previously described method (Karuna, et al., 2011).

2.2.2.3 Lipopolysaccharide binding protein (LBP) assay

Plasma LBP was analysed by Christopher Groves, a member of the research group at OCDEM. LBP was measured using the Hycult Biotech HK503 ELISA. Plasma samples (stored at -80°C) and reagents (stored at 2-4°C) were brought up to room temperature (18-25°C). Microwell plates (Hycult Biotech) were pre-coated with primary LBP antibody. One µl of diluted (1:1000) samples was added to the microwells and incubated at room temperature for one hour. The plates were washed four times with wash buffer. One hundred microlitre of diluted biotinylated tracer was added to each well. The plates were then covered and incubated for one hour at room temperature. The plates were again washed four times with wash buffer. One hundred µl of diluted streptavidin-peroxidase was then added to each well and incubated for one hour at room temperature. Bound LBP was detected by adding 100 µl of TMB substrate to each well, incubated for 30 minutes at room temperature and the reaction stopped by adding 100 µl of stop solution. Absorbance was measured at 450 nm using VersaMax plate reader (Molecular Devices). The sigmoidal standard curve was fitted by nonlinear regression analysis (using SoftMax Pro software) and LBP concentrations calculated in µg/ml. Intra-assay CV was 10% and inter-assay CV was 6%.

2.2.2.4 Serum cytokines

Serum cytokines (IL-6, IL-1, TNFα and IL1-ra) were analysed by Joshua Chai (through collaboration with Professor Robin Choudhury), at the Department of Cardiovascular Medicine, John Radcliffe Hospital, Oxford. Serum cytokines were analysed using a Luminex™ Multiplex bead-based system using Milliplex™ MAP kits, from the Human Cytokine/Chemokine panel.

2.2.2.5 White Blood Cell (WBC) count and Erythrocyte Sedimentation Rate (ESR)

The WBC count and ESR were analysed through collaboration with Dan Smith at the haematology laboratory, John Radcliffe hospital, Oxford. The WBC count was analysed using a Sysmex© XE2100 analyser. The ESR was measured using the Vitech Scientific© Interrliner.

2.2.3 Glycan analysis

Glycan analysis comprises of two parts.

- **Release of the WBC membrane and plasma glycans**

  I and another member (Amanda Bennett) of the research group at OCDEM carried out the **WBC membrane glycan release** at the Diabetes Research Laboratory at OCDEM. The details of the method used are provided below. The **plasma glycan release** was carried out by Mislav Novokmet (through collaboration with Dr Olga Gornik and Professor Gordan Lauc) at the Glycobiology Laboratory, Genos Ltd, Zagreb, Croatia, using a previously reported method (Royle, et al., 2008).

- **Hydrophilic Interaction High Performance Liquid Chromatography (HILIC-HPLC) of released glycans**

  HILIC-HPLC of released glycans was carried out by Mislav Novokmet at the Glycobiology Laboratory, Genos Ltd, Zagreb, Croatia (Royle, et al., 2008).

2.2.3.1 WBC membrane glycan release

As discussed in Chapter 1 (section 1.6.2), HNF1A regulates plasma protein fucosylation. Fucosylation of selectin ligands is necessary for neutrophil adhesion and migration during an acute inflammatory response. *HNF1A* haploinsufficiency could lead to hypofucosylation of selectin ligands present on the WBC membranes. To investigate this possible effect, WBC membrane glycans were isolated at different time points during the acute inflammatory response in healthy controls and subjects with HNF1A-MODY and type 2 diabetes (this study is described in chapter 6).

I carried out the optimisation of WBC membrane glycan release protocol in close collaboration with Dr Olga Gornik from the Glycobiology Laboratory of Genos Ltd, Zagreb, Croatia. After optimisation, I performed the WBC membrane glycan release of samples from HNF1A-MODY subjects and controls. The WBC membrane glycan release of samples from type 2 diabetes subjects was performed by Amanda Bennett, a member of the research group. The details of the optimisation and the final optimised WBC membrane glycan release protocol are provided below.

Optimisation steps

WBC membrane glycan release protocol was finalised after 2 optimisation steps. These steps are described below and illustrated in **figure-2.1** and **2.2**.

(a) Optimisation 1

A single blood draw was taken (from a healthy volunteer) and divided into three parts. WBC isolation and membrane glycan release was performed at Oxford. Dried released membrane glycans were sent to Genos Ltd, Croatia for high performance liquid chromatography (HPLC) analysis. Unacceptable variation in measured glycan peaks was noted between the three samples (illustrated in **figure-2.1**) and the protocol went through a second stage of optimisation.

(b) Optimisation 2

In the second optimisation, WBC isolation and membrane glycan release was carried out in parallel at Oxford and Genos Ltd, Croatia. Three blood draws were taken and each divided in three parts (as illustrated in **figure-2.2**). Three samples served as controls and were not treated with glycan release enzyme (N-glycosidase F [PROzyme]). Variation in glycan peaks was again observed within the samples. However, the difference within the samples was more marked in samples processed at Genos Ltd, Croatia compared with those processed at Oxford. This was thought to be due to an additional step, the washing of isolated WBC with PBS, performed at Oxford. It was observed that differences within the samples decreased by adding multiple washings of the isolated WBC with PBS.

Optimisation. 1

1 blood draw
(divided in three parts)

3 WBC separations

3 WBC membrane glycan samples isolated

Released glycans shipped to the Glycobiology Laboratory,
Genos Ltd, Zagreb, Croatia for HILIC-HPLC

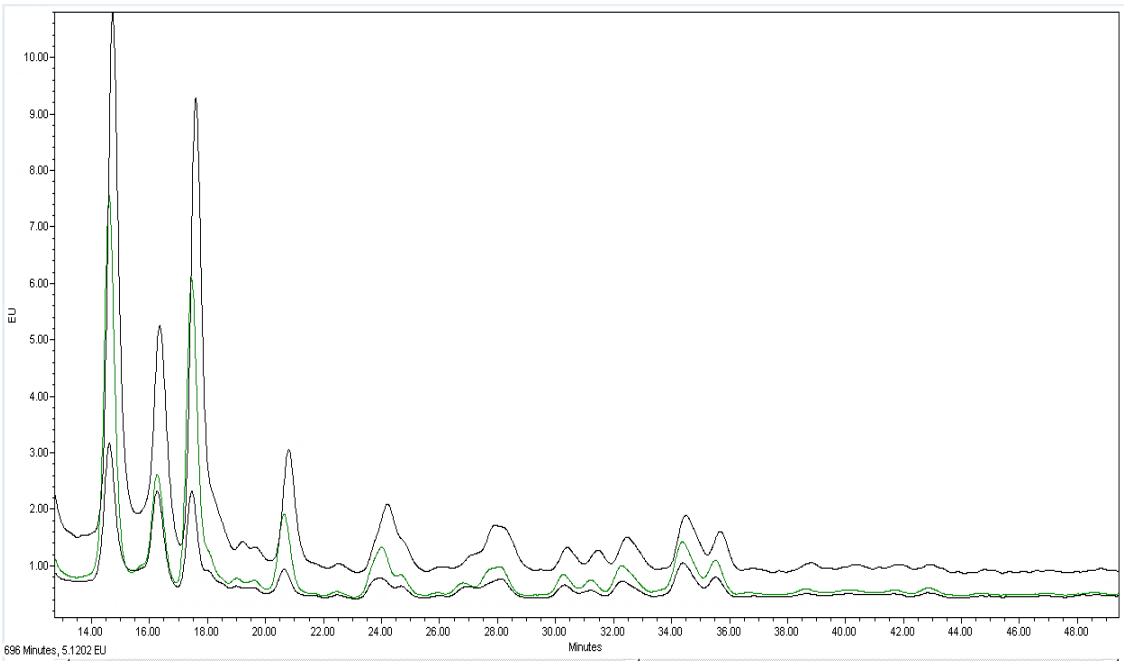


Figure 2.2. Optimisation (1) of WBC membrane glycan release protocol

Optimisation 2

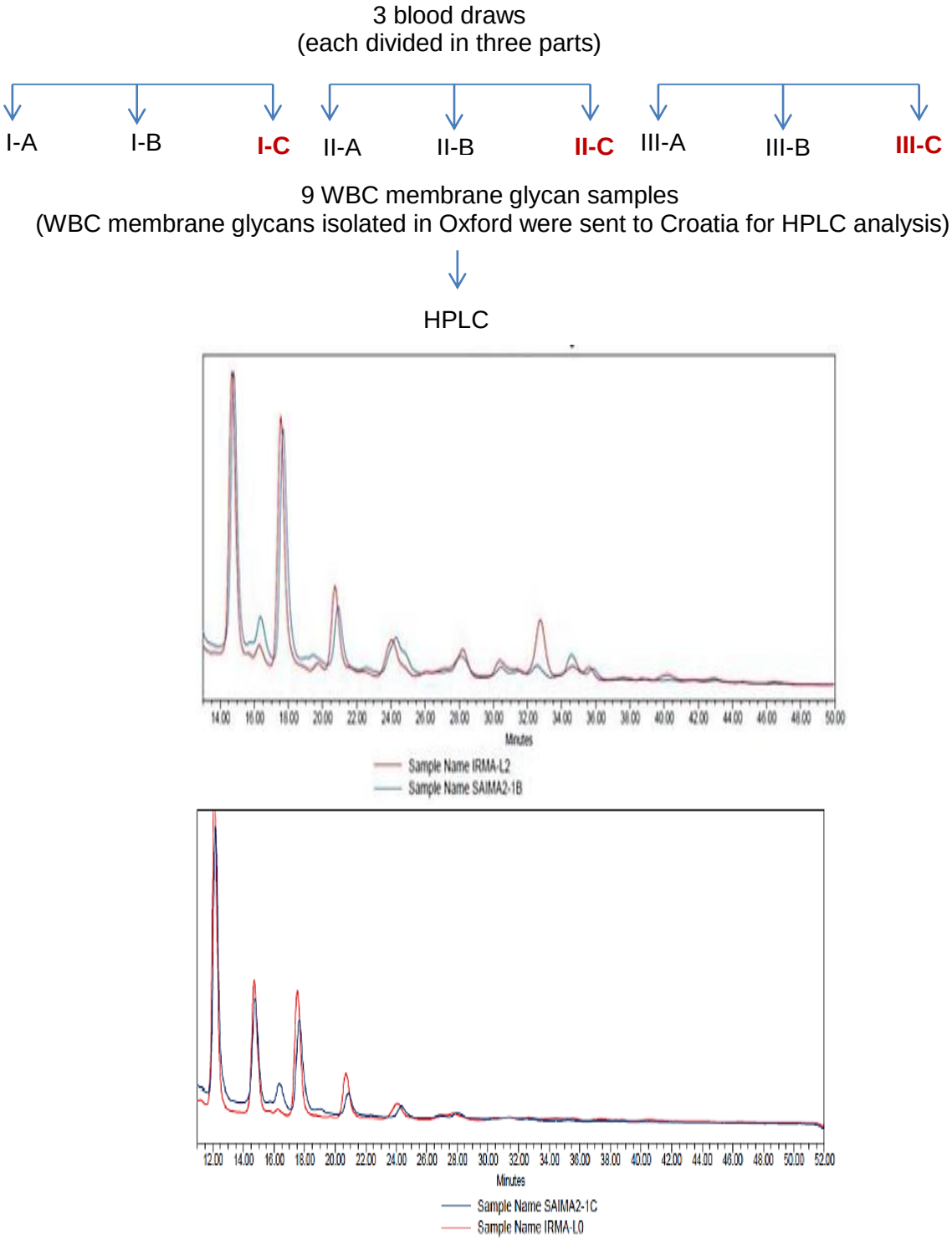


Figure 2.2. Optimisation (2) of WBC membrane glycan release protocol.

This optimisation was carried out in parallel at Zagreb and Oxford. The Samples I-C, II-C and III-C in the flow chart served as controls and were not treated with glycan release enzyme. The graphs show results of HPLC analysis. The red lines in the graph, IRMA-L2 (sample treated with enzyme) and IRMA-L0 (control sample not treated with enzyme), refer to the membrane glycans isolated in Zagreb. The blue lines in the graph, Saima2-1B (treated with enzyme) and Saima2-1C (control sample not treated with enzyme), refer to membrane glycans isolated in Oxford.

Optimised WBC membrane glycan release protocol

The WBC membrane glycan release protocol comprises of two steps: WBC isolation and WBC membrane glycan release. These steps are described below and illustrated in **Figure 2.3.**

(a) WBC isolation

For isolation of WBC, the protocol by Norgen Biotek corporation (Lam, et al.) was used. Five volumes of red blood cell (RBC) lysis buffer (Qiagen) were added to a blood sample collected with EDTA, and incubated at room temperature for 3 to 5 minutes. During the incubation, the sample was vortexed several times to aid in mixing. The sample was then centrifuged at 250g for 3 minutes and the supernatant decanted.

Two additional volumes of the RBC lysis buffer were then added to the pelleted white blood cells and mixed by gentle vortexing for 10 seconds. The tubes were again centrifuged at 250g for 3 minutes and the supernatant decanted. Pelleted cells were washed by adding 5ml of PBS (Biosciences) and voxtexing vigorously for 30 seconds. The sample was centrifuged at 250g for 10 minutes and the supernatant decanted. To decrease contamination, washing with PBS was repeated 5 times.

(b) WBC membrane glycan release

For WBC membrane glycan release, a previously described protocol for release of N-glycans from plasma, was adapted and optimised for release of WBC membrane glycans (Royle, et al., 2008)

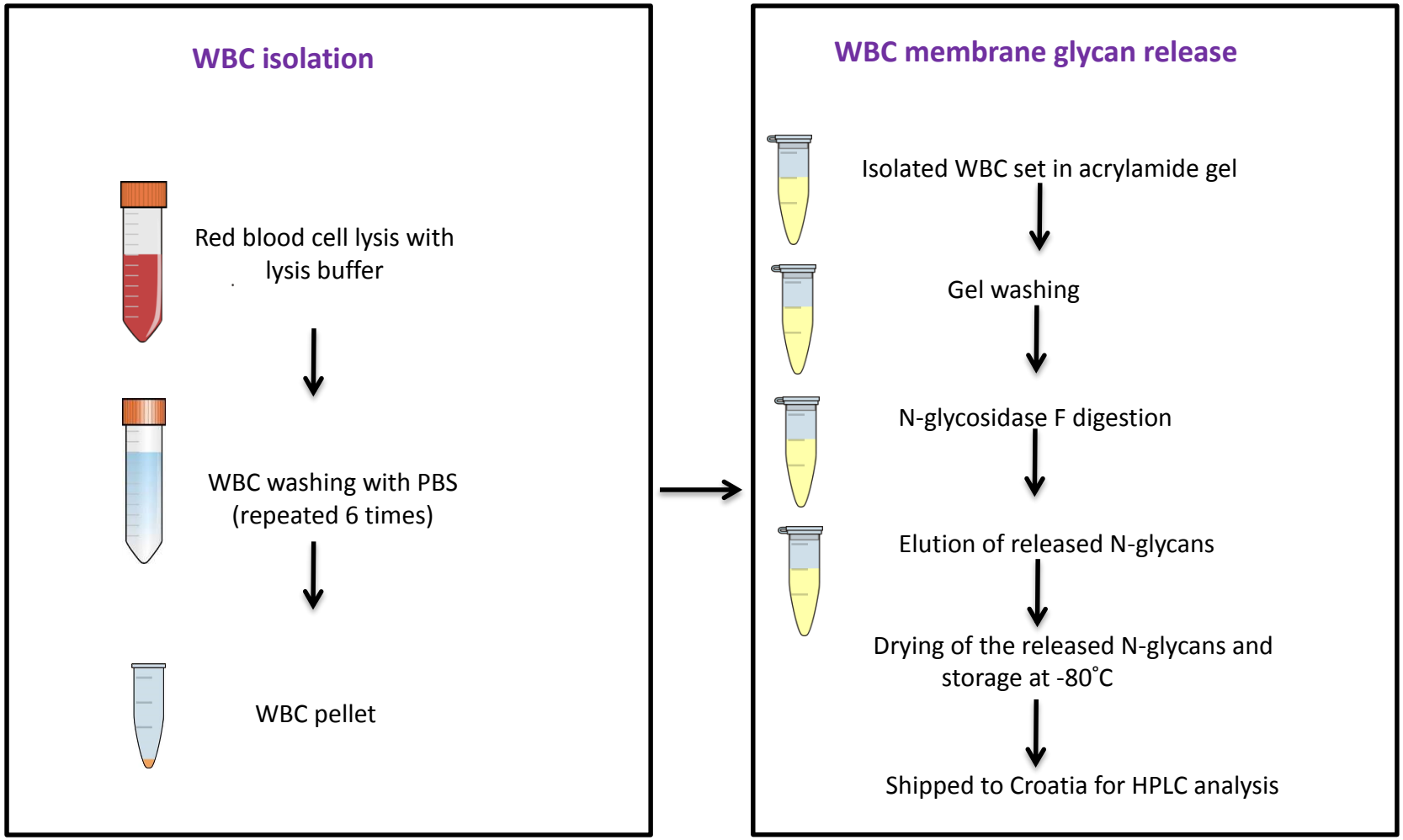


Figure 2.3: Flow diagram: Optimised WBC membrane glycan release protocol.

WBC=White blood cells, PBS=Phosphate buffer saline HPLC=High performance liquid chromatography.

One hundred microlitres of acrylamide gel was prepared using 61.6µl 30% acrylamide/bis-acrylamide (Sigma), 25µl 1.5M Tris pH7.5 (Sigma), 1µl 1% ammonium persulphate (Sigma) and 0.018g urea (Sigma). Seventy microlitres of this mixture was added to approximately 10^6 cells. One microlitre of Tetramethylethylenediamine (TEMED, Sigma) was added and allowed to set for 15 minutes. The gel was then washed with 1ml of Acetonitrile (ACN; Sigma), and allowed to shake for 10 minutes. Afterwards the ACN was removed, and the washing procedure was repeated with 1ml of enzyme buffer (20mM NaHCO3 pH7), followed by 1ml of ACN, 1ml of enzyme buffer and finally 1ml of acetonitrile. N-glycans were released by adding a mixture of 99µl of enzyme buffer and 1µl of N-glycosidase F (PROzyme) to the acrylamide gel. The tube was sealed and incubated at 37°C for 18 hours. The released N-glycans were collected by adding 3×200µl of ultrapure water, 200µl of ACN, 200µl of water and finally 200µl of ACN to the gel. The solution was collected in a clean eppendorf tube (1.5ml size). The solution was then dried overnight at medium heat setting in SpeedVac (Thermo Scientific). The dried membrane glycans were sent to Genos Ltd, Croatia where further glycan analysis (HILIC-HPLC) was carried out by Mislav Novokmet.

2.2.3.2 Hydrophilic Interaction Chromatography (HILIC)-HPLC of released glycans

Released glycans were subjected to hydrophilic interaction high performance liquid chromatography (HILIC) on a 2500 mm i.d. 5 mm particle packed TSKgel Amide 80 column (Tosoh Bioscience, Stuttgart, Germany) at 30 °C with 50 mM formic acid adjusted to pH 4.4 with ammonia solution as solvent A and ACN as solvent B. Separation method used linear gradient of 65–53% acetonitrile (v/v) at

flow rate of 0.8 ml/min in a 60 min analytical run. HPLCs were equipped with a Waters temperature control module and a Waters 2475 fluorescence detector set with excitation and emission wavelengths of 330 and 420 nm, respectively. The system was calibrated using an external standard of hydrolyzed and 2-AB-labeled glucose oligomers from which the retention times for the individual glycans were converted to glucose units (GU). Glycans were analyzed on the basis of their elution positions and measured in glucose units then compared with reference values in NIBRT's ''GlycoBase v3.0 '' database available at (http://glycobase.nibrt.ie) for structure assignment.

2.3 STATISTICAL ANALYSIS

A brief outline of statistical methods is given below and details are provided in the individual chapters. Power and sample size calculations are also provided in the individual chapters.

The Shapiro-Wilk test was used to assess the normality of variable distribution. If the data were normally distributed, Student's T-test was used to compare means between two groups. For non-normal distributions, Mann-Whitney U was used to compare differences between two independent groups. The Chi square test for contingency tables (2×2 table) was used to compare categorical variables. Where the individual cell count in a 2×2 table was less than five, Fisher's exact test was used.

Contingency tables (2×2) were used to calculate sensitivity and specificity, positive and negative predictive values and likelihood ratios (as shown in **tables 2.8** and **2.9**). Receiver operating characteristic (ROC) curve analysis was

performed to assess the discriminative accuracy of different biomarkers investigated.

The computations were performed using IBM SPSS Statistics Version 20.0 and $P<0.05$ was considered significant.

	Disease +ve	**Disease –ve**	**Row total**
Test +ve	A True positive	B False positive	A+B
Test -ve	C False negative	D True negative	C+D
Column total	A+C	B+D	

Table 2.8. Contingency (2×2) table

Sensitivity	A/(A+C)%
Specificity	D/(B+D)%
Predictive value	
Positive predictive value	A/(A+B)%
Negative predictive value	D/(C+D)%
Likelihood ratio	
Positive Likelihood ratio	(A/[A+C])/ (B/[B+D]) Sensitivity/(1-specificity)
Negative Likelihood ratio	(C/[A+C])/ (D/[B+D]) (1-sensitivity)/specificity

Table 2.9. Calculation of measures of biomarker performance using 2×2 contingency table

CHAPTER 3

Evaluation of Apolipoprotein M as a biomarker for HNF1A-MODY

3.1 Introduction

Mutations in *HNF1A* are the most common cause of MODY in the UK, accounting for 52% of diagnosed MODY cases (Shields, et al., 2010). A correct molecular diagnosis of HNF1A-MODY is important for selecting the optimal treatment, predicting the clinical course of disease and for identification of other family members at risk. Despite the clinical value of a correct diagnosis, 80% of the MODY cases in the UK are estimated to be misdiagnosed as having type 1 or type 2 diabetes (Shields, et al., 2010).

As outlined in chapter 1, the accuracy with which HNF1A-MODY is diagnosed can be improved by using non-genetic biomarkers that facilitate prioritisation of patients for molecular diagnostic testing. Over the last decade, several candidate biomarkers have been investigated (Mughal, et al., 2013b). One such biomarker is apolipoprotein M (apoM).

3.1.1 Apolipoprotein M (apoM)

ApoM, a 25 kDa apolipoprotein, was described by Xu *et al* in 1999 (Xu and Dahlback, 1999). *APOM* is expressed in both liver and kidney (Faber, et al., 2006). ApoM is found mainly associated with high density lipoprotein (HDL) cholesterol (Xu and Dahlback, 1999).

3.1.1.1 Biological functions of apoM

Anti-atherosclerotic and vasculoprotective role of apoM

ApoM contributes to the anti-atherosclerotic and anti-oxidative properties of

HDL-cholesterol. ApoM promotes formation of preβ-HDL, a precursor of mature HDL and an important mediator of reuptake of cholesterol from arterial walls and transport back to the liver (a process known as reverse cholesterol transport). It was demonstrated by two independent studies that overexpression of human and mouse *Apom* protected against development of atherosclerosis in transgenic mice (Dahlback, et al., 2008; Wolfrum, et al., 2005). ApoM-containing HDL is more efficient in mediating cholesterol efflux from macrophages (Dahlback, et al., 2008). ApoM is strongly correlated with total cholesterol, a well-known risk factor for coronary heart disease (Axler, et al., 2007; Plomgaard, et al., 2009). ApoM also acts as a carrier of an important vasoprotective lipid mediator sphingosine 1 phosphate in HDL and contributes to the vasculoprotective properties of HDL (Christoffersen, et al., 2011).

ApoM: a marker of sepsis

It has recently been shown that serum apoM concentration decreases during acute and chronic inflammation (Christoffersen and Nielsen, 2012; Kumaraswamy, et al., 2012). Decreased apoM concentration has been reported in patients with chronic infections such as HIV and in patients with septicaemia (Kumaraswamy, et al., 2012). The decrease in the apoM concentration was consistent with disease severity. Stimulation of acute inflammatory response in mice also results in decreased *Apom* expression in the liver and the kidney (Feingold, et al., 2008). These findings suggest that apoM is a negative acute phase protein and that apoM could serve as a marker to assess the severity of sepsis (Christoffersen and Nielsen, 2012).

3.1.1.2 Regulation of *APOM* expression

APOM expression is regulated by various transcription factors, one of which is hepatocyte nuclear factor 1 alpha (HNF1A) (Richter, et al., 2003) (**Figure 3.1**). Liver receptor homolog-1 (LRH-1) and Forkhead box A2 (FOXA2) (Venteclef, et al., 2008; Wolfrum, et al., 2008) are two other transcription factors that up regulate *APOM* expression. The human *APOM* promoter has a dual specificity regulatory region that can bind HNF1A and pro-inflammatory transcription factors such as c-Jun and JunB (Mosialou, et al., 2011). HNF1A up regulates while c-Jun and JunB repress *APOM* expression.

3.1.2 Investigation of apoM as a biomarker for HNF1A-MODY

ApoM was investigated as a biomarker for HNF1A-MODY following the observation of reduced *Apom* expression in *Hnf1a* homozygous knockout mice (Richter et al., 2003). As discussed in chapter 1 (section 1.5.1), three independent studies evaluated apoM as a biomarker for HNF1A-MODY and reported inconsistent results (Cervin, et al., 2010; Richter, et al., 2003; Skupien, et al., 2007).

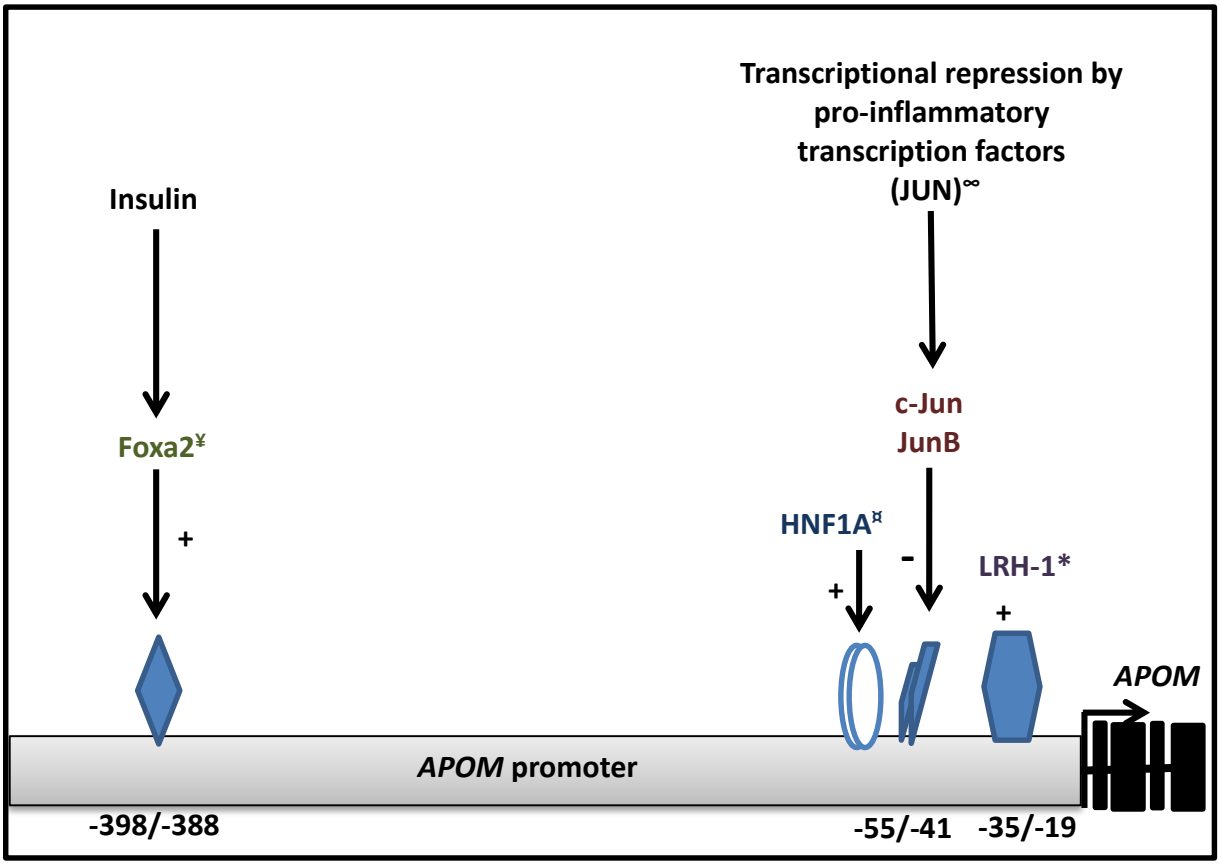


Figure 3.1: Transcriptional regulation of human *APOM* expression

(¤Richter *et al* 2003, *Venteclef *et al* 2008, ¥Wolfrum *et al* 2008, ∞Mosialou *et al* 2011)

Initially, Richter *et al* reported 36% lower serum apoM concentration in subjects with HNF1A-MODY compared with healthy controls (Richter, et al., 2003). However, neither follow up studies could confirm this finding (Cervin, et al., 2010; Skupien, et al., 2007). Follow up studies also included a type 2 diabetes group and did not observe a significant difference in serum apoM concentration in subjects with HNF1A-MODY compared with those with type 2 diabetes. Each of the three studies reported different mean serum apoM concentration in healthy controls (chapter 1: table 1.6).

The reason for variability in the findings of the three studies is not clear. Possible reasons for this inconsistency could be the differences in sample size and baseline characteristics of the subjects, as well as due to differences in the specificity of techniques and antibodies used for apoM detection.

1. The first study by Richter *et al* had a small sample size compared with later two studies (Richter, et al., 2003) (chapter 1: table 1.6). They used western blot and densitometry for serum apoM measurement. Densitometry is poorly quantitative and can lead to variable results.

2. The second study by Skupien *et al* used dot blot technique for serum apoM measurement (Skupien, et al., 2007). Dot blot is not very specific as it lacks information about protein molecular weight.

3. Finally Cervin *et al* used a sandwich ELISA, a more sensitive and quantitative technique for protein quantification compared with the

western blot (Cervin, et al., 2010). Cervin *et al* reported low serum apoM only in female carriers of the *HNF1A* mutation. In this study, they did not observe a significant difference in serum apoM concentration in subjects with type 2 diabetes compared with the healthy controls. However using the same ELISA in another study authors have observed low serum apoM concentration in subjects with type 2 diabetes compared with healthy controls (Plomgaard, et al., 2009).

3.2 Aims

The aim of the current study was to re-examine the use of apoM as a biomarker for HNF1A-MODY employing a recently described, highly sensitive and specific ELISA (Karuna, et al., 2011). As none of the previous studies examined subjects with type 1 diabetes (an important differential diagnosis for HNF1A-MODY), a type 1 diabetes group was included, along with type 2 diabetes and healthy controls.

3.3 Patients and Methods

3.3.1 Sample Size

Sample size estimation for the current study was carried out using the results reported by Richter *et al* (Richter, et al., 2003). Richter *et al* reported mean apoM concentration of 4.0µmol/L in healthy controls. No standard deviation was reported. The authors observed that subjects with HNF1A-MODY had approximately 36% lower apoM concentration compared with healthy controls (an estimated mean of 2.6µmol/L in HNF1A-MODY). As no standard deviation was reported, the sample size for the current study was calculated using

various values of assumed apoM common standard deviation (**Table 3.1**).

It was estimated that a sample size of 58 healthy controls and 58 HNF1A-MODY subjects will have 80% power to detect a difference in means of 1.4 μmol/L (the difference between a control mean μ1 of 4.0 and a HNF1A-MODY mean, μ2, of 2.6 μmol/L) assuming that the common standard deviation is 3.0 using a two group t-test with a 0.05 two-sided significance level.

Access was available to serum samples of 100 healthy controls and 69 HNF1A-MODY subjects so the study was sufficiently powered to show a difference in mean apoM concentration, of 1.4 μmol/L, in between the two groups.

3.3.2 Study Participants

Subjects were recruited from UK and Poland. I carried out data collection (for UK HNF1A-MODY, type 1 and type 2 diabetes subjects), statistical analysis and interpretation of the results.

UK subjects comprised subjects with HNF1A-MODY (n=22), type 1 diabetes (n=50), type 2 diabetes (n=50), recruited from YDX cohort (chapter 2: section 2.1.1).

Assumed common standard deviation σ	**2.0**	**2.5**	**3.0**
Test significance level α	0.05	0.05	0.05
1 or 2 sided test	2	2	2
Control mean apoM (µmol/L) µ1	4	4	4
HNF1A-MODY mean (µmol/L) µ2	2.6	2.6	2.6
Difference in means (µ1-µ2)	1.4	1.4	1.4
Effect size (δ = µ1-µ2/ σ)	0.70	0.56	0.46
Power (%)	80	80	80
Subjects needed			
Healthy controls	26	41	58
HNF1A-MODY	26	41	58

Table 3.1: Sample size calculation for evaluation of apoM as HNF1A-MODY biomarker

Healthy controls (n=100) from the Oxford Biobank were made available for this study through collaboration with Dr Fredrik Karpe (chapter 2: section 2.1.2). Type 2 diabetes was defined as: C-peptide positive, no requirement for permanent insulin within 3 months of diagnosis and negative glutamic acid decarboxylase antibodies (GAD; positive level defined as >14 WHO units/ml) (Bingley et al., 1997). Type 1 diabetes was defined as permanent insulin treatment since diagnosis with additional evidence of severe beta cell dysfunction (C-peptide≤0.2nmol/L), positive GAD antibodies or both. The clinical and biochemical characteristics of UK subjects are given in **table 3.2**.

Polish subjects included subjects with HNF1A-MODY (n=47) and type 2 diabetes (n=70). Subjects with type 2 diabetes did not require permanent insulin within 1 year after the diagnosis and did not meet clinical criteria for MODY or type 1 diabetes. The clinical and biochemical characteristics of Polish subjects are described in chapter 2 (section 2.1.1 and table 2.3).

Both UK and Polish cases of HNF1A-MODY had a heterozygous loss-of-function mutation confirmed by sequencing in a certified diagnostic centre. *HNF1A* variants identified in these subjects were considered pathogenic if they met one or more of these criteria: (a) previously published reports (b) rare, conserved variants with likely damaging consequence on protein function or (c) co-segregation of the variant with a MODY phenotype within the family. The type and position of mutations present in UK and Polish subjects are illustrated in **figure 3.2** and **3.3.**

Characteristics	HNF1A-MODY	Type 1 diabetes	Type 2 diabetes	Healthy controls	P value
Males (%)	36	44	64	50	<0.0001
Diabetes Duration (years)	9.0 (2.5-28.5)	15.4 (10.8-19.5)	16.0 (7.2-23.0)	-	<0.0001
Age of Diagnosis (years)	29.5 (20.7-36.2)	25.5 (17.7-29.2)	40.0 (37.0-43.0)	-	<0.0001
BMI (kg/m^2)	24.2 (22.1-27.6)	25.7 (22.5-28.0)	32.7 (28.3-36.9)	25.2 (22.6-27.1)	<0.0001
Fasting glucose (mmol/L)	6.3 (5.7-8.0)	11.7 (6.2-15.7)	8.6 (6.6-10.9)	5.3 (5.0-5.6)	<0.0001
HDL (mmol/L)	1.7 (1.4-2.0)	1.4 (1.2-2.1)	1.1 (1.0-1.3)	1.3 (1.1-1.6)	<0.0001

Table 3.2: Clinical and biochemical characteristics of UK subjects. Data are shown as median (interquartile range). P values were determined by Chi-Square test and Kruskal−Wallis test. Clinical and biochemical characteristics of Polish subjects are shown in chapter 2 table 2.3.

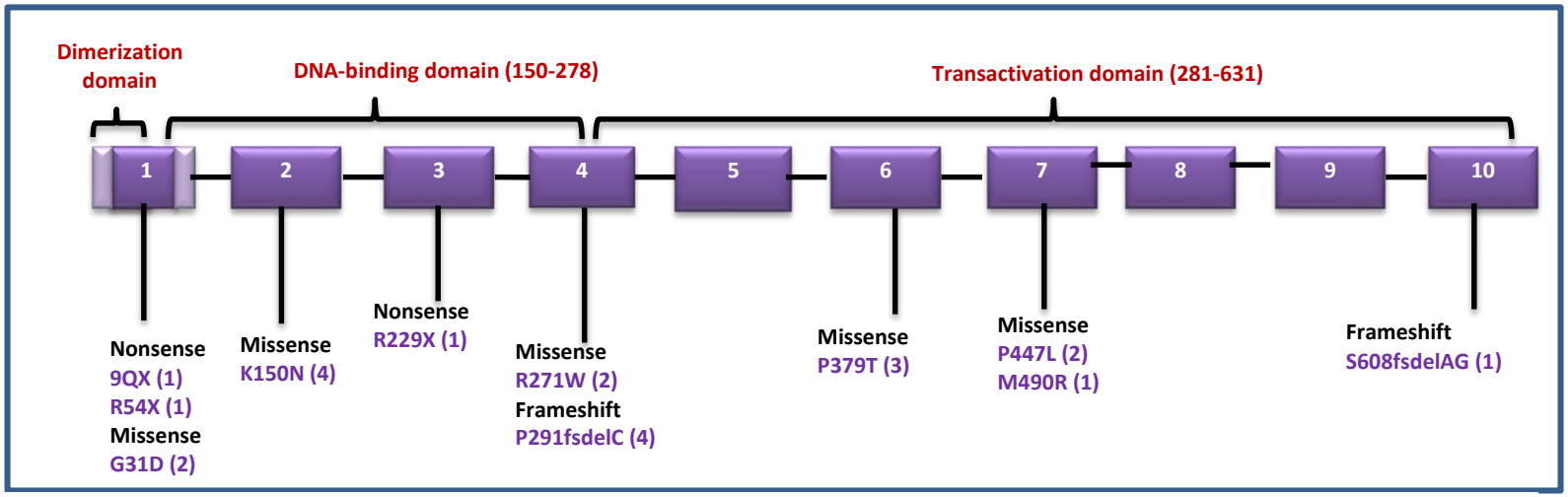


Figure 3.2: Location of mutations within the 10 exons, promoter, and introns of *HNF1A* in the UK-subjects

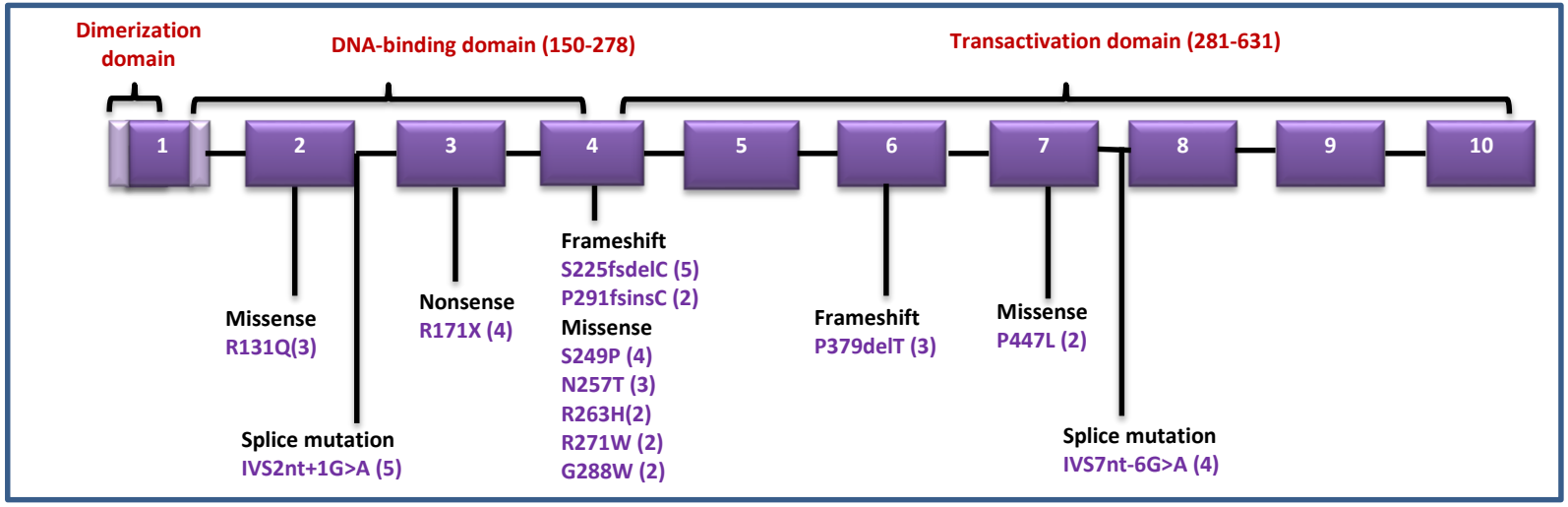


Figure 3.3: Location of mutations within the 10 exons, promoter, and introns of *HNF1A* in the Polish-subjects

The precise position of mutation was not available for seven Polish subjects

3.3.3 Apolipoprotein M assay

Serum apoM was measured by Rebekka Park (via collaboration with Professor Markus Stoffel) at the Institute of Molecular Systems Biology, Zurich, Switzerland. Serum apoM was measured using an ELISA provided by Roche (Karuna et al., 2011).

3.3.4 Statistical Analysis

The Shapiro-Wilk test was used to assess the normality of variable distribution. Diabetes duration, age of diagnosis, BMI, fasting glucose and HDL were not normally distributed ($p<0.05$). The Mann-Whitney U test was used to compare these variables and data are reported as median (interquartile range). ApoM and apoM/HDL ratio were normally distributed ($p>0.05$) and the independent samples T-test was used to compare these variables and data are reported as mean (SD).The correlation between apoM concentration and the other quantitative traits, such as total cholesterol, was calculated using the Spearman correlation test. The performance of apoM and apoM/HDL ratio as a diagnostic test for HNF1A-MODY was evaluated using receiver operating characteristic (ROC) curve analysis. The C-statistic (area under the curve) was used to evaluate the discriminative accuracy of apoM and apoM/HDL ratio.

As discussed in chapter 1 (section 1.5.3), high sensitivity C-reactive protein (hsCRP) is a promising biomarker for HNF1A-MODY. The combined contribution of apoM and hsCRP in discriminating HNF1A-MODY from type 1 and type 2 diabetes was evaluated. Binary logistic regression analysis was used to determine individual and combined contributions of apoM and hsCRP

for the prediction of HNF1A-MODY. Two regression models were made using either apoM or hsCRP and a third regression model was made containing both apoM and hsCRP. The goodness of fit test (Hosmer-Lemeshow) was used to assess model fit. Predicted probabilities were obtained from the logistic regression models. ROC curves were plotted and the C-statistic was used to evaluate the discriminative accuracy of apoM, hsCRP and combined apoM and hsCRP for diabetes aetiology.

Analysis was performed using SPSS Version 20.0 and graphs created using SigmaPlot Version 12.5. P<0.05 was considered significant.

3.4 Results

Similar findings for the biochemical characteristics and measures of biomarker performance were observed for UK and Polish subjects. Therefore, the results were combined and are discussed collectively. Independent data for UK and Polish subjects are also provided in **table 3.3** and the distribution of apoM levels is illustrated in **figure 3.4**.

3.4.1 ApoM results

Mean (SD) serum apoM concentration (μmol/L) was markedly lower in HNF1A-MODY (0.86 [0.29]) than type 1 diabetes (1.37 μmol/L [0.26], P=3.10×10^{-18}) (**Table 3.4** and **Figure 3.5**). No significant differences were observed in the serum apoM concentration of HNF1A-MODY compared with type 2 diabetes (0.89 μmol/L [0.28], P=0.13).

Characteristics	UK subjects		Polish subjects	
	HNF1A-MODY	**Type 2 diabetes**	**HNF1A-MODY**	**Type 2 diabetes**
Gender (male/female)	**8/14**	**32/18**	**18/29**	**50/20**
Diabetes Duration (years)	**9.1 (26.2)**	**16.2 (16.5)**	**7.1 (14.5)**	**10.5 (8.2)**
P value[§] vs. HNF1A-MODY		0.24		0.09
Age of Diagnosis (years)	**29.5 (15.5)**	**40.2 (6.1)**	**18.0 (15.1)**	**40.5 (9.2)**
P value[§] vs. HNF1A-MODY		3.8×10^{-4}		1.6×10^{-9}
BMI (kg/m^2)	**24.2 (5.5)**	**32.7 (8.5)**	**22.8 (5.2)**	**30.8 (7.2)**
P value[§] vs. HNF1A-MODY		3.2×10^{-6}		1.5×10^{-12}
Fasting glucose (mmol/L)	**6.3 (2.3)**	**8.6 (4.3)**	**5.8 (2.5)**	**6.8 (1.8)**
P value[§] vs. HNF1A-MODY		0.03		0.04
ApoM (µmol/L)*	**0.76 (0.33)**	**0.89 (0.26)**	**0.85 (0.34)**	**0.89 (0.26)**
P value[#] vs. HNF1A-MODY		0.09		0.50
HDL (mmol/L)	**1.7 (0.63)**	**1.1 (0.30)**	**1.2 (0.63)**	**1.0 (0.40)**
P value[§] vs. HNF1A-MODY		5.2×10^{-5}		0.02
Ratio (apoM/HDL)*	**0.47 (0.26)**	**0.79 (0.29)**	**0.66 (0.25)**	**0.79 (0.27)**
P value[#] vs. HNF1A-MODY		1.0×10^{-4}		0.01

Table 3.3. Clinical and biochemical characteristics of UK and Polish HNF1A-MODY and type 2 diabetes subjects. Data are shown as median (interquartile range) unless otherwise stated. *Mean (SD). *P* value compares type 2 diabetes with HNF1A-MODY subjects and was calculated by [#]Student's t-test or [§]Mann-Whitney U test

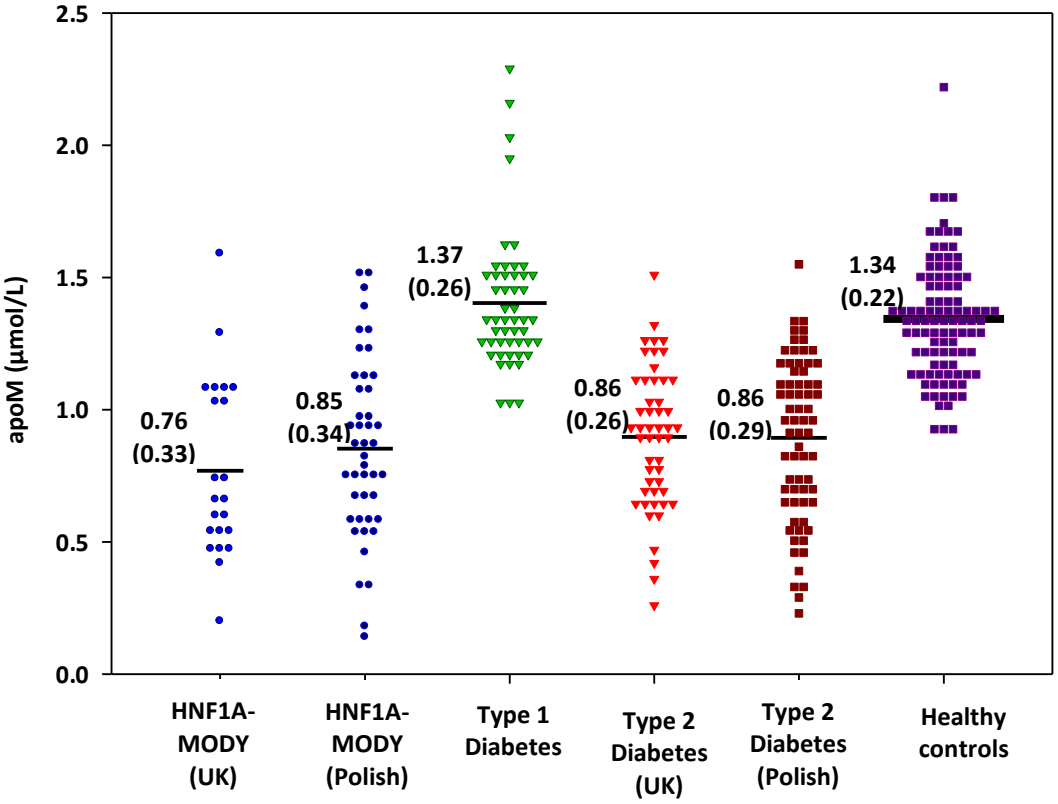


Figure 3.4. Dot histograms illustrating distribution of apoM concentration in UK and Polish study groups. Mean values are highlighted by black dashed line.

Characteristics	HNF1A-MODY	Type 1 diabetes	Type 2 diabetes	Healthy controls
Gender (male/female)	**26/43**	**22/28**	**82/38**	**50/50**
Diabetes Duration (years)	**9.0 (2.0-20.0)**	**15.4 (10.8-19.3)**	**12.0 (7.0-20.0)**	**-**
P value§ vs. HNF1A-MODY		0.01	0.29	-
Age of Diagnosis (years)	**20.0 (17.0-32.0)**	**25.5 (18.0-29.0)**	**40.0 (36.0-44.0)**	**-**
P value§ vs. HNF1A-MODY		0.83	5.2×10^{-12}	-
BMI (kg/m²)	**22.8 (20.5-25.7)**	**25.7 (22.5-28.0)**	**31.5 (28.0-35.8)**	**25.2 (22.7-27.1)**
P value§ vs. HNF1A-MODY		0.003	1×10^{-17}	0.003
Fasting glucose (mmol/L)	**6.2 (5.3-7.8)**	**11.7 (6.8-15.7)**	**7.2 (6.3-9.0)**	**5.3 (5.6-5.0)**
P value§ vs. HNF1A-MODY		6.7×10^{-5}	0.01	3.5×10^{-6}
ApoM (µmol/L)*	**0.86 (0.29)**	**1.37 (0.26)**	**0.89 (0.28)**	**1.34 (0.22)**
P value# vs. HNF1A-MODY		3.1×10^{-18}	0.13	7.2×10^{-19}
HDL (mmol/L)	**1.4 (1.0-1.7)**	**1.4 (1.2-2.1)**	**1.1 (0.9-1.3)**	**1.4 (1.1-1.6)**
P value§ vs. HNF1A-MODY		0.05	2.3×10^{-4}	0.53
Ratio (apoM/HDL)*	**0.66 (0.26)**	**0.80 (0.28)**	**0.79 (0.28)**	**0.98 (0.24)**
P value# vs. HNF1A-MODY		3.3×10^{-6}	2.3×10^{-5}	1.8×10^{-16}

Table 3.4 Clinical and biochemical characteristics of UK and Polish subjects combined

Data for UK and Polish HNF1A-MODY and type 2 diabetes groups were combined and shown collectively in this table. Data are shown as median (interquartile range) unless otherwise stated. *Mean (SD). P value compares type 2 diabetes, type 1 diabetes and healthy controls with HNF1A-MODY subjects and was calculated by #Student's t-test or §Mann-Whitney U

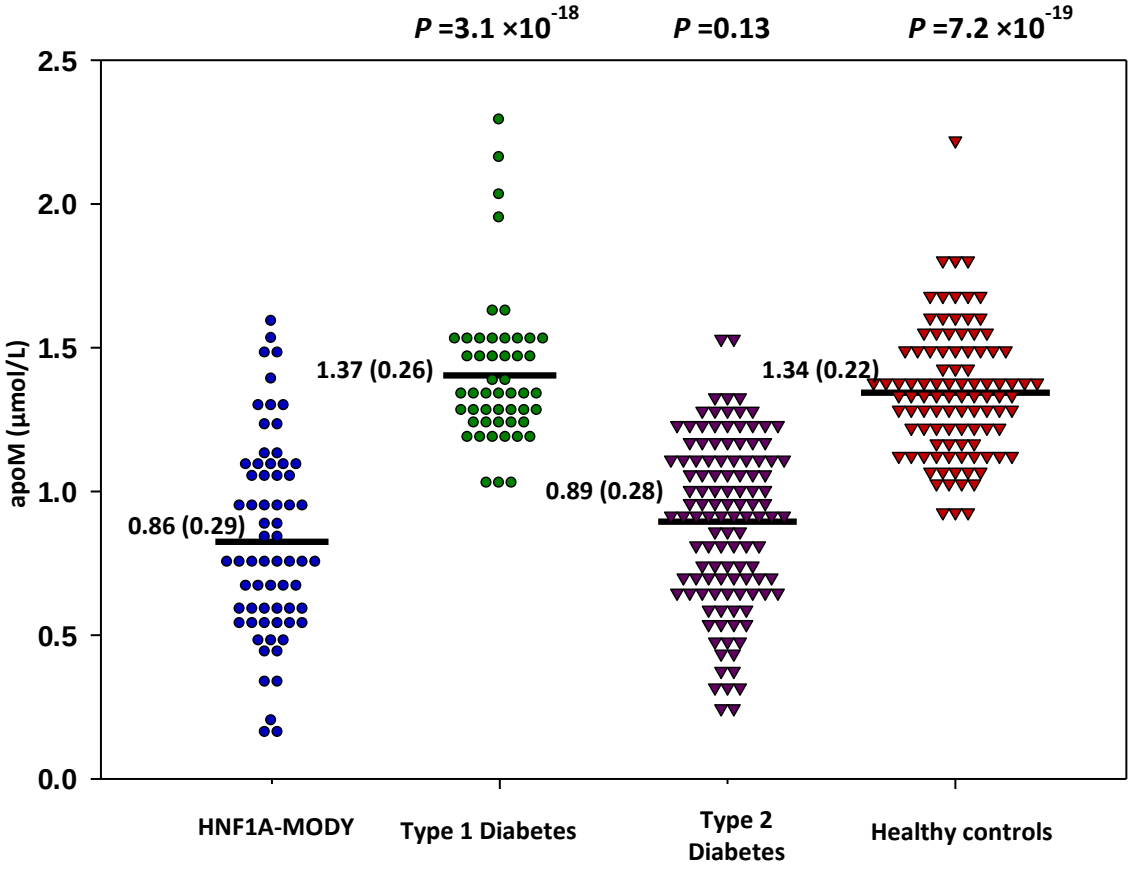


Figure 3.5. Dot histograms illustrating apoM concentration in study groups

Data for UK and Polish HNF1A-MODY and type 2 diabetes groups were combined and shown collectively. P values are calculated by Student's T test in comparison with subjects with HNF1A-MODY. Mean values are highlighted by black dashed line

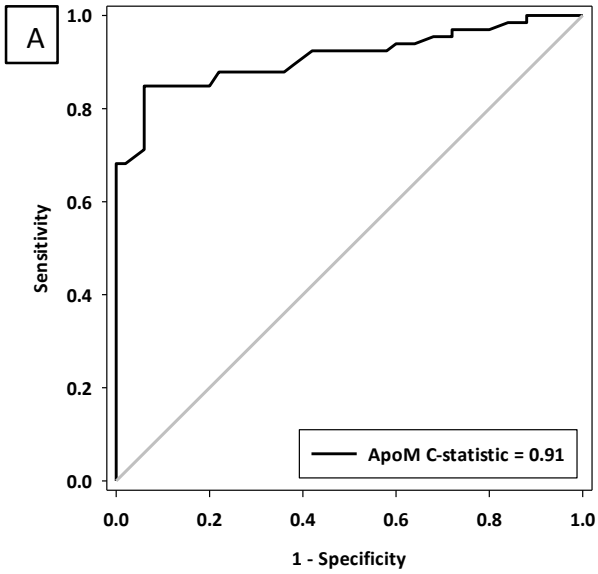
Serum apoM concentration in controls (1.34 μmol/L [0.22]) was similar to type 1 diabetes (*P*=0.14) and significantly higher than observed in HNF1A-MODY and type 2 diabetes ($p<10^{-18}$). Serum apoM concentration was markedly lower in type 2 diabetes than type 1 diabetes ($P=7.70\times10^{-21}$).

Although serum apoM concentration in HNF1A-MODY was significantly different from type 1 diabetes patients, this does not automatically lead to the inference that apoM could be used clinically to discriminate HNF1A-MODY from type 1 diabetes. ROC curve analysis was performed to assess the discriminative potential of apoM concentration. The ROC curve derived C-statistic was 0.91 indicating apoM can discriminate well between HNF1A-MODY and type 1 diabetes (**Figure 3.6**). The C-statistic for HNF1A-MODY vs. type 2 diabetes was 0.57 indicating that apoM does not distinguish these two groups (**Figure 3.6**).

Serum apoM concentration correlated well with total cholesterol (r=0.46, $P=3.7\times10^{-11}$) and HDL (r=0.40, $P=1.2\times10^{-13}$) (Axler, et al., 2007). Adjustment for total cholesterol did not affect the magnitude or significance in apoM differences observed between the study groups.

3.4.2 HDL and apoM/HDL ratio

There was no significant difference in median (interquartile range) HDL (mmol/L) in subjects with HNF1A-MODY (1.4 [1.0-1.7]) compared with controls (1.4 [1.1-1.6], *P*=0.53) and those with type 1 diabetes (1.4 [1.2-2.1], *P*=0.05).



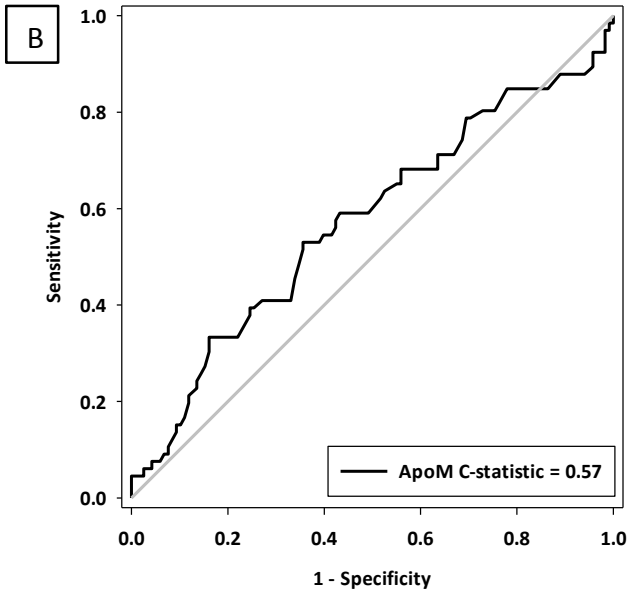


Figure 3.6: ROC curves illustrating the performance of the apoM to discriminate (A) HNF1A-MODY and type 1 diabetes (B) HNF1A-MODY and type 2 diabetes

As previously reported (McDonald, et al., 2012), HDL was significantly higher in HNF1A-MODY as compared with type 2 diabetes (1.1 [0.9-1.3], P=2.30×10-4). ApoM constitutes 5% of HDL. Moreover, since apoM and HDL levels are correlated, we hypothesised that if there was decreased apoM production, apoM might make up a lower proportion of the HDL in HNF1A-MODY and that the apoM/HDL ratio might be a superior biomarker for HNF1A-MODY. Mean (SD) apoM/HDL ratio was lower in HNF1A-MODY (0.60 [0.26]) compared with type 1 diabetes (0.80 [0.28], $P=3.30\times10^{-6}$), type 2 diabetes (0.79 [0.28], $P=2.30\times10^{-5}$) and controls (0.98 [0.24], $P=1.80\times10^{-16}$). The apoM/HDL ratio however performed less well than apoM alone in differentiating HNF1A-MODY from type 1 diabetes (C-statistic=0.79) (**Figure 3.7**). The C-statistic for apoM/HDL to discriminate HNF1A-MODY from type 2 diabetes was 0.68 (**Figure 3.7**).

3.4.3 Other measures for apoM performance as HNF1A-MODY biomarker

Sensitivity, specificity, predictive values, likelihood ratios and post-test probabilities were calculated for different apoM thresholds and are shown in **table 3.5**. A cut-off apoM concentration of 1.14 μmol/L provided highest sensitivity and specificity, positive and negative predictive values and positive and likelihood ratio for discriminating HNF1A-MODY from type 1 diabetes (84.8%, 94.0%, 94.9%, 82.4%, 14.1 and 0.16 respectively) (Altman and Bland, 1994a; Altman and Bland, 1994b; Deeks and Altman, 2004).

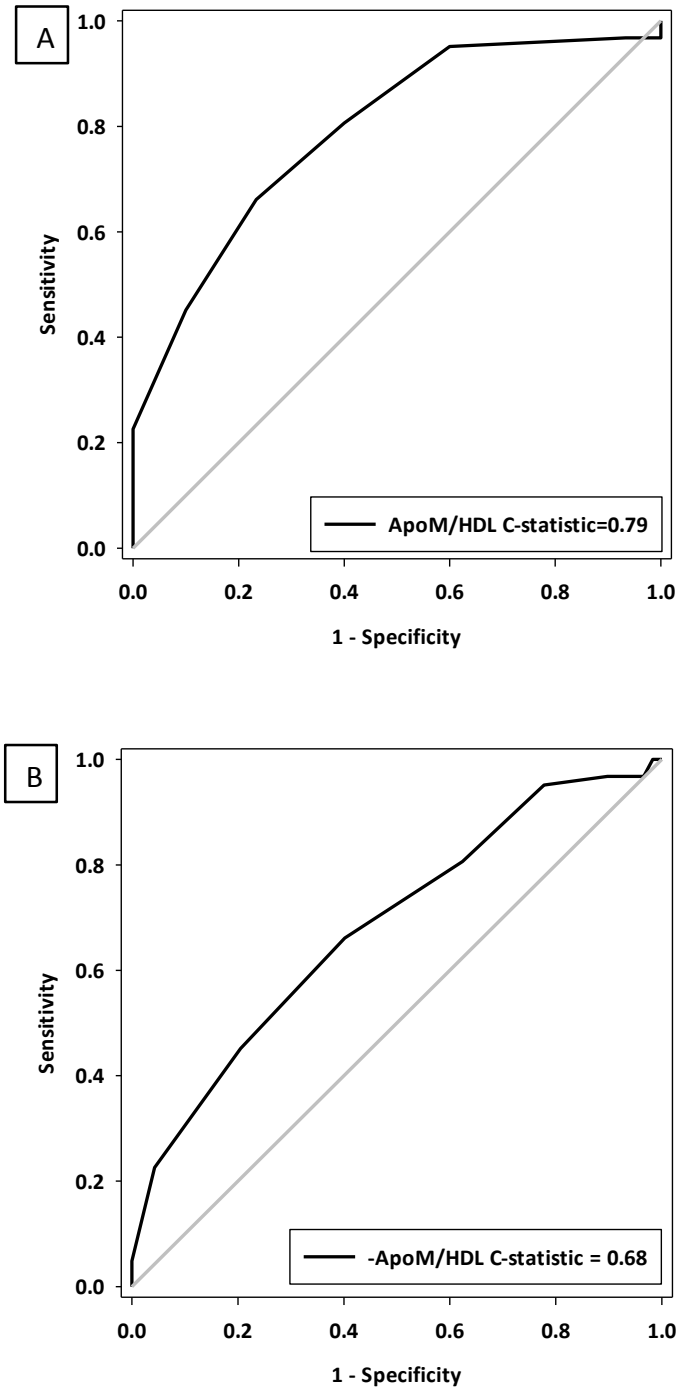


Figure 3.7: ROC curves illustrating the performance of the apoM/HDL ratio to discriminate (A) HNF1A-MODY and type 1 diabetes (B) HNF1A-MODY and type 2 diabetes

ApoM (µmol/L) Threshold for *HNF1A* sequencing	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Positive likelihood ratio	Positive post-test probability * (%)	Negative likelihood ratio	Negative post-test probability* (%)
1.08	78.7	94	94.5	77.0	13.1	11.5	0.22	0.21
1.10	80.3	94	94.6	78.3	13.3	11.5	0.21	0.20
1.12	81.8	94	94.7	79.6	13.6	12.0	0.19	0.18
1.14	84.8	94	94.9	82.4	14.1	12.3	0.16	0.15
1.16	84.8	92	93.3	82.1	10.6	9.5	0.16	0.15
1.18	84.8	90	91.8	81.8	8.4	7.8	0.16	0.15

Table 3.5: Measures of apoM performance at various thresholds for discriminating HNF1A-MODY and type 1 diabetes

*Based on pre-test probability of 1% misdiagnosed HNF1A-MODY cases in clinically-labelled type 1 diabetes . Sensitivity=the ability of the test to identify those who have the disease; Specificity=the ability of the test to identify those who do not have the disease; Positive predictive value=the probability of having the disease if a person tests positive; Negative predictive value=the probability of not having the disease if a person tests negative; Positive likelihood ratio=the ratio of the likelihood that a positive result will occur in subjects with the disease to the likelihood that the same result will occur in subjects without the disease; Negative likelihood ratio: the ratio of the likelihood that a negative result will occur in subjects with the disease to the probability that the same result will occur in subjects without the disease; Post-test probability=the probability of the disease presence given a positive or negative test result

Post-test diagnostic probabilities were calculated using data from an aetiological investigation of young adults with diabetes from the UK that estimated pre-test probabilities of 1% for HNF1A-MODY among those clinically diagnosed as having type 1 diabetes (Thanabalasingham, et al., 2012b). Using cut-off apoM concentration of 1.14 μmol/L, post-test probability for a positive test was 12.3%.

3.4.4 Combined effect of apoM and hsCRP

HsCRP is a promising biomarker for HNF1A-MODY (Owen, et al., 2010; Thanabalasingham, et al., 2011). Logistic regression models were used to calculate the combined contribution of apoM and hsCRP in discriminating HNF1A-MODY from both type 1 and type 2 diabetes. For HNF1A-MODY vs. type 1 diabetes, adding hsCRP to the model using apoM as a predictor of HNF1A-MODY resulted in a small improvement in C-statistic from 0.91 to 0.94, p=0.048 (**Figure 3.8**). For HNF1A-MODY vs. type 2 diabetes, there was no significant benefit in adding apoM measurements to a model using hsCRP as a predictor of HNF1A-MODY (an increase in C-statistic from 0.88 to 0.90, p=0.16) (**Figure 3.9**).

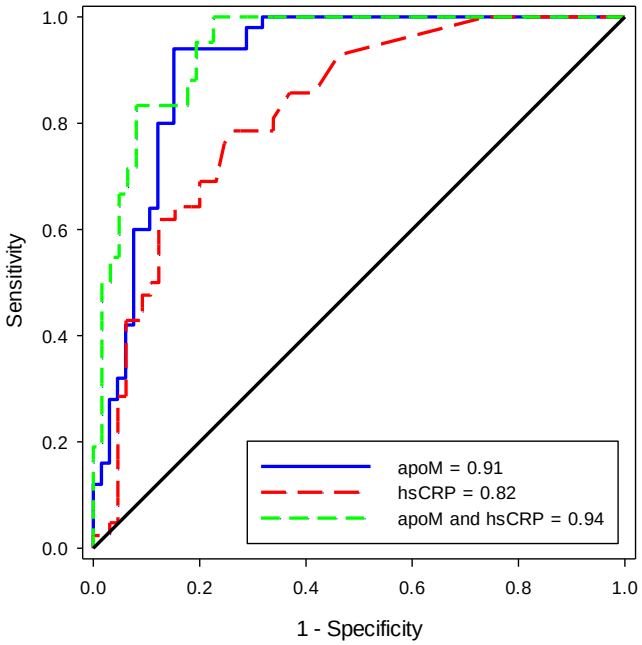


Figure 3.8. ROC curve illustrating the capacity of logistic regression models to distinguish between HNF1A-MODY and type 1 diabetes using apoM and hsCRP

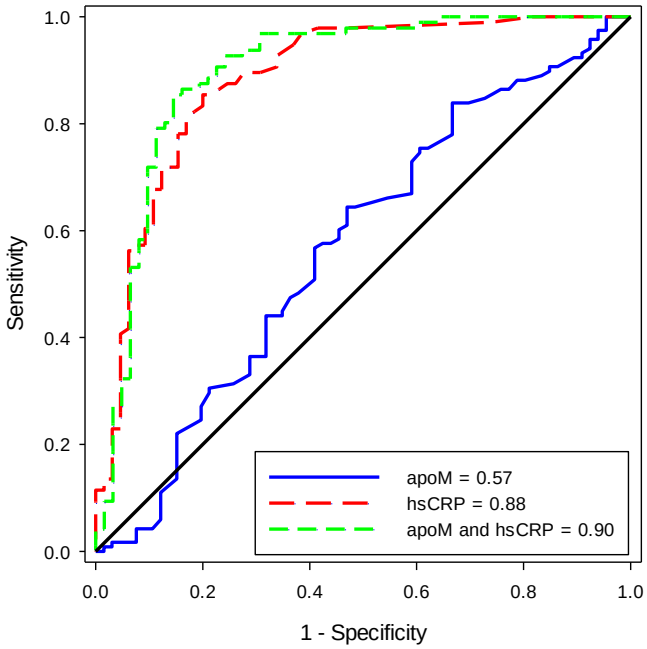


Figure 3.9. ROC curve illustrating the capacity of logistic regression models to distinguish between HNF1A-MODY and type 2 diabetes using apoM and hsCRP

3.5 Discussion

This study confirms the result of an initial report that subjects with HNF1A-MODY have lower serum apoM concentration than controls (Richter, et al., 2003). Serum apoM concentration was lower in subjects with HNF1A-MODY than those with type 1 diabetes, and apoM provided good discrimination between these two patient groups. As low apoM concentration was also observed in subjects with type 2 diabetes, apoM does not distinguish HNF1A-MODY from type 2 diabetes. A lower apoM/HDL ratio was observed in the HNF1A-MODY group compared with all other study groups, but this was not highly discriminative between the groups.

A cut-off apoM concentration of 1.14 μmol/L confers a sensitivity of 84.8% and specificity of 94.0% for the discrimination of HNF1A-MODY from type 1 diabetes. It also showed high positive and negative predictive values of 94.9% and 82.4%. However, it should be taken into account that a test's predictive value is affected by the prevalence of a disease in a population (Altman & Bland, 1994a). MODY is a disease of low prevalence (prevalence of 1% in those diagnosed has having type 1 diabetes) and these estimates of predictive values could be higher than those in a clinical setting. Likelihood ratios are measures of diagnostic test performance that are not affected by the disease prevalence (Deeks & Altman, 2004). The cut-off apoM concentration of 1.14 μmol/L showed a positive likelihood ratio of 14.1. It was estimated that the post-test probability of presence of an underlying functional *HNF1A* variant would be 12% in a patient clinically diagnosed as type 1 diabetes, and who is found to have an apoM concentration of ≤1.14 (μmol/L). If the same patient

has an apoM concentration of >1.14 μmol/L, the post-test probability of having unrecognized HNF1A-MODY would be 0.1%.

It is of interest that apoM can discriminate HNF1A-MODY from type 1 diabetes. Recently described biomarkers for HNF1A-MODY, high sensitivity C-reactive protein (hsCRP), C-peptide and islet autoantibodies either do not provide good discrimination from type 1 diabetes or their performance is dependent on diabetes duration (McDonald, et al., 2011a; Owen, et al., 2010; Thanabalasingham, et al., 2012b). Unlike C-peptide and islet autoantibodies, apoM is not likely to be affected by duration of diabetes and might possibly have value as HNF1A-MODY biomarker during the honeymoon period when endogenous insulin secretion persists in those with type 1 diabetes.

Low apoM in type 2 diabetes has been previously observed (Plomgaard, et al., 2009). Indirect evidence suggests this is probably caused by the combination of chronic inflammation and hyperinsulinaemia found in type 2 diabetes. The *APOM* promoter contains a dual specificity regulatory region that binds both HNF1A and pro-inflammatory transcription factors (c-Jun, JunB) (Mosialou, et al., 2011). HNF1A leads to activation and Jun leads to repression of ApoM promoter activity. Inflammation-induced over-expression of Jun in HepG2 cells lead to transcriptional repression of *APOM* (Mosialou, et al., 2011) (**Figure 3.1**). In murine models, *APOM* expression was reduced by hyperinsulinaemia via a FOXA2-mediated mechanism (Wolfrum, et al., 2008).

The features of a clinically useful biomarker would include reproducibility of

results and a widely available, economical assay. Richter *et al* used western blot for apoM measurement which although specific is not highly quantitative and has low throughput. Skupien *et al* employed dot blot method, less specific then western blot, using commercially available antibodies. Cervin *et al* used an ELISA that was developed in-house and is not widely available. In 2008, another study reported development of a mass spectrometry-based assay (Copeland, 2008). A mass spectrometry-based assay would require expensive specialist equipment and is unlikely to be available for clinical use. The mass spectrometry-based assay has not been used in any later on research studies investigating apoM. The apoM assay used in this study is an ELISA developed by Roche (Basel, Switzerland). Based on average values from healthy controls, it is estimated that this ELISA is 1.5 fold more sensitive than the ELISA used by Cervin *et al.* This ELISA currently has limited availability. However, with increasing interest in apoM as a marker for cardiovascular risk (Christoffersen, et al., 2011; Karuna, et al., 2011), apoM may be more routinely measured.

This study used strict selection criteria for subject recruitment. Both type 1 and type 2 diabetes subjects included in this study either did not meet clinical criteria for MODY diagnostic testing or had been tested and were negative for common MODY mutations. This narrow selection of subjects could have led to a spectrum effect. As discussed in chapter 1 (table 1.5), the spectrum effect is a source of variation in results of studies evaluating biomarkers. A spectrum effect results if cases and controls lying on extremes of disease spectrum are included and patients with mild, difficult to diagnose, disease are omitted.

Therefore, it cannot be assumed that the performance of the biomarker would be as good in a clinical setting where a physician might come across patients with the full range of symptoms. Further validation of apoM in unselected datasets of young-onset diabetes patients is required before use in clinical setting as a biomarker for HNF1A-MODY.

In conclusion, this study demonstrates that serum apoM concentration is lower in HNF1A-MODY than in both controls and type 1 diabetes. Serum apoM provides good discrimination between HNF1A-MODY and type 1 diabetes and can help prioritise patients with early-onset diabetes for definitive molecular testing.

Chapter 4

Use of high sensitivity C-reactive protein and desialylated glycan 9 index for differential diagnosis of HNF1A-MODY in subjects with young-onset diabetes

4.1 Introduction

As discussed in chapter 1, high sensitivity C-reactive protein (hsCRP) and plasma glycan measurements have been identified as putative HNF1A-MODY biomarkers (Owen, et al., 2010; Thanabalasingham, et al., 2013). Both were initially identified by genome-wide association studies (GWAS) reporting association of common variants near *HNF1A* with small changes in CRP levels and plasma N-glycans (oligosaccharide structures covalently attached to plasma proteins at asparagine) (Lauc, et al., 2010; Reiner, et al., 2008; Ridker, et al., 2008). The GWAS findings are supported by functional work showing that HNF1A is part of a transcriptional complex regulating CRP expression (Nishikawa, et al., 2008; Toniatti, et al., 1990a). Similarly, the functional follow-up of the N-glycome GWAS results showed that HNF1A affects plasma protein fucosylation (addition of sugar “fucose” to protein bound glycans) by regulating fucose synthesis, upregulating enzymes necessary for antennary fucosylation and down regulating those needed for core fucosylation (Lauc, et al., 2010).

Taking these GWAS findings further, work carried out by my supervisors research group has shown that subjects with HNF1A-MODY have lower baseline levels of hsCRP and DG9-glycan index (a ratio of triantennary glycans with and without antennary fucose) compared with healthy controls and those with other subtypes of diabetes. In pilot and replication studies, hsCRP showed promise as a diagnostic biomarker with a ROC curve-derived C-statistic of 0.79-0.97 for distinguishing HNF1A-MODY from type 2 diabetes (Owen, et al., 2010; Thanabalasingham, et al., 2011). Similarly, it was observed that DG9-glycan index provided excellent discrimination between

HNF1A-MODY and both type 1 and type 2 diabetes (C-statistic of >0.90) (Thanabalasingham, et al., 2013).

Previous studies investigating hsCRP and DG9-glycan index were cross-sectional and included clearly defined groups of MODY, type 1 and type 2 diabetes (selection criteria detailed in chapter 1: section 1.5.4). MODY was excluded from the type 1 and type 2 diabetes groups as the subjects either did not meet clinical criteria for MODY diagnostic testing or had undergone genetic sequencing and did not have a mutation in *HNF1A, HNF4A* or *GCK* (Owen, et al., 2010; Thanabalasingham, et al., 2013). Use of clearly defined groups of patients is a common approach used by initial studies evaluating diagnostic tests. However, this approach decreases the number of false negatives and false positives resulting in overestimation of the test sensitivity and specificity and lack of reproducibility of results by follow up studies (Lachs, et al., 1992; Mulherin and Miller, 2002; Whiting, et al., 2004). As explained in chapter 1 (table 1.5 and section 1.5.4), this phenomenon is known as the “spectrum effect” and occurs when cases and controls lying on extremes of the disease spectrum are included and mild, difficult to diagnose, patients are omitted. Therefore, it is important to determine the “true estimates” of sensitivity and specificity of hsCRP and DG9-glycan index in a group of patients that have not been previously investigated for MODY and that closely represent actual young-onset diabetes clinic population.

4.2 Aims

This study aims to determine the performance of hsCRP and DG9-glycan

index for identifying HNF1A-MODY in a group of subjects previously uninvestigated for MODY, clinically diagnosed as having young-onset non-autoimmune diabetes. This study also aims to determine the frequency of the commonest MODY subtypes in subjects with young-onset non-autoimmune diabetes.

4.3 Subjects and Methods

4.3.1 Subjects

Subjects for the current study were selected from the dataset described in chapter 2 (section 2.1.4) comprising of North European subjects (n=396) from Ulm, Germany, clinically diagnosed and treated as having young-onset non-autoimmune diabetes. None of the subjects had been previously investigated for MODY. From this dataset, subjects meeting the following selection criteria were selected: age of onset of diabetes <30 years, negativity for islet autoantibodies (Glutamic acid decarboxylase, Islet cell antibodies and islet autoantigen 2) and not receiving insulin or a positive C-peptide (>0.2nmol/L) if on insulin treatment. Complete data were not available for all subjects: for example, data for presence or absence of islet autoantibodies was unavailable for 95 subjects. These subjects were not included in the study. Remaining subjects meeting the above-mentioned criteria (n=208) were selected for the current study. The characteristics of the subjects selected are provided in **table 4.1**.

	Subjects (n=208)
% Male	53%
Age at sampling (years)	28 (2-68)
Age at diagnosis (years)	26 (2-30)
Diabetes duration	0 (0-47)
BMI (kg/m^2)	26 (16-53)
Treatment (number of subjects)	
Diet/Insulin/OAD/OAD and insulin	1/3/202/2

Table 4.1. Characteristics of the German subjects selected for evaluation of hsCRP and DG9-glycan index. Data presented as median (range). OAD=Oral antidiabetic agents.

4.3.2 Methods

The two hundred and eight subjects selected, underwent Sanger sequencing for variants in the most common MODY genes (*HNF1A, GCK* and *HNF4A*) and hsCRP and plasma glycan measurement. Details of the genetic sequencing, hsCRP assay and glycan measurement are provided in chapter 2 (section 2.2). Subjects with hsCRP value ≥10mg/L were not excluded during analysis of the results. I carried out data collection, statistical analysis and interpretation of the results.

In-silico analysis was performed to predict the functional effect of variants using bioinformatics programs: Sorting Intolerant From Tolerant (SIFT); Mutation Taster; CONsensus DELeteriousness score of missense single nucleotide variants (CONDEL); Alamut (Adzhubei, et al., 2010; Gonzalez-Perez and Lopez-Bigas, 2011; Schwarz, et al., 2010) (http://www.interactive-biosoftware.com). Details of these software are provided in chapter 2 (section 2.2).

Statistical Analysis

The Mann-Whitney U test was used to compare quantitative variables between groups. Previously defined thresholds of hsCRP (≤0.40mg/L) and DG9-glycan index (≤0.16) were used to calculate the sensitivity, specificity and positive and negative likelihood ratios for HNF1A-MODY (Owen, et al., 2010; Thanabalasingham, et al., 2012a). All computations were performed using IBM SPSS Statistics Version 20.0 and $P<0.05$ was considered significant.

4.4 Results

Among 208 subjects sequenced, four non-synonymous (G292fs, T260M, D135N, P290R), one splice site (c.955+5G>A) and two synonymous (P112P, T513T) *HNF1A* variants were identified in nine subjects (non-synonymous or splice variants in seven subjects and synonymous variants in two subjects). These are illustrated in **figure 4.1**. G292fs was identified in three un-related probands. The synonymous variant P112P was found in two probands and one of these probands carried a second synonymous variant T513T. All other variants were identified in one proband each. Among these, three (G292fs, T260M, D135N) have been previously reported as causal for HNF1A-MODY (Bellanne-Chantelot, et al., 2008; Glucksmann, et al., 1997; Lehto, et al., 1997; Yamagata, et al., 1996b) and two were novel (P290R, c.955+5G>A). No *HNF4A* or *GCK* variants were identified.

Details of *HNF1A* variant position, bioinformatics prediction, conservation across species and assessment of amino acid change and hsCRP levels for the subjects carrying these variants are provided in **Table 4.2** and discussed below.

4.4.1 Assessment of pathogenicity of *HNF1A* variants

A. Previously reported variants

G292fs

G292fs (previously known as P291fsinsC), is the most common HNF1A-MODY causal variant reported in at least 234 families worldwide (Colclough, et al., 2013).

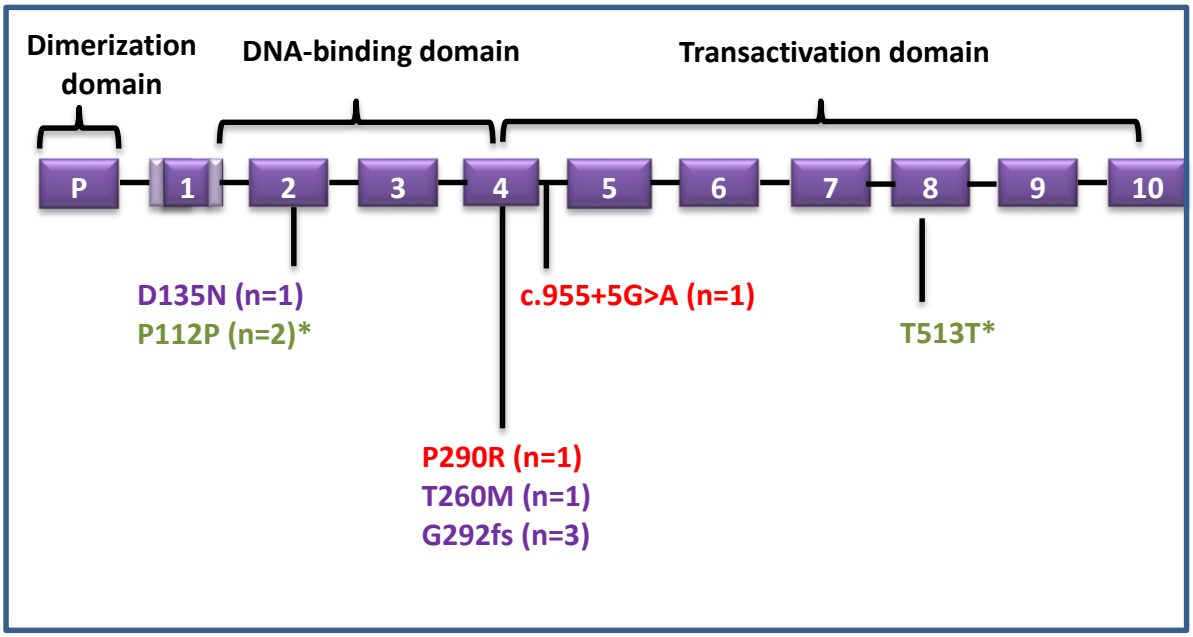


Figure 4.1. Location of variants within the 10 exons and introns of *HNF1A* identified in the study evaluating hsCRP and DG-9glycan index as HNF1A-MODY biomarker
Colour scheme: Previously reported variants (purple), Novel variants (red), Synonymous variants (green). *One of the subjects carrying the synonymous variant P112P also carried the synonymous variant T513T

Region	Protein level/cDNA level	Previously reported	Prediction using SIFT	Prediction using MT	Prediction using Condel	Conservation across species	Amino acid change	HsCRP mg/L	DG-9 Glycan index	Interpretation
Exon 2	p.D135N c.403G>A	Bellanne-Chantelot *et al* 2008	Damaging	Disease causing	Deleterious	Highly conserved nucleotide (phyloP: 5.61 [-14.1;6.4]). Highly conserved amino acid	Small physicochemical difference between Aspartic acid (D) and Asparagine (N) (Grantham dist.: 23 [0-215])	0.02	0.14	Likely pathogenic
Exon 4	p.T260M c.779C>T	Reported in 12 families to date; cosegregates with diabetes where families tested	Damaging	Disease causing	Deleterious	Highly conserved nucleotide (phyloP: 5.53 [-14.1;6.4]). Highly conserved amino acid	Moderate physicochemical difference between Thrreonine (T) and Methionine (M) (Grantham dist.: 81 [0-215])	2.85	0.07	Likely pathogenic
Exon 4	p.P290R c.869C>G	Novel	Tolerated	Polymorphism	Neutral	Weakly conserved nucleotide, (phyloP: 0.85 [-14.1;6.4]). Moderately conserved amino acid	Moderate physicochemical difference between Proline (P) and Arginine (R) (Grantham dist.: 103 [0-215])	0.63	0.35	Likely benign
Exon 4	p.G292fs c.872dupC (identified in three subjects)	Common HNF1A-MODY causal variant	NA	NA	NA	NA	This duplication creates a frame shift starting at codon Gly292. The new reading frame ends in a STOP codon 24 positions downstream. The mRNA produced might be targeted for NMD	0.02 0.02 0.09	0.17 0.08 0.08	Likely pathogenic
Intron 4	c.955+5G>A	Novel	NA	NA	NA	NA	NA	0.38	0.14	Likely pathogenic substitution in intron 4. Weakens consensus of existing splice site and strengthens alternative site 8n downstream.

Table 4.2. Bioinformatic interpretation of non-synonymous and splice site *HNF1A* variants identified in German subjects

MT=Mutation Taster, PhyloP=a measure of evolutionary conservation for the specific base, Grantham distance=a chemical distance that measures difference between two amino acids taking into account their volume, polarity and composition of side chain, NMD=nonsense mediated decay. PhyloP score and Grantham distance were calculated using bioinformatics software Alamut. Conservation of species was assessed considering 11 species (Human, Chimp, Orangutan, Macaque, Rat, Mouse, Dog, Cat, Cow, Chicken and Frog). N/A= Not available (As SIFT and CONDEL predict missense variants only, so prediction was not available for frameshift variant)

In-silico analysis performed in the current study predicted the variant to be likely disease causing (**Table 4.2**). G292fs was identified in three un-related probands in this study. All three subjects carrying this variant had a low hsCRP (lower than previously reported threshold of 0.40 mg/L). Two of the subjects had a low DG9-glycan index (lower than previously reported threshold of 0.16). One subject had a DG9-glycan index of 0.17.

T260M

T260M has been identified in 12 families and co-segregates with MODY phenotype (Lehto et al., 1997) (Glucksmann et al., 1997). *In-silico* analysis performed in the current study predicted the variant to be likely disease causing (**Table 4.2**). The subject carrying this variant had an hsCRP of 2.85 mg/L (higher than previously reported thresholds of 0.40 mg/L) and a DG9-glycan index of 0.07 (lower than previously reported thresholds of 0.16).

D135N

D135N has been reported as MODY causing variant in a large French study of 356 *HNF1A* variants identified in unrelated probands (Bellanne-Chantelot, et al., 2008). Information for the co-segregation of the variant with disease phenotype in the family members was not provided. The authors stated that all *HNF1A* variants identified in their study were considered as pathogenic based on the following criteria: nature of the amino acid change, conservation of the residue across species, absence of the variant in 300 control subjects of Euro-Caucasian origin and co-segregation of the variant with young-onset diabetes, when relatives were available. *In-silico* analysis performed in the

current study predicted the variant to be likely disease causing (**Table 4.2**). The subject carrying this variant had a low hsCRP and a low DG9-glycan index (0.02 mg/L and 0.14 respectively).

The family history of the subjects carrying these previously reported variants was suggestive of MODY (**Figure 4.2**). The subjects had a two-generation history of young-adult onset diabetes and treatment with oral antidiabetic agents. Based on previous reports and bioinformatics prediction, all previously reported variants were considered as likely pathogenic.

B. Novel variants

P290R

P290R was predicted as likely benign by all bioinformatics programs used (**Table 4.2**). The subject with this variant had higher levels of both hsCRP and DG9-glycan index (0.63 mg/L and 0.35; higher than previously reported thresholds of 0.40 mg/L and 0.16 respectively). Based on bioinformatics prediction, P290R was considered as likely benign.

c.955+5G>A

The second novel variant, c.955+5G>A is located five nucleotides downstream of the exon-intron junction and hence likely to affect splicing. The subject with c.955+5G>A had low hsCRP level and a low DG-9 glycan index (0.38 mg/L and 0.14).

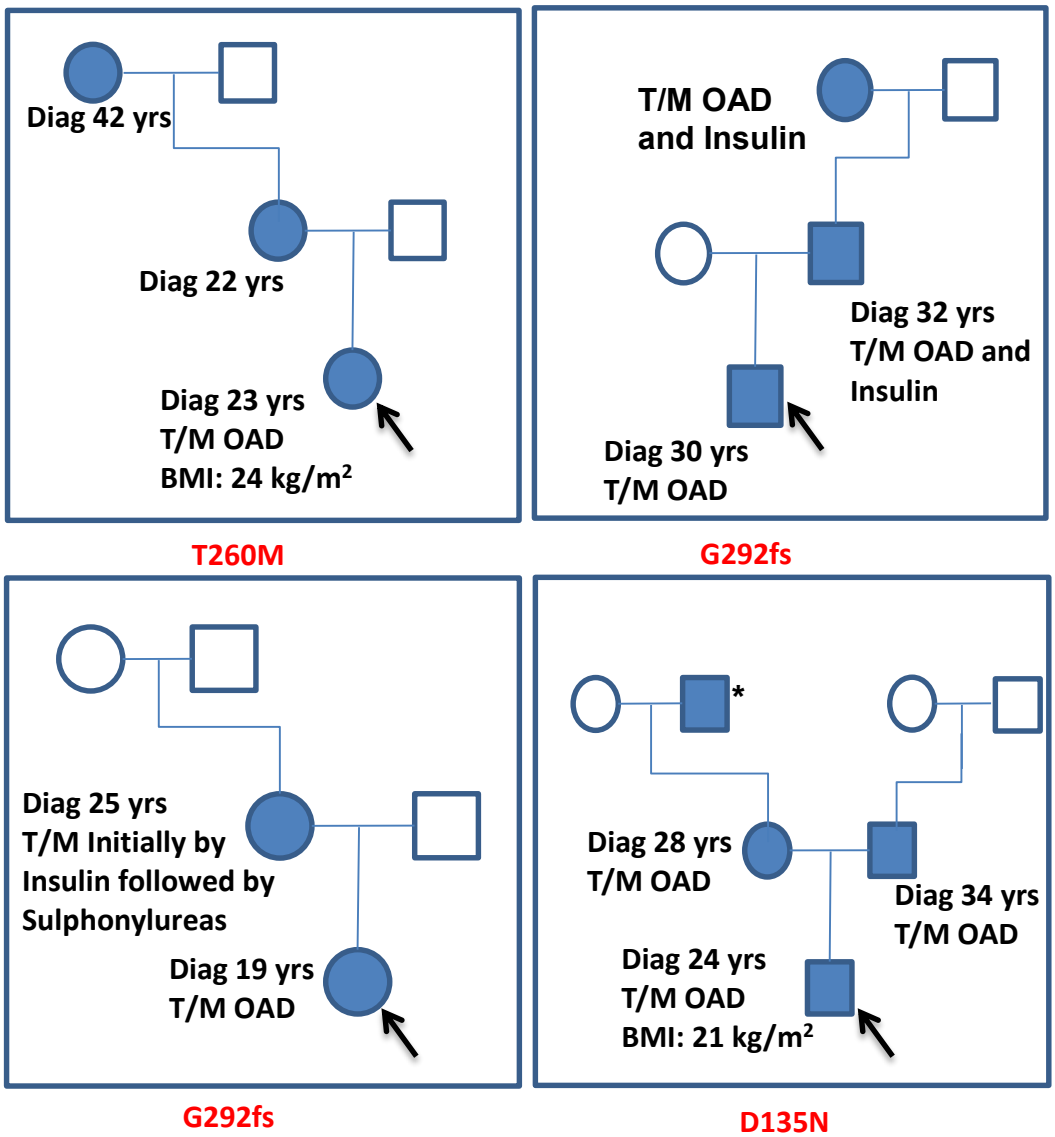


Figure 4.2. Family tree of four probands with *HNF1A* variants.
T/M= treatment, OAD= Oral antidiabetic agents, * Age of onset unknown
Family history was unavailable for other subjects with *HNF1A* variants.

Due to unavailability of family data, co-segregation of novel variants with MODY phenotype could not be confirmed. As described in chapter 2 (section 2.2), functional work to assess the definitive causal role of these variants is being carried out by members of the research group.

4.4.2 Clinical features of subjects with HNF1A-MODY

The selection criteria used in this study (negative result for islet autoantibodies and a positive c-peptide) meant that subjects predominantly had non-autoimmune diabetes and the main differential diagnoses were type 2 diabetes and MODY. The subjects (n=6) with known causal or likely pathogenic *HNF1A* variants (G292fs identified in 3 probands, D135N, T260M, c.955+5G>A one in each proband) were considered as having HNF1A-MODY. All other subjects were considered as having type 2 diabetes. The subject carrying the P290R variant, predicted as likely benign by bioinformatics, was not included in the HNF1A-MODY group and was therefore included in the type 2 diabetes group. Clinical features of both groups of subjects are provided in **table 4.3**.

Median (range) age of diagnosis for subjects with HNF1A-MODY was 23 (10-30) years (**Table 4.3** and **Figure 4.3**). Median (range) BMI (kg/m^2) for subjects with HNF1A-MODY was 24 (21-25). Five out of six subjects with HNF1A-MODY were taking oral antidiabetic agents. There was no significant difference in age at sampling, age at diagnosis, BMI, diabetes duration and treatment of diabetes between subjects with HNF1A-MODY and those with type 2 diabetes (**Table 4.3**).

	HNF1A-MODY (n=6)	Type 2 diabetes (n=202)	*P* value
% Male	66%	52%	0.68
Age at sampling (years)	24 (19-49)	28 (2-68)	0.32
Age at diagnosis (years)	23 (10-30)	26 (2-30)	0.26
Diabetes duration	0 (0-39)	0 (0-47)	0.98
BMI (kg/m^2)	24 (21-25)	26 (16-53)	0.39
hsCRP (mg/L)	0.05 (0.02-2.85)	2.49 (0.01-100.37)	0.003
DG9-Glycan index	0.11 (0.07-0.17)	0.23 (0.05-0.45)	0.0004
GP14/(GP13+GP14)	0.36 (0.30-0.40)	0.52 (0.25-0.78)	0.0001
GP14/(GP15+GP14)	0.83 (0.78-0.85)	0.92 (0.73-0.97)	0.00004
DG7/(DG5+DG6)	0.02 (0.02-0.05)	0.04 (0.02-0.10)	0.08
Pharmacological Treatment (number of subjects)			
No pharmacological Treatment	0	1	
Taking Insulin only	1	4	
Taking oral antidiabetic agents only	5	195	
Taking oral antidiabetic agents and insulin	0	2	

Table 4.3. Clinical and biochemical characteristics of German subjects with *HNF1A* variants and those with type 2 diabetes. Data presented as median (range). The subject carrying the P290R variant, predicted as likely benign by bioinformatics, was not included in the HNF1A-MODY group.

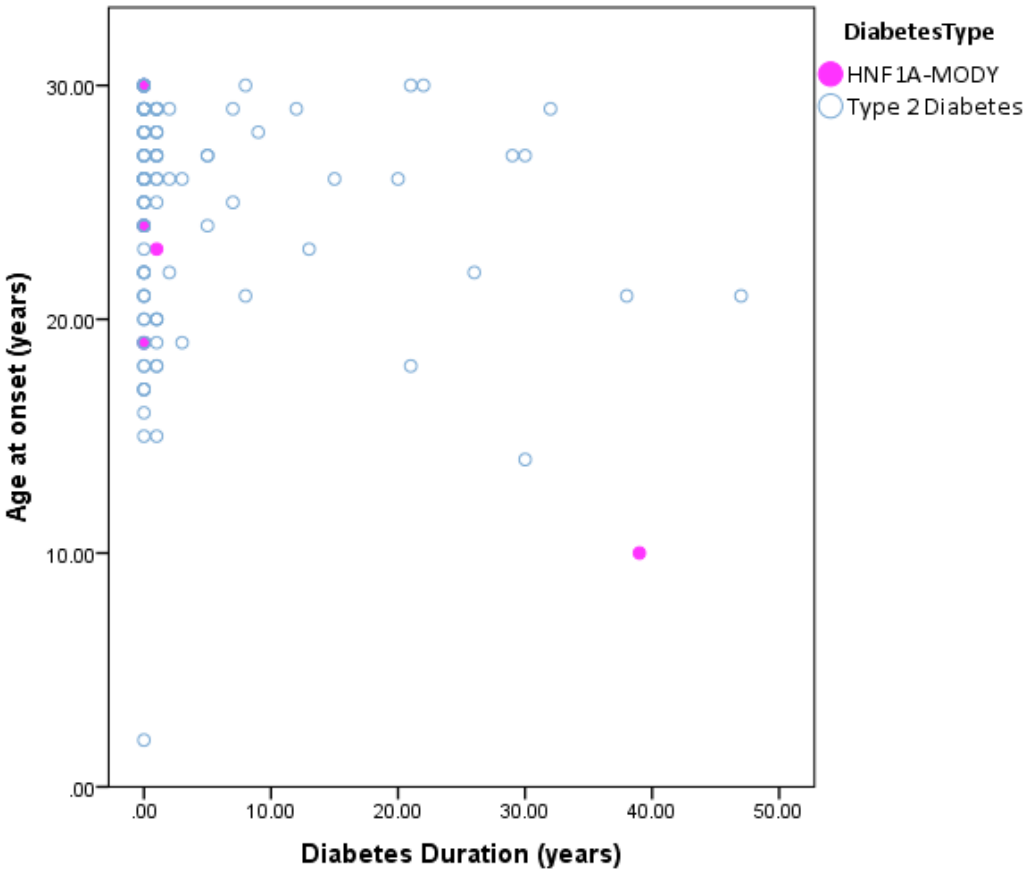


Figure 4.3. Scatter plot to illustrate age at onset and diabetes duration of subjects with HNF1A-MODY and those with type 2 diabetes

4.4.3 Use of hsCRP to identify HNF1A-MODY cases

Median (range) serum hsCRP (mg/L) was significantly lower in HNF1A-MODY (0.05 [0.02-2.85]) compared with type 2 diabetes (2.49 [0.01-100.37], p=0.003) (**Table 4.3** and **Figure 4.4**). Using the previously suggested threshold of hsCRP of ≤0.40 mg/L, the sensitivity, specificity, positive and negative likelihood ratios were 83%, 78%, 3.9 and 0.2 (**Table 4.4**).

4.4.4 Use of DG9-glycan index to identify HNF1A-MODY cases

Median (range) DG9-glycan index was significantly lower in HNF1A-MODY (0.11 [0.07-0.16]) as compared with type 2 diabetes (0.23 [0.05-0.45], p=0.0004) (**Table 4.3** and **Figure 4.4**). Using the previously suggested threshold for DG9-glycan index of ≤0.16, the sensitivity, specificity, positive and negative likelihood ratios for HNF1A-MODY were 83%, 82%, 4.7 and 0.2 respectively (**Table 4.4**).

4.4.5 Combined use of hsCRP and DG9-glycan index

Using criteria of either hsCRP ≤0.40 mg/L or DG9-glycan index of ≤0.16, the sensitivity, specificity, positive and negative likelihood ratios were 100%, 63%, 2.7 and 0.0 respectively (**Table 4.4**).

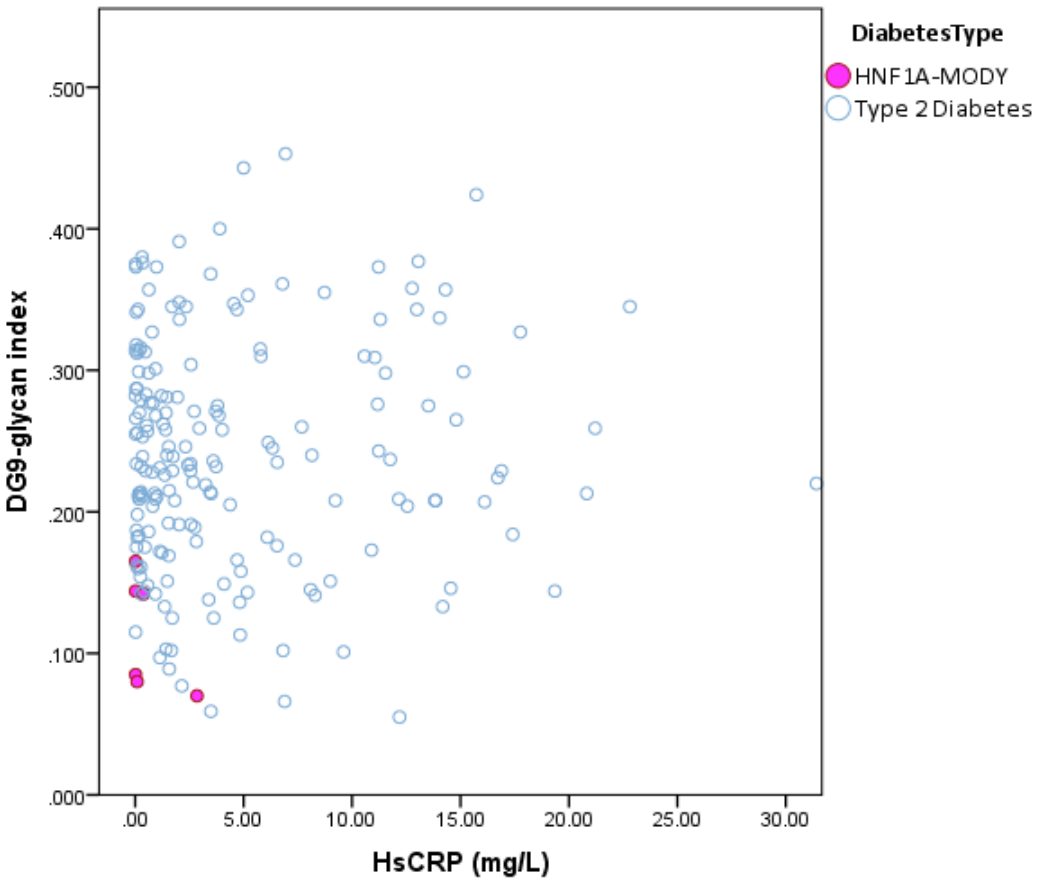


Figure 4.4. Scatter plot to illustrate hsCRP levels and DG-9 glycan index in subjects with HNF1A-MODY and those with type 2 diabetes

Threshold for *HNF1A* sequencing	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Positive likelihood ratio	Positive post-test probability* (%)	Negative likelihood ratio	Negative post-test probability* (%)
hsCRP ≤0.20 mg/L	66	85	12	98	4.5	15.8	0.3	1.5
hsCRP ≤0.40 mg/L	83	78	10	99	3.9	14.0	0.2	0.8
hsCRP ≤1.00 mg/L	83	67	9	99	2.5	9.6	0.2	1.0
DG9-glycan index ≤0.16	83	82	13	99	4.7	16.4	0.2	0.8
hsCRP ≤0.40 mg/L OR DG9-glycan index ≤0.16	100	63	7	100	2.7	10.1	0.0	0.0

Table 4.4. Use of previously defined thresholds of hsCRP and DG9-glycan index to identify HNF1A-MODY

*Based on pre-test probability of 4% misdiagnosed HNF1A-MODY cases in clinically-labelled type 2 diabetes (Thanabalasingham, et al., 2012b). Sensitivity=the ability of the test to identify those who have the disease; Specificity=the ability of the test to identify those who do not have the disease; Positive predictive value=the probability of having the disease if a person tests positive; Negative predictive value=the probability of not having the disease if a person tests negative; Positive likelihood ratio=the ratio of the likelihood that a positive result will occur in subjects with the disease to the likelihood that the same result will occur in subjects without the disease; Negative likelihood ratio= the ratio of the likelihood that a negative result will occur in subjects with the disease to the probability that the same result will occur in subjects without the disease; Post-test probability=the probability of the disease presence given a positive or negative test result

4.4.6 Novel glycan measures

The full glycome profile was available for all the subjects investigated in this study (**Table 4.5**). The performance of novel glycan measures was assessed. The glycan ratios GP14 to (GP13+GP14) and GP14 to (GP14+GP15) offered good discrimination between HNF1A-MODY and type 2 diabetes (C-statistic of 0.83 and 0.86 respectively) (**Figure 4.5**). Using a cut-off of 0.40 for GP14 to (GP13+GP14), the sensitivity was 89% and specificity was 86%. Using a cut-off of 0.85 for GP14 to (GP15+GP14), the sensitivity was 98% and specificity was 86%.

The DG7 to (DG5+DG6) ratio reported in the previous study (reporting a C-statistic >0.99) did not perform well in this study (C-statistic of 0.70 in this study) (Thanabalasingham, et al., 2013).

Glycan measure	HNF1A-MODY (n=6)	Type 2 diabetes (n=202)	P value
GP1	0.18 (0.11-0.25)	0.11 (0.08-0.15)	0.08
GP2	2.60 (2.47-3.25)	2.39 (1.87-3.05)	0.282
GP3	1.96 (1.55-2.29)	2.01 (1.71-2.26)	0.748
GP4	4.32 (4.12-6.10)	4.23 (3.65-4.91)	0.183
GP5	2.10 (1.81-2.36)	1.92 (1.69-2.15)	0.242
GP6	3.53 (3.49-4.43)	3.63 (3.18-4.15)	0.588
GP7	7.89 (7.50-8.56)	7.16 (6.52-7.81)	0.066
GP8	11.50 (10.0-12.30)	10.60 (9.45-11.61)	0.127
GP9	37.00 (35.59-39.21)	39.67 (37.77-41.89)	0.019
GP10	5.97 (4.65-6.94)	6.44 (5.58-7.48)	0.346
GP11	2.30 (2.18-3.04)	2.19 (1.86-2.52)	0.127
GP12	2.58 (2.22-3.14)	2.40 (2.12-2.62)	0.147
GP13	8.21 (6.96-9.10)	6.75 (5.52-7.90)	0.071
GP14	5.24 (4.30-6.09)	7.31 (6.34-8.49)	0.003
GP15	0.93 (0.84-1.04)	0.64 (0.48-0.76)	0.003
GP16	1.55 (1.07-1.64)	1.31 (1.13-1.53)	0.642
DG1	0.17 (0.14-0.23)	0.14 (0.10-0.20)	0.125
DG2	2.91 (2.44-3.42)	2.50 (1.93-3.08)	0.225
DG3	2.53 (2.08-3.14)	2.55 (2.28-2.82)	0.968
DG4	5.08 (4.57-6.38)	4.56 (4.01-5.37)	0.131
DG5	49.01 (47.00-51.70)	49.66 (47.85-51.93)	0.64
DG6	12.85 (11.59-14.18)	12.78 (11.21-14.24)	0.963
DG7	2.02 (1.40-2.65)	2.49 (1.98-3.12)	0.051
DG8	15.55 (13.24-17.68)	13.86 (12.34-15.86)	0.154
DG9	2.22 (1.53-3.07)	4.27 (3.36-5.36)	0.005
DG10	0.87 (0.72-1.22)	1.10 (0.91-1.25)	0.211
DG11	3.57 (2.78-3.91)	2.70 (2.34-3.05)	0.046
DG12	0.57 (0.36-0.73)	0.84 (0.63-1.13)	0.007
DG13	1.55 (0.92-1.64)	1.11 (0.81-1.48)	0.218

Table 4.5. Complete glycome profile of HNF1A-MODY and type 2 diabetes subjects. Data presented as median (25th-75th centiles). P value calculated using Mann-Whitney U tests

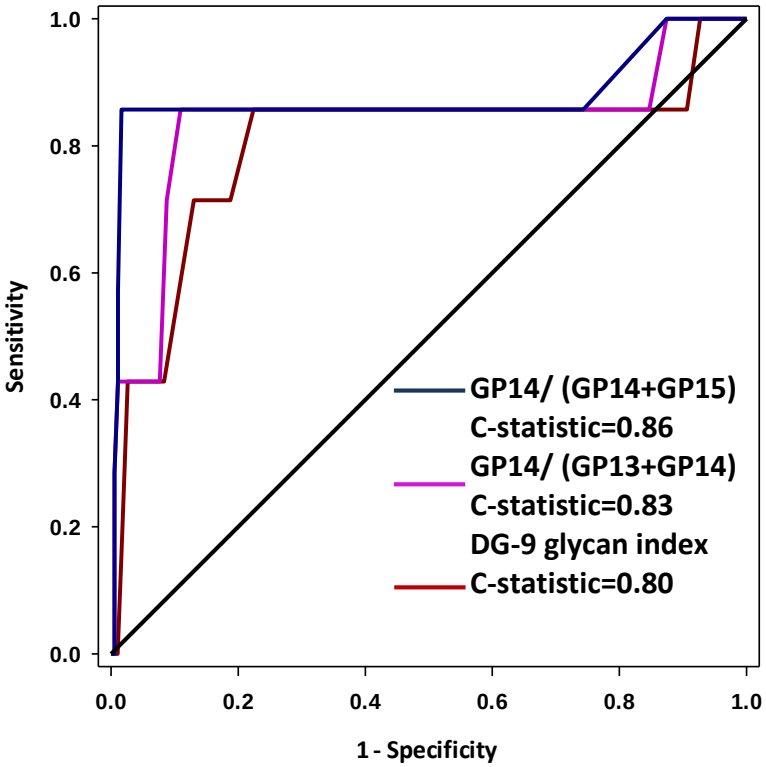


Figure 4.5. ROC curves illustrating the performance of GP14/ (GP13+GP14) and GP14/ (GP14+GP15) to discriminate HNF1A-MODY and type 2 diabetes

4.5 Discussion

In this study, I determined the performance of hsCRP and DG9-glycan index for identifying HNF1A-MODY in a group of subjects clinically diagnosed with young-onset non-autoimmune diabetes that had not been previously investigated for MODY. This study confirms previous observations that both hsCRP and DG9-glycan index have clinical value to be used as biomarkers for HNF1A-MODY (Owen, et al., 2010; Thanabalasingham, et al., 2012a; Thanabalasingham, et al., 2011). Using the previously suggested threshold of DG9-glycan index ≤0.16 in this unselected dataset, sensitivity was 83% and specificity was 81%. HsCRP ≤0.4mg/L had a sensitivity of 83% and a specificity of 78%.

The performance of DG9-glycan index (using the previously suggested threshold of ≤0.16) in this unselected group of subjects was comparable with the previously reported figures of sensitivity and specificity (88% and 81% respectively for distinguishing HNF1A-MODY from type 2 diabetes) (Thanabalasingham, et al., 2013). HsCRP (using previously suggested threshold of ≤0.4mg/L) had both a higher sensitivity and a higher specificity in the current dataset compared with the previously reported figures (71% and 77%, respectively, for distinguishing HNF1A-MODY from type 2 diabetes, excluding CRP values≥10mg/L and 65% and 82%, respectively, if subjects with CRP≥10 mg/l are not excluded) (Owen, et al., 2010). These results indicate that both hsCRP and DG9-glycan index can be used as useful adjuncts to the current clinical guidelines for the identification of HNF1A-MODY.

Five non-synonymous or splice variants in *HNF1A* were identified. Based on previous reports and bioinformatics prediction, all *HNF1A* variants except one novel variant (P290R) were considered as likely pathogenic. P290R was predicted as likely benign and was associated with both a higher hsCRP and DG9-glycan index than the previously suggested threshold. All subjects with known HNF1A-MODY causal or likely pathogenic *HNF1A* variants had either hsCRP or DG9-glycan index below the diagnostic threshold of 0.40 mg/L and 0.16 respectively. This indicates that hsCRP and DG9-glycan index could have a role in assessing pathogenicity of *HNF1A* variants.

As outlined in chapter 1, *HNF1A* variants can occur as neutral variants with no clinical symptoms, variants increasing susceptibility to type 2 diabetes and variants resulting in a clinical phenotype of MODY (Miedzybrodzka, et al., 1999; Weedon, et al., 2005; Winckler, et al., 2007). Even the variants that result in a MODY phenotype in one individual can display reduced penetrance and do not necessarily lead to HNF1A-MODY phenotype in every carrier. This issue of reduced penetrance has been raised in early years when HNF1A-MODY was first defined (Miedzybrodzka, et al., 1999), and was recently highlighted by a large scale sequencing study in which *HNF1A* variants previously reported in MODY or rare, conserved and protein damaging variants were identified in older, unaffected individuals (Flannick, et al., 2013). Therefore, in the light of these observations in MODY, as well as for other Mendelian diseases and genetic disorders, it follows

that a particular genotype is not predictive of a characteristic phenotype in every carrier (Cooper, et al., 2013). There is another possible explanation for the presence of previously reported MODY variants in older, asymptomatic individuals (Flannick, et al., 2013). Most of the *HNF1A* variants previously reported in MODY were identified in patients referred for genetic testing due to presence of an HNF1A-MODY phenotype. It is likely that some of these variants were not truly causal for MODY and were naively assumed as causal because they were identified in symptomatic individuals.

In the light of the above, the question arises whether the variants identified in the current study were truly judged as pathogenic. In these cases, as the probands had clinical features in keeping with MODY (young-onset, antibody negative, c-peptide positive, normal BMI, positive parental history of young-onset diabetes where available) and low levels of hsCRP or DG-9 glycan index there is a high likelihood that these variants were causal for HNF1A-MODY. At the same time, it is also possible that these variants could display reduced penetrance in another carrier resulting in late onset diabetes or no diabetes at all.

This study reports the prevalence of MODY in subjects with young-onset non-autoimmune diabetes (age of onset < 30yrs) of 3.3% (95% CI 1.6%-6.7%). This is comparable to the prevalence reported in a previous study by our research group (Thanabalasingham, et al., 2012b). In the previous study, comprising subjects with clinically defined type 2 diabetes (n=244), subjects diagnosed with

diabetes up to 30 years and those without features of metabolic syndrome diagnosed up to 45 years were sequenced for *HNF1A* and *HNF4A*. Ten *HNF1A* and two *HNF4A* mutations were found. A prevalence of MODY of 4% (comparable to the prevalence observed in the current study) was observed among those with apparent type 2 diabetes diagnosed by the age of 45 years. A prevalence of MODY of 25% was observed among those with apparent type 2 diabetes diagnosed up to the age of 30 years, which is higher than the prevalence observed in the current study. However, it could have been an overestimate as it was based on a smaller subset of total study subjects.

Other estimates of MODY prevalence have been reported. The SEARCH study, a large US population-based registry of diabetes in youth (age of onset of diabetes <20 years), reported a prevalence of MODY of 8% among those with non-autoimmune diabetes (negative islet autoantibodies and C-peptide ≥0.8 ng/ml [≥0.26 nmol/l]) (Pihoker, et al., 2013). Another, Polish population-based study of children aged 0-18 years, reported a prevalence of monogenic diabetes (including MODY, neonatal diabetes and Wolfram and Alström syndromes) of 3.1–4.2% (with GCK-MODY being the most frequent type, amounting to 83% of patients with monogenic diabetes) (Fendler, et al., 2012). These variations in prevalence estimates could be due to differences in the selection criteria and the age of the subjects included.

In conclusion, this study evaluated the use of hsCRP and DG9-glycan index for

identifying HNF1A-MODY in a group of unselected subjects, where all study participants were sequenced. The results of this study confirm that hsCRP and DG9-glycan index have potential clinical value to identify those at high risk of carrying a pathogenic *HNF1A* variant.

Chapter 5

To evaluate the use of high sensitivity C-reactive protein for identifying South Asians with HNF1A-MODY

5.1 Introduction

South Asians (individuals of Indian, Pakistani, Bangladeshi, Nepali or Sri Lankan descent) comprise the largest ethnic minority group in UK accounting for 4% of the total UK population (2001). Epidemiological studies have consistently shown that South Asians have a higher risk of having diabetes compared with other ethnic groups. In a survey of patients with known diabetes conducted in 1985 at Southall, an area of west London with a large number of South Asians, the prevalence of diabetes in South Asians was found to be at least 3.8 times higher than that in Europeans (Mather and Keen, 1985). A more recent cross-sectional screening study set in Leicestershire, UK showed that the prevalence of diabetes in South Asians and Europeans was 9.0% vs. 3.9% among males and 7.4% vs. 3.3% among females (Khunti, et al., 2013). Similarly, a higher prevalence of diabetes has been reported for immigrant South Asians residing in other countries such as the USA and Singapore (Gupta, et al., 2011; Kanaya, et al., 2010; Yeo, et al., 2006). The majority of cases are due to type 2 diabetes, which presents at an earlier age and lower BMI than Europeans (1994).

The cause of the higher type 2 diabetes prevalence in South Asians is not clear. Factors associated with an increased risk of type 2 diabetes (Hu, 2011), have been shown to be more prevalent in South Asians compared with other ethnic groups (Fischbacher, et al., 2004; Lip, et al., 1996; Misra, et al., 2009). These include lifestyle factors, such as a lack of physical activity and unhealthy diet (high intake of refined carbohydrates and saturated fat), and metabolic factors. South

Asians are referred to as ‘metabolically obese’ (Bakker, et al., 2013). They have an increased propensity for insulin resistance compared with Europeans (Bakker, et al., 2013). Higher insulin resistance can result from several mechanisms. South Asians have a higher percentage of body fat for a given BMI, stored largely in visceral and deep subcutaneous fat compartments, observed to be correlated with insulin resistance. Studies have shown that South Asians have differences in adipose tissue function compared with Europeans such as an increased adipocyte size, free fatty acid release and an increase in liver fat content leading to insulin resistance (Bakker, et al., 2013). The dysfunctional adipose tissue also releases pro-inflammatory cytokines, leading to a state of chronic low-grade inflammation that is associated with insulin resistance via inhibition of the insulin-signalling cascade (Plomgaard, et al., 2005). As discussed later in this chapter, chronic low-grade inflammation in South Asians is manifested by higher CRP levels compared with Europeans (Chambers, et al., 2001; Forouhi, et al., 2001).

Although life style and metabolic factors are important determinants of diabetes (Hu, 2011), these do not explain all the excess increased risk of type 2 diabetes in South Asians (Kanaya, et al., 2014). From a genetic perspective, most of the type 2 diabetes risk variants identified so far in Europeans are shared by South Asians with no systematic difference in allele frequency and effect size (Kooner, et al., 2011). The increased susceptibility to type 2 diabetes in South Asians suggests the possibility of population-specific risk variants not yet reported, as most of the GWAS on type 2 diabetes have been performed on Europeans, or

low frequency or rare variants for type 2 diabetes not yet identified. A few recent studies focussed on South Asians reported two new diabetes susceptibility loci (within *TMEM163* and *SGCG*) potentially specific to South Asian populations (Saxena, et al., 2013; Tabassum, et al., 2013). Identification of more population specific or rare variants may help to understand the cause of an increased risk of type 2 diabetes in South Asians.

5.1.1 MODY in South Asians

MODY occurs in UK Asian families: however, it is rarely diagnosed. A study investigating inherited childhood diabetes in the UK showed that MODY due to *HNF1A* and *GCK* mutations was present in UK South Asian children with a similar clinical phenotype to that observed in white UK children (Porter, et al., 2006). The authors noted that MODY had not been previously described in the UK Asian paediatric population. To investigate whether South Asian families were offered genetic testing, the authors examined the UK MODY diagnostic lab database (containing records of all referrals for genetic testing for diabetes in the UK since 1996) and found that only 8 out of 1369 (0.5%) UK index case referrals for MODY testing were of South Asian origin (Porter, et al., 2006). These numbers suggest a low referral for genetic testing given that South Asians comprise 4% of the UK population and are four times more likely to have diabetes. A lower referral also seems paradoxical, as the clinically similar features at diabetes onset of type 2 diabetes and MODY in South Asians would be expected to lead to a higher rather than a lower referral for genetic testing. Most of the research studies on MODY in UK or elsewhere have predominantly consisted of subjects with European

ancestry, and there is a lack of studies in other ethnic groups. Clinical and biochemical characteristics of South Asian individuals with MODY have not been described. Therefore, one reason for the low referral could be the lack of awareness among clinicians that MODY occurs in South Asians or that which South Asians might have MODY.

Three studies have examined MODY in South Asians residing in India (Anuradha, et al., 2011; Radha, et al., 2009; Sahu, et al., 2007). Details of the number of subjects used in these studies, genes sequenced and variants identified are provided in **Table 5.1**. These studies sequenced patients clinically classified as having MODY for variants in the common MODY genes (*HNF1A, HNF4A* and *GCK*). Anuradha and Radha selected patients for genetic sequencing if they had an age at diagnosis of diabetes ≤ 25 years, control of hyperglycaemia for a minimum period of 2 years without insulin, absence of ketonuria at any time and a three-generation family history of diabetes.

Study	Genes sequenced	Study design and number of subjects investigated	Results as reported	HNF1A/HNF4A-MODY subjects identified
Sahu *et al,* 2007	*HNF1A*	**96** young-onset type 2 diabetes patients tested for most common *HNF1A* mutation c.872dupC (previously described as P291fsinsC). Patients with strong clinical suspicion of having HNF1A-MODY (32 of total 96 subjects) sequenced for the entire *HNF1A* gene	**c.872dupC** was not detected in any patient. One *HNF1A* variant previously reported in association with MODY (**R200W**) and 7 previously described polymorphisms (**L17L, I27L, A98V, G288G, L459L, S487N, and T515T**) were identified among those sequenced for the entire *HNF1A* gene.	1 of 32 (3%) of those with strong clinical suspicion of HNF1A-MODY
Radha *et al* 2009	*HNF1A*	**96** patients clinically classified as having MODY sequenced for the entire *HNF1A* gene	Seven novel likely pathogenic (**-538G/C, R114C, V134V, R171G, E235Q, G245R and R263H**) and 2 previously reported *HNF1A* variants (**R271Q, A301T**) were identified. In addition, 2 novel (**-373C/T and V103M**) and 10 previously reported *HNF1A* polymorphisms (**L17L, I27L, A58A, T74T, D80E, A98V, G288G, L459L, N487S, IVS7+7A/G**) were identified.	9 of 96 (9.6%) clinically suspected MODY patients
Anuradha *et al* 2011	*HNF4A and GCK*	**87** patients from the same cohort as stated above in Radha *et al* 2009 (excluding the 9 HNF1A-MODY patients) sequenced for *HNF4A* and *GCK*	Three likely pathogenic *HNF4A* variants (**-1009 G/C, -129 T/C and -79 C/T**) and 8 previously reported polymorphisms (**T19T, A58A, T130I, V255M, rs2425637, rs2144908, rs1884614, rs3212195**) were reported.	3% of those clinically classified as having MODY

Table 5.1 Studies investigating MODY in South Asians

Subjects included in studies by Radha and Anuradha *et al* were recruited from the same cohort and there was an overlap of subjects. There was no overlap of subjects included in the study by Sahu *et al* and those used by Radha and Anuradha *et al*.

Similar clinical criteria (age of onset <25 years, family history of diabetes and BMI of 19.6±2.7 kg/m2) were used by Sahu *et al* for selection of patients for genetic sequencing.

The pathogenicity of *HNF1A* variants was assessed by co-segregation of the variant with MODY phenotype in the study by Sahu *et al*. Co-segregation analysis was performed for part of the variants in the study by Radha and Anuradha *et al*. Additionally, functional work was performed for two variants by Radha et al. A molecular diagnosis of MODY was made in 3-12% of those with a strong clinical suspicion of MODY. As the authors investigated patients clinically classified as having MODY, they could not determine the proportion of MODY found in the general diabetes South Asian population

5.1.2 Use of hsCRP as HNF1A-MODY biomarker in South Asians

As discussed in earlier chapters, HNF1A-MODY is associated with low hsCRP levels and hsCRP has been shown to have value as a biomarker for differentiating HNF1A-MODY and type 2 diabetes (McDonald et al., 2011; Owen et al., 2010; Thanabalasingham et al., 2011). However, the studies investigating hsCRP included mainly North European subjects and its potential role as a biomarker in other ethnic groups has not yet been investigated. Due to the clinically similar presentation of type 2 diabetes (young age at onset and lower BMI), diagnosing MODY is more challenging in South Asians compared with Europeans. It would be a great advantage if hsCRP could be used as a diagnostic adjunct in South Asian subjects.

Population studies have suggested that CRP levels differ among adults from different ethnic backgrounds (Shah, et al., 2010). CRP levels have been found to be higher in Indian Asians compared with Europeans (Chambers, et al., 2001; Forouhi, et al., 2001). Chambers *et al* observed that the difference in CRP between the two ethnic groups remained statistically significant after adjusting for age, smoking, heart disease and BMI but disappeared when adjusted for high waist-hip ratio or insulin resistance score in South Asians (Chambers, et al., 2001). It is not known if CRP levels would be higher or similar in South Asians with HNF1A-MODY compared with their European counterparts and also if CRP would provide similar discrimination between type 2 diabetes and HNF1A-MODY in South Asians to that seen in Europeans.

5.2 Aims

The aims of the research in this chapter are to:

1. evaluate whether low hsCRP can identify South Asian subjects at high risk of having HNF1A-MODY;
2. estimate a minimum prevalence of HNF1A-MODY in South Asians with diabetes diagnosed as young adults.

5.3 Subjects and Methods

5.3.1 Subjects

South Asian subjects with diabetes were selected from 3 independent cohorts.

These were the London Life Sciences Prospective Population study (LOLIPOP; a population-based study of multi-ethnic men and women ascertained from GP surgeries in West London, including both healthy controls and subjects with diabetes), the Young Diabetes in Oxford study (YDX; including subjects diagnosed with diabetes ≤45 years of age, ascertained from Oxfordshire, UK) and young South Asians with diabetes from Birmingham (comprising South Asian subjects diagnosed with diabetes ≤40 years of age). Detailed characteristics of these cohorts have been provided in chapter 2, section 2.1.

5.3.2 Study design

This study consisted of two parts. The study design is illustrated in **figure 5.1**.

1. Subjects selected for a low hsCRP or an age of onset of diabetes ≤30 years

In the first part of the study, subjects from the 3 cohorts listed above were selected for *HNF1A* sequencing based on the following two criteria: (a) early onset diabetes (age <50 years) with CRP ≤1.0 mg/L or (b) an age of onset of diabetes ≤30 years irrespective of CRP levels. Rationale for using CRP ≤1.0 mg/L was the previous observation of CRP ≤1.0 mg/L in 80% of HNF1A-MODY subjects (Owen et al., 2010).

Data (CRP levels, age at sampling, BMI, fasting glucose, HbA1c and lipid measures) were available for 749 subjects with diabetes from the LOLIPOP study.

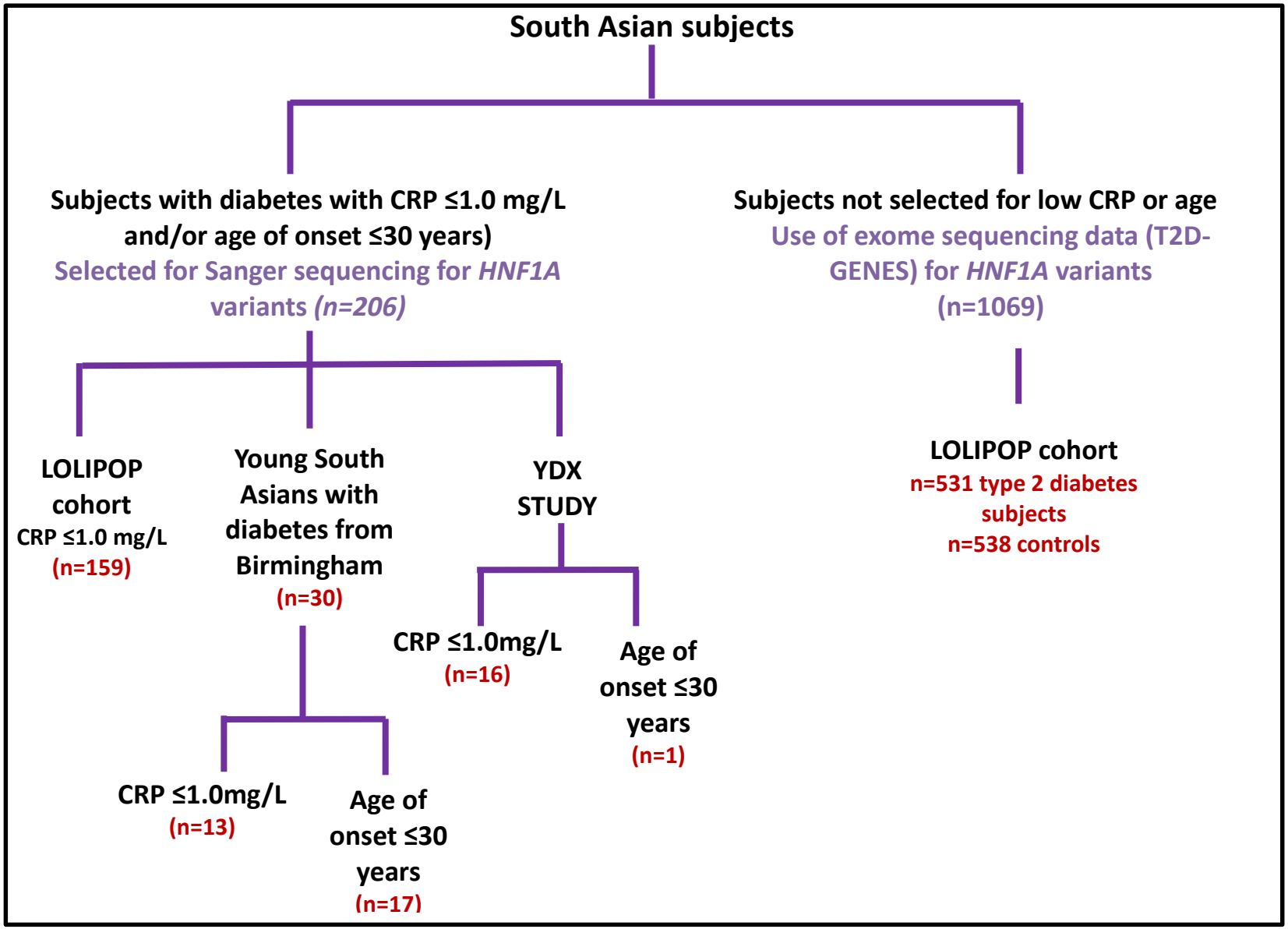


Figure 5.1. Summary of study design.

All these subjects had an age at sampling <50 years. Age at diagnosis of diabetes was not available for the LOLIPOP cases. Subjects with hsCRP ≤1.0mg/L (n=159) were selected for Sanger sequencing for *HNF1A*. Data were available for 40 South Asian subjects with young-onset diabetes from the YDX study and 17 subjects (16 subjects with CRP ≤1.0 mg/L and one subject with CRP >1.0mg/L but an age at diagnosis of diabetes ≤30 years) were selected for Sanger sequencing. Data were available for 72 South Asian subjects from Birmingham with young-onset diabetes and 30 subjects (13 subjects with CRP ≤1.0 mg/L and 17 subjects with CRP >1.0mg/L but an age at diagnosis of diabetes ≤30 years) were selected for sequencing.

Overall, 206 subjects were selected from 861 South Asian subjects for Sanger sequencing. The clinical and biochemical characteristics of subjects selected are given in **Table 5.2**.

2. Subjects not selected for a low CRP or age of onset of diabetes

As the first part of this study included Sanger sequencing of subjects predominantly having CRP ≤1.0mg/L, I wanted to determine whether using low CRP as the selection criteria identified most of the HNF1A-MODY cases. Moreover, I wanted to determine the prevalence of HNF1A-MODY in a group of South Asian subjects not selected for low CRP or age. For this, I used data available for South Asian subjects from a large-scale Exome sequencing project performed by the "Type 2 Diabetes Genetic Exploration by Next-generation sequencing in multi-Ethnic Samples (T2D-GENES)" consortium.

	Subjects with CRP ≤1.0 mg/L (n=188)			Subjects with age at diagnosis of diabetes ≤30 years n=18	
	LOLIPOP n=159	**Birmingham N=13**	**YDX n=16**	**Birmingham N=17**	**YDX n=1**
Age at sampling (years)*	45.3 (35.0-49.9)	39.0 (24.0-69.0)	49.4 (32.1-70.3)	41.0 (21.0-57.0)	25
Age of onset (years)*	NA	31 (20-39)	36 (26-45)	26 (19-30)	21
BMI (kg/m^2)	26.0 (23.9-28.7)	NA	24.9 (22.3-26.0)	NA	30.7
HbA1c (%)	7.2 (6.2-8.4)	NA	7.4 (6.6-8.3)	NA	6.0
Glucose (mmol/L)	7.6 (6.5-9.3)	NA	7.9 (5.5-8.8)	NA	4.5
HDL-Cholesterol (mmol/L)	1.16 (1.05-1.34)	NA	1.25 (1.00-1.45)	NA	1.30
Total Cholesterol (mmol/L)	4.9 (4.2-5.6)	NA	4.2 (3.8-4.7)	NA	4.7
Triglycerides (mmol/L)	1.51 (1.10-2.31)	NA	1.27 (0.71-1.60)	NA	1.40
HsCRP (mg/L)*	0.40 (0.00-1.00)	0.25 (0.02-0.87)	0.14 (0.01-0.84)	6.20 (1.77-28.46)	13.20

Table 5.2: Clinical and biochemical characteristics of South Asian subjects selected for Sanger sequencing. Data are presented as median (interquartile range). NA=data not available *=Criteria used for selection (Age at sampling, age at onset and hsCRP) are shown as median (range)

T2D-GENES comprises five major ancestry groups: African-American, East Asian, European, Hispanic and South Asian. The South Asian subjects included 531 subjects with diabetes and 538 controls without diabetes from the LOLIPOP cohort. Data were available for *HNF1A* variants identified in these subjects as well as hsCRP levels and clinical and biochemical characteristics. The clinical and biochemical characteristics of South Asian subjects from the LOLIPOP cohort used for exome sequencing are given in **Table 5.3**.

There was an overlap of 33 subjects with diabetes from the LOLIPOP cohort who underwent both Sanger and exome sequencing.

5.3.3 Methods

Details of Sanger and exome sequencing are provided in chapter 2 (section 2.2). *In-silico* assessment of variant effect on protein function was performed using bioinformatics tools: Sorting Intolerant From Tolerant (SIFT), Mutation Taster, CONsensus DELeteriousness score of missense single nucleotide variants (CONDEL) and Alamut (Ng and Henikoff, 2001) (Gonzalez-Perez and Lopez-Bigas, 2011) (Schwarz, et al., 2010) (http://www.interactive-biosoftware.com/alamut.html). Two splice prediction tools, MaxEntScan and GeneSplicer, were used to predict the potential effects on splicing of a non-coding variant (Pertea, et al., 2001; Yeo and Burge, 2004). Details of these programmes are provided in chapter 2 (section 2.2).

	Subjects without diabetes n=538	Subjects with diabetes n=531	P value
Age at sampling (years)*	66.1 (37.0-79.0)	53.6 (26.8-64.3)	<0.001
BMI (kg/m2)	26.6 (24.5-29.0)	26.8 (24.5-28.7)	0.39
HbA1c (%)	5.7 (5.3-6.0)	7.6 (6.6-9.0)	<0.001
Glucose(mmol/L)	5.2 (4.9-5.4)	8.3 (7.0-10.7)	<0.001
Total Cholesterol (mmol/L)	4.8 (4.1-5.7)	4.7 (4.0-5.6)	0.04
HDL-Cholesterol (mmol/L)	1.21 (1.05-1.41)	1.10 (0.98-1.33)	<0.001
Triglycerides (mmol/L)	1.37 (0.96-1.95)	1.70 (1.18-2.41)	<0.001
HsCRP (mg/L)*	2.1 (0.0-51.5)	2.0 (0.0-79.0)	0.28

Table 5.3. Clinical and biochemical characteristics of South Asian subjects from the LOLIPOP cohort used for exome sequencing as part of T2DGENES study. P value were determined by the Mann-Whitney U test. Data are presented as median (interquartile range). *=Data shown as median (range).

The variants were considered as “likely disease causing” or “pathogenic” based on the following criteria: co-segregation of variant with MODY phenotype when family members were available; previously published reports with supporting evidence of variant pathogenicity; presence of the variants resulting in a premature stop codon; variants likely to result in exon skipping (variants at canonical splice sites); rare, conserved missense variants with likely damaging consequence on protein function; non-coding variants with potential effect on splicing.

The Mann-Whitney U test was used to analyse the data. Computations were performed using IBM SPSS Statistics Version 20.0. and $P<0.05$ was considered significant.

5.4 Results

5.4.1 Variants identified through Sanger sequencing

Two hundred and six subjects (n=188 with CRP ≤1.0 mg/L and n=18 with an age at diagnosis of diabetes ≤30 years and CRP >1.0 mg/L) underwent Sanger sequencing for *HNF1A*. Seven *HNF1A* variants were found, all in subjects with CRP ≤1.0 mg/L. All variants were identified in one proband each. Details of variant position, bioinformatics prediction, conservation across species, assessment of amino acid change and hsCRP levels for the subjects carrying these variants are provided in **Table 5.4**. Among these were two previously reported and five novel *HNF1A* variants. One variant (G339S) was identified in a subject from the YDX study and access to family members was available.

Region	cDNA/ Protein level	Previously reported	SIFT Human Protein	Mutation taster	Condel	Conservation across species	Amino acid change	HsCRP (mg/L)	Interpretation
Exon 1	c.14delT p.L5Rfs17	Novel frameshift	N/A	Disease causing	N/A	N/A	This deletion creates a frame shift starting at codon Leu5. The new reading frame ends in a STOP codon 16 positions downstream. The mRNA produced might be targeted for NMD.	0.4	Likely pathogenic
Exon 2	c.307G>A p.V103M	Radha *et al* 2009	Damaging	Disease causing	Deleterious	Highly conserved nucleotide (phyloP: 4.97 [-14.1;6.4]). Highly conserved amino acid, up to Frog	Small physicochemical difference between Val and Met (Grantham dist.: 21 [0-215])	0.4	Likely pathogenic
Exon 4	c.872dupG p.292Rfs25	Novel frameshift duplication (1 bp) in exon 4	N/A	Disease causing	N/A	N/A	This duplication creates a frame shift starting at codon Gly292. The new reading frame ends in a STOP codon 24 positions down-stream. The mRNA produced might be targeted for NMD.	0.5	Likely pathogenic
Exon 5	c.1015G>A p.G339S	Novel missense	Tolerated	Polymorphism	Neutral	Weakly conserved nucleotide (phyloP: 0.69 [-14.1;6.4]). Moderately conserved amino acid	Small physicochemical difference between Gly and Ser (Grantham dist.: 56 [0-215])	0.8	Likely benign
Exon 6	c.1135C>T p.P379S	Bellanne-Chantelot *et al* 2008	Damaging	Disease causing	Deleterious	Highly conserved nucleotide (phyloP: 5.37 [-14.1;6.4]). Highly conserved amino acid, up to Frog	Moderate physicochemical difference between Pro and Ser (Grantham dist.: 74 [0-215])	0.8	Likely pathogenic
Exon 8	c.1544C>T p.T515M	Novel missense	Damaging	Disease causing	Deleterious	Moderately conserved nucleotide (phyloP: 3.76 [-14.1;6.4]). Highly conserved amino acid, up to Chicken	Moderate physicochemical difference between Thr and Met (Grantham dist.: 81 [0-215])	ND	Likely pathogenic
Exon 10	c.1751T>C p.L584P	Novel missense	Damaging	Disease causing	Neutral	Moderately conserved nucleotide (phyloP: 2.22 [-14.1;6.4]). Highly conserved amino acid, up to Frog	Moderate physicochemical difference between Leu and Pro (Grantham dist.: 98 [0-215])	0.1	Likely pathogenic

Table 5.4. List of *HNF1A* variants found by Sanger sequencing in South Asians

PhyloP=a measure of evolutionary conservation for the specific base base, Grantham distance=a chemical distance that measures difference between two amino acids taking into account their volume, polarity and composition of side chain, NMD=nonsense mediated decay, PhyloP score and Grantham distance were calculated using bioinformatic software Alamut. Conservation of species was assessed considering 11 species (Human, Chimp, Orangutan, Macaque, Rat, Mouse, Dog, Cat, Cow, Chicken and Frog). N/A= Not available (As SIFT and CONDEL predict missense variants only, so prediction was not available for frameshift variants), ND=not detected, below the lower limit of detection of the assay

All other variants were identified from subjects in the LOLIPOP cohort and access to family members was not available. None of the variants was identified in the dataset of South Asian subjects from Birmingham.

A. Previously reported variants

1) V103M

One of the missense variants, V103M, was previously identified by Radha *et al* in six unrelated South Asian patients with diabetes and was labelled by these authors as a “polymorphism” (Radha et al., 2009). It is not clear why this variant was considered as a “polymorphism” as the authors did not provide any information on co-segregation (or lack thereof) of the variant with the disease phenotype in the family members nor was the functional effect of the variant assessed with laboratory approaches. *In-silico* analysis performed in the current study (using SIFT, CONDEL, Mutation Taster and Alamut) predicted the variant to be likely “disease causing”. HsCRP level for this variant was 0.4 mg/L.

Based on bioinformatics prediction, V103M was considered as likely pathogenic in the current study.

2) P379S

The second variant, P379S, has been reported as disease-causing in a large French study describing the spectrum of *HNF1A* mutations identified in 356 unrelated HNF1A-MODY patients (Bellanne-Chantelot et al., 2008). The variant P379S was identified in two unrelated subjects. Information for the co-segregation

of the variant with the disease phenotype in the family members was not provided. The authors stated that all *HNF1A* variants identified in their study were considered as pathogenic based on the following criteria: nature of the amino acid change, conservation of the residue across species, absence of the variant in 300 control subjects of Euro-Caucasian origin and co-segregation of the variant with young-onset diabetes, when relatives were available. *In-silico* analysis performed in the current study predicted the variant to be “likely disease causing”. The hsCRP level for this variant was 0.8 mg/L. Based on bioinformatics prediction, P379S was considered as likely pathogenic in the current study.

B. Novel variants

1) L584P and T515M

Two novel missense variants, L584P and T515M, were predicted as likely pathogenic by bioinformatics (**Table 5.4**). HsCRP was 0.1 mg/L and undetectable respectively, in subjects carrying these variants.

2) c.14delT and c.872dupG

The other two novel variants, c.14delT and c.872dupG, comprise a single base deletion and duplication respectively, resulting in frameshifts. These variants result in a STOP codon 16 and 24 positions down-stream respectively, and hence are highly likely to affect protein synthesis. Both variants were considered as likely pathogenic. HsCRP levels were 0.4 and 0.5 mg/L respectively.

3) G339S

One of the novel variants, G339S, identified in a subject from the YDX study, was predicted as likely benign by all bioinformatics programs used (**Table 5.4**). Family data were also available and the family tree is illustrated in **Figure 5.2**. The proband has a strong three-generation and bilineal family history of diabetes. Both parents and brother of the proband have been diagnosed with diabetes, with her mother diagnosed at a younger age and lower BMI than her father. All family members are overweight or obese. The proband's mother, father and sister were tested for the variant (G339S). Her brother was not available for the study. The mother and sister of the proband were found to carry the same *HNF1A* variant as identified in the proband. The proband's sister (current age 29 years) does not have diabetes (HbA1c=44mmol/mol). There was no history of sensitivity to Sulphonylureas for any of the family members. Overall, the family data were not conclusive. Both the proband and her mother have a young age at diagnosis of diabetes suggestive of MODY. However, the presence of an unaffected relative with the same variant is less supportive of a MODY phenotype. Diabetes develops by age 25 years in 63% of *HNF1A* mutation carriers and by age 50 years in 93% (Shepherd, et al., 2001). However, it is possible that the sister could develop diabetes at a later age. HsCRP level for this variant was 0.8 mg/L. Based on the bioinformatics prediction, the variant (G339S) was considered as "likely benign".

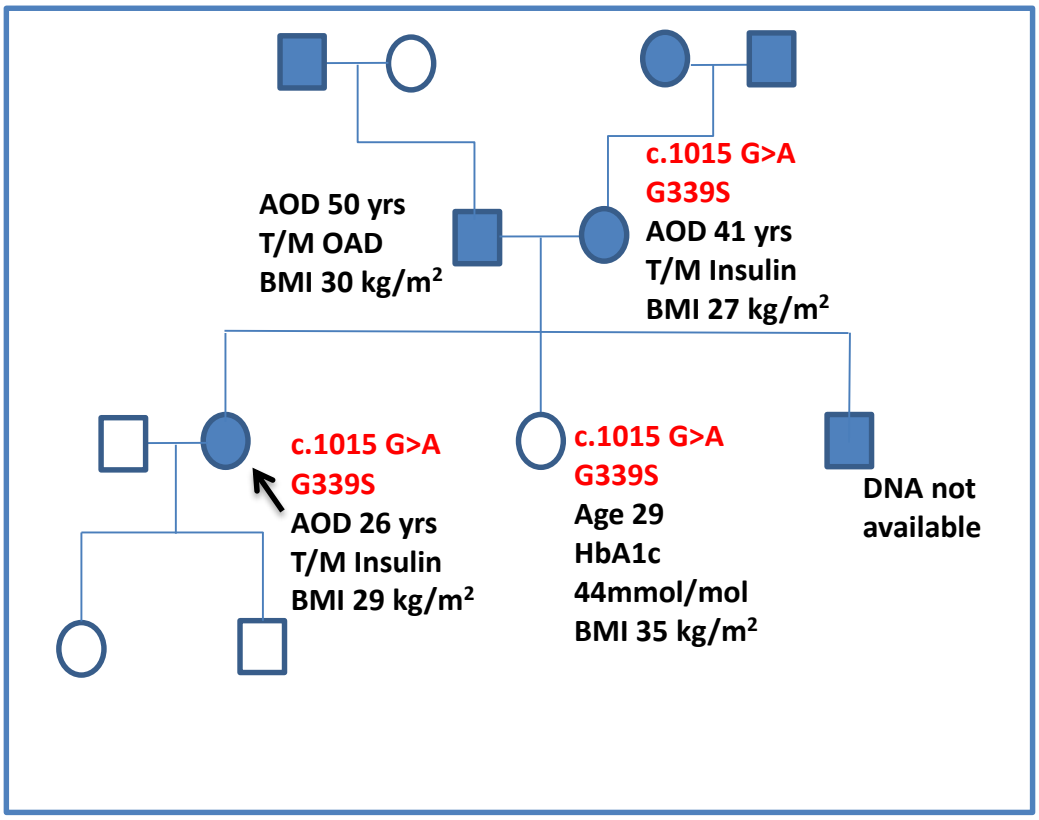


Figure 5.2 Pedigree of South Asian subject carrying *HNF1A* variant p.G339S
AOD=age of diagnosis, T/M=diabetes treatment, OAD=oral antidiabetic agent, BMI=body mass index

This provides a minimum prevalence of HNF1A-MODY of 0.7% (6 out of 861 subjects [95% CI 0.3%-1.5%]) among South Asians diagnosed as having diabetes by the age of 50 years. The characteristics of the subjects with and without likely pathogenic *HNF1A* variants are shown in **Table 5.5**

5.4.2 Variants identified through exome sequencing

Out of 1069 South Asian subjects undergoing exome sequencing, 36 *HNF1A* variants with MAF of <2% in South Asians were identified. Out of the 36 variants identified there were 12 non-coding (2 upstream, 1 variant within 10 base pairs of exon-intron junction, 6 intronic, 2 downstream), 11 synonymous and 13 non-synonymous missense variants. Details of variant position, bioinformatics prediction, conservation across species, assessment of amino acid change, number of cases and controls with these variants and associated hsCRP levels are provided in **Tables 5.6** (non-synonymous variants), **5.7** (synonymous variants) and **5.8** (non-coding variants). None of the non-coding variants was in the canonical splice nucleotides. One of the non-coding variants was within 10 base pairs of exon-intron junction and bioinformatics prediction for this variant is provided below. Synonymous variants were considered as “likely benign”. Variants in non-coding regions more than 10 base pairs away from the exon intron junction and synonymous variants were not included for further analysis in this study. Family data were not available for any of the variants identified through exome sequencing.

	Subjects with diabetes with CRP ≤1.0 (mg/L) and without any likely pathogenic *HNF1A* variants n=182	P value**	HNF1A-MODY n=6	P value***	Subjects with diabetes not sequenced for *HNF1A* n=655
Age at sampling (years)*	45.5 (24.0-70.0)	0.52	45.4 (35.8-48.0)	0.29	44.6 (30.6-70.9)
BMI (kg/m^2)	26.3 (23.9-28.7)	0.18	25.5 (20.3-26.2)	0.004	29.1 (26.3-32.9)
Fasting glucose (mmol/L)	7.6 (6.4-9.3)	0.27	9.6 (6.9-11.6)	0.74	8.4 (7.2-11.1)
HbA1c (%)	7.3 (6.2-8.4)	0.40	8.0 (6.4-9.0)	0.88	7.8 (6.7-9.2)
Total Cholesterol (mmol/L)	4.8 (4.1-5.5)	0.42	5.3 (4.6-5.7)	0.96	5.1 (4.3-6.0)
Triglycerides (mmol/L)	1.49 (1.11-2.24)	0.66	1.42 (0.88-2.19)	0.13	1.93 (1.34-2.90)
HDL (mmol/L)	1.17 (1.04-1.34)	0.23	1.35 (1.08-1.77)	0.05	1.10 (0.97-1.28)
Insulin (pmol/L)	12.0 (7.3-17.9)	0.22	8.8 (2.0-11.3)	0.03	16.2 (11.0-24.5)
HsCRP (mg/L)*	0.4 (0.0-1.0)	0.50	0.4 (0.0-0.8)	3.00×10^{-4}	4.75 (0.0-166.0)

Table 5.5. Clinical and biochemical characteristics of South Asian subjects with likely pathogenic *HNF1A* variants (HNF1A-MODY), without *HNF1A* variants and those that were not selected for Sanger sequencing. Data are presented as median (interquartile range) unless stated otherwise. *Data are shown as median (range). P values were calculated with the use of the Mann–Whitney U test. **represents P value for subjects without any likely pathogenic *HNF1A* variants vs. HNF1A-MODY, ***represents P value for subjects not sequenced for *HNF1A* vs. HNF1A-MODY. HNF1A-MODY subjects do not include variant (G339S) considered as likely benign.

Region	Variant (cDNA/ Protein level)	1000 Genomes /EVS/ Previously reported	hsCRP mg/L	Age*	Subjects	SIFT Human Protein	MT	CONDEL	Conservation across species	Amino acid change	Interpretation
Missense variants											
Exon 1	c.290C>T p.A97V	R/R/NR	2.1	70	1 control	Damaging	Disease causing	Deleterious	Moderately conserved nucleotide (phyloP: 3.35 [-14.1;6.4]). Highly conserved amino acid, up to Frog	Small physicochemical difference between Ala and Val (Grantham dist.: 64 [0-215])	Likely benign[δ]
Exon 2	c.467C>T p.T156M	R/R/NR	1.0	65	1 control	Damaging	Disease causing	Deleterious	Weakly conserved nucleotide (phyloP: 1.82 [-14.1;6.4])	Moderate physicochemical difference between Thr and Met (Grantham dist.: 81 [0-215])	Likely benign[δ]
Exon 4	c.872C>A p.P291Q	NR/NR/NR	0.6-10 1.3-5.9	51-64 50-74	3 controls 4 cases	Tolerated	Polymorphism	Neutral	Weakly conserved nucleotide (phyloP: 1.98 [-14.1;6.4]). Moderately conserved amino acid	NA	Likely benign
Exon 6	c.1190A>G p.N397S	NR/NR/NR	9.0	53	1 control	Tolerated	Polymorphism	Neutral	Not conserved nucleotide (phyloP: -0.12 [-14.1;6.4]). Moderately conserved amino acid	Small physicochemical difference between Asn and Ser (Grantham dist.: 46 [0-215]).	Likely benign
Exon 7	c.1313T>C p.L438P	NR/NR/NR	2.3	51	1 case	Damaging	Disease causing	Deleterious	Highly conserved nucleotide (phyloP: 4.48 [-14.1;6.4]). Highly conserved amino acid	Moderate physicochemical difference between Leu and Pro (Grantham dist.: 98 [0-215]).	Likely pathogenic
Exon 7	c.1322C>A p.T441K	R/R/NR	3.4 NA	71 65	2 controls	Tolerated	Disease causing	Neutral	Weakly conserved nucleotide (phyloP: 1.50 [-14.1;6.4]). Highly conserved amino acid, up to Chicken	Moderate physicochemical difference between Thr and Lys (Grantham dist.: 78 [0-215])	Likely benign
Exon 8	p.L518F	NR/NR/NR	1.9	66	1 control	Tolerated	NA	Neutral	NA	NA	NA
Exon 8	c.1571T>C p.I524T	NR/NR/NR	1	43	1 control	Tolerated	Disease causing	Deleterious	Highly conserved nucleotide (phyloP: 4.64 [-14.1;6.4]). Highly conserved amino acid	Moderate physicochemical difference between Ile and Thr (Grantham dist.: 89 [0-215])	Uncertain significance

Exon 8	c.1605C>A p.S535R	NR/NR/NR	7.2	54	1 case	Damaging	Disease causing	Deleterious	Weakly conserved nucleotide (phyloP: 1.34 [-14.1;6.4]). Highly conserved amino acid	Moderate physicochemical difference between Ser and Arg (Grantham dist.: 110 [0-215])	Likely pathogenic
Exon 9	c.1693C>G p.L565V	NR/NR/NR	5.4	73	1 control	Tolerated	Polymorphism	Neutral	Weakly conserved nucleotide (phyloP: 1.17 [-14.1;6.4]). Weakly conserved amino acid	Small physicochemical difference between Leu and Val (Grantham dist.: 32 [0-215])	Likely benign
Exon 9	c.1748G>A p.R583Q	R/R/R (Owen et al. 2003, Urhammer et al. 1997)	0.1	70	1 control	Tolerated	Polymorphism	Neutral	Weakly conserved nucleotide (phyloP: 1.34 [-14.1;6.4]). Highly conserved amino acid	Small physicochemical difference between Arg and Gln (Grantham dist.: 43 [0-215])median: 4.32)	Likely benign
Exon 9	c.1751T>C p.L584P	NR/NR /NR	0.1	48	1 case	Damaging	Disease causing	Neutral	Moderately conserved nucleotide (phyloP: 2.22 [-14.1;6.4]). Highly conserved amino acid	Moderate physicochemical difference between Leu and Pro (Grantham dist.: 98 [0-215])	Likely pathogenic
Exon 10	c.1816G>A p.G606S	NR/NR/R (Colclough *et al* 2013) (Thanabalasingham et al 2012)	NA	52	1 case	Tolerated	Polymorphism	Neutral	Weakly conserved nucleotide (phyloP: 1.42 [-14.1;6.4]). Moderately conserved amino acid	Small physicochemical difference between Gly and Ser (Grantham dist.: 56 [0-215])	Likely benign

Table 5.6: List of missense variants identified through Exome sequencing in South Asian subjects

Age*=Age at sampling, EVS=Exome Variant Server, [#]=Base change not available, R=Reported, NA=Not available, NR=Not reported, MT=Mutation Taster, PhyloP=a measure of evolutionary conservation for the specific base, Grantham distance=a chemical distance that measures difference between two amino acids taking into account their volume, polarity and composition of side chain, NMD=nonsense mediated decay. PhyloP score and Grantham distance were calculated using bioinformatics software Alamut. Conservation of species was assessed considering 11 species (Human, Chimp, Orangutan, Macaque, Rat, Mouse, Dog, Cat, Cow, Chicken and Frog). For variants identified in multiple cases and controls, age at sampling and CRP levels are shown as "range", [δ]=variants considered as likely benign as the subjects had not developed diabetes by the age of 65 and 70 years.

Region	Variant (cDNA/ Protein level)	1000 Genomes/ EVS	hsCRP level (mg/L)	Subjects	Amino acid change	Interpretation
Synonymous variants						
Exon 1	c.150C>T/p.C50C	R/NR	5.5	1 case	Synonymous substitution	Likely benign
Exon 1	c.153C>T/p.G51G	R/NR	8.2	1 control	Synonymous substitution	Likely benign
Exon 1	c.204G>A/p.R68R	NR/NR	1.5	1 case	Synonymous substitution	Likely benign
Exon 2	c.402C>T/p.V134V	NR/NR	0.1	1 control	Synonymous substitution	Likely benign
Exon 2	c.519G>A/p.V173V	NR/NR	0.1-8.8 5.0	3 cases 1 control	Synonymous substitution	Likely benign
Exon 3	c.663G>A/p.Q221Q	NR/NR	0.3	1 control	Synonymous substitution	Likely benign
Exon 5	c.1062G>A/p.T354T	R/R	NA	1 control	Synonymous substitution	Likely benign
Exon 6	c.1206C>T/p.N402N	NR/NR	2.1	1 control	Synonymous substitution	Likely benign
Exon 7	c.1464C>G/p.P488P	NR/NR	2.8	1 control	Synonymous substitution	Likely benign
Exon 7	c.1491G>A/p.Q497Q	NR/NR	0.1	1 control	Synonymous substitution	Likely benign
Exon 9	c.1659C>T/p.S553S	NR/NR	2.5 4.6	2 controls	Synonymous substitution	Likely benign

Table 5.7: List of Synonymous variants identified through Exome sequencing in South Asian subjects

EVS=Exome Variant Server, R=Reported, NA=Not available, NR=Not reported. For variants identified in multiple cases and controls, age at sampling and CRP levels are shown as "range"

Region	Variant (cDNA level*)	hsCRP level (mg/L)	Age at sampling (years)	No of subjects	Interpretation
Upstream variants					
Upstream substitution	c.-40	0.2	46	1 case	NA
5' UTR substitution	c.-4	0.4-10.2 0.2-5.3	42-59 44-74	6 cases 7 controls	NA
Intronic Variants					
Intron 2	c.526+17	0.3	58	1 case	NA
Intron 3	c.713+14	0.7 0.2	50 46	1 control 1 case	NA
Intron 5	c.1108-27 (Het)	0.2-8.8 NA	46-74 67	4 cases 1 control	NA
Intron 5	c.1108-27 (Hom)	4.7	62	1 case	NA
Intron 7	c.1502-39	1	46	1 control	NA
Intron 8	c.1624-44	5.9	NA	1 control	NA
Intron 9	c.1768+44	0.3-2.4 0.1-51.5	54-60 40-73	3 cases 6 controls	NA
Intron 9	c.1769-6	5.0	59	1 case	Likely benign
Downstream variants					
3' UTR substitution	c.40	0.3	56	1 case	NA
Downstream substitution	c.2917	3.5	70	1 control	NA

Table 5.8: List of variants in non-coding DNA identified through exome sequencing in South Asian subjects
For variants identified in multiple cases and controls, age at sampling and CRP levels are shown as "range". NA=not available.

Details of missense variants identified are given below.

A. Missense variants identified in non-diabetic controls only

1) **A97V**

A97V was identified in one control, euglycaemic at 70 years. This variant was predicted as “likely disease causing” by bioinformatics. The hsCRP was 2.1 mg/L. This variant has been reported in Exome variant server (African American ancestry, MAF=0.02).

2) **T156M**

T156M was identified in one control, euglycaemic at 65 years. This variant was predicted as “likely disease causing” by bioinformatics. The hsCRP was 1.0 mg/L. This variant has been reported in EVS (African American ancestry, MAF=0.02).

3) **N397S**

N397S was identified in one control, euglycaemic at 53 years. This variant was predicted as “likely benign” by bioinformatics. The hsCRP was 9.0 mg/L. This variant has not been reported previously.

4) **T441K**

T441K was identified in two controls, euglycaemic at age 71 and 65 years. This variant was predicted as “likely disease causing” by mutation taster and “likely benign” by SIFT and CONDEL. The hsCRP was 3.4 mg/L in one

subject and was not available for the second subject. This variant has been reported in EVS (European American ancestry, MAF=0.01).

5) L518F

L518F was identified in one control, euglycaemic at 66 years. The hsCRP was 1.9 mg/L. This variant was predicted as “likely benign” by bioinformatics. It has not been reported previously.

6) I524T

I524T was identified in one control, euglycaemic at 43 years. The hsCRP was 1.0 mg/L. This variant was predicted as “likely benign” by SIFT and as “likely disease causing” by mutation taster and CONDEL. It has not been reported previously.

7) L565V

L565V was identified in one control, euglycaemic at 73 years. The hsCRP was 5.4 mg/L. This variant was predicted as “likely benign” by bioinformatics. It has not been reported previously.

8) R583Q

R583Q was identified in one control, euglycaemic at 70 years. This variant was predicted as “likely benign” by bioinformatics. The hsCRP was 0.1 mg/L. R583Q has been previously reported in association with MODY,

identified in one British and two Danish subjects, but in these reports was not associated with a typical MODY phenotype (Owen, Stride, Ellard, & Hattersley, 2003; Urhammer et al., 1997). R583Q has also been reported in 1000 Genomes and Exome Variant Server database (EVS: reported in European American ancestry, MAF=0.06).

Out of the above eight variants, seven (A97V, T156M, N397S, T441K, L518F, L565V and R583Q) were identified in subjects euglycaemic at age more than 50 years. Two variants, A97V and T156M, were predicted as likely pathogenic and five variants, N397S, T441K, L518F, L565V and R583Q, were predicted as likely benign by bioinformatics. The penetrance of *HNF1A* mutations for causing a MODY phenotype has been estimated to be 63% by age 25, 93.6% by age 50 and 98.7% by age 75 (Shepherd et al., 2001). The fact that these subjects had not developed diabetes by the age of 50 does not favour these variants as being pathogenic. Therefore, these variants were considered as unlikely to be pathogenic in this study. Having said that, as discussed in chapter 1, *HNF1A* variants map across a broad spectrum of functional and clinical severity. *HNF1A* variants range from neutral variants, to those associated with an increased risk of type 2 diabetes and finally the variants leading to a MODY phenotype (Flannick, et al., 2013; Miedzybrodzka, et al., 1999; Voight, et al., 2010; Weedon, et al., 2005; Winckler, et al., 2007). Even the variants that result in a MODY phenotype in certain members of a family can display reduced penetrance and consequently a lack of a MODY phenotype in others family members carrying the same variant

(Miedzybrodzka, et al., 1999). Therefore, it is possible that some of the above listed rare, conserved variants identified in non-diabetic controls display reduced penetrance in this study and may be found to be associated with a MODY phenotype in other families.

One missense variant, I524T, was identified in a subject without diabetes with age at sampling 43 years and predicted as likely pathogenic by Mutation Taster and CONDEL and as neutral (likely benign) by SIFT. Given the prediction by two bioinformatics tools, for this variant, as being likely pathogenic and the possibility that this subject could still develop young-onset diabetes, no conclusive decision could be made on the functional effect of this variant. Functional studies are required to further determine the molecular impact of this variant.

B. Missense variant identified in cases and controls

One missense variant, P291Q, was identified in three cases and four non-diabetic controls. The variant was predicted as “likely benign” by bioinformatics. HsCRP levels ranged from 0.6-10.1 mg/L in cases and from 1.3-5.9 mg/L in controls. This variant has not been reported previously.

C. Missense variants identified only in cases

Four missense variants were identified in subjects with diabetes.

1) **L438P**

L438P was identified in one subject with diabetes. The hsCRP was 2.3

mg/L. This variant was predicted as “likely disease causing” by all bioinformatics programs. It has not been reported previously.

2) S535R

S535R was identified in one subject with diabetes. The hsCRP was 7.2 mg/L. This variant was predicted as “likely disease causing” by all bioinformatics programs. It has not been reported previously.

3) L584P

L584P was common to both the Sanger sequencing and exome sequencing experiments and has been described in section 5.4.1.

4) G606S

G606S was identified in one subject with diabetes. HsCRP was not available. This variant was predicted as “likely benign” by all bioinformatics programs. G606S has been previously reported as a potentially MODY causing variant in a South Asian subject from the YDX study (Thanabalasingham et al., 2012). The patient had a two-generation family history of young-onset type 2 diabetes with long periods of control on sulphonylureas (Thanabalasingham et al., 2012). DNA testing of the family members was not performed. Based on the bioinformatics prediction and inconclusive information available from the previous report, this variant was considered as “likely benign” in the current study.

Seven out of 13 missense *HNF1A* variants in the exome sequencing study were identified in *HNF1A* exons 8-10. Variants in these exons have been proposed to have reduced penetrance with diabetes presenting at a later age compared with variants in exons 1-7 (Harries, et al., 2006). Three of the seven *HNF1A* variants identified in exons 8-10 in this study were in subjects with diabetes of which two were predicted as likely pathogenic. As age at diagnosis of diabetes was not available for any of the subjects, it is not known if subjects with variants in exons 8-10 were diagnosed at a later age.

D. Splice variant

1) c.1769-6 C>G

Out of the 12 non-coding variants, only one variant, c.1769-6 C>G, was within 10 base pairs of exon-intron junction. It was identified in a subject with diabetes. HsCRP was 5.0 mg/L. *In-silico* analysis for this variant was carried out using two splice prediction tools, MaxEntScan and GeneSplicer (Pertea, et al., 2001; Yeo and Burge, 2004). Both tools predicted a small drop in the affinity of the splicing machinery for the altered splice acceptor site. The MaxEntScan score dropped from 7.35 to 6.83, whereas the score from GeneSplicer dropped from 9.44 to 8.30. Based on the bioinformatics prediction, this variant was considered as unlikely to affect splicing. However, this variant is located in the polypyrimidine tract, a regulatory signal required for spliceosome assembly and efficient intron splicing, and potential effect on splicing cannot be definitively ruled out.

The location of all likely pathogenic *HNF1A* variants identified through Sanger and exome sequencing within the 10 exons of the gene are illustrated in **figure 5.3**.

Overall, in this second group of South Asian subjects that had not been selected for low CRP, three likely pathogenic *HNF1A* variants were identified in three subjects with diabetes. This gives a proportion of HNF1A-MODY of 0.5% (95% CI 0.1%-1.7%) amongst those with apparent type 2 diabetes. Two out of the three subjects had CRP>1.0 mg/L.

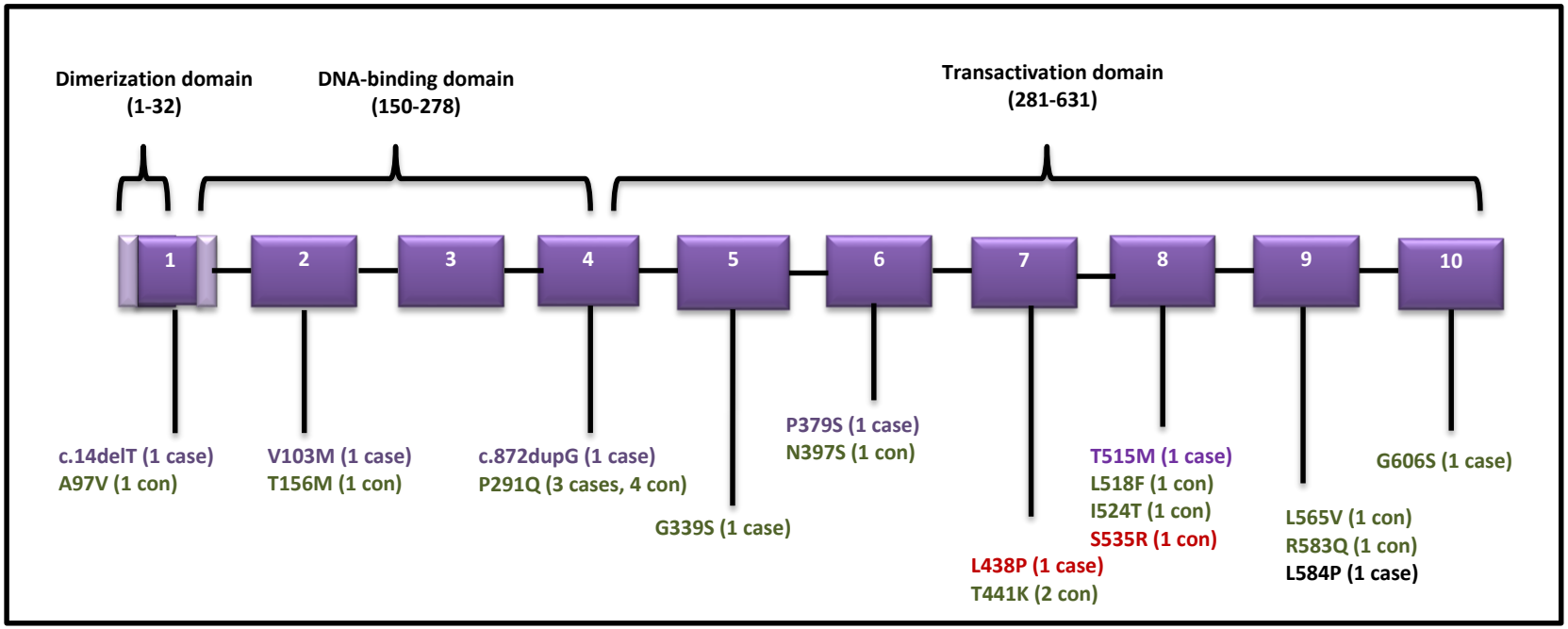


Figure 5.3: Location of all *HNF1A* variants identified within the 10 exons of the gene in South Asian subjects. The functional domains of *HNF1A* are shown with numbers in brackets representing the codons. **Likely pathogenic *HNF1A* variants** identified through Sanger sequencing are shown in purple. **Likely pathogenic *HNF1A* variants** identified through exome sequencing are shown in red. **One likely pathogenic *HNF1A* variant identified through both exome and Sanger sequencing** is shown in black. **Variants not likely to have a functional effect,** identified through both exome and Sanger sequencing, are shown in green. Numbers and text in the brackets next to the variants, represent the number of subjects with diabetes (cases) and without diabetes (con) carrying these variants.

5.5 Discussion

Low CRP has been identified as a useful biomarker for HNF1A-MODY in various studies including mainly European subjects, but has not been investigated in other ethnic groups (McDonald, et al., 2011b; Owen, et al., 2010; Thanabalasingham, et al., 2011). In this study, I evaluated the use of low CRP for identifying South Asians subjects at high risk of having HNF1A-MODY, an ethnic group with high type 2 diabetes prevalence in young, lean subjects making differentiation from MODY challenging.

This study consisted of two parts. In the first part, South Asian subjects at high risk of having HNF1A-MODY, determined mainly by CRP levels ≤1.0 mg/L or in a few subjects using the age of onset of diabetes <30 years irrespective of CRP levels, were selected for Sanger sequencing. Six likely pathogenic *HNF1A* variants were identified, giving a proportion of HNF1A-MODY of 0.7% (95% CI 0.3%-1.5%) amongst those with apparent type 2 diabetes. A prevalence of MODY of 4% has been previously observed among Europeans clinically diagnosed as having apparent type 2 diabetes by the age of 45 years (Thanabalasingham, et al., 2012b).

One reason for this observation could simply be the approximately four times higher prevalence of type 2 diabetes found in South Asians compared with Europeans (Khunti, et al., 2013; Mather and Keen, 1985). If the overall population prevalence of HNF1A-MODY in South Asians is the same as in Europeans, the larger numbers of people with type 2 diabetes would lead to an approximately four times lower proportion of MODY in a given subset of

South Asian subjects with diabetes, as observed in this study. Secondly, it is possible that due to concurrent insulin resistance, HNF1A-MODY South Asians have higher CRP levels than those observed in Europeans and by using CRP ≤1.0 mg/L, some of the MODY subjects have been missed.

As the first part of the study involved sequencing of subjects mainly with low CRP, I wanted to understand if using low CRP meant I had found most of the HNF1A-MODY cases. For this, I used exome sequencing results from a group of South Asian subjects that had not been selected for low CRP. In the exome sequencing, three likely pathogenic *HNF1A* variants were identified giving a proportion of HNF1A-MODY of 0.5% (95% CI 0.1%-1.7%) amongst those with apparent type 2 diabetes. Two out of three subjects had CRP>1.0 mg/L, indicating that we might have missed HNF1A-MODY by using low CRP in the first part of the study. However, using a higher CRP cut-off would also have led to testing of a larger number of subjects with type 2 diabetes. It is worth noting that if the lower cut-off of CRP of 0.5mg/L were used in the first group of subjects, five out of 6 “likely disease causing” *HNF1A* variants would have been picked up avoiding testing of 41% of the subjects.

The findings of this study suggest that using CRP alone is not sufficient for identifying potential HNF1A-MODY cases. Even if we did not ascertain all the HNF1A-MODY cases with our approach, the true number of cases is likely to be small and there would be a large number of false positives who would undergo sequencing. Characteristics such as lower BMI, younger age at diagnosis, lower HbA1c, parent with diabetes or not being treated with oral

hypoglycaemic agents or insulin have been shown to be useful to discriminate MODY from type 2 diabetes in Europeans (Shields, et al., 2012). Similarly, additional relevant phenotypic information in South Asian subjects, in addition to low CRP, is likely to be needed to improve identification of HNF1A-MODY cases.

The low proportion of HNF1A-MODY subjects identified in this study indicates that the study did not have an adequate sample size for estimation of sensitivity and specificity of CRP as a HNF1A-MODY biomarker in South Asians. Using the Burderer formula (for calculating sample size for diagnostic test), a significance level of 0.05, confidence interval of 95%, previously reported sensitivity of hsCRP of 83% (Thanabalasingham, et al., 2011), prevalence of HNF1A-MODY of 0.5-0.7% amongst those with apparent type 2 diabetes observed in this study and a study precision of 0.1, a very large sample size of approximately 7,740 South Asian subjects with diabetes would be needed to adequately assess the performance of CRP as HNF1A-MODY biomarker in South Asians. This sample size estimation again suggests that additional clinical or biochemical features such a lower BMI or a lower age at diagnosis would be needed to enrich dataset of South Asian subjects for HNF1A-MODY, to decrease the sample size for a future study evaluating hsCRP as biomarker.

This study also adds to the increasing evidence of challenges faced while predicting the functional effect of novel variants. For example, during analysis of exome sequencing data, two missense variants predicted as likely disease

causing by bioinformatics tools were identified in subjects euglycaemic at the age of 65 and 70 years. Another highly conserved missense variant was identified in a subject not diagnosed with diabetes by the age of 43 years. Lack of a HNF1A-MODY phenotype has been previously reported within members of the same family (euglycaemic at age of 87 and 46 and carrying the same mutation as those expressing a HNF1A-MODY phenotype) (Miedzybrodzka, et al., 1999). A recent study by Flannick *et al* reported similar observations of identifying *HNF1A* variants known as causal for MODY or rare, conserved and protein damaging variants in the asymptomatic general population (Flannick, et al., 2013). Similar findings have been observed in other Mendelian diseases and in large-scale sequencing studies where asymptomatic individuals have been shown to harbour a surprisingly large number of loss of function (MacArthur, et al., 2012) or potentially deleterious missense variants (Genomes Project, et al., 2010; Xue, et al., 2012). Incomplete penetrance is one of the several possible explanations for these observations, leading to mild or no effects on health, despite the presence of a conserved putative pathogenic variant (Cassa, et al., 2013; Cooper, et al., 2013).

All these observations suggest that there is marked heterogeneity in the clinical phenotype of HNF1A-MODY variants, hence complicating assessment of causality of novel variants. The causality of novel variants can be investigated through phenotypic analysis (such as age of diagnosis, clinical history of a glycaemic improvement on Sulphonylureas, levels of biomarkers), use of bioinformatics, segregation in family members and molecular studies. One of the limitations of this study was unavailability of detailed clinical

phenotype and access to family members for most of the variants identified. Therefore, the likely functional effect of the variants was predicted using bioinformatics tools, which have limitations. As observed in this study, for some of the variants, there was inconsistency in the results of the bioinformatics tools used. Functional studies are being carried out by members of my supervisor's research group to further determine the role of the novel missense *HNF1A* variants identified in this study. These functional studies comprise of following: assessment of HNF1A transcriptional activity by using a luciferase reporter assay; quantifying *HNF1A* protein expression (total, cytoplasmic and nuclear) by western blot analysis; describing cellular localisation of variant proteins using immunofluorescence and determining the effect of variants in the DNA binding or dimerization domains on the ability of the transcription factor to bind DNA (by using DNA binding assays).

Another limitation of this study is that non-coding variants (except c.1769-6) and synonymous variants were excluded from analysis. Both variant types can affect splicing by modifying auxiliary exonic splicing elements and by excluding these variants from analysis potential causal *HNF1A* mutations might have been missed.

In conclusion, this is the first study investigating the use of low CRP (≤1. 0 mg/L) to identify South Asians at high risk of having HNF1A-MODY. Using low CRP, a proportion of HNF1A-MODY of 0.7% amongst those with apparent type 2 diabetes was observed, which is lower than that observed in Europeans. In the second part of the study using a dataset of subjects not

selected for low CRP, two out of three subjects with diabetes with likely pathogenic *HNF1A* variants had CRP>1.0 mg/L. This suggests that potential HNF1A-MODY cases might be missed using low CRP alone. Future studies could use the strategy of investigating younger, leaner South Asian subjects with diabetes and combine age of onset, BMI and a lower CRP cut-off than used in this study as a way of identifying highest-risk individuals. This needs to be followed up with family studies to look at co-segregation, response to treatment change and molecular studies to confirm the functional impact of variants.

CHAPTER 6

Investigation of the acute inflammatory response in HNF1A-MODY

6.1 Introduction

In Chapter 1, I discussed the acute inflammatory response, the components (including C-reactive protein; CRP and glycans) which are regulated by HNF1A and the theoretical effects of *HNF1A* haploinsufficiency on the working of a normal acute inflammatory response. These will be briefly reviewed in the first part of this chapter and the later parts will describe the study investigating the acute inflammatory response in HNF1A-MODY.

6.1.1 HNF1A-facilitated C-reactive protein production

CRP is a major acute phase protein mainly synthesized in the liver, and to some extent in extra hepatic sites such as mononuclear cells, adipose tissue, kidney and gut (Yeh, 2005). CRP levels rise during an acute inflammatory response or in chronic systemic inflammatory diseases and are hence used as a marker of inflammation in clinical practice (Pepys and Hirschfield, 2003). CRP plays important roles during an acute inflammatory response such as: it activates the complement system, facilitates phagocytosis, induces the expression of inflammatory cytokines and also mediates resolution of the inflammatory response (Du Clos, 2013).

As discussed in chapter 1, work carried out by my supervisors' research groups has shown that baseline levels of CRP are significantly lower in HNF1A-MODY compared with other forms of diabetes and non-diabetic controls (Owen et al., 2010). This is likely to be because CRP is transcriptionally regulated by HNF1A (Nishikawa et al., 2008). This finding raised important questions about whether,

in subjects with HNF1A-MODY, CRP rises normally under stimulation and if it can be used a marker of the acute inflammatory process in those with HNF1A-MODY.

6.1.2 HNF1A and plasma protein fucosylation

Previous work has shown that patients with HNF1A-MODY have a lower ratio of fucosylated to non-fucosylated plasma glycans compared with healthy controls and patients with type 1 and type 2 diabetes (Thanabalasingham, et al., 2013). This is because HNF1A regulates fucose biosynthesis genes and enzymes catalysing the antennary fucosylation of protein bound N-glycans (Lauc, et al., 2010). Fucosylation is important for the selectin-mediated white blood cell (WBC; Leukocyte) rolling during an acute inflammatory response (Schottelius, et al., 2003).

A decreased expression of *HNF1A* is also associated with an increase in highly branched glycans (Zoldos, et al., 2012). Although not well studied, changes in glycan profile such as an increase in highly sialylated branched glycans and an increase in the ratio of antennary to core fucosylated glycans were found in an observational study in two patients with severe acute inflammation (Gornik, et al., 2007).

Together, these findings suggest that, during acute inflammation, patients with HNF1A-MODY could have an altered plasma glycan profile and hypofucosylation of leukocyte selectin ligands.

6.1.3 The Acute Inflammatory Response

The acute inflammatory response is the response of the body to inflammatory stimuli such as physical or chemical injury or microbial invasion (Ward and Lentsch, 1999). In the case of microbial invasion such as by gram-negative bacteria, bacterial surface molecules are recognized by specialized receptors on macrophages and neutrophils. One of the most studied bacterial surface molecules is lipopolysaccharide (LPS or endotoxin, terms used interchangeably in this chapter), which is also used experimentally in human and animal studies to stimulate and study the acute inflammatory response (Andreasen, et al., 2008; Bahador and Cross, 2007). LPS is recognized by toll like receptor 4 (TLR-4), a transmembrane cell surface receptor on leukocytes including monocytes, macrophages and neutrophils (**Figure 6.1**) (Lu, et al., 2008). LPS recognition by TLR-4 is facilitated by other blood borne or macrophage surface proteins such as LPS-binding protein (LBP), cluster of differentiation 14 (CD14) and myeloid differentiation-2 (MD2) (Lu, et al., 2008).

The LPS-TLR-4 complex triggers a cascade of intracellular events leading to activation of NF-kB and MAPK signalling pathways and production of pro-inflammatory cytokines (e.g. IL-1, IL-6 and TNFα). Pro-inflammatory cytokines stimulate endothelial cells to express adhesion molecules attracting neutrophils to the site of injury or infection. Pro-inflammatory cytokines also stimulate production of acute phase proteins such as CRP from the liver.

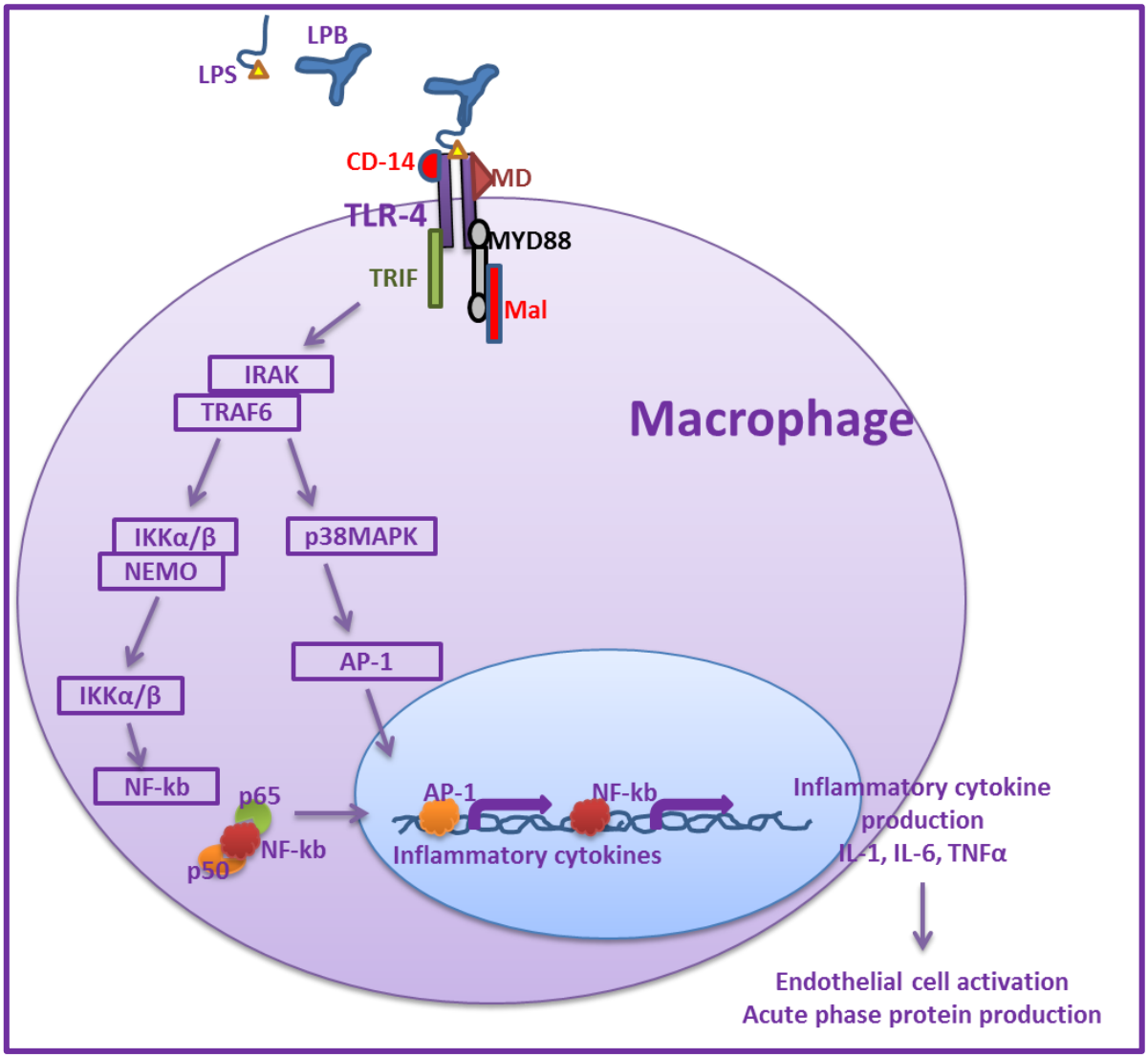


Figure 6.1 Summary of LPS-mediated cytokine production

LPS=Lipopolysaccharide, LBP=Lipopolysaccharide binding protein, TLR4=Toll-like receptor 4, MD-2=Myeloid differentiation-2, CD14=cluster of differentiation 14, MYD88=Myeloid differentiation primary response 88, MAL=MyD88 adapter-like protein, IKK=I kappa B kinase, TRIF=TIR-domain-containing adapter-inducing interferon-β, TRAF=Tumor necrosis factor receptor-associated factor, NF-kb=Nuclear factor kappa-light-chain-enhancer of activated B cells, NEMO=nuclear factor- B essential modulator, AP-1=Activator protein 1, MAPK=mitogen-activated protein kinase

The acute inflammatory response gradually subsides with microbial clearance, decreased production of pro-inflammatory mediators and production of anti-inflammatory cytokines and lipid mediators (Spite and Serhan, 2010).

6.2 Aims

Given the role of HNF1A in regulating important components of the acute inflammatory response, I undertook this study to investigate the dynamics of an acute inflammatory response in HNF1A-MODY using a standardised model of acute inflammation.

The main aims of this study were to investigate the changes in CRP, plasma glycans and WBC membrane glycans in HNF1A-MODY during an acute inflammatory response. To investigate further elements of the inflammatory response in HNF1A-MODY, other important mediators of an acute inflammatory response such as serum cytokines and LBP were also measured.

6.3 Subjects and Methods

6.3.1 Sample size estimate

Studies using an LPS-stimulated acute inflammatory response show that 2ng/kg of LPS generates a CRP rise to 30-40 mg/L in healthy controls (van Eijk, et al., 2007) (Hudgins, et al., 2003). Baseline CRP was 50% lower in HNF1A-MODY compared with healthy controls (Owen et al., 2010). Using a similar baseline difference, I estimated that a sample size of 6 subjects in each group (HNF1A-MODY and healthy controls) would have 90% power to detect a 50% reduction in peak CRP levels at one tailed alpha of 0.05 (**Table 6.1**). If subjects with HNF1A-MODY demonstrated a lower than baseline difference in peak CRP

levels (for example 40% lower than baseline difference), 10 subjects in each group would be required to have 80% power at one tailed alpha of 0.05 (**Table 6.1**).

A larger CRP difference is observed between HNF1A-MODY and type 2 diabetes patients, so by aiming to recruit 10 patients in each study group, the study was powered to show a CRP peak 40% lower than that in controls.

6.3.2 Subjects

Subject recruitment and study protocol was carried out at Oxford Centre for Diabetes, Endocrinology and Metabolism (OCDEM). Subjects with HNF1A-MODY were recruited from patients known to the OCDEM clinical service. Subjects with type 2 diabetes were recruited from the Young Diabetes in Oxford study, the Diabetes Alliance for Research in England study and from the OCDEM clinical research unit database. Healthy adults were identified from the Oxford Biobank or by local advertisement. Details of the above-mentioned cohorts are provided in chapter 2 (section 2.1).

Inclusion criteria used are listed below:

- age range of 18-60 was used for all study groups;
- HNF1A-MODY was defined as presence of *HNF1A* mutations (pathogenicity of *HNF1A* variants was determined by co-segregation of the variant with MODY phenotype in the family members);

	Sample size calculated using observed baseline difference in CRP levels between HNF1A-MODY and controls*	Sample size calculated using a lower than baseline difference (40% lower than baseline difference)
Expected mean (SD) CRP mg/L, 24 hours post 2ng/kg LPS in controls[#]	40 (10)	40 (10)
Expected mean (SD) CRP mg/L, 24 hours post 2ng/kg LPS in HNF1A-MODY	20 (10)	28 (10)
Effect size (HNF1A-MODY vs. Healthy controls)	2.0	1.2
α (one tailed)	0.05	0.05
Power of the study	90%	80%
Healthy controls	6	10
HNF1A-MODY	6	10

Table 6.1 Sample size calculation for the study evaluating acute inflammatory response in HNF1A-MODY

#van Eijk et al., 2007, * Owen et al 2010

- type 2 diabetes was defined as per standard clinical criteria for type 2 diabetes subjects ([http://cks.nice.org.uk/diabetes-type-2], in addition subjects had no requirement for insulin or were C-peptide positive and negative for islet autoantibodies if on insulin treatment);
- non-diabetic controls were defined as subjects with normal fasting glucose.

Subjects with HNF1A-MODY were group matched for gender and BMI to the non-diabetic controls. As patients with type 2 diabetes are mostly obese and have later age of onset, we did not aim to match the HNF1A-MODY and type 2 diabetes subjects for age and BMI.

Subjects were excluded if:

- CRP was more than 10mg/L at screening visit;
- they had a known hypersensitivity to polyethylene glycol (a component of endotoxin preparation);
- pregnant or breastfeeding;
- having conditions (e.g. infection or inflammatory diseases) that raise CRP levels or taking medications (e.g. glucocorticoids and aspirin) that lower CRP levels.

Subjects were advised to inform the research team if they developed any febrile illness in a 14-day period prior to the study. In such instances, the participation of subjects in the study was postponed and they were invited later to allow a complete resolution of the illness. Informed consent was obtained from all

subjects. Appropriate approval was sought and gained from Oxfordshire Local Research Ethics Committee.

Subject characteristics

The clinical characteristics of the participants at the time of screening are shown in **Table 6.2**. All subjects had a baseline CRP of <10.0 mg/L. None of the subjects were smokers. Healthy controls and subjects with HNF1A-MODY were group matched for gender, age and BMI and there was no significant difference between the two groups for these variables (p=0.99, 0.68 and 0.32 respectively). Patients with type 2 diabetes were older than HNF1A-MODY [median (range) years: 54 (40-59) vs. 40 (24-54), p=0.009] and controls [54 (40-59) vs. 40 (20-52), p=0.003]. However, there was no significant difference in the BMI between type 2 diabetes and HNF1A-MODY [median (range) kg/m^2 28.9 (19.3-40.0) vs. 24.7 (20.2-40.0), p=0.99] and controls [28.9 (19.3-40.0) vs. 23.7 (19.3-38.8), p=0.24). Six subjects with HNF1A-MODY and all of the type 2 diabetes subjects were taking statins.

These subjects were instructed to stop taking statins a week before and during the study. Eight subjects with HNF1A-MODY and four with type 2 diabetes were taking sulphonylureas. Two subjects with HNF1A-MODY and four with type 2 diabetes were taking metformin. Anti-diabetic medicines were not stopped before or during the study.

	HNF1A-MODY (n=9)	P*	Controls (n=10)	P**	Type 2 diabetes (n=7)
Males %	55%	0.99	60%	0.99	71%
Age (years)	40 (24-54)	0.68	40 (20-52)	0.01	54 (40-59)
BMI (kg/m^2)	24.7 (20.2-40.0)	0.32	23.7 (19.3-38.8)	0.24	28.9 (19.3-40.0)
Baseline Glucose (mmol/L)	9.9 (5.6-15.3)	0.01	4.8 (4.1-4.8)	0.01	7.0 (5.5-10.4)
Alcohol intake (units/week)	0.5 (0.5-20.0)	0.51	1.0 (0.0-4.0)	0.12	0.1 (0.0-10.0)
Sulphonylureas (%)	88	-	-	-	57
Statins (%)	66	-	-	-	100
Metformin (%)	22	-	-	-	57

Table 6.2 Clinical characteristics of the study groups at the time of screening. Data are shown as median (range) unless stated otherwise *represents P value for HNF1A-MODY vs. controls, **represents P value for type 2 diabetes vs. controls

6.3.4 Model for investigating the acute inflammatory response in HNF1A-MODY

Different models have been described in the literature for induction of acute inflammatory response in humans. These include severe exercise, administration of typhoid or influenza vaccine, use of pro-inflammatory cytokines or by administration of LPS. For investigation of acute inflammatory response in HNF1A-MODY, I initially used typhoid vaccine as an inflammatory stimulus. However, due to lack of a sufficient inflammatory response generated by typhoid vaccine (details discussed in the next section), a stronger inflammatory stimulus, LPS was used. The two models used are discussed below.

6.3.4.1 Evaluation of typhoid vaccine as an acute inflammatory stimulus

Initially typhoid vaccine was used as an acute inflammatory stimulus. It has been shown previously that the use of routine typhoid vaccine causes transient changes in markers of inflammation (Clapp, et al., 2004; Hingorani, et al., 2000). This model has been used to study the effects of acute inflammation on the vascular endothelium by researchers in the Department of Cardiovascular Medicine in Oxford [Dr Rajesh Kharbanda (Hingorani, et al., 2000)], and this local expertise was one rationale for initially choosing this method.

To investigate if an adequate inflammatory response was generated by typhoid vaccine, hsCRP levels and WBC count were measured for two healthy controls. It was found that typhoid vaccine did not induce a rise in the hsCRP levels in either study participant (baseline hsCRP mg/L: 0.06 and 1.08, 48 hours hsCRP: 0.04 and 1.11 for subjects A and B respectively).

During an acute inflammatory response, CRP levels are expected to rise to a maximum at 24-48 hours. However, this was not observed after administration of typhoid vaccine. Similarly, typhoid vaccine induced only a slight increase in WBC count (baseline WBC count n×10^9 cells/L: 3.21 and 4.59, 4 hours WBC count: 4.59 and 4.89 for subjects A and B respectively).

As typhoid vaccine did not generate a good inflammatory response, the study protocol was modified to use LPS for inducing an acute inflammatory response in the study participants.

6.3.4.2 Experimental endotoxemia as a model of acute inflammatory response

Experimental human endotoxemia is a well-tested model of inflammation, and has been used in a large number of studies involving over 2000 subjects (Andreasen, et al., 2008; Bahador and Cross, 2007). It has also been used to study inflammatory response in subjects with type 2 diabetes (Andreasen, et al., 2010). Endotoxin administration induces a febrile systemic inflammatory response with a rise in body temperature, increase in WBC count, increased production of cytokines and CRP. Published work shows that at the dose selected (2ng/kg), endotoxin generates a CRP rise to approximately 30-40 mg/L (Hudgins, et al., 2003; van Eijk, et al., 2007).

6.3.5 Study protocol

The study protocol included a screening visit, study day 1, day 2 and day 8. The study protocol is illustrated in **Figure 6.2**.

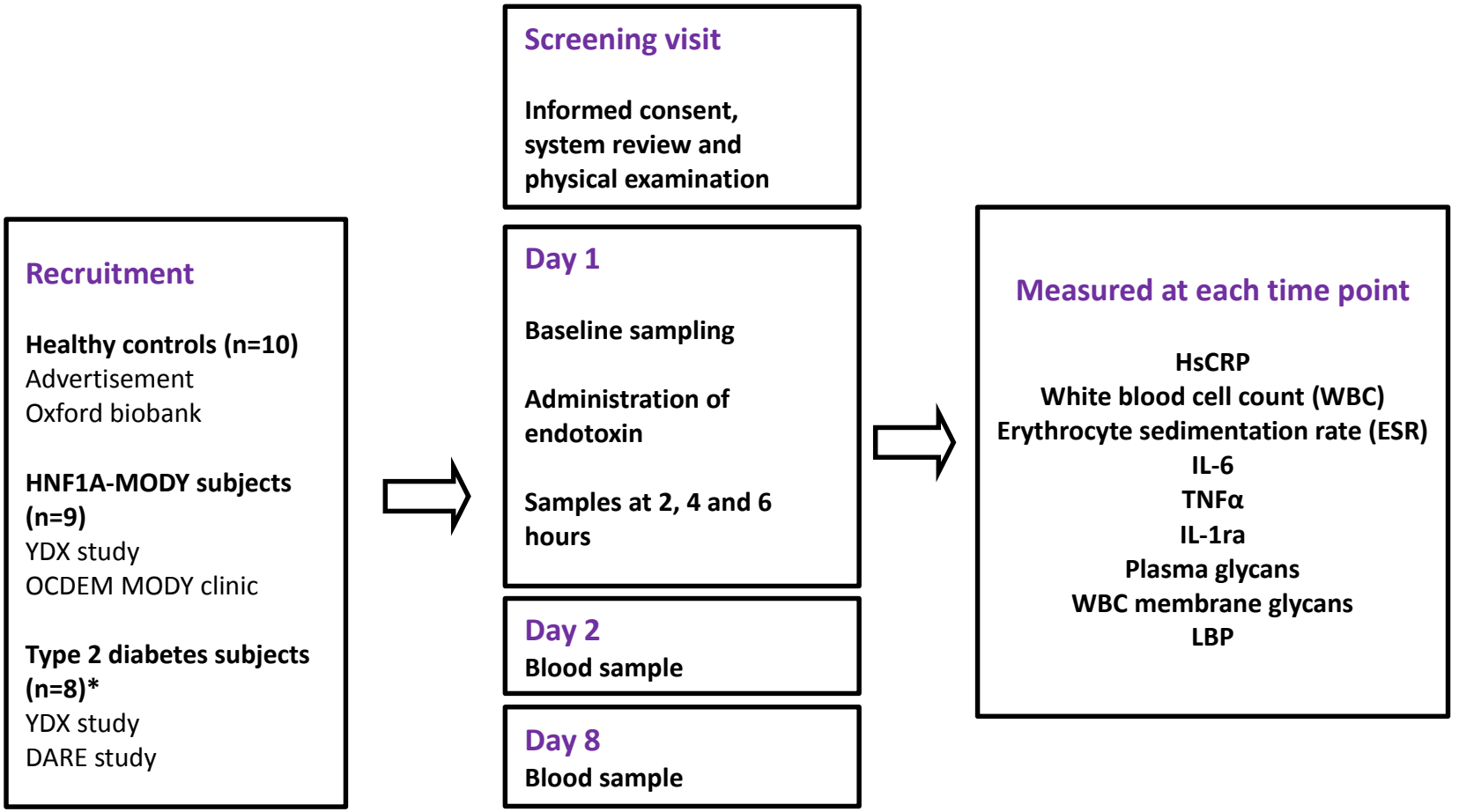


Figure 6.2 Study flow diagram: Investigation of acute inflammatory response in HNF1A-MODY

*One subject with type 2 diabetes experienced sudden nausea and redness of hands and feet during administration of LPS. Full dose of LPS could not be administered and the results of this subject were not included in the analysis.

6.3.5.1 Screening visit

To detect any underlying medical condition, subjects were invited for a screening visit prior to administration of endotoxin. This visit included detailed medical history, physical examination (height and weight measurement, cardiovascular system examination) and electrocardiography. HsCRP was measured to ensure that the subjects recruited did not have any acute or chronic underlying inflammation

6.3.5.2 Day 1

On day 1, baseline blood sampling was performed. Subjects were administered a single intravenous dose of endotoxin (2ng/kg). Samples were taken at 2, 4 and 6 hours post endotoxin administration.

Endotoxin preparation and administration

Endotoxin is purified lipopolysaccharide prepared from Escherichia coli, 0:113 (U.S. Standard Reference Endotoxin). Endotoxin was kindly provided by Critical Care Medicine Department, National Institutes of Health, Bethesda, Maryland, USA. The endotoxin was supplied as a sterile, white, lyophilized powder in a clear glass 5mL vial. Each vial contained 10,000 endotoxin units (EU) or 1000ng of Reference Endotoxin. Vials were stored at 2-8˚C in the Clinical Research Unit laboratory, OCDEM.

On the day of administration, vials were reconstituted as recommended with 5mL of Sterile Water for Injection. The vial was then sealed with Parafilm and vortexed for 30 minutes. When reconstituted, each mL contained 200ng of endotoxin. Two ng/kg of endotoxin was administered. The required amount of endotoxin was

taken from the vial with a 1mL syringe and filtered through a pre-moisturized 0.22µl sterile filter into a 10ml syringe already filled with 8ml of sterile water for injection. Endotoxin was slowly administered intravenously over 1 min followed by 5ml of normal saline to ensure complete delivery.

The most common side effects of endotoxin are 'flu like symptoms (fever, headache, malaise, arthralgia, myalgia) that begin approximately an hour after the administration (Andreasen, et al., 2008; Bahador and Cross, 2007). LPS also causes nausea and vomiting. To decrease the likelihood of unpleasant side effects, all subjects were given Paracetamol (1g) and metoclopramide (10mg) before the administration of endotoxin. For hydration, normal saline (100ml/hour) was administered for 4 hours.

Patient Monitoring

The study participants remained in the Clinical Research Unit for 7 hours after the administration of endotoxin. They underwent continuous monitoring of heart rate, peripheral oxygen saturation and non-invasive monitoring of blood pressure every 15 minutes for first 4 hours and then every 30 minutes. If systolic blood pressure fell >20 mm Hg from baseline or below 100 mmHg or heart rate dropped to <60/min, the foot end of the bed was raised and 500 ml of normal saline over 30 minutes was given and response assessed.

6.3.5.3 Day 2 and 8

A single blood sample was taken at 24 hours and another on day 8 post endotoxin administration.

6.3.5.4 Haematological and biochemical analysis

As written above, blood samples were taken before and after administration of endotoxin. On day 1, baseline blood sampling was performed for fasting glucose, hsCRP, plasma and membrane glycans, WBC count, ESR, cytokines (IL-6, TNF-α and IL-1ra). Both cytokines and WBC rise to peak levels at 2-4 hours and then start to decrease (Andreasen, et al., 2008; Hudgins, et al., 2003). To observe the rise and fall in cytokines and WBC levels, blood sampling was performed at 2, 4 and 6 hours after administration of endotoxin. HsCRP levels rise to a peak at 24-48 hours and drop back to baseline 3-7 days after the removal of inflammatory stimulus (Hudgins, et al., 2003; van Eijk, et al., 2007). To observe the peak hsCRP levels and return to baseline, blood sampling was performed at 24 hours and on day 8 post endotoxin administration. Plasma and WBC membrane glycans have not been previously investigated during a stimulated acute inflammatory response and the time line of possible changes in glycan profile are not known. Samples for plasma glycans were taken at all time points during the study. Samples for WBC membrane glycans were taken at 0, 2, 4, 24 hours and day 8 of the study. Details of laboratory methods used are provided in chapter 2.

6.3.6 Statistical analysis

The Fisher's exact test was used to compare categorical variables. The Mann–Whitney U test was used to compare quantitative variables. Change in the variables over time was analysed with analysis of variance with repeated measures (RM.ANOVA). Area under the curve (AUC) was calculated using the trapezoidal method. All computations were performed using IBM SPSS Statistics Version 20.0. and $P<0.05$ was considered significant. Power analysis was conducted using the software package, G*Power (Faul, et al., 2007).

6.4 Results

6.4.1 Flu-like symptoms

Volunteers manifested varying degrees of flu-like symptoms approximately 1 hour after the intravenous administration of LPS. The number of patients in each study group experiencing flu-like symptoms is shown in **table 6.3**. Symptoms were at their maximum 90 min after the administration of LPS. Headache was the most common symptom. One male subject with type 2 diabetes experienced sudden nausea and redness of hands and feet during intravenous administration of endotoxin (intravenous bolus injection administered slowly over one minute). This subject could not be administered a full dose of endotoxin and his results are not included in the analysis.

All symptoms disappeared 6 hours after the administration of LPS and all subjects were symptom free at the time of leaving the Clinical Research Unit.

6.4.2 Response of physiological variables to LPS

Endotoxin administration induced a significant decrease in mean arterial pressure (MAP) in all groups ($p=0.01$ as tested by RM.ANOVA). Systolic blood pressure dropped below 100mmHg in four subjects (two controls and one subject each in HNF1A-MODY and type 2 diabetes groups), 1-2 hours post LPS administration. The blood pressure rose back to baseline levels after the saline flow/minute was increased and the foot end of the bed was raised.

	Healthy controls (n=10)	HNF1A-MODY (n=9)	Type 2 diabetes (n=7)
Headache	5	3	3
Chills	2	2	1
Myalgia	0	1	2
Nausea	2	1	1
Vomiting	0	0	1
Backache	0	0	2

Table 6.3 Clinical response to endotoxin: number of subjects in each group having flu-like symptoms after LPS administration

Heart rate increased significantly in all groups post endotoxin administration (p=0.0001). There was no significant difference in MAP fall or pulse rise over time in between the three groups as tested with RM.ANOVA (p=0.10 and p=0.62 for HNF1A-MODY vs. controls, p=0.31 and p=0.44 for type 2 diabetes vs. controls, p=0.64 and p=0.81 for HNF1A-MODY vs. type 2 diabetes). The change in MAP and heart rate following LPS administration is shown in **figure 6.3**. As all participants were given 1g paracetamol before LPS administration, the rise in temperature which usually occurs following LPS administration was not observed in this study.

6.4.3 WBC and neutrophil response

LPS induced an increase in the number of WBC's, primarily the neutrophils, peaking 6 hours after LPS administration and returning to baseline values within 24 hours. Baseline and peak WBC and neutrophil count and AUC for all groups are provided in **Table 6.4**. Change in WBC count following LPS administration is illustrated in **figure 6.3**.

No difference in WBC count over time was observed in between the three groups (HNF1A-MODY vs. controls [p=0.29], type 2 diabetes vs. controls [p=0.44], HNF1A-MODY vs. type 2 diabetes [p=0.73] as tested with RM.ANOVA).

6.4.4 Erythrocyte sedimentation rate (ESR)

As illustrated in **figure 6.3**, there was no significant difference in ESR in between the three groups during the acute inflammatory response (HNF1A-MODY vs. controls [p=0.51], type 2 diabetes vs. controls [p=0.62], HNF1A-MODY vs. type 2 diabetes [p=0.86] as tested with RM.ANOVA).

	HNF1A-MODY (n=9)	P*	Healthy controls (n=10)	P **	Type 2 diabetes (n=7)
WBC (10^9/ml)					
0hrs	5.7 (3.7-10.1)	0.10	4.4 (2.9-7.2)	0.10	6.0 (4.3-7.2)
6hrs	10.4 (7.3-17.8)	0.46	10.3 (5.4-15.2)	0.69	10.1 (5.0-14.0)
Neutrophils (10^9/ml)					
0hrs	3.2 (1.8-9.1)	0.01	2.4 (1.4-4.3)	0.03	3.6 (2.4-4.1)
6hrs	7.8 (6.4-11.5)	0.68	8.7 (3.9-12.6)	0.92	8.7 (4.8-10.6)
ESR					
0hrs	2 (2-17)	0.90	2 (2-15)	0.14	5 (2-5)
24hrs	6 (2-17)	0.23	3 (2-15)	0.57	5 (2-13)
Day 8	3 (2-12)	0.47	2 (2-13)	0.42	2 (2-16)
CRP (mg/L)					
0hrs	0.09 (0.02-2.18)	0.34	0.29 (0.03-4.21)	0.03	3.20 (0.39-7.49)
24hrs	30.01 (15.10-51.11)	0.25	32.10 (24.60-53.51)	0.06	39.70 (28.81-59.50)
AUC	300 (143-544)	0.28	339 (233-617)	0.05	443 (304-657)
IL-6 (pg/ml)					
0hrs	14.2 (9.1-15.2)	0.002	4.5 (0.0-15.2)	0.60	12.0 (0.0-14.5)
2hrs	479.7 (294.6-1184.8)	0.36	741.1 (299.0-1862.8)	0.77	801.8 (174.9-3553.2)
AUC	1090 (707-3246)	0.12	1866 (705-6253)	0.77	2133 (460-10110)
TNFα (pg/ml)					
0hrs	0.0# (0.0-14.9)	0.77	0.0# (0.0-364.1)	0.18	12.8 (0.0-438.7)
2hrs	286.7 (166.4-460.2)	0.02	367.0 (266.3-1213.3)	0.43	504.0 (135.7-2202.1)
AUC	711 (400-1085)	0.03	942 (681-4810)	0.43	1249 (423-6419)
IL-1ra (ng/ml)∞					
0hrs	0.6 (0.3-3.9)	0.99	0.8 (0.2-2.6)	0.24	0.9 (0.4-4.9)
4hrs	92.9 (58.1-173.3)	0.16	126.3 (44.3-824.8)	0.38	82.1 (53.6-740.8)
AUC	305 (175-494)	0.36	351 (161-1728)	0.43	286 (230-1572)
LBP (µg/ml)					
0hrs	11.0 (5.2-19.5)	0.06	7.4 (0.0-12.4)	0.02	11.2 (7.8-21.5)
24hrs	30.0 (7.6-41.7)	0.56	29.9 (14.0-40.4)	0.18	32.9 (20.5-47.3)
AUC	547 (208-776)	0.50	513 (251-746)	0.36	524 (335-837)

Table 6.4 Between-group comparison of measured variables at baseline and peak levels in the acute inflammatory response study

Data are shown as median (range). *represents P value for HNF1A-MODY vs. controls, **represents P value for type 2 diabetes vs. controls, # represents values below the detection limit of the assay. P values were calculated with the use of the Mann–Whitney U test ∞ for ease of interpretation IL-1ra is shown as "ng/ml" rather than pg/ml

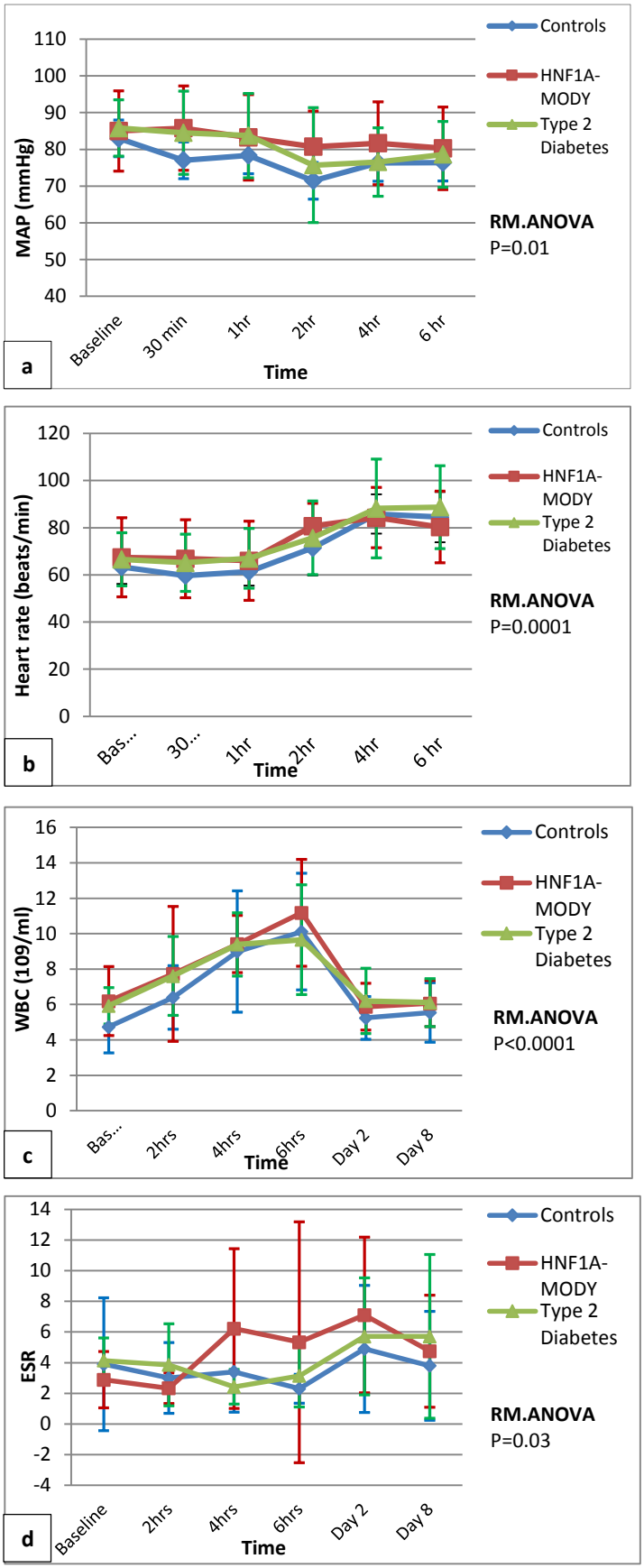


Figure 6.3: Mean (SD) Mean arterial pressure (MAP), heart rate, white blood cell (WBC) count and erythrocyte sedimentation rate (ESR) for healthy controls, HNF1A-MODY and type 2 diabetes for each time point. P values refer to the effect of time on outcome variables in all groups calculated by RM.ANOVA. There was no significant difference in MAP fall or pulse rise over time in between the three groups. There was also no significant difference in WBC rise over time or ESR in between the groups.

6.4.5 CRP

LPS elicited an increase in CRP in all three groups ($p<0.0001$ as tested by RM.ANOVA) rising to a maximum at 24 hours (**Table 6.4** and **Figure 6.4**). Median (range) peak CRP (mg/L) values in subjects with HNF1A-MODY (30.0 [15.1-51.1]) were comparable to controls (32.1 [24.6-53.5], $p=0.31$ as tested by RM.ANOVA).

There was no significant difference in AUC for CRP among HNF1A-MODY and controls ($p=0.28$). Subjects with type 2 diabetes had higher baseline and peak CRP levels compared with controls and those with HNF1A-MODY (**Table 6.4** and **Figure 6.4**). Median (range) peak CRP (mg/L) values were higher in type 2 diabetes (39.7 [28.8-59.5]) than in controls ($p=0.04$ as tested by RM.ANOVA) and HNF1A-MODY ($p=0.008$ as tested by RM.ANOVA). AUC for hsCRP was higher in type 2 diabetes vs. controls ($p=0.05$) and for type 2 diabetes vs. HNF1A-MODY ($p=0.02$).

6.4.6 Serum cytokines

As illustrated in **table 6.4** and **figure 6.5**, pro and anti-inflammatory cytokines (IL-6, TNFα and IL1-ra) increased significantly in response to endotoxemia in all groups ($p<0.0001$). IL-6 and TNFα reached a peak level at 2 hours and IL-1ra at 4 hours after LPS administration. There was a tendency toward lower levels of all cytokines in subjects with HNF1A-MODY compared with healthy controls and subjects with type 2 diabetes. This was most marked for TNFα. Median (range) peak TNFα (pg/ml) was significantly lower in HNF1A-MODY (286.7 [166.4-460.2]) than in controls (367.0 [266.3-1213.3], $p=0.02$).

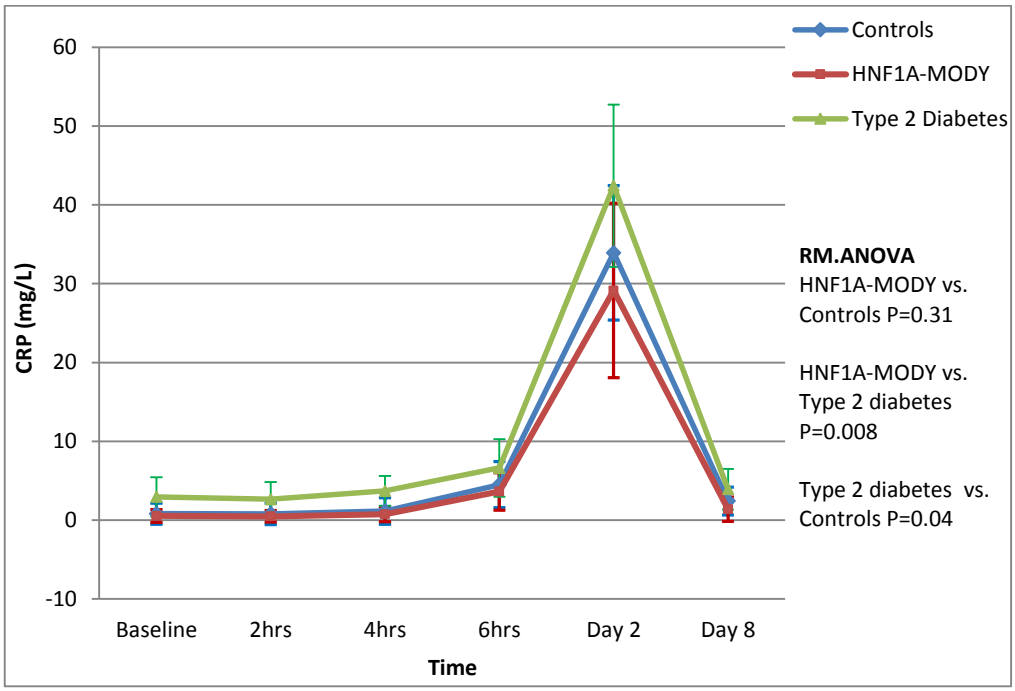


Figure 6.4: Mean (SD) CRP for healthy controls, HNF1A-MODY and type 2 diabetes for each time point. LPS elicited a significant increase in CRP levels in all groups ($p<0.0001$ as tested by RM.ANOVA). P values in the graph refer to difference between groups calculated by RM.ANOVA.

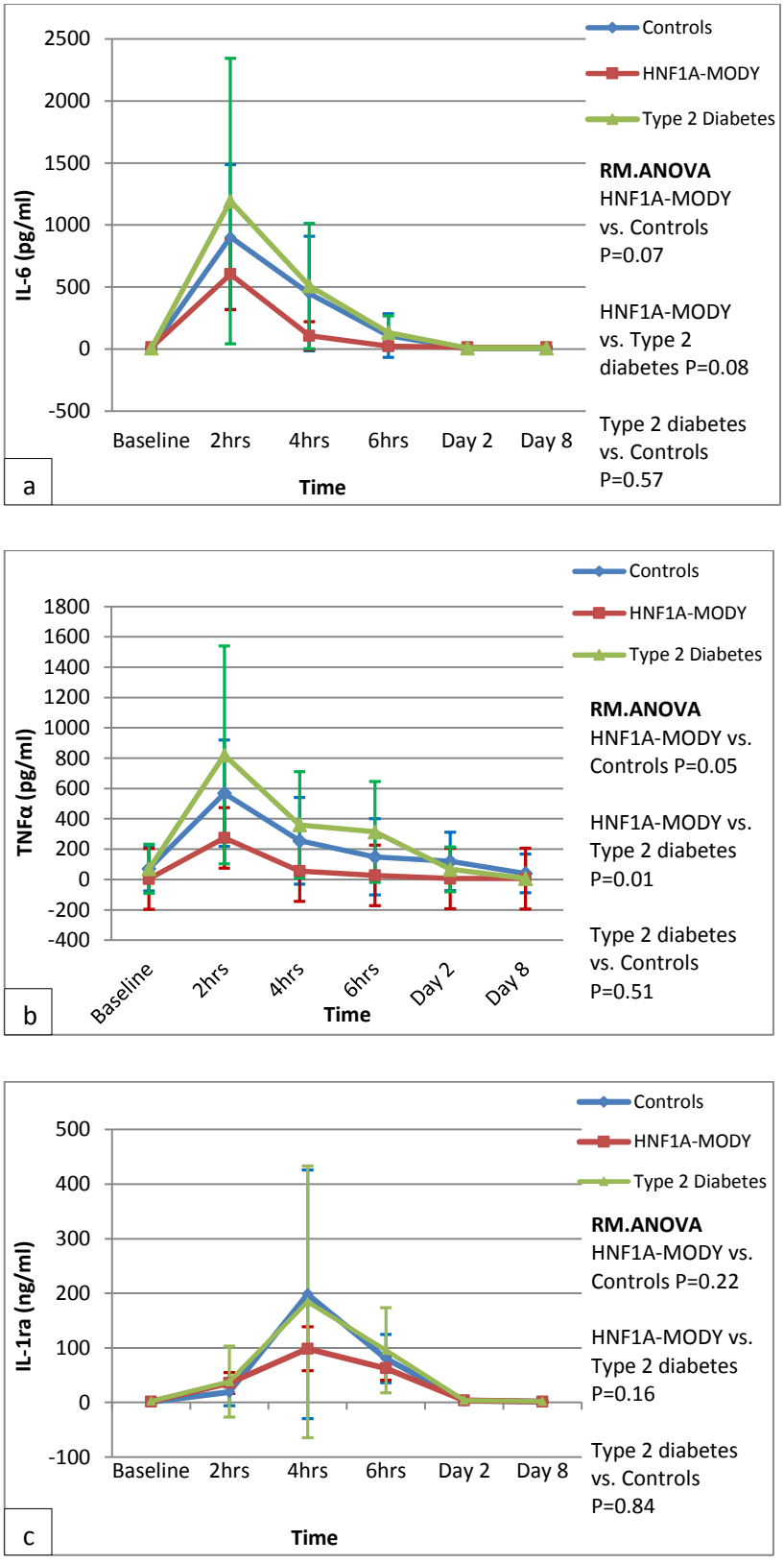


Figure 6.5: Mean (SD) IL-6, TNFα and IL-1ra for healthy controls, HNF1A-MODY and type 2 diabetes for each time point. LPS elicited a significant increase in IL-6, TNFα and IL-1ra in all groups ($p<0.0001$ as tested by RM.ANOVA). P values in the graph refer to difference between groups calculated by RM.ANOVA

The reason for an attenuated cytokine response in HNF1A-MODY is not clear. One possible explanation relates to the use of sulphonylurea drugs, which are the first line treatment in HNF1A-MODY. Seven out of 8 of the HNF1A-MODY subjects were taking gliclazide (a type of sulphonylurea), compared with 4 out of 7 of the type 2 diabetes group. Previous examination of the effects of sulphonylurea exposure has suggested that the cytokines TNFα and IL-6 may be reduced in patients taking sulphonylureas (Desfaits, Serri, & Renier, 1998). Examining the effect of sulphonylureas on cytokines in this study showed that there was no significant difference in TNFα and IL-6 in subjects using sulphonylureas compared with those (including controls) not using sulphonylureas (**figures 6.6**).

There was no significant difference in serum cytokines in subjects with type 2 diabetes compared with controls.

6.4.7 Lipopolysaccharide binding protein (LBP)

As shown in **figure 6.7**, LPS induced a significant increase in LBP level in all groups ($p<0.0001$ as tested by RM.ANOVA). There was no significant difference in LBP rise over time in between the three groups (HNF1A-MODY vs. controls [$p=0.36$], HNF1A-MODY vs. type 2 diabetes [$p=0.47$], type 2 diabetes vs. controls [$p=0.10$] as tested by RM.ANOVA). Baseline and peak LBP levels and AUC are provided in **table 6.4**.

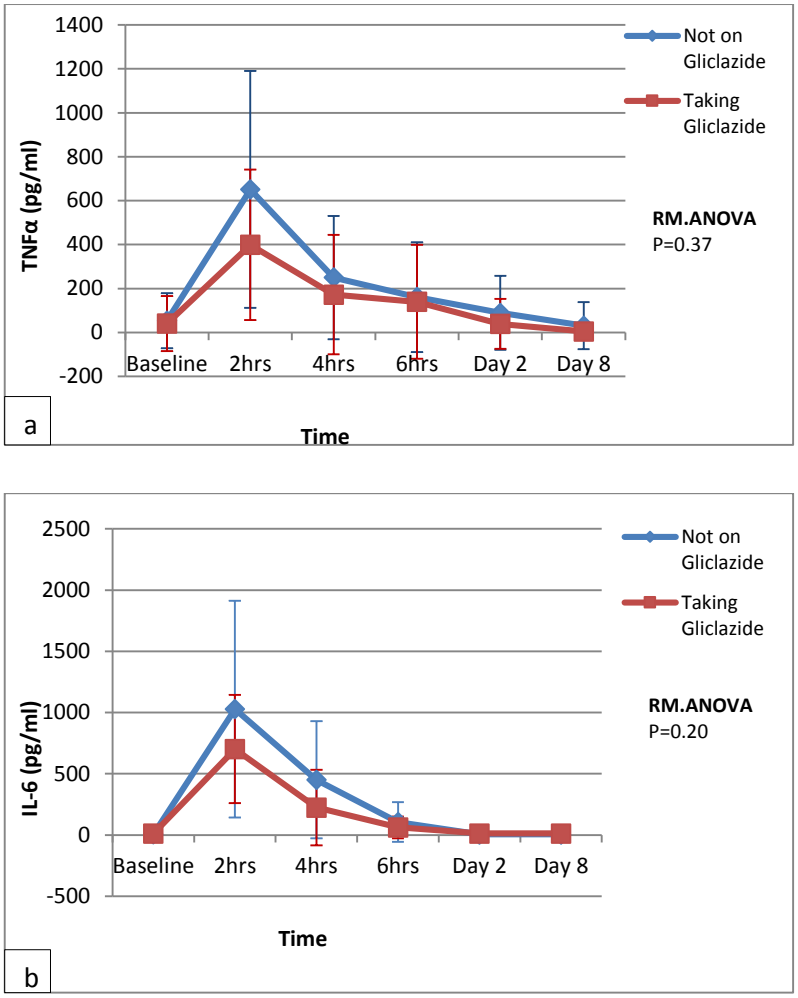


Figure 6.6. Figure illustrating TNFα and IL-6 levels in subjects with (n=12) and without (n=14) Gliclazide treatment. P values refer to effect of time on TNFα and IL-6 in all groups calculated by RM.ANOVA.

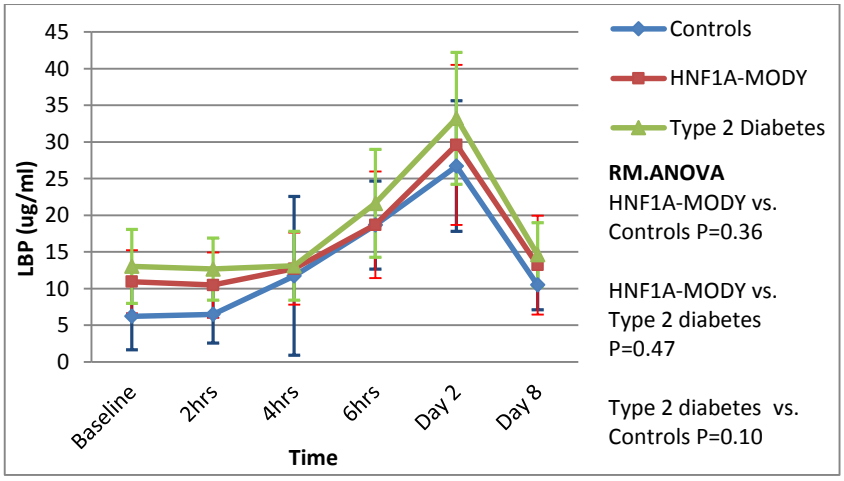


Figure 6.7: Mean (SD) LBP for healthy controls, HNF1A-MODY and type 2 diabetes for each time point. LPS elicited a significant increase in LBP levels in all groups ($p<0.0001$ as tested by RM.ANOVA). P values in the graph refer to difference between groups calculated by RM.ANOVA.

6.4.8 Plasma glycans

Chromatograms from fluorescently labelled plasma glycans were separated into 42 peaks.

Unlike WBCs, CRP, cytokines or LBP, plasma glycans in individual glycan peaks did not demonstrate any change over time during the acute inflammatory response.

Baseline levels of plasma glycans (abbreviated as PL.GP), PL.GP 29, 30 and 36 were found to be significantly different in HNF1A-MODY compared with controls. PL.GP 30 and 36, containing antennary fucosylated glycans, were significantly lower in HNF1A-MODY compared with controls ($p=1.6\times10^{-4}$ and $p=0.01$ respectively, as tested by RM.ANOVA) and type 2 diabetes ($p=3.0\times10^{-6}$ and $p=0.03$ respectively, as tested by RM.ANOVA). These results are consistent with the previous report (Thanabalasingham et al., 2013), showing that subjects with HNF1A-MODY have lower levels of antennary fucosylated glycans. Levels of PL.GP30 and 36 at different time points during the study and AUC are given in **table 6.5** and baseline levels are illustrated in **figure 6.8**.

Consistent with the previous observation (Zoldos, et al., 2012), PL.GP 29, a highly branched tetraantennary glycan, was significantly higher in HNF1A-MODY compared with controls (P=0.003). PL.GP 29 was also higher in HNF1A-MODY compared with subjects with type 2 diabetes. Levels of PL.GP29 at different time points during the study and AUC are given in **table 6.5** and baseline levels illustrated in **figure 6.8**.

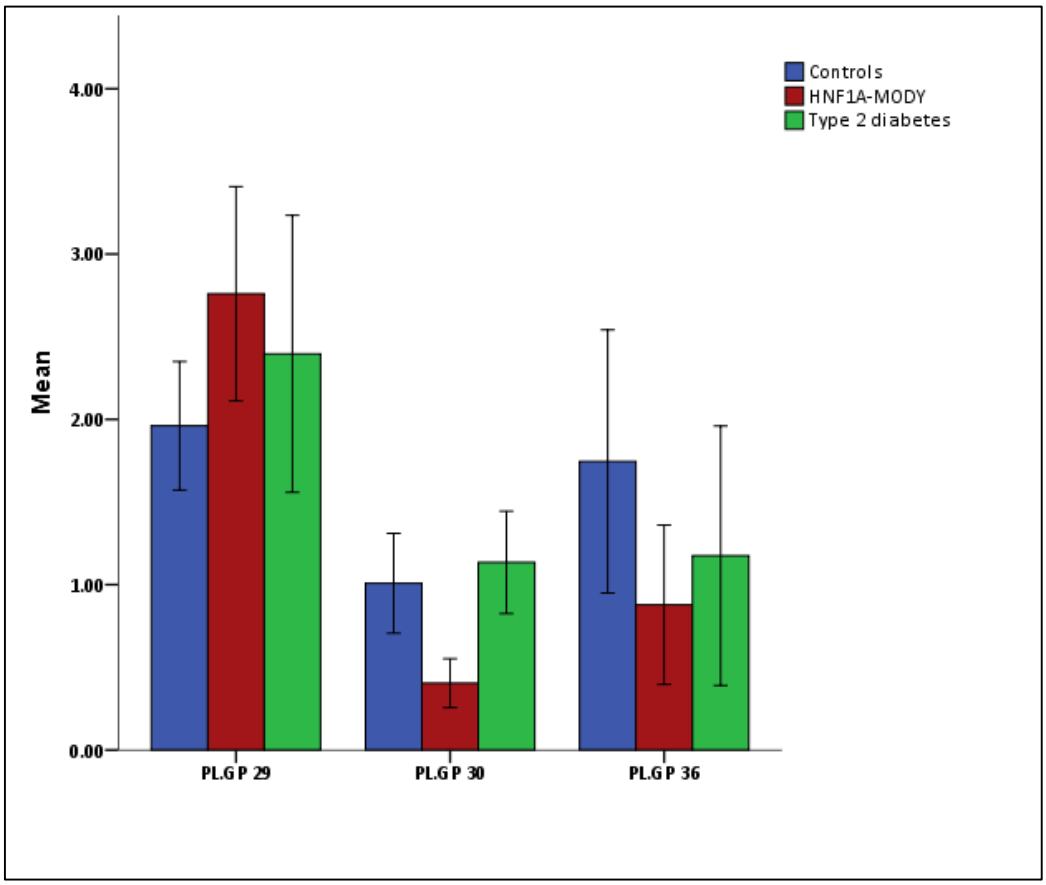


Figure 6.8. Bar chart showing means with standard-deviation error bars for baseline levels of plasma glycan 29, 30 and 36

	HNF1A-MODY (n=9)	P*	Healthy controls (n=10)	P**	Type 2 diabetes (n=7)
PL.GP 29					
0hrs	2.75 (1.63-3.59)	0.019	2.00 (1.37-2.65)	0.150	2.67 (1.02-3.28)
2hrs	2.71 (2.08-3.08)	0.008	1.93 (1.56-2.82)	0.156	2.52 (1.63-3.09)
4hrs	2.56 (2.08-3.53)	0.006	2.01 (1.53-2.41)	0.081	2.57 (1.76-3.07)
6 hrs	2.73 (1.94-3.18)	0.011	2.09 (0.80-2.50)	0.192	2.85 (1.25-3.28)
Day 2	2.92 (2.35-3.52)	0.002	2.22 (1.72-2.62)	0.380	2.40 (1.48-3.19)
AUC	62.9 (33.8-77.1)	0.003	50.3 (23.4-61.4)	0.120	65.4 (50.5-79.8)
PL.GP 30					
0hrs	0.38 (0.23-0.63)	0.001	0.97 (0.65-1.64)	0.351	1.24 (0.67-1.47)
2hrs	0.43 (0.21-0.70)	0.002	0.94 (0.48-1.39)	0.320	1.29 (0.81-1.36)
4hrs	0.37 (0.20-0.64)	0.001	0.95 (0.84-1.60)	0.191	1.29 (0.80-1.41)
6hrs	0.44 (0.23-0.62)	0.001	0.96 (0.56-1.53)	0.152	1.33 (0.91-1.58)
Day 2	0.48 (0.21-0.63)	0.001	1.09 (0.76-1.50)	0.740	1.08 (0.82-1.31)
AUC	30.6 (20.5-32.0)	0.001	24.2 (13.7-36.4)	0.126	10.9 (5.5-14.8)
PL.GP 36					
0hrs	1.14 (0.13-1.37)	0.041	1.83 (0.52-2.86)	0.153	0.92 (0.38-2.27)
2hrs	1.02 ((0.30-1.57)	0.300	1.35 (0.51-3.05)	0.825	0.93 (0.42-2.87)
4hrs	1.16 (0.15-1.33)	0.042	1.80 (0.55-2.75)	0.826	1.41 (0.60-2.79)
6 hrs	0.66 (0.46-1.48)	0.131	1.39 (0.21-2.67)	0.994	1.32 (0.38-2.94)
Day 2	1.13 (0.38-1.61)	0.011	1.78 (0.87-2.97)	0.381	1.23 (0.39-2.98)
AUC	31.9 (10.6-69.8)	0.010	35.6 (25.2-65.1)	0.580	21.0 (11.3-34.7)
Memb.Gly 25					
0hrs	0.57 (0.49-0.69)	0.042	0.41 (0.20-0.60)	0.660	0.4 (0.32-0.66)
2hrs	0.59 (0.43-0.74)	0.062	0.43 (0.31-0.64)	0.191	0.51 (0.44-0.81)
4hrs	0.57 (0.48-0.81)	0.040	0.48 (0.27-0.57)	0.154	0.57 (0.41-0.62)
Day 2	0.53 (0.34-0.62)	0.111	0.35 (0.27-0.60)	0.223	0.52 (0.33-0.58)
AUC	12.6 (9.8-14.3)	0.003	9.8 (6.5-13.6)	0.084	13.3 (10.6-16.8)
Memb.Gly 34					
0hrs	2.57 (1.68-2.80)	0.116	2.13 (0.85-2.44)	0.561	2.14 (1.92-3.12)
2hrs	2.77 (2.58-3.15)	0.046	2.44 (1.18-2.91)	0.470	2.32 (2.18-2.61)
4hrs	2.74 (2.38-3.04)	0.003	2.17 (1.43-2.38)	0.562	2.23 (2.00-2.83)
Day 2	2.34 (2.04-2.81)	0.013	1.91 (1.18-2.30)	0.801	1.97 (1.55-2.05)
AUC	50.2 (46.4-59.1)	0.003	48.2 (36.6-55.7)	0.572	60.8 (53.7-67.9)
Memb.Gly 44					
0hrs	1.66 (1.08-1.90)	0.061	1.35 (0.78-1.55)	0.880	1.36 (1.00-2.13)
2hrs	1.52 (1.10-2.57)	0.250	1.33 (0.94-1.91)	0.562	1.13 (0.94-2.11)
4hrs	1.87 (1.37-2.28)	0.021	1.14 (0.54-1.71)	0.663	1.22 (1.09—1.70)
Day 2	1.58 (1.16-2.34)	0.038	1.08 (0.49-1.64)	0.931	1.09 (0.74-1.52)
AUC	26.7 (24.3-38.2)	0.003	27.1 (16.6-39.9)	0.935	41.1 (35.6-46.4)

Table 6.5: Between-group comparison of plasma and membrane glycan levels at baseline and post LPS administration

Data are shown as median (Range). P values were calculated with the use of the Mann–Whitney U test. *represents P value for HNF1A-MODY vs. controls, **represents P value for type 2 diabetes vs. controls

There was no significant difference in the baseline levels of any other plasma glycan peaks between HNF1A-MODY and controls. Additionally there was no significant difference over time in plasma glycans in subjects with diabetes (combining both HNF1A-MODY and type 2 diabetes) and healthy controls

Changes in glycan profile, such as an increase in the ratio of antennary to core fucosylated glycans, have been previously observed during inflammation (Zoldos, et al., 2012). To investigate these changes, plasma glycan peaks representing core fucosylated, antennary fucosylated, highly branched and sialylated glycans were aggregated and analysed. For example, PL.GP 38 and 39, representing antennary fucosylated glycans, were added to derive a measure of antennary fucosylated glycans. PL.GP 13-19 and PL.GP 22, representing monosialylated glycans, were added to generate a measure of monosialylated glycans.

An increase in the ratio of antennary to core fucosylated glycans was observed in all groups rising to a maximum at 24 hours (**Figure 6.9**). No systematic response was seen in the degree of branching and sialylation when testing the three subject groups separately or when combining subjects in all three groups. There was no significant difference in any of these measures in between the three study groups.

Levels of all 42 plasma glycans are provided in Appendix **Table 1**.

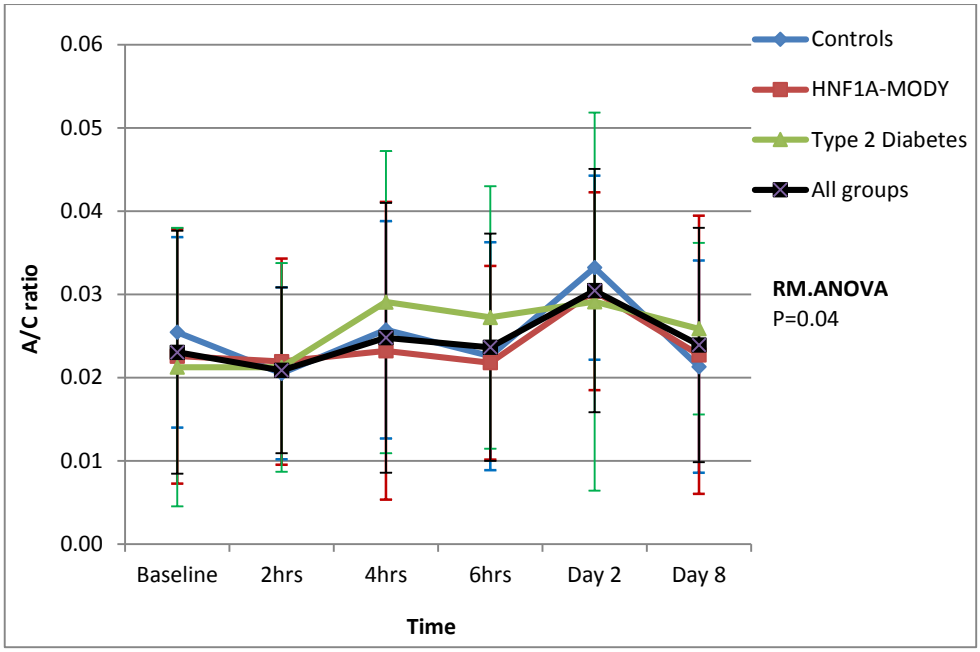


Figure 6.9: Mean (SD) ratio of antennary to core fucosylated (A/C ratio) glycans for healthy controls, HNF1A-MODY, type 2 diabetes and for all groups combined for each time point. P value refers to change in concentration over time for all groups combined calculated by RM.ANOVA.

6.4.9 WBC Membrane glycans

Chromatograms from fluorescently labelled membrane glycans were separated into 44 peaks.

Membrane glycans (abbreviated as Mem.GP), Mem.GP 16, 25, 28 and 34, showed a change in concentration over time during the inflammatory response. These glycan groups rose to a peak at 2-4 hours in all patient groups ($p<0.0001$ as tested by RM.ANOVA). Mem.GP 25 was higher in subjects with HNF1A-MODY compared with controls at baseline and at 4 hours post LPS administration ($p=0.04$ for both time points, AUC HNF1A-MODY vs. controls $P=0.003$). Mem.GP 34 was also significantly higher in HNF1A-MODY compared with controls at 2, 4 and 24 hours post LPS administration ($p=0.04$, 0.003 and 0.01 respectively, AUC HNF1A-MODY vs. controls $p=0.003$) (**table 6.5** and **figure 6.10**). Although Mem.GP 16 and 28 showed a change in concentration over time, they did not differ in between the groups.

Another membrane glycan, Mem.GP 44, was higher in HNF1A-MODY compared with controls at 4 and 24 hours post LPS administration (HNF1A-MODY vs. controls: $p=0.02$ and 0.03 respectively at 4 and 24 hours, AUC $P=0.003$) (**table 6.5** and **figure 6.11**). Mem.GP 44 did not demonstrate a significant change in concentration over time ($p=0.35$ as calculated by RM.ANOVA). Mem.GP 34 and 44 were also significantly higher in subjects with HNF1A-MODY compared with those with type 2 diabetes.

Levels of all 44 WBC membrane glycans are provided in Appendix **Table 2**.

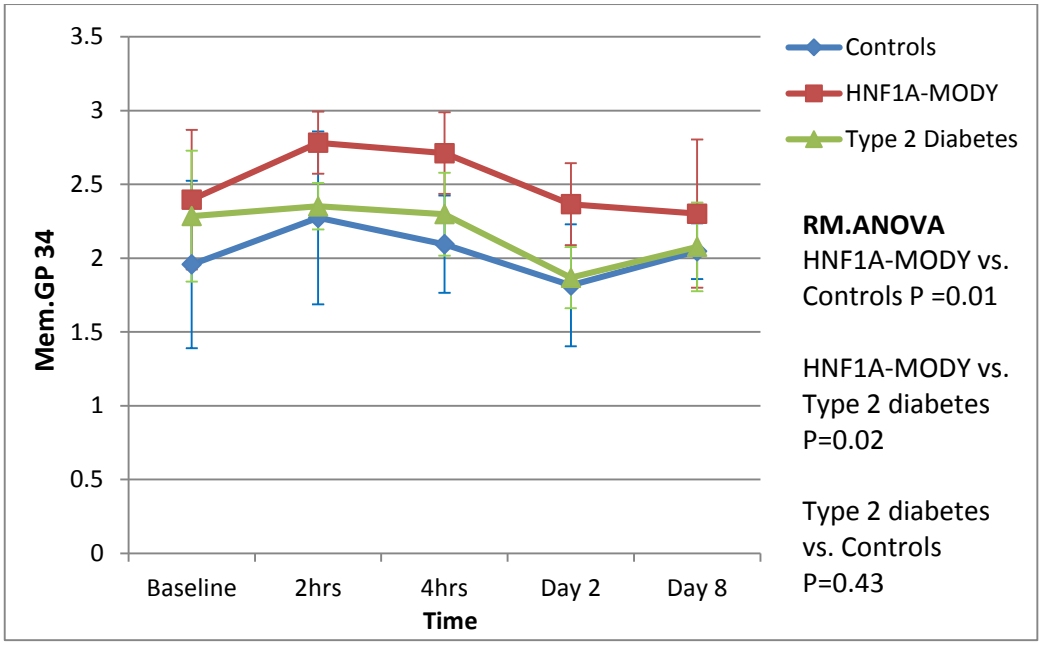


Figure 6.10: Mean (SD) Mem.GP 34 for healthy controls, HNF1A-MODY and type 2 diabetes for each time point. LPS elicited a significant increase in Mem.GP 34 in all groups ($p<0.0001$ as tested by RM.ANOVA). P values in the graph refer to difference between groups calculated by RM.ANOVA.

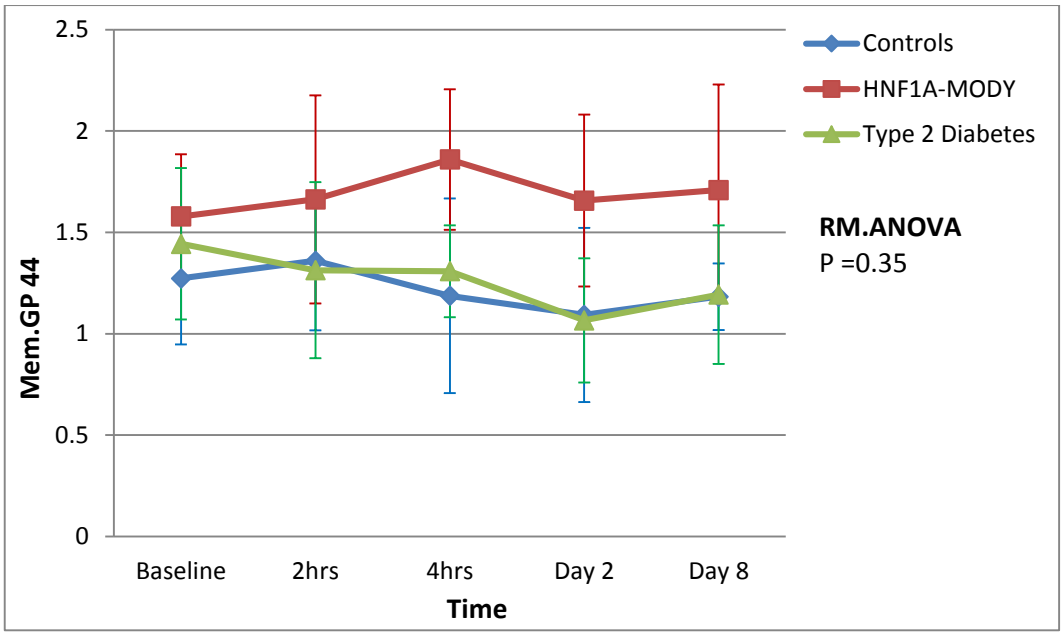


Figure 6.11: Mean (SD) Mem.GP 44 for healthy controls, HNF1A-MODY and type 2 diabetes for each time point. P value refers to change in Mem.GP 44 levels over time for all groups calculated by RM.ANOVA.

6.5 Discussion

HNF1A-MODY has been shown to be associated with low baseline CRP and lower concentration of antennary fucosylated glycans. As discussed previously, both CRP and glycans are important for an acute phase response and have been shown to undergo a change in concentration and/or structure during acute inflammation. This study aimed to investigate whether subjects with HNF1A-MODY demonstrate any abnormalities in CRP, plasma or membrane glycan profile, or show any alteration of other markers of inflammation during a moderate acute inflammatory response. For this, I used LPS-induced human endotoxemia, a standardised model of moderate acute inflammation. Despite low baseline CRP, subjects with HNF1A-MODY do not have a clinically significant alteration of CRP response compared with controls. Consistent with the known effect of HNF1A on fucosylation, a lower baseline concentration of antennary fucosylated glycans was observed, but there was no variation during the time course of the inflammatory response. In accordance with the previous report of an association of HNF1A-MODY with an increase in glycan branching, a significant increase in highly branched glycans was observed in HNF1A-MODY in the present study. A surprising finding was that despite a normal CRP response (a cytokine-mediated process), subjects with HNF1A-MODY appeared to have an attenuated cytokine response.

The role of HNF1A in regulation of *CRP* expression has been investigated in a number of in vitro studies (Nishikawa, et al., 2008; Toniatti, et al., 1990b). HNF1A forms a complex with the cytokine stimulated transcription factors (c-FOS and STAT3) which then binds with *CRP* response element to stimulate CRP

production. Most of the CRP present in serum is produced in the liver (Gabay, 1999). However some CRP is also produced in non-hepatic tissues such as adipocytes, gut, macrophages, coronary artery smooth muscle cells, epithelial cells of the respiratory tract and renal epithelium (Yeh, 2005). *HNF1A* expression is greatest in the liver, islets, gut and kidney while lower expression has been observed in other tissues such as visceral adipose tissue (Harries, et al., 2006). Therefore, it is possible that the intact CRP rise in HNF1A-MODY could be due to HNF1A-independent mechanisms of CRP production in hepatic or non-hepatic sites. Another explanation could be an increased production of wild type HNF1A during the stimulated state hence overcoming *HNF1A* haploinsufficiency and consequent normal CRP rise in HNF1A-MODY.

The a-priori sample size calculations in this study were based on the hypothesis that the difference in peak CRP levels between subjects with HNF1A-MODY and healthy controls would be of similar magnitude to as that seen at baseline (i.e. 50% lower than healthy controls). In this study, the peak CRP in HNF1A-MODY subjects was approximately 6% lower. This could indicate an attenuated response that the study was underpowered to demonstrate. A post hoc power analysis revealed that on the basis of the difference in peak mean CRP between healthy controls and HNF1A-MODY observed in the present study, a sample size of 49 subjects in each group would be needed to show a significant difference at 80% statistical power. Having said that, such a small difference in peak CRP levels is not likely to have clinical implications for HNF1A-MODY patients. No studies or anecdotal reports exist to suggest impaired acute inflammatory response in HNF1A-MODY.

CRP is downstream of IL-6 in the inflammation pathway. It seems paradoxical that despite a low baseline CRP and an attenuated cytokine response, subjects with HNF1A-MODY had a normal CRP rise. It is not clear how *HNF1A* haploinsufficiency can affect cytokine response. Cytokines are mainly produced by macrophages. HNF1A has so far not been shown to affect the proteins involved in cytokine production signalling cascade (**Figure 6.1**) (Servitja, et al., 2009) and blood cells are not known to be a major site of *HNF1A* expression, although the Human Protein Atlas data (http://www.proteinatlas.org/) suggests that this does occur in the lung macrophages. Cytokines operate in a complex network. Once cytokines are produced, some cytokines such as IL-1 and TNFα stimulate production of other cytokines. *HNF1A* knockout mice show downregulation of genes encoding IL-1 receptor (IL-1 receptor type 1 and IL-1 receptor accessory protein) (Servitja, et al., 2009). How much this could affect cytokine production is not known.

Another plausible explanation for an attenuated cytokine response in HNF1A-MODY is use of sulphonylureas, exposure to which has been associated with reduced cytokine production (Desfaits, et al., 1998; Kewcharoenwong, et al., 2013; Mamputu, et al., 2006). Sulphonylureas are the first line treatment in HNF1A-MODY and consequently 8 out of 9 HNF1A-MODY subjects were taking gliclazide (a common sulphonylurea) compared with 4 out of 7 of the type 2 diabetes group. Studies in patients with type 2 diabetes investigating the effect of gliclazide observed reduced TNFα and IL-6 secretion in patients taking gliclazide and in LPS-stimulated human macrophages (Desfaits, et al., 1998;

Mamputu, et al., 2006). As most MODY subjects were taking gliclazide, it was not possible to compare cytokine production among those taking and not taking gliclazide. This requires further investigation possibly by comparative studies investigating effect of sulphonylureas exposure on LPS-stimulated cytokine production in monocytes derived from patients with HNF1A-MODY, type 2 diabetes and controls.

Only one previous study has investigated acute inflammatory response in otherwise healthy subjects with type 2 diabetes using human endotoxemia (Andreasen, et al., 2010). A low dose of LPS (0.3ng/kg, compared with 2 ng/kg in this study) was used. In this case, the authors observed an attenuated cytokine response (TNFα, lL-6 and IL1-ra) in subjects with type 2 diabetes compared with controls. An attenuated cytokine response was not observed among subjects with type 2 diabetes in the current study. The studies may not be comparable, however, because of the difference in endotoxin dose used.

This is the first study to investigate change in plasma and WBC membrane glycans using a standardised model of acute inflammation. As observed previously (Thanabalasingham, et al., 2013), baseline antennary fucosylated plasma glycans were low in HNF1A-MODY subjects compared with controls and subjects with type 2 diabetes. Change in plasma glycans (increase in ratio of antennary to core fucosylated glycans) previously associated with inflammation (Gornik, et al., 2007) was also observed in this study. This previously published description of plasma glycan profile during inflammation was based on only two individuals and a non-standardised setting.

Membrane glycans, Mem.GP 34 and 44, were higher in subjects with HNF1A-MODY compared with controls and those with type 2 diabetes. Both Mem.GP 34 and 44 represent highly branched glycan structures. This finding accords with an earlier observation demonstrating an increase in highly branched glycans in HNF1A-MODY (Ohtsubo, et al., 2011; Zoldos, et al., 2012).

White cell membrane glycans, Mem.GP 16, 25, 28 and 34, showed a change in concentration over the course of the inflammatory response. These membrane glycans could represent WBC selectins or selectin ligands that are upregulated during an acute inflammation. Due to technical difficulties experienced during the interpretation of chromatogram peaks by our collaborator group in Croatia, the precise composition of Mem.GP 16, 25, 28 and 34 could not be determined. Therefore, the reason for change in concentration of these membrane glycans was not clear at the time of writing of this chapter. The pattern of increase and decrease in the concentration of Mem.GP 16, 25, 28 and 34 during the inflammatory response was similar in the three groups of subjects, indicating consistency in the protocol used for isolation of membrane glycans. No significant differences were observed in any other membrane glycans in HNF1A-MODY compared with controls.

In essence, the plasma and membrane glycan data is in keeping with the CRP result in that, despite previously reported differences in baseline levels of CRP and glycans, no significant perturbation is observed for these measures during a stimulated state of inflammation in HNF1A-MODY.

In conclusion, the subjects with HNF1A-MODY maintain the ability to generate peak CRP levels comparable to the controls. Attenuated cytokine response was observed in HNF1A-MODY, the mechanism for which remains uncertain but may be related to the use of sulphonylureas. This study confirms previous reports of change in plasma glycan profile during an acute inflammation by using a standardised model of inflammatory response.

Chapter 7

General discussion and conclusions

From the original description of MODY as a distinct diabetes subtype in 1975 (Tattersall and Fajans, 1975) to the identification of causal genes, clinical characterisation and defining optimum treatment to the identification of biomarkers for improving diagnosis, we have come a long way in our understanding of MODY. The work presented in this thesis adds to this increasing continuum of knowledge, specifically for the most common form of MODY due to mutations in *HNF1A*, in two particular aspects. Firstly, this research work evaluates the biomarkers required for an improved diagnosis of HNF1A-MODY. The perturbations in these biomarkers suggested that the acute inflammatory response might be impaired in HNF1A-MODY. This was investigated in the second part of this thesis.

7.1 Evaluation of HNF1A-MODY biomarkers

A correct molecular diagnosis of HNF1A-MODY has significant clinical implications for the patients and their family members. A correct diagnosis is important for optimal treatment and preventing unnecessary treatment with insulin, predicting clinical course and identifying at-risk family members. However, the majority of HNF1A-MODY cases remain misdiagnosed. To facilitate prioritisation of patients for genetic testing, a number of studies have investigated up to 12 biomarkers. Some of the previous candidate biomarkers investigated could not demonstrate sufficient sensitivity or specificity or their performance was dependent on matching for certain variables between cases and controls. For some biomarkers, the initial positive findings could not be replicated by the follow-up studies. Lastly a few have shown promising results in initial studies and follow-up studies are yet to be performed. In this thesis,

biomarkers in the last two categories were evaluated.

Apolipoprotein M (apoM), a component of high-density lipoprotein (HDL) cholesterol regulated by HNF1A, is one such biomarker for which initial study findings were not replicated by the follow up studies. Initially, lower serum apoM concentration was observed in HNF1A-MODY compared with subjects without diabetes (Richter, et al., 2003). This finding could not be replicated in two follow up studies nor was a difference in apoM concentration observed between HNF1A-MODY and other diabetes subtypes (Cervin, et al., 2010; Skupien, et al., 2007). These inconsistent results made it difficult to determine the role of apoM as HNF1A-MODY biomarker. One explanation for the observed inconsistency in the results of the previous studies was the difference in the techniques used for determining apoM concentration.

To evaluate the role of apoM as HNF1A-MODY biomarker, the study described in chapter 3 set out to measure apoM concentration in subjects with HNF1A-MODY, type 1 and type 2 diabetes and controls using a highly sensitive and specific ELISA. As per the initial observation, it was found that apoM was significantly lower in HNF1A-MODY compared with non-diabetic controls. Moreover, apoM was markedly lower in HNF1A-MODY compared with those with type 1 diabetes. The receiver operating characteristic (ROC) curve derived C-statistic was 0.91 indicating that apoM can discriminate well between HNF1A-MODY and type 1 diabetes. No significant difference was observed in apoM concentration between HNF1A-MODY and those with type 2 diabetes.

These results add apoM to the list of other biomarkers proposed for differentiating HNF1A-MODY from type 1 diabetes: C-peptide, islet autoantibodies and DG9-

glycan index (Besser, et al., 2011; McDonald, et al., 2011a; Thanabalasingham, et al., 2013). There are advantages of the use of apoM compared with C-peptide and islet autoantibodies. Unlike C-peptide, apoM concentration is unlikely to change with the clinical course of diabetes. Although, the results of a UK study show that <1% of MODY subjects are positive for islet autoantibodies, islet autoantibodies have been observed in a higher proportion of MODY subjects in other reports (Lehto, et al., 1999; Schober, et al., 2009; Thanabalasingham, et al., 2012b).

This study included carefully defined groups of subjects, which could have led to an overestimate of apoM performance. Follow up studies in unselected group of subjects are required to assess apoM performance in a scenario more typical of a young-adult diabetes clinic.

One limitation to the use of apoM in clinical care is that the ELISA used for apoM measurement in the study described in chapter 3, is currently restricted to research use. There is growing interest in apoM both as a marker of sepsis and as a carrier of sphingosine 1 phosphate in HDL-cholesterol, an important vasoprotective lipid mediator, and this may lead to an increase in the apoM assay availability (Christoffersen, et al., 2011; Kumaraswamy, et al., 2012).

In addition to apoM, two other biomarkers, hsCRP and DG9-glycan index, were studied in this thesis. HsCRP and DG9-glycan index have shown promise to discriminate between HNF1A-MODY and type 2 diabetes and between HNF1A-MODY and both type 1 and type 2 diabetes respectively (McDonald, et al., 2011b; Thanabalasingham, et al., 2013; Thanabalasingham, et al., 2011). It was hypothesised that given the strict selection criteria used, the initial studies

investigating these biomarkers may have been prone to “spectrum bias” resulting in inflated estimates of sensitivity, specificity and other measures of biomarker performance. The study described in chapter 4 set out to determine the “true performance” of the biomarkers, hsCRP and DG9-glycan index, for identifying HNF1A-MODY in an unselected group of subjects with non-autoimmune diabetes diagnosed by the age of 30 years. This study also aimed to determine the frequency of the most common MODY subtypes.

All the participants were sequenced for the three most common MODY genes (*HNF1A, GCK* and *HNF4A*), so that the performance of the biomarkers could be compared with the gold standard test of sequencing. Four likely disease-causing *HNF1A* variants were identified (in six subjects out of 208 subjects sequenced), giving a proportion of MODY of 3.3% among those with young-onset non-autoimmune diabetes. Using previously reported cut-offs for hsCRP and DG9-glycan index (0.16 and 0.4mg/L), both biomarkers demonstrated a sensitivity of 83% (identifying five out of six HNF1A-MODY cases) and a specificity of 78 and 81% respectively.

This prospective evaluation confirmed the results of the earlier studies evaluating hsCRP and DG9-glycan index (Owen, et al., 2010; Thanabalasingham, et al., 2013) and support the use of hsCRP as an adjunct to the current clinical criteria used to select patients for *HNF1A* sequencing. A great advantage of using hsCRP is that it is available as a routine laboratory test. The use of DG9-glycan index is currently limited, as it requires an initial laboratory separation of plasma glycans followed by high performance liquid chromatography (HPLC), requiring specialist equipment. Therefore, the clinical use of DG9-glycan index is

dependent on the development of a suitable assay. This currently requires more work on understanding which of several glycan structures altered in HNF1A-MODY provides optimum discrimination between diabetes subtypes and design of a suitable high-throughput assay for this structure such as an ELISA. This work is being pursued in other joint products between my supervisors and our collaborators in Croatia.

As all subjects with likely disease causing *HNF1A* variants had either a low DG9-glycan index or a low hsCRP, this indicates that these biomarkers have potential to be used as a useful adjunct in interpreting the clinical significance of novel variants. With the increasing availability of DNA sequencing, both in research and clinical setting, more people will have *HNF1A* gene sequenced and more novel *HNF1A* variants are likely to be identified. The challenge will progressively shift towards would be the interpretation of the clinical significance of novel variants identified on sequencing. Examples of these would be rare, conserved variants identified in asymptomatic individuals or variants presenting with a MODY phenotype in a proband but not causing diabetes in another adult family member carrying the same variant. The hsCRP levels and DG9-glycan index might prove useful in such instances as one assessment of the functional effect of the novel *HNF1A* variants and to inform the diabetes risk to the individuals and potentially to the family members.

The results of this first prospective pilot study have paved the way for a larger prospective evaluation study being carried out by our research group. Efforts are underway to collect a dataset of approximately 1200 subjects with young-onset diabetes. All the subjects will undergo *HNF1A* sequencing, hsCRP and glycan

analysis. The results will help to further explore the performance of these biomarkers for identifying HNF1A-MODY.

An important step in the development of a biomarker is to determine if it can be used consistently across ethnic groups. South Asians comprise 4% of the UK population (2001). Only one previous study investigated MODY among South Asians in the UK. This study observed a low referral of South Asians for genetic testing (Porter, et al., 2006). A low referral seems paradoxical given the fact that South Asians have a four times higher prevalence of diabetes, mainly type 2 diabetes, that presents at a younger age and lower BMI compared with the Europeans making differentiation from MODY challenging (1994).

The study described in chapter 5 set out to evaluate the use of low CRP for identifying HNF1A-MODY among South Asians with young-onset diabetes. This study comprised of two groups of subjects. In the first group, South Asian subjects at a high risk of HNF1A-MODY, determined mainly by CRP levels ≤1.0 mg/L or in a few subjects using the age of onset of diabetes ≤30 years irrespective of CRP levels were sequenced for *HNF1A*. Six likely pathogenic *HNF1A* variants were identified, giving a proportion of MODY of 0.7% (6 out of 861 subjects) among South Asians with diabetes diagnosed by the age of 50 years. This was lower than the previously reported estimates of MODY in Europeans (4% among those with apparent type 2 diabetes), but is consistent with the greater prevalence of type 2 diabetes seen in South Asians. In order to determine whether using low CRP as the primary selection criteria identified most of the HNF1A-MODY cases, the study went on to evaluate CRP in a second group of subjects with and without diabetes not selected for low CRP. In the

second group, three likely pathogenic *HNF1A* variants were identified (in 3 out of 531 subjects with diabetes), giving a proportion of MODY of 0.5% amongst those with apparent type 2 diabetes. Two out of the three subjects had CRP more than 1.0 mg/L, indicating that by using low CRP in the first group of the subjects, some HNF1A-MODY cases might have been missed. The results of this study suggest that additional phenotypic and biochemical features will assist in selecting those at highest risk of monogenic diabetes. Future studies are required to determine which clinical and biochemical characteristics optimally discriminate MODY from young-onset type 2 diabetes in South Asians. These studies would need to establish a dataset of South Asians with MODY and with young-onset type 2 diabetes in order to compare and define the characteristics of the two diabetes subtypes.

During the analysis of the results of the studies described in chapters 4 and 5, the results of a large scale sequencing study were published (Flannick, et al., 2013). Flannick *et al* investigated the presence and phenotypic effects of the variants previously reported in association with MODY or rare, conserved variants, in randomly selected subjects from population-based cohorts. The authors observed that 3 in 200 individuals carry variants previously reported in association with MODY (including *HNF1A* variants) and remain euglycaemic through middle age. Case reports support the findings of Flannick *et al*. Miedzybrodzka *et al* reported a family with a broad heterogeneity in the clinical presentation of HNF1A-MODY (Miedzybrodzka, et al., 1999). In this family, five subjects carrying a single conserved, likely protein-damaging variant, T620I, had an age at diagnosis of diabetes of less than 30 years. Two family members carrying the same variant were diagnosed at age 67 and 52 years while two

members of the family were euglycaemic by the age of 46 and 87 years. Similarly, the type and position of the *HNF1A* variants also affect the age at diagnosis of diabetes (Bellanne-Chantelot, et al., 2008; Harries, et al., 2006). In addition, there are *HNF1A* variants that are reported in association with type 2 diabetes (Voight, et al., 2010; Winckler, et al., 2007). Together, these observations indicate that *HNF1A* variants range across a broad spectrum of functional and clinical severity.

In line with the above observations, in the study described in chapter 5, I identified *HNF1A* variants, predicted by bioinformatics as conserved and with likely damaging effect on protein function, in subjects without diabetes by the age of 65 and 70 years. Molecular functional characterisation is required to determine the impact of these variants on HNF1A function. Ongoing research studies carried out by our research group are performing molecular functional analyses of protein-altering *HNF1A* variants identified from the studies described in this thesis as well as through analyses of large-scale whole exome sequence data from the major ethnic groups. These studies will then investigate the relationship of the in vivo functional severity with the clinical phenotype and biomarker profiles. The results will be used to update MODY diagnostic guidelines.

7.2 Investigation of the acute inflammatory response in HNF1A-MODY

HNF1A is part of a transcriptional complex regulating hepatic CRP production. HsCRP is an acute phase protein, used an indicator of inflammation in the clinical practice. Moreover, it is not just a passive bystander in the process of inflammation, but plays an important role in inflammation such as activation of

the complement system and opsonisation of pathogens for phagocytosis (Du Clos, 2013; Pepys and Hirschfield, 2003).

HNF1A also regulates protein fucosylation (Lauc, et al., 2010). Fucosylation is important for cell adhesion and leukocyte migration into the inflamed tissues during an acute inflammation (Lauc et al., 2010). Moreover, although the clinical significance is not known, changes in glycan profile such as an increase in the ratio of antennary to core fucosylated glycans and an increase in the highly sialylated branched glycans have been observed in inflammation (Gornik, et al., 2007).

The second part of my thesis investigated the acute inflammatory response in HNF1A-MODY. A lipopolysaccharide (LPS) stimulated model of acute inflammation was used to compare CRP, plasma and membrane glycans and other biochemical and haematological components of an acute inflammatory response in HNF1A-MODY, controls and those with type 2 diabetes. It was observed that despite low baseline levels, HNF1A-MODY subjects had an equivalent CRP rise comparable to that in the controls. Possible explanations for this observation could be HNF1A independent hepatic or extrahepatic CRP production or that the process of acute inflammation overrides the requirement for HNF1A for hsCRP production. Another explanation could be that the acute inflammation induces compensatory expression from the wild-type allele resulting in a normal CRP rise in HNF1A-MODY. This requires further investigation as the transcriptional complex formation of HNF1A with two other transcription factors, C-fos and STAT3, is so far known to be essential for cytokine driven hepatic hsCRP production (Nishikawa, et al., 2008).

A change in the plasma glycan profile (an increase in the ratio of antennary to core fucosylated glycans) previously reported to be associated with inflammation, was also observed in this study (Gornik, et al., 2007). The previous observation was based on the results from two individuals during a state of severe acute inflammation. This is the first study to confirm these changes in the glycan profile during an acute inflammatory response using a standardised model of acute inflammation.

Two white cell membrane glycan peaks containing highly branched glycans were found to be higher in subjects with HNF1A-MODY compared with the controls and those with type 2 diabetes at baseline and post LPS administration. The higher levels of these membrane glycan peaks in HNF1A-MODY are consistent with the results of a previous study showing a significant increase in the levels of highly branched plasma glycans in HNF1A-MODY (Zoldos, et al., 2012). A novel finding was that some membrane glycans (Mem.GP 16, 25, 28 and 34), also showed a change in concentration over the course of the inflammatory response. The change in concentration suggests that these glycan peaks contain structures, which are upregulated during the white cell response to inflammation. Identifying these structures (using Mass Spectrometry) would give us a novel insight into the way white cells behave during inflammation.

Another finding of this study was an attenuated response of the pro-inflammatory cytokines, IL-6 and TNFα, in HNF1A-MODY. It seems paradoxical that despite an attenuated cytokine response, CRP levels rose to the same extent as in controls. Low cytokines in MODY have not been reported before. One possible explanation for the attenuated cytokine response in HNF1A-MODY could be

exposure to sulphonylureas, the first line treatment in HNF1A-MODY, which has been previously reported to be associated with a decreased cytokine production (Desfaits, et al., 1998; Mamputu, et al., 2006). One way of further investigating this would be to investigate the effect of sulphonylurea exposure on LPS-stimulated cytokine production in an *in-vitro* system using cultured monocytes derived from patients with HNF1A-MODY, type 2 diabetes and controls.

7.3 Concluding remarks

The research work presented in this thesis contributes to the existing knowledge on HNF1A-MODY biomarkers and takes the biomarkers investigated, apoM, hsCRP and DG9-glycan index, a step further towards their clinical translation and use for prioritising subjects for genetic testing. Rather than becoming redundant due to increased availability of genetic sequencing, the difficulties described above with interpretation of genetic variants suggest that biomarkers have great potential to become part of the armoury of ways that we assess the clinical significance of alteration in the genome. This research work also emphasises the need for future studies defining the optimum clinical and biochemical features to identify HNF1A-MODY cases in ethnic groups where MODY has not been well characterised. Finally, this thesis provides the first mechanistic insight of the impact of HNF1A regulation on the components of an acute inflammatory response.

Appendices

Complete profile of plasma and membrane glycan levels at baseline and after Lipopolysaccharide administration

	HNF1A-MODY	Controls	Type 2 diabetes	P
PL.GP.1				0.35
0hr	3.25 (2.00-4.55)	3.62 (1.88-6.69)	3.52 (3.09-5.96)	
2h	3.82 (1.75-4.16)	4.06 (1.82-6.04)	3.28 (3.06-4.22)	
4hr	3.22 (2.05-4.86)	3.53 (2.22-6.67)	3.76 (1.99-4.45)	
6hr	3.41 (1.79-4.37)	4.31 (1.77-6.10)	3.26 (2.58-5.79)	
24 hr	3.09 (1.71-4.69)	3.32 (1.73-6.19)	3.39 (2.98-4.81)	
D8	3.31 (1.84-4.62)	3.92 (1.87-6.58)	3.38 (2.29-3.95)	
PL.GP.2				0.85
0hr	1.85 (1.67-2.35)	2.02 (1.43-2.38)	2.03 (1.42-2.62)	
2h	1.89 (1.62-2.46)	1.97 (1.68-2.27)	1.79 (1.44-2.77)	
4hr	1.82 (1.53-2.79)	2.03 (1.38-2.28)	1.73 (1.47-2.22)	
6hr	1.88 (1.63-2.08)	1.98 (1.58-2.91)	1.86 (1.44-2.39)	
24 hr	1.72 (1.55-1.95)	1.69 (1.29-2.15)	2.00 (1.26-2.58)	
D8	1.85 (1.58-2.59)	2.01 (1.46-2.41)	1.78 (1.56-2.07)	
PL.GP.3				0.48
0hr	0.12 (0.07-0.24)	0.11 (0.04-0.21)	0.115 (0.09-0.14)	
2h	0.13 (0.09-0.19)	0.09 (0.06-0.19)	0.11 (0.07-0.15)	
4hr	0.13 (0.08-0.24)	0.11 (0.05-0.23)	0.10 (0.09-0.17)	
6hr	0.10 (0.05-0.19)	0.08 (0.04-0.2)	0.12 (0.09-0.13)	
24 hr	0.10 (0.07-0.18)	0.08 (0.04-0.21)	0.11 (0.08-0.15)	
D8	0.11 (0.07-0.19)	0.11 (0.06-0.18)	0.09 (0.04-0.16)	
PL.GP.4.				0.27
0hr	3.65 (2.98-5.88)	4.23 (3.32-6.99)	3.77 (2.28-7.30)	
2h	3.89 (2.67-5.29)	4.49 (2.92-6.51)	3.68 (2.58-4.92)	
4hr	3.67 (2.98-5.23)	4.31 (3.27-6.98)	3.74 (2.26-5.45)	
6hr	3.66 (2.70-5.06)	4.37 (3.17-6.97)	3.28 (2.64-6.86)	
24 hr	3.82 (2.59-3.98)	3.86 (3.02-6.12)	3.63 (2.12-5.99)	
D8	3.83 (2.75-5.48)	4.22 (2.51-6.91)	3.66 (2.61-4.82)	
PL.GP.5				0.31
0hr	1.77 (1.49-2.85)	2.17 (0.97-4.05)	1.99 (1.14-3.01)	
2h	1.94 (1.39-2.48)	2.33 (1.01-3.21)	1.98 (1.27-2.24)	
4hr	2.19 (1.48-2.48)	2.19 (1.17-3.54)	2.05 (1.04-2.51)	
6hr	1.70 (1.42-2.54)	2.43 (0.94-3.48)	1.80 (1.29-2.95)	
24 hr	1.65 (1.36-2.68)	1.97 (0.93-2.89)	1.89 (1.03-2.56)	
D8	1.80 (1.39-2.59)	2.34 (1.02-3.76)	1.88 (1.13-2.43)	
PL.GP.6				0.48
0hr	1.23 (0.94-2.25)	1.13 (0.89-1.49)	1.54 (0.81-1.75)	
2h	1.33 (0.91-1.85)	1.22 (0.89-1.37)	1.24 (0.91-1.88)	
4hr	1.24 (0.81-2.26)	1.18 (0.88-1.54)	1.17 (0.86-1.65)	
6hr	1.30 (0.91-1.83)	1.19 (0.89-1.62)	1.35 (0.94-1.59)	
24 hr	1.13 (1.00-10.68)	1.01 (0.78-1.28)	1.41 (0.75-1.73)	
D8	1.20 (0.89-1.86)	1.27 (0.84-1.45)	1.16 (0.79-1.56)	
PL.GP.7				0.81
0hr	1.38 (0.94-1.62)	1.30 (1.07-1.68)	1.47 (0.93-2.02)	
2h	1.30 (1.11-1.59)	1.32 (0.99-1.97)	1.15 (1.06-2.06)	
4hr	1.22 (0.88-1.84)	1.32 (1.00-1.55)	1.19 (1.00-11.74)	
6hr	1.23 (1.03-1.39)	1.24 (0.99-1.87)	1.29 (1.08-1.71)	
24 hr	1.15 (0.94-1.55)	1.16 (0.99-1.43)	1.33 (0.88-1.97)	
D8	1.32 (0.87-1.75)	1.21 (0.91-2.55)	1.29 (1.03-1.66)	
PL.GP.8				0.97
0hr	2.93 (2.39-3.85)	2.89 (1.90-3.89)	2.91 (1.95-3.72)	
2h	2.75 (2.15-4.49)	3.22 (1.98-4.07)	3.15 (2.08-3.96)	
4hr	3.03 (1.95-3.41)	2.76 (1.82-3.65)	3.05 (2.08-3.87)	
6hr	3.11 (2.20-3.83)	3.09 (2.19-3.83)	3.14 (1.68-3.79)	

24 hr	2.94 (1.96-3.42)	3.02 (1.55-3.68)	3.28 (1.87-3.82)	
D8	2.99 (2.31-4.28)	3.04 (2.25-3.66)	2.85 (1.5-3.25)	
PL.GP.9				0.25
0hr	0.21 (0.13-0.41)	0.16 (0.11-0.31)	0.20 (0.16-0.25)	
2h	0.19 (0.17-0.26)	0.17 (0.1-0.28)	0.16 (0.12-0.27)	
4hr	0.26 (0.15-0.39)	0.165 (0.12-0.3)	0.18 (0.14-0.31)	
6hr	0.21 (0.12-0.33)	0.14 (0.11-0.39)	0.18 (0.14-0.21)	
24 hr	0.18 (0.13-0.25)	0.17 (0.1-0.26)	0.20 (0.13-0.21)	
D8	0.19 (0.14-0.33)	0.18 (0.12-0.24)	0.16 (0.11-0.20)	
PL.GP.10+11				0.26
0hr	4.81 (3.99-8.32)	5.25 (2.99-7.58)	4.54 (1.97-7.11)	
2h	5.68 (3.87-6.18)	4.90 (3.93-6.62)	4.52 (2.17-5.07)	
4hr	4.97 (4.20-8.09)	5.41 (2.89-6.75)	4.58 (2.09-5.56)	
6hr	4.66 (3.84-6.75)	5.14 (3.59-8.54)	4.20 (2.27-6.71)	
24 hr	4.46 (3.74-5.87)	4.61 (2.65-5.45)	4.15 (1.81-6.12)	
D8	4.62 (4.09-6.74)	4.90 (2.23-6.91)	4.44 (2.96-5.07)	
PL.GP.12				0.74
0hr	1.27 (0.71-1.95)	1.09 (0.91-1.37)	1.03 (0.62-1.89)	
2h	1.23 (0.88-1.35)	1.18 (0.88-1.47)	0.88 (0.68-2.12)	
4hr	1.22 (0.75-1.89)	1.06 (0.96-1.36)	0.87 (0.69-1.99)	
6hr	1.12 (0.83-1.66)	1.15 (0.9-1.54)	0.93 (0.79-1.74)	
24 hr	1.08 (0.71-1.26)	1.00 (0.88-1.21)	0.95 (0.62-1.79)	
D8	1.25 (0.7-1.62)	1.07 (0.71-1.72)	1.00 (0.80-1.61)	
PL.GP.13				0.62
0hr	1.67 (1.4-2.22)	1.6 (1.08-2.18)	1.59 (1.27-2.34)	
2h	1.67 (1.11-1.97)	1.68 (1.34-2.05)	1.59 (1.25-1.81)	
4hr	1.75 (1.44-2.27)	1.62 (0.96-1.93)	1.65 (0.69-1.91)	
6hr	1.57 (1.49-1.88)	1.61 (1.28-2.32)	1.56 (1.26-2.18)	
24 hr	1.52 (1.26-1.86)	1.58 (0.96-1.77)	1.52 (1.27-2.13)	
D8	1.61 (1.34-2.42)	1.64 (1.38-1.83)	1.52 (1.00-1.85)	
PL.GP.14				0.64
0hr	0.69 (0.46-0.79)	0.61 (0.12-0.87)	0.77 (0.34-1.52)	
2h	0.61 (0.5-0.84)	0.64 (0.43-1.15)	0.59 (0.40-1.81)	
4hr	0.64 (0.5-0.88)	0.62 (0.44-0.87)	0.70 (0.40-1.19)	
6hr	0.62 (0.53-0.81)	0.73 (0.43-1.02)	0.68 (0.15-1.08)	
24hr	0.58 (0.49-0.89)	0.61 (0.36-0.77)	0.64 (0.4-1.58)	
D8	0.60 (0.51-0.90)	0.62 (0.47-1.34)	0.70 (0.56-1.25)	
PL.GP.15				0.60
0hr	18.9 (16.3-23.2)	18.3 (14.1-21.4)	20.4 (13.9-25.9)	
2h	17.8 (15.1-25.0)	19.6 (13.9-22.6)	20.4 (13.6-26.9)	
4hr	18.8 (15.0-22.5)	17.1 (14.7-20.9)	19.9 (13.9-22.2)	
6hr	19.5 (15.9-21.2)	18.4 (14.5-25.2)	18.7 (13.1-24.2)	
24 hr	18.5 (13.6-21.6)	18.6 (14.3-19.7)	21.5 (13.4-24.8)	
D8	19.9 (16.5-25.6)	18.4 (15.9-24.2)	18.7 (12.3-22.2)	
PL.GP.16				0.11
0hr	0.69 (0.47-1.38)	0.55 (0.45-0.83)	0.67 (0.57-0.92)	
2h	0.70 (0.58-0.92)	0.67 (0.40-1.05)	0.67 (0.46-0.88)	
4hr	0.86 (0.55-1.38)	0.63 (0.41-0.97)	0.69 (0.50-0.94)	
6hr	0.73 (0.51-1.05)	0.57 (0.43-0.78)	0.72 (0.52-0.81)	
24 hr	0.67 (0.50-0.91)	0.55 (0.41-0.95)	0.72 (0.51-0.87)	
D8	0.70 (0.52-1.02)	0.66 (0.45-0.93)	0.65 (0.51-0.75)	
PL.GP.17+18				0.73
0hr	6.35 (4.46-9.39)	6.05 (5.3-7.99)	6.19 (3.58-8.39)	
2h	6.03 (5.05-7.03)	6.49 (5.40-7.49)	5.74 (4.04-7.70)	
4hr	6.01 (4.39-9.09)	6.42 (5.07-7.56)	5.59 (3.85-7.80)	
6hr	6.51 (4.75-7.54)	6.24 (5.39-9.68)	5.41 (4.24-7.68)	
24 hr	5.51 (4.39-7.08)	5.90 (4.78-6.42)	6.33 (3.48-7.28)	
D8	6.97 (4.18-7.51)	6.15 (4.16-8.26)	5.73 (5.26-6.98)	
PL.GP.19				0.96
0hr	2.51 (1.21-3.56)	2.16 (1.56-2.98)	1.81 (1.32-4.64)	

2h	2.28 (1.59-2.91)	2.29 (1.47-3.85)	1.58 (1.15-4.74)	
4hr	2.15 (1.30-3.83)	2.16 (1.63-2.92)	1.71 (1.28-4.74)	
6hr	2.29 (1.41-2.84)	2.10 (1.43-3.47)	1.74 (1.26-4.47)	
24 hr	2.06 (1.17-2.59)	1.91 (1.32-2.77)	1.71 (1.22-4.35)	
D8	2.20 (1.20-3.46)	2.07 (1.41-4.70)	1.75 (1.35-4.38)	
PL.GP.20+21				0.90
0hr	3.09 (2.54-3.68)	2.99 (2.37-3.81)	3.02 (2.65-3.48)	
2h	3.26 (2.06-3.82)	3.25 (2.41-3.93)	2.98 (2.71-4.08)	
4hr	3.01 (2.62-3.62)	3.17 (2.58-3.97)	2.95 (2.59-3.89)	
6hr	3.29 (2.84-3.84)	3.33 (2.49-3.96)	3.06 (2.89-3.24)	
24 hr	3.00 (1.95-4.03)	3.19 (2.63-3.99)	3.01 (2.60-3.19)	
D8	2.92 (2.64-3.63)	3.31 (2.01-3.81)	3.31 (2.85-3.80)	
PL.GP.22				0.29
0hr	1.28 (0.94-1.62)	1.02 (0.84-1.62)	1.32 (1.00-1.91)	
2h	1.09 (0.95-1.48)	1.07 (0.83-1.46)	1.25 (0.98-2.30)	
4hr	1.11 (0.95-1.55)	1.03 (0.84-1.57)	1.28 (1.04-1.36)	
6hr	1.04 (0.96-1.49)	1.19 (0.83-1.60)	1.34 (0.99-1.59)	
24 hr	1.00 (0.96-1.65)	0.99 (0.73-1.36)	1.20 (0.98-1.80)	
D8	1.29 (0.95-1.74)	1.20 (0.84-1.60)	1.29 (1.00-1.49)	
PL.GP.23				0.90
0hr	22.0 (15.5-25.1)	21.8 (15.9-29.1)	21.0 (11.8-29.3)	
2h	21.9 (15.3-25.6)	20.2 (17.7-24.4)	22.5 (16.9-27.7)	
4hr	21.7 (15.0-24.3)	22.1 (17.3-27.8)	23.1 (16.5-29.0)	
6hr	23.3 (20.3-26.5)	21.3 (10.8-26.0)	22.7 (14.5-26.8)	
24 hr	23.5 (15.7-26.4)	23.0 (19.9-30.2)	20.1 (17.0-27.9)	
D8	21.4 (14.8-23.6)	21.6 (13.6-27.1)	24.0 (19.8-25.5)	
PL.GP.24				0.07
0hr	0.68 (0.57-0.72)	0.64 (0.48-0.86)	0.84 (0.64-1.15)	
2h	0.67 (0.46-0.73)	0.64 (0.57-1.06)	0.74 (0.60-1.23)	
4hr	0.61 (0.58-0.90)	0.64 (0.49-0.87)	0.76 (0.61-0.94)	
6hr	0.61 (0.57-0.75)	0.61 (0.53-1.02)	0.79 (0.61-1.04)	
24 hr	0.63 (0.53-0.72)	0.61 (0.55-0.83)	0.81 (0.56-1.13)	
D8	0.65 (0.51-0.97)	0.64 (0.50-1.25)	0.67 (0.61-0.90)	
PL.GP.25				0.21
0hr	2.65 (1.91-4.08)	2.8 (2.45-4.2)	3.455 (1.98-4.11)	
2h	2.82 (1.55-4.22)	2.99 (2.38-3.89)	3.41 (2.64-4.23)	
4hr	2.73 (1.94-3.86)	2.95 (2.53-4.31)	3.74 (2.38-4.34)	
6hr	3.10 (1.81-3.69)	2.93 (1.95-4.18)	3.35 (2.43-4.76)	
24 hr	2.93 (1.99-3.46)	3.09 (2.47-4.1)	3.13 (2.49-3.96)	
D8	2.69 (1.75-3.83)	2.94 (1.96-4.23)	3.96 (2.64-4.71)	
PL.GP.26				0.41
0hr	1.58 (0.84-2.03)	1.08 (0.79-2.21)	1.04 (0.79-2.70)	
2h	1.62 (0.77-2.72)	1.07 (0.73-2.41)	1.18 (0.62-2.57)	
4hr	1.37 (0.97-2.08)	1.15 (0.82-2.07)	1.18 (0.80-2.53)	
6hr	1.63 (0.92-2.55)	0.96 (0.76-2.15)	1.06 (0.64-2.69)	
24 hr	1.44 (0.87-2.63)	1.00 (0.77-1.91)	0.98 (0.66-2.52)	
D8	1.24 (0.74-2.64)	1.01 (0.75-2.04)	1.00 (0.77-2.63)	
PL.GP.27+28				0.16
0hr	3.31 (2.33-4.69)	2.60 (1.75-3.86)	2.86 (1.65-3.85)	
2h	3.25 (2.68-3.78)	2.62 (1.91-3.88)	2.84 (2.34-3.81)	
4hr	3.01 (2.60-4.58)	2.64 (2.03-3.57)	2.89 (2.51-3.44)	
6hr	3.22 (2.72-4.01)	2.86 (1.47-3.67)	3.01 (2.01-3.86)	
24 hr	3.31 (2.69-4.59)	2.86 (2.16-3.73)	2.81 (2.24-3.28)	
D8	3.18 (2.51-4.42)	2.74 (1.62-3.64)	3.09 (2.54-3.18)	
PL.GP.29				0.03
0hr	2.75 (1.63-3.59)	2.00 (1.37-2.65)	2.67 (1.02-3.28)	
2h	2.71 (2.08-3.08)	1.93 (1.56-2.82)	2.52 (1.63-3.09)	
4hr	2.56 (2.08-3.53)	2.01 (1.53-2.41)	2.57 (1.76-3.07)	
6hr	2.73 (1.94-3.18)	2.09 (0.80-2.50)	2.85 (1.25-3.28)	
24 hr	2.92 (2.35-3.52)	2.22 (1.72-2.62)	2.40 (1.48-3.19)	

D8	2.70 (1.79-3.4)	2.10 (1.32-2.82)	2.30 (1.72-3.27)	
PL.GP.30				1×10^{-5}
0hr	0.38 (0.23-0.63)	0.97 (0.65-1.64)	1.24 (0.67-1.47)	
2h	0.43 (0.21-0.70)	0.94 (0.48-1.39)	1.29 (0.81-1.36)	
4hr	0.37 (0.20-0.64)	0.95 (0.84-1.60)	1.29 (0.80-1.41)	
6hr	0.44 (0.23-0.62)	0.96 (0.56-1.53)	1.33 (0.91-1.58)	
24 hr	0.48 (0.21-0.63)	1.09 (0.76-1.50)	1.08 (0.82-1.31)	
D8	0.40 (0.29-0.53)	0.91 (0.69-1.42)	1.25 (0.88-1.56)	
PL.GP.31				0.36
0hr	0.79 (0.35-1.02)	0.65 (0.27-0.86)	0.61 (0.24-0.73)	
2h	0.72 (0.47-0.95)	0.50 (0.36-0.86)	0.53 (0.42-0.89)	
4hr	0.71 (0.35-1.04)	0.55 (0.44-0.80)	0.64 (0.38-0.81)	
6hr	0.76 (0.44-0.91)	0.58 (0.19-0.84)	0.68 (0.31-0.83)	
24 hr	0.82 (0.44-0.95)	0.61 (0.51-0.86)	0.64 (0.33-0.83)	
D8	0.69 (0.36-0.96)	0.60 (0.27-0.80)	0.69 (0.47-0.86)	
PL.GP.32				0.94
0hr	0.14 (0.05-0.18)	0.15 (0.03-0.28)	0.12 (0.08-0.19)	
2h	0.11 (0.08-0.23)	0.16 (0.05-0.23)	0.11 (0.1-0.15)	
4hr	0.15 (0.07-0.19)	0.17 (0.06-0.23)	0.12 (0.1-0.29)	
6hr	0.11 (0.08-0.22)	0.14 (0.03-0.23)	0.13 (0.08-0.22)	
24 hr	0.15 (0.10-0.26)	0.18 (0.12-0.22)	0.13 (0.07-0.25)	
D8	0.11 (0.08-0.21)	0.14 (0.04-0.23)	0.17 (0.07-0.29)	
PL.GP.33				0.35
0hr	5.06 (1.26-5.87)	3.67 (1.26-5.24)	2.87 (0.95-4.15)	
2h	3.99 (2.02-6.32)	2.76 (1.53-5.36)	2.57 (1.24-5.17)	
4hr	3.95 (1.36-6.56)	3.59 (1.73-5.01)	3.42 (1.65-4.84)	
6hr	3.98 (1.88-5.90)	3.41 (0.62-4.98)	3.23 (1.15-5.14)	
24 hr	5.01 (1.69-6.21)	3.71 (2.99-5.32)	3.17 (1.24-5.71)	
D8	4.18 (1.42-5.74)	3.54 (0.94-4.52)	3.02 (2.21-4.69)	
PL.GP.34				0.70
0hr	0.35 (0.04-0.7)	0.29 (0.08-0.45)	0.33 (0.07-0.39)	
2h	0.29 (0.11-0.70)	0.26 (0.11-0.45)	0.22 (0.15-0.60)	
4hr	0.29 (0.05-0.87)	0.34 (0.13-0.50)	0.34 (0.12-0.95)	
6hr	0.33 (0.12-0.45)	0.29 (0.02-0.46)	0.44 (0.08-0.60)	
24 hr	0.37 (0.11-0.65)	0.32 (0.21-0.47)	0.36 (0.11-0.53)	
D8	0.27 (0.06-0.65)	0.31 (0.06-0.41)	0.36 (0.15-0.60)	
PL.GP.35				0.42
0hr	1.14 (0.26-1.49)	0.90 (0.24-1.17)	0.50 (0.12-1.26)	
2h	0.93 (0.33-1.44)	0.60 (0.23-1.24)	0.53 (0.26-1.43)	
4hr	0.73 (0.28-1.61)	0.80 (0.33-1.35)	0.79 (0.19-1.47)	
6hr	0.94 (0.32-1.31)	0.73 (0.04-1.17)	0.71 (0.13-1.32)	
24 hr	1.36 (0.40-1.67)	0.88 (0.40-1.52)	0.63 (0.13-1.81)	
D8	1.00 (0.29-1.38)	0.73 (0.18-1.00)	0.82 (0.36-0.95)	
PL.GP.36				0.09
0hr	1.14 (0.13-1.37)	1.83 (0.52-2.86)	0.92 (0.38-2.27)	
2h	1.02 (0.3-1.57)	1.35 (0.51-3.05)	0.93 (0.42-2.87)	
4hr	1.16 (0.15-1.33)	1.80 (0.55-2.75)	1.41 (0.6-2.79)	
6hr	0.66 (0.46-1.48)	1.39 (0.21-2.67)	1.32 (0.38-2.94)	
24 hr	1.13 (0.38-1.61)	1.78 (0.87-2.97)	1.23 (0.39-2.98)	
D8	0.80 (0.23-1.39)	1.43 (0.25-2.03)	1.225 (0.8-3.21)	
PL.GP.37				0.40
0hr	0.40 (0.05-0.49)	0.32 (0.05-0.39)	0.215 (0.06-0.33)	
2h	0.29 (0.13-0.51)	0.25 (0.07-0.41)	0.14 (0.08-0.45)	
4hr	0.36 (0.06-0.53)	0.30 (0.12-0.36)	0.28 (0.1-0.45)	
6hr	0.30 (0.12-0.43)	0.27 (0.02-0.42)	0.25 (0.07-0.40)	
24 hr	0.41 (0.10-0.46)	0.29 (0.21-0.43)	0.23 (0.06-0.46)	
D8	0.28 (0.07-0.47)	0.24 (0.04-0.37)	0.24 (0.13-0.38)	
PL.GP.38				0.23
0hr	0.14 (0.01-0.27)	0.26 (0.03-0.40)	0.14 (0.04-0.33)	
2h	0.13 (0.03-0.24)	0.16 (0.06-0.37)	0.11 (0.08-0.43)	

4hr	0.13 (0.02-0.26)	0.21 (0.05-0.40)	0.21 (0.07-0.58)	
6hr	0.13 (0.05-0.20)	0.19 (0.01-0.34)	0.23 (0.04-0.38)	
24 hr	0.17 (0.05-0.25)	0.23 (0.09-0.41)	0.22 (0.04-0.42)	
D8	0.10 (0.04-0.23)	0.15 (0.03-0.27)	0.23 (0.10-0.48)	
PL.GP.39				0.87
0hr	0.60 (0.10-0.72)	0.57 (0.11-0.74)	0.36 (0.08-0.74)	
2h	0.43 (0.22-0.78)	0.47 (0.17-0.67)	0.33 (0.12-0.67)	
4hr	0.48 (0.10-0.80)	0.51 (0.24-0.91)	0.51 (0.16-0.71)	
6hr	0.42 (0.19-0.69)	0.44 (0.04-0.84)	0.45 (0.10-0.93)	
24 hr	0.65 (0.18-0.79)	0.63 (0.35-1.00)	0.51 (0.11-0.85)	
D8	0.45 (0.11-0.77)	0.46 (0.08-0.69)	0.43 (0.24-0.72)	
PL.GP.40				0.60
0hr	0.33 (0.03-0.67)	0.37 (0.05-0.63)	0.12 (0.05-0.29)	
2h	0.16 (0.09-0.70)	0.20 (0.05-0.64)	0.11 (0.05-0.52)	
4hr	0.40 (0.04-0.73)	0.29 (0.09-0.60)	0.20 (0.07-0.54)	
6hr	0.22 (0.05-0.46)	0.25 (0.03-0.55)	0.16 (0.05-0.46)	
24 hr	0.53 (0.07-0.74)	0.29 (0.15-0.57)	0.25 (0.05-0.56)	
D8	0.28 (0.04-0.45)	0.22 (0.03-0.44)	0.15 (0.10-0.70)	
PL.GP.41				0.88
0hr	0.59 (0.05-1.04)	0.64 (0.10-1.06)	0.24 (0.07-0.57)	
2h	0.29 (0.15-1.19)	0.39 (0.10-1.05)	0.23 (0.10-1.170)	
4hr	0.58 (0.06-1.37)	0.58 (0.14-1.19)	0.43 (0.10-1.08)	
6hr	0.33 (0.11-0.75)	0.45 (0.04-0.94)	0.39 (0.07-0.86)	
24 hr	0.89 (0.14-1.24)	0.68 (0.23-1.04)	0.54 (0.07-1.04)	
D8	0.39 (0.08-0.99)	0.45 (0.05-0.8)	0.37 (0.17-1.27)	
PL.GP.42				0.80
0hr	0.33 (0.04-0.57)	0.46 (0.04-0.72)	0.15 (0.04-0.52)	
2h	0.17 (0.11-0.75)	0.28 (0.05-0.79)	0.19 (0.06-0.83)	
4hr	0.36 (0.04-0.69)	0.39 (0.12-1.15)	0.29 (0.07-1.06)	
6hr	0.21 (0.08-0.43)	0.35 (0.03-0.64)	0.30 (0.04-0.69)	
24 hr	0.44 (0.10-0.60)	0.54 (0.18-0.91)	0.40 (0.04-0.95)	
D8	0.18 (0.09-0.73)	0.30 (0.02-0.61)	0.30 (0.13-1.18)	

Appendix Table 1: Complete profile of plasma glycan levels at baseline and after LPS administration.

Data are shown as median (Range). P values refer to difference between groups calculated by RM.ANOVA.

	HNF1A-MODY	Controls	Type 2 diabetes	P
Mem.GP.1				0.22
0hr	0.84 (0.52-1.5)	0.86 (0.51-2.23)	0.76 (0.48-1.35)	
2h	0.44 (0.18-0.72)	0.75 (0.48-2.63)	0.76 (0.27-0.96)	
4hr	0.44 (0.1-1.00)	0.59 (0.43-3.07)	0.37 (0.22-0.87)	
24 hr	0.63 (0.55-2.53)	1.36 (0.59-2.16)	1.33 (0.76-2.46)	
D8	0.52 (0.19-1.59)	0.99 (0.56-1.45)	0.99 (0.58-3.40)	
MEM.GP.2				0.27
0hr	0.09 (0.06-0.17)	0.10 (0.02-0.26)	0.13 (0.07-0.22)	
2h	0.18 (0.08-0.54)	0.20 (0.08-0.34)	0.15 (0.09-0.21)	
4hr	0.18 (0.12-0.33)	0.17 (0.10-0.53)	0.13 (0.11-0.32)	
24 hr	0.14 (0.11-0.21)	0.17 (0.07-0.38)	0.33 (0.12-1.28)	
0hr	0.17 (0.06-0.26)	0.12 (0.10-0.14)	0.10 (0.07-0.25)	
MEM.GP.3				0.15
0hr	5.41 (2.6-10.61)	6.83 (4.30-15.39)	6.91 (2.75-9.96)	
2h	2.70 (1.12-5.41)	3.91 (2.10-15.46)	4.02 (2.37-5.60)	
4hr	2.02 (0.72-4.70)	4.61 (2.22-11.28)	2.31 (1.94-5.90)	
24 hr	4.81 (3.21-9.10)	7.90 (4.52-13.51)	9.48 (3.63-13.21)	
D8	5.41 (1.60-9.11)	7.00 (4.24-12.64)	6.88 (5.10-8.20)	
MEM.GP.4.				0.39
0hr	2.26 (1.56-2.95)	2.01 (1.26-2.52)	2.40 (1.71-3.00)	
2h	2.13 (1.01-2.64)	1.99 (1.87-2.53)	2.34 (1.75-4.01)	
4hr	1.69 (1.35-2.06)	1.31 (1.02-1.93)	2.15 (1.71-2.81)	
24 hr	2.23 (1.68-2.98)	2.21 (0.93-2.97)	1.70 (1.49-2.65)	
D8	2.09 (1.41-2.34)	1.96 (1.61-3.12)	2.21 (1.53-3.01)	
MEM.GP.5				0.08
0hr	4.33 (3.1-6.98)	5.63 (3.51-11.83)	4.83 (2.99-6.16)	
2h	2.71 (1.91-4.81)	4.22 (2.42-9.16)	3.80 (2.86-4.64)	
4hr	2.735 (1.48-4.53)	4.68 (3.33-8.52)	3.15 (2.44-4.95)	
24 hr	4.33 (3.52-6.56)	6.13 (3.61-10.08)	6.11 (3.40-8.27)	
D8	4.55 (2.6-7.95)	5.94 (4.61-7.05)	5.83 (4.24-7.31)	
MEM.GP.6				0.32
0hr	1.30 (0.97-1.57)	1.32 (0.71-1.69)	1.12 (0.77-1.46)	
2h	1.13 (0.86-1.23)	0.90 (0.77-1.13)	1.03 (0.87-1.07)	
4hr	0.92 (0.79-1.07)	0.90 (0.67-1.33)	0.94 (0.80-1.20)	
24 hr	1.08 (0.52-1.47)	1.11 (0.73-1.49)	1.02 (0.70-1.67)	
D8	1.14 (0.78-1.68)	0.93 (0.73-1.35)	0.68 (0.54-1.37)	
MEM.GP.7				0.03
0hr	0.57 (0.54-0.96)	0.45 (0.25-1.21)	0.50 (0.39-0.78)	
2h	0.70 (0.56-0.83)	0.33 (0.28-0.62)	0.54 (0.30-0.62)	
4hr	0.62 (0.47-0.76)	0.26 (0.22-0.89)	0.44 (0.38-0.68)	
24 hr	0.62 (0.38-0.72)	0.48 (0.26-0.82)	0.47 (0.39-0.68)	
D8	0.51 (0.35-1.22)	0.35 (0.28-0.68)	0.33 (0.31-0.61)	
MEM.GP.8				0.06
0hr	4.75 (4.13-7.41)	5.70 (3.94-10.94)	4.80 (3.57-6.33)	
2h	3.83 (3.22-5.89)	5.07 (3.51-8.71)	4.69 (4.03-5.93)	
4hr	4.43 (3.04-6.51)	6.04 (5.45-9.73)	5.00 (4.06-6.91)	
24 hr	5.42 (4.27-6.55)	6.79 (4.45-9.79)	6.22 (5-8.25)	
D8	5.43 (3.89-8.41)	6.87 (5.32-7.63)	6.86 (4.57-9.38)	
MEM.GP.9				0.04
0hr	1.31 (0.77-1.79)	0.95 (0.49-1.85)	1.09 (0.73-1.37)	
2h	1.1 (0.87-1.43)	0.81 (0.55-0.93)	1.02 (0.59-2.56)	
4hr	1.11 (0.66-1.48)	0.71 (0.49-1.63)	0.88 (0.74-1.23)	
24 hr	1.25 (0.77-1.45)	0.84 (0.63-1.14)	1.12 (0.71-1.36)	
D8	1.14 (0.76-2.31)	0.90 (0.40-1.10)	0.64 (0.46-0.93)	
MEM.GP.10				0.03
0hr	2.18 (1.56-2.30)	1.69 (0.88-2.38)	1.55 (1.29-2.44)	
2h	2.39 (1.63-2.79)	1.73 (1.06-2.86)	2.03 (1.26-2.63)	
4hr	2.17 (1.78-2.50)	1.77 (1.58-3.41)	2.07 (1.25-3.15)	
24 hr	1.92 (1.55-2.110)	1.55 (0.98-2.18)	1.73 (1.38-2.29)	
D8	1.82 (1.10-2.43)	1.18 (0.97-1.64)	1.44 (0.74-1.7)	
MEM.GP.11				0.05
0hr	4.4 (3.3-7.2)	5.7 (3.4-11.5)	4.5 (3.2-6.6)	
2h	3.5 (2.9-6.7)	5.1 (3.5-9.3)	5.3 (4.5-6.5)	
4hr	4.6 (3.2-8.5)	7.2 (6-11.1)	5.8 (4.0-7.8)	
24 hr	5.3 (3.8-6.5)	6.3 (4.2-10.3)	6.1 (4.6-9.2)	
D8	5.4 (3.4-9.6)	7.0 (5.2-7.6)	6.8 (3.7-10.2)	

MEM.GP.12				0.70
0hr	5.18 (3.64-7.65)	4.81 (3.27-8.03)	5.73 (2.4-8.16)	
2h	6.55 (4.24-7.31)	5.55 (3.67-7.54)	6.16 (4.69-8.14)	
4hr	5.97 (4.16-6.44)	5.73 (4.02-8.83)	5.52 (3.92-9.04)	
24 hr	4.43 (3.13-7.3)	4.91 (2.61-9.66)	5.06 (4.84-7.18)	
D8	4.73 (2.44-6.9)	4.27 (2.64-5.98)	4.10 (2.73-7.62)	
MEM.GP.13				0.89
0hr	0.84 (0.71-1.44)	0.84 (0.68-1.87)	0.86 (0.69-1.63)	
2h	0.60 (0.52-0.86)	0.62 (0.41-0.97)	0.71 (0.47-0.91)	
4hr	0.5 (0.46-1.02)	0.52 (0.21-0.79)	0.55 (0.49-0.87)	
24 hr	0.81 (0.6-0.99)	0.95 (0.45-1.31)	0.67 (0.52-0.96)	
D8	0.8 (0.47-1.05)	0.74 (0.64-1.39)	0.53 (0.46-2.17)	
MEM.GP.14				0.76
0hr	0.94 (0.67-1.41)	1.00 (0.49-1.94)	1.12 (0.87-1.89)	
2h	0.88 (0.78-1.02)	0.98 (0.53-1.98)	1.04 (0.58-2.25)	
4hr	0.91 (0.7-1.03)	0.86 (0.37-1.44)	0.96 (0.68-2.51)	
D8	1.00 (0.72-1.19)	1.07 (0.43-1.3)	0.97 (0.54-1.6)	
24 hr	0.87 (0.71-1.61)	0.92 (0.73-2.02)	0.87 (0.71-1.75)	
MEM.GP.15				0.09
0hr	1.89 (1.27-3.23)	2.15 (1.49-3.67)	1.66 (1.39-2.59)	
2h	1.46 (1.24-2.26)	1.78 (1.27-3.51)	1.91 (1.58-2.56)	
4hr	1.80 (1.22-3.15)	2.47 (2.06-3.84)	2.10 (1.81-3.1)	
24 hr	2.17 (1.56-2.41)	2.65 (1.93-3.58)	2.29 (1.72-3.14)	
D8	1.95 (1.41-4.64)	2.49 (2.12-3.19)	2.92 (1.33-4.32)	
MEM.GP.16				0.66
0hr	1.48 (0.91-5.39)	1.43 (0.94-2.86)	1.59 (0.85-4.67)	
2h	3.03 (1.86-7.71)	2.60 (1.24-8.05)	2.69 (1.52-5.49)	
4hr	3.37 (2.38-7.66)	4.08 (1.53-7.94)	4.28 (1.60-5.29)	
24 hr	1.42 (1.04-2.77)	1.50 (0.97-6.17)	2.49 (1.23-9.17)	
D8	1.77 (0.92-5.68)	1.41 (1.08-3.03)	1.29 (1.07-3.07)	
MEM.GP.17				0.60
0hr	0.27 (0.18-0.36)	0.28 (0.13-0.38)	0.27 (0.26-0.60)	
2h	0.26 (0.23-0.35)	0.27 (0.2-0.35)	0.25 (0.24-0.34)	
4hr	0.27 (0.19-0.29)	0.23 (0.18-0.25)	0.27 (0.22-0.28)	
24 hr	0.26 (0.22-0.34)	0.26 (0.16-0.32)	0.25 (0.22-0.29)	
D8	0.26 (0.19-0.4)	0.25 (0.21-0.35)	0.27 (0.18-0.31)	
MEM.GP.18				0.95
0hr	1.36 (0.98-1.86)	1.37 (1.09-1.77)	1.29 (1.04-2.01)	
2h	1.34 (1.03-1.63)	1.48 (0.97-1.69)	1.40 (1.08-2.04)	
4hr	1.29 (0.97-1.53)	1.23 (0.9-1.63)	1.25 (1.00-2.01)	
24 hr	1.18 (1.05-1.44)	1.28 (1.02-1.77)	1.18 (0.69-1.51)	
D8	1.37 (0.81-1.81)	1.32 (1.13-1.75)	1.21 (0.95-2.08)	
MEM.GP.19				0.12
0hr	2.61 (2.28-2.82)	3.03 (2.31-3.23)	2.66 (2.17-3.12)	
2h	2.46 (1.92-2.88)	2.71 (2.37-3.36)	2.55 (2.19-3.16)	
4hr	2.71 (2.28-3.08)	3.00 (2.28-3.24)	2.64 (2.39-3.46)	
24 hr	2.89 (2.66-3.20)	2.96 (2.05-3.58)	2.39 (2.30-2.60)	
D8	2.93 (2.25-4.07)	3.01 (2.52-3.42)	2.91 (2.25-3.16)	
MEM.GP.20				0.06
0hr	1.20 (1.04-1.96)	1.10 (0.46-1.58)	1.49 (1.12-2.19)	
2h	1.26 (1.07-1.93)	1.25 (0.90-1.60)	1.54 (1.18-2.2)	
4hr	1.27 (1.01-1.80)	1.00 (0.71-1.32)	1.35 (1.13-2.31)	
24 hr	1.52 (0.85-1.69)	1.24 (0.55-1.57)	1.46 (0.76-1.92)	
D8	1.35 (1.1-1.87)	1.18 (1.01-1.59)	1.42 (1.15-2.14)	
MEM.GP.21				0.28
0hr	0.39 (0.21-0.45)	0.43 (0.1-0.53)	0.39 (0.31-0.58)	
2h	0.30 (0.29-0.4)	0.44 (0.33-0.47)	0.35 (0.33-0.4)	
4hr	0.35 (0.28-0.42)	0.39 (0.21-0.45)	0.32 (0.26-0.42)	
24 hr	0.39 (0.18-0.53)	0.41 (0.3-0.53)	0.39 (0.24-0.43)	
D8	0.54 (0.21-0.62)	0.45 (0.4-0.48)	0.37 (0.22-0.53)	
MEM.GP.22				0.23
0hr	3.27 (2.67-4.75)	3.64 (2.25-4.66)	3.92 (3.12-5.29)	
2h	3.67 (3.37-4.4)	4.09 (2.63-4.48)	4.41 (3.38-5.87)	
4hr	3.78 (3.56-4.06)	3.52 (2.48-4.36)	4.65 (3.10-5.41)	
24 hr	3.78 (3.05-4.41)	3.71 (1.79-4.90)	3.38 (2.51-4.44)	
D8	3.57 (3.36-4.06)	4.01 (3.05-4.38)	3.99 (3.46-5.04)	
MEM.GP.23				0.21

0hr	3.27 (2.55-4.08)	3.21 (1.42-3.41)	2.94 (2.80-3.53)	
2h	3.20 (2.39-3.88)	3.10 (1.71-3.88)	3.05 (2.72-3.18)	
4hr	3.26 (2.35-3.53)	2.60 (1.75-3.31)	2.92 (2.41-3.33)	
24 hr	3.27 (2.85-3.52)	2.72 (1.8-3.51)	2.55 (1.98-3.08)	
D8	3.1 (2.51-3.95)	2.90 (2.71-3.69)	2.95 (2.02-3.38)	
MEM.GP.24				0.61
0hr	2.88 (2.68-3.56)	3.18 (1.89-3.4)	3.32 (2.72-3.92)	
2h	3.28 (2.73-3.8)	3.16 (2.13-3.95)	3.25 (2.82-3.91)	
4hr	3.32 (2.92-3.7)	3.11 (2.27-3.45)	3.36 (2.74-4.01)	
24 hr	3.27 (2.66-3.49)	3.06 (1.96-3.7)	3.13 (2.04-4.24)	
D8	3.14 (2.78-3.63)	3.22 (2.99-3.48)	3.15 (2.80-3.66)	
MEM.GP.25				0.02
0hr	0.57 (0.49-0.69)	0.41 (0.20-0.60)	0.40 (0.32-0.66)	
2h	0.59 (0.43-0.74)	0.43 (0.31-0.64)	0.51 (0.44-0.81)	
4hr	0.57 (0.48-0.81)	0.48 (0.27-0.57)	0.57 (0.41-0.62)	
24 hr	0.53 (0.34-0.62)	0.35 (0.27-0.60)	0.52 (0.33-0.58)	
D8	0.57 (0.49-0.75)	0.39 (0.26-0.56)	0.40 (0.28-0.60)	
MEM.GP.26				0.22
0hr	6.23 (4.82-7.46)	6.12 (2.82-6.56)	5.63 (5.31-7.27)	
2h	6.47 (5.23-7.21)	6.20 (3.48-7.85)	5.58 (4.94-6.96)	
4hr	6.27 (5.49-7.2)	5.85 (4.08-6.29)	5.28 (4.83-7.06)	
24 hr	6.44 (4.89-6.71)	5.29 (3.6-6.7)	4.65 (3.78-6.33)	
D8	6.17 (5.36-7.42)	5.82 (4.3-6.93)	5.35 (4.72-6.46)	
MEM.GP.27				0.13
0hr	2.61 (2.12-2.77)	2.59 (1.58-2.7)	2.62 (2.11-2.86)	
2h	2.79 (2.48-3.27)	2.66 (1.57-3.23)	2.51 (2.40-3.12)	
4hr	2.79 (2.6-3.22)	2.63 (1.89-3.09)	2.43 (2.38-2.84)	
24 hr	2.84 (2.3-2.95)	2.26 (1.89-2.63)	2.25 (1.88-2.52)	
D8	2.48 (2.21-2.9)	2.37 (2.27-2.55)	2.365 (1.91-2.74)	
MEM.GP.28				0.53
0hr	3.46 (2.58-5.01)	3.60 (1.47-5.35)	3.41 (2.86-6.49)	
2h	5.25 (4.22-7.03)	4.87 (1.82-5.64)	4.47 (3.13-5.22)	
4hr	5.05 (3.98-5.6)	4.52 (2.25-6.65)	4.68 (3.28-6.87)	
24 hr	3.78 (2.69-4.93)	3.36 (2.55-3.64)	3.62 (2.45-5.54)	
D8	3.66 (2.78-4.99)	3.54 (2.47-3.85)	3.07 (2.86-5.04)	
MEM.GP.29				0.21
0hr	9.02 (6.51-10.30)	8.73 (5.10-10.50)	8.71 (7.70-9.80)	
2h	10.31 (7.6-12.60)	9.61 (5.80-12.70)	9.52 (8.31-10.70)	
4hr	10.32 (8.0-12.40)	9.21 (6.60-10.80)	9.11 (8.14-10.30)	
24 hr	9.14 (7.48-11.06)	7.22 (4.70-9.20)	6.83 (5.12-9.20)	
D8	8.58 (6.24-10.81)	8.23 (5.50-9.20)	7.73 (5.71-10.21)	
MEM.GP.30				0.57
0hr	2.16 (1.44-2.57)	2.32 (1.15-2.6)	2.05 (1.92-2.15)	
2h	2.33 (1.56-2.60)	2.24 (1.47-3.23)	2.02 (1.85-2.48)	
4hr	2.25 (1.72-2.52)	2.04 (1.65-2.40)	2.01 (1.68-2.19)	
24 hr	2.24 (1.57-2.41)	2.28 (1.21-2.32)	1.64 (1.41-2.08)	
D8	1.86 (1.55-2.56)	2.23 (1.37-2.46)	1.93 (1.60-2.32)	
MEM.GP.31				0.19
0hr	1.37 (1.00-1.88)	1.23 (0.55-1.69)	1.22 (1.01-1.71)	
2h	1.28 (1.1-1.55)	1.26 (0.72-1.47)	1.27 (1.06-1.58)	
4hr	1.31 (1.08-1.59)	1.09 (0.86-1.43)	1.12 (0.95-1.63)	
24 hr	1.52 (1.09-1.66)	1.21 (0.59-1.67)	0.86 (0.73-1.50)	
D8	1.40 (1.17-1.88)	1.35 (1.05-1.59)	1.33 (0.84-1.54)	
MEM.GP.32				0.21
0hr	1.07 (0.84-1.45)	1.07 (0.78-1.15)	0.96 (0.77-1.26)	
2h	1.03 (0.79-1.12)	0.96 (0.62-1.34)	0.93 (0.84-1.2)	
4hr	0.97 (0.88-1.16)	0.88 (0.65-0.98)	0.93 (0.79-1.21)	
24 hr	1.05 (0.80-1.120)	1.09 (0.59-1.30)	0.87 (0.61-1.04)	
D8	1.12 (0.75-1.26)	1.06 (0.81-1.15)	0.95 (0.65-1.35)	
MEM.GP.33				0.29
0hr	1.34 (0.97-2.02)	1.42 (0.56-1.66)	1.29 (1.15-2.12)	
2h	1.66 (1.24-1.83)	1.46 (0.74-1.68)	1.43 (1.29-1.71)	
4hr	1.45 (1.06-1.84)	1.3 (0.92-1.37)	1.25 (1.08-1.99)	
24 hr	1.43 (1.00-1.85)	1.34 (0.76-1.74)	1.08 (1.01-1.41)	
D8	1.32 (0.99-2.12)	1.39 (1.15-1.61)	1.34 (0.99-1.74)	
MEM.GP.34				0.01
0hr	2.57 (1.68-2.80)	2.13 (0.85-2.44)	2.14 (1.92-3.12)	

2h	2.77 (2.58-3.15)	2.44 (1.18-2.91)	2.32 (2.18-2.61)	
4hr	2.74 (2.38-3.04)	2.17 (1.43-2.38)	2.23 (2.00-2.83)	
24 hr	2.34 (2.04-2.81)	1.91 (1.18-2.3)	1.97 (1.55-2.05)	
D8	2.31 (1.55-2.99)	2.15 (1.64-2.16)	2.05 (1.58-2.48)	
MEM.GP.35				0.25
0hr	1.33 (1.12-1.83)	1.37 (0.67-1.53)	1.32 (1.17-2.27)	
2h	1.65 (1.32-1.78)	1.60 (1.00-1.76)	1.47 (1.28-1.81)	
4hr	1.52 (1.27-1.83)	1.38 (1.10-1.51)	1.38 (1.08-2.04)	
24 hr	1.40 (1.13-2.05)	1.26 (0.78-1.53)	1.29 (1.04-1.42)	
D8	1.26 (0.90-1.94)	1.40 (1.11-1.62)	1.39 (1.19-1.70)	
MEM.GP.36				0.55
0hr	1.12 (0.92-1.50)	1.10 (0.58-1.44)	1.15 (1.06-2.12)	
2h	1.30 (0.94-1.45)	1.25 (0.76-1.70)	1.16 (1.02-1.63)	
4hr	1.26 (0.98-1.45)	1.06 (0.79-1.30)	1.10 (1.01-1.92)	
24 hr	1.21 (0.89-1.76)	1.13 (0.62-1.46)	1.05 (0.75-1.29)	
D8	1.18 (0.93-1.57)	1.29 (1.08-1.60)	1.24 (0.92-1.50)	
MEM.GP.37				0.02
0hr	0.59 (0.45-1.02)	0.70 (0.36-0.94)	0.99 (0.34-1.51)	
2h	0.90 (0.62-1.59)	0.80 (0.48-1.17)	0.92 (0.35-1.27)	
4hr	0.83 (0.49-1.40)	0.85 (0.52-0.94)	1.06 (0.50-1.42)	
24 hr	0.69 (0.35-1.38)	0.71 (0.35-0.84)	0.93 (0.51-1.43)	
D8	0.57 (0.4-1.00)	0.65 (0.59-0.87)	0.94 (0.44-1.13)	
MEM.GP.38				0.30
0hr	1.30 (0.95-1.64)	1.23 (0.8-1.44)	1.14 (1.00-2.38)	
2h	1.25 (1.08-1.46)	1.19 (0.83-1.49)	1.23 (0.98-1.95)	
4hr	1.29 (0.97-1.45)	1.05 (0.83-1.37)	1.09 (0.96-1.86)	
24 hr	1.32 (1.05-1.51)	1.08 (0.65-1.41)	1.02 (0.66-1.72)	
D8	1.32 (0.91-1.56)	1.22 (1.18-1.42)	1.10 (0.94-1.78)	
MEM.GP.39				0.53
0hr	0.68 (0.48-0.91)	0.81 (0.2-0.90)	0.71 (0.69-1.19)	
2h	0.75 (0.66-0.98)	0.82 (0.33-1.04)	0.68 (0.52-1.11)	
4hr	0.78 (0.66-0.91)	0.62 (0.30-0.90)	0.74 (0.59-0.95)	
24 hr	0.83 (0.52-0.95)	0.70 (0.25-0.89)	0.52 (0.38-0.79)	
D8	0.67 (0.54-0.93)	0.76 (0.50-0.92)	0.71 (0.63-0.77)	
MEM.GP.40				0.27
0hr	0.56 (0.40-0.77)	0.55 (0.18-0.77)	0.62 (0.57-1.03)	
2h	0.63 (0.54-0.88)	0.63 (0.24-0.76)	0.58 (0.50-0.82)	
4hr	0.64 (0.53-0.83)	0.56 (0.30-0.61)	0.66 (0.52-0.85)	
24 hr	0.60 (0.39-0.65)	0.45 (0.27-0.78)	0.44 (0.39-0.65)	
D8	0.56 (0.42-0.85)	0.60 (0.49-0.70)	0.6 (0.53-0.67)	
MEM.GP.41				0.30
0hr	1.64 (1.1o-2.02)	1.86 (0.60-2.07)	1.55 (1.41-2.75)	
2h	1.88 (1.70-2.64)	1.89 (0.81-2.40)	1.72 (1.30-2.43)	
4hr	2.05 (1.76-2.34)	1.54 (0.76-2.07)	1.84 (1.48-2.32)	
24 hr	1.84 (1.30-2.06)	1.45 (0.77-1.90)	1.29 (1.06-1.62)	
D8	1.56 (1.06-2.53)	1.71 (1.34-2.03)	1.59 (1.27-2.02)	
MEM.GP.42				0.23
0hr	1.92 (1.27-2.38)	2.13 (0.80-2.32)	1.77 (1.52-3.42)	
2h	2.09 (1.74-2.95)	2.22 (0.97-2.61)	1.86 (1.38-2.36)	
4hr	2.25 (1.93-2.55)	1.60 (0.74-2.56)	1.89 (1.66-2.65)	
24 hr	2.19 (1.33-3.09)	1.82 (0.67-2.65)	1.46 (1.07-1.84)	
D8	1.87 (1.40-3.03)	1.85 (1.60-2.21)	1.71 (1.33-2.17)	
MEM.GP.43				0.10
0hr	1.34 (0.95-1.70)	1.42 (0.57-1.76)	0.76 (0.48-1.35)	
2h	1.53 (1.33-2.34)	1.66 (0.62-1.89)	0.76 (0.27-0.96)	
4hr	1.82 (1.55-1.98)	1.15 (0.46-1.76)	0.37 (0.22-0.87)	
24 hr	1.48 (1.00-2.29)	1.10 (0.40-1.95)	1.33 (0.76-2.46)	
D8	1.34 (0.99-2.17)	1.30 (0.97-1.67)	0.99 (0.58-3.40)	
MEM.GP.44				0.004
0hr	1.66 (1.08-1.90)	1.35 (0.78-1.55)	0.13 (0.07-0.22)	
2h	1.52 (1.10-2.57)	1.33 (0.94-1.91)	0.15 (0.09-0.21)	
4hr	1.87 (1.37-2.28)	1.14 (0.54-1.71)	0.13 (0.11-0.32)	
24 hr	1.58 (1.16-2.34)	1.08 (0.49-1.64)	0.33 (0.12-1.28)	
D8	1.64 (0.86-2.37)	1.16 (0.95-1.42)	0.10 (0.07-0.25)	

Appendix Table 2: Complete profile of membrane glycan levels at baseline and after LPS administration. Data are shown as median (Range). P values refer to difference between groups calculated by RM.ANOVA.

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