Identification of hepatocyte nuclear factor 1β-associated disease

Submitted by Rhian Lynne Clissold to the University of Exeter as a thesis for the degree of Doctor of Medicine in August 2017

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

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ABSTRACT

Heterozygous mutations and deletions of the gene that encodes the transcription factor hepatocyte nuclear factor 1β (*HNF1B*) are the commonest known monogenic cause of developmental kidney disease. However, diagnosis remains challenging due to phenotypic variability and frequent absence of a family history. There is also no consensus as to when *HNF1B* genetic testing should be performed. This thesis includes work looking at the identification of HNF1B-associated disease.

An HNF1B score was developed in 2014 to help select appropriate patients for genetic testing. The aim in chapter 2 was to test the clinical utility of this score in a large number of referrals for *HNF1B* genetic testing to the UK diagnostic testing service for the *HNF1B* gene. An HNF1B score was assigned for 686 referrals using clinical information available at the time of testing; performance of the score was evaluated by receiver-operating characteristic curve analysis. Although the HNF1B score discriminated between patients with and without a mutation/deletion reasonably well, the negative predictive value of 85% reduces its clinical utility.

HNF1B-associated disease is due to an approximate 1.3 Mb deletion of chromosome 17q12 in about 50% of individuals. This deletion includes *HNF1B* plus 14 additional genes and has been linked to an increased risk of neurodevelopmental disorders, such as autism. The aim in chapter 3 was to compare the neurodevelopmental phenotype of patients with either an *HNF1B* intragenic mutation or 17q12 deletion to determine whether haploinsufficiency of the *HNF1B* gene is responsible for this aspect of the phenotype. Brief behavioural screening showed high levels of psychopathology and impact in children with a deletion. 8/20 (40%) patients with a deletion had a clinical diagnosis of a neurodevelopmental disorder compared to 0/18 with a mutation, P=0.004. 17q12 deletions were also associated with more autistic traits. Two independent clinical geneticists were

able to predict the presence of a deletion with a sensitivity of 83% and specificity of 79% when assessing facial dysmorphic features as a whole. These results demonstrate that the 17q12 deletion but not *HNF1B* intragenic mutations are associated with neurodevelopmental disorders; we conclude that the *HNF1B* gene is not involved in the neurodevelopmental phenotype of these patients.

Extra-renal phenotypes frequently occur in HNF1B-associated disease, including diabetes mellitus and pancreatic hypoplasia. Faecal elastase-1 levels have only been reported in a small number of individuals, the majority of which have diabetes. In chapter 4 we measured faecal elastase-1 in patients with an *HNF1B* mutation or deletion regardless of diabetes status and assessed the degree of symptoms associated with pancreatic exocrine deficiency. We found that faecal elastase-1 deficiency is a common feature of HNF1B-associated renal disease even when diabetes is not present and pancreatic exocrine deficiency may be more symptomatic than previously suggested. Faecal elastase-1 should be measured in all patients with a known *HNF1B* molecular abnormality complaining of chronic abdominal pain, loose stools or unintentional weight loss.

Hypomagnesaemia is a common feature of HNF1B-associated disease and is due to renal magnesium wasting. The aim in chapter 5 was to measure both serum and urine magnesium and calcium levels in individuals with an *HNF1B* molecular defect and compare to a cohort of patients followed up in a general nephrology clinic in order to assess their potential as biomarkers for HNF1B-associated disease. The results of this pilot study show that using a cut-off for serum magnesium of ≤0.75 mmol/L was 100% sensitive and 87.5% specific for the presence of an *HNF1B* mutation/deletion. All individuals in the HNF1B cohort had hypermagnesuria with fractional excretion of magnesium >4%; a cut-off of ≥4.1% was 100% sensitive and 71% specific. This suggests serum magnesium levels and fractional excretion of magnesium are highly sensitive biomarkers for HNF1B-associated renal disease; if these results are confirmed in a larger study of patients with congenital anomalies of the kidneys or urinary tract they could be implemented as cheap screening tests for *HNF1B* genetic testing in routine clinical care.

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ABBREVIATIONS

ADHD	Attention deficit hyperactivity disorder
ASD	Autism spectrum disorder
ASQ	Autism Spectrum Quotient
AUC	Area under the curve
CAKUT	Congenital anomaly of the kidneys or urinary tract
CBP	CREB-binding protein
cDNA	Complementary deoxyribonucleic acid
CI	Confidence interval
CNI	Calcineurin inhibitor
CNV	Copy-number variant
CT	Computed tomography
DAWBA	Development and Well-Being Assessment
DNA	Deoxyribonucleic acid
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, 4th Edition
eGFR	Estimated glomerular filtration rate
ESRD	End-stage renal disease
FECa	Fractional excretion of calcium
FEMg	Fractional excretion of magnesium
GDNF	Glial-derived neurotrophic factor

HNF1A/B	Hepatocyte nuclear factor 1α/β
IQR	Interquartile range
IQ	Intelligence quotient
ISD-10	International Statistical Classification of Diseases and Related Health Problems 10th Revision
MLPA	Multiplex ligation-dependent probe amplification
MODY	Maturity-onset diabetes of the young
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
NHS	National Health Service
NIHR	National Institute for Health Research
NMD	Nonsense-mediated decay
NODAT	New-onset diabetes after transplantation
NPV	Negative predictive value
OR	Odds ratio
PCAF	p300/CBP-associated factor
PCBD1	Pterin-4 alpha-carbinolamine dehydratase 1
PCR	Polymerase chain reaction
PPI	Proton pump inhibitor
PPV	Positive predictive value
PTH	Parathyroid hormone
RCAD	Renal cysts and diabetes syndrome

RCC	Renal cell carcinoma
ROC	Receiver-operating characteristic
SDQ	Strengths and Difficulties Questionnaire
SPK	Simultaneous pancreas and kidney
TRPM6	Transient receptor potential melastatin type 6
UCPCR	Urinary C-peptide/creatinine ratio
UK	United Kingdom

CHAPTER 1

Introduction

1.1 Structure and aims of thesis

This thesis aims to explore some of the issues surrounding disease recognition in individuals with heterozygous mutations and deletions in the gene that encodes the transcription factor hepatocyte nuclear factor 1β (*HNF1B*).

The introduction in chapter 1 involves a comprehensive review of HNF1B-associated disease at the time of commencing this Doctor of Medicine programme, with an emphasis on both the renal and extra-renal clinical characteristics.

Diagnosis of HNF1B-associated disease is challenging due to phenotypic variability and frequent absence of a family history despite an autosomal dominant inheritance pattern. An HNF1B score was developed in 2014 to help select appropriate patients for genetic testing. The aim in chapter 2 was to test the clinical utility of this score in a large number of referrals for *HNF1B* genetic testing to the UK diagnostic testing service for the *HNF1B* gene.

HNF1B-associated disease is due to an approximate 1.3 Mb deletion of chromosome 17q12 in about 50% of individuals. This 17q12 deletion includes *HNF1B* plus 14 additional genes and has been linked to an increased risk of neurodevelopmental disorders, such as autism; this is a source of concern for patients and their families. The aim in chapter 3 was to systematically compare the neurodevelopmental phenotype of individuals with either an *HNF1B* intragenic mutation or 17q12 deletion to determine whether haploinsufficiency of the *HNF1B* gene is responsible for this aspect of the phenotype. This should allow for the provision of more accurate information on diagnosis and prognosis in the clinical setting.

There is still no consensus as to when *HNF1B* genetic testing should be performed. In view of the phenotypic variability that is seen in HNF1B-associated disease and the

expense attached to genetic testing, additional tools before genetic screening would be helpful to improve both recognition of the condition and cost-effectiveness of genetic testing. Chapters 4 and 5 are concerned with the assessment of potential biomarkers for HNF1B-associated disease. Faecal elastase-1 levels have only been reported in a small number of individuals with HNF1B-associated disease, the majority of which have diabetes. The aim in chapter 4 was to measure faecal elastase-1 in patients regardless of diabetes status, as well as assess the degree of symptoms associated with pancreatic exocrine deficiency. Hypomagnesaemia is a common feature of HNF1B-associated disease and is due to renal magnesium wasting. It is unknown if hypermagnesuria is also seen in patients even when serum magnesium measurements fall within the normal reference range. The aim in chapter 5 was to measure both serum and urine magnesium and calcium levels in individuals with an *HNF1B* molecular defect and compare to a cohort of patients followed up in a general nephrology clinic in order to assess their potential as biomarkers for HNF1B-associated disease.

The discussion in chapter 6 provides an overview of the main findings of each chapter including the clinical impact of the results, limitations of the data and areas for future research within the context of the most current literature.

1.2 Overview of the literature:

HNF1B-associated renal and extra-renal disease—an expanding clinical spectrum

Rhian L. Clissold[a], Alexander J. Hamilton[a], Andrew T. Hattersley[a], Sian Ellard[a] and Coralie Bingham[b]

[a] Institute of Biomedical and Clinical Science, University of Exeter Medical School, Exeter, UK

[b] Exeter Kidney Unit, Royal Devon and Exeter Hospital, Exeter, UK

Authors' contributions

I reviewed the literature for the article and wrote the manuscript. Alexander Hamilton helped with figure preparation. All authors contributed to discussions of the content and review of the manuscript before submission.

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INTRODUCTION

Hepatocyte nuclear factor 1β (HNF1B) is a homeodomain-containing transcription factor. HNF1B has known functions in nephron development and heterozygous mutations and deletions are the most common known monogenic cause of developmental renal disease.[1-4] Renal cysts are the most commonly observed clinical feature in HNF1B-associated disease, and numerous affected individuals additionally exhibit early-onset diabetes mellitus; this led to the description of the renal cysts and diabetes (RCAD) syndrome.[5] Since initial reporting of the early cases in 1997, it has become evident that additional clinical features are also associated with molecular defects in *HNF1B*.[6-8] These features include pancreatic hypoplasia,[9, 10] genital tract malformations,[11] abnormal liver function,[7, 12] hypomagnesaemia,[13] hyperuricaemia, and early-onset gout.[14] HNF1B-associated disease is, therefore, considered to be a multi-system disorder (Figure 1.1).

The discovery of *HNF1B* gene mutations as a cause of developmental renal disease arose from unexpected findings in the study of maturity-onset diabetes of the young (MODY).[6-8] MODY is a monogenic form of early-onset diabetes mellitus that is typically diagnosed before the age of 25 years. MODY is inherited in an autosomal dominant manner and results from pancreatic β-cell dysfunction.[15] The most common cause of MODY is mutation of the gene encoding the transcription factor HNF1A,[16] which binds to the same DNA sequence as HNF1B, and both proteins show >80% sequence homology.[17] This made *HNF1B* a good candidate gene for MODY cases without a known genetic cause and a mutation associated with early-onset diabetes mellitus was first reported in a Japanese family in 1997.[6] Renal disease was also present in the three affected individuals in this family, which ranged in severity from persistent proteinuria to chronic renal failure. Bilateral renal cysts were subsequently identified in the individual with proteinuria.[7] The association of diabetes mellitus with non-diabetic renal disease was strengthened by the identification of two additional families with a heterozygous mutation in *HNF1B*.[8, 11] Many of the affected family members additionally had abnormal liver function and genital

malformations, providing the first evidence that a mutation in *HNF1B* could result in a multi-system disease.

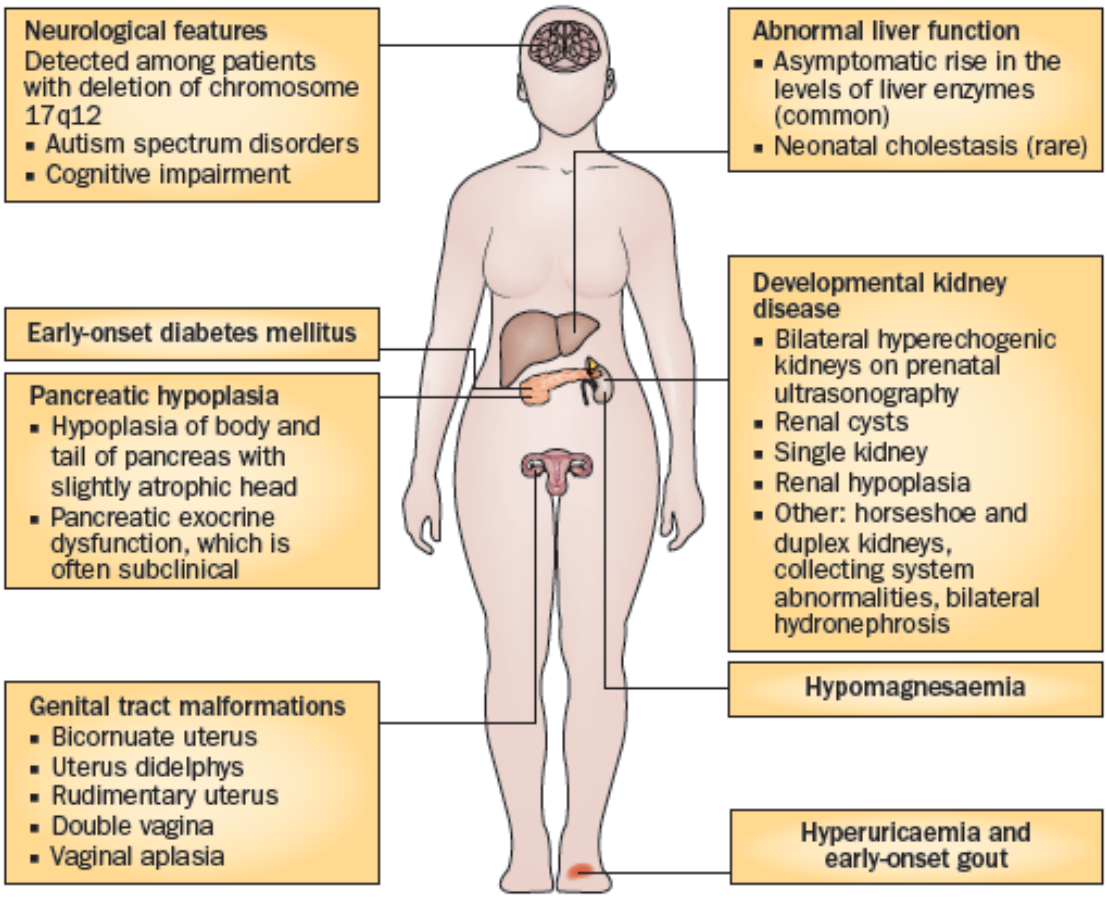

Figure 1.1 Renal and extra-renal phenotypes frequently observed among patients with hepatocyte nuclear factor 1β-associated disease

This Review focuses on HNF1B-associated renal developmental disease. We first provide an outline of the molecular genetics of this disorder. We next discuss functional studies, including the use of animal models in determining the expression of *HNF1B* during embryonic development. The various phenotypes thus far associated with *HNF1B* mutations and deletions are described and finally, we highlight key areas for future research.

MOLECULAR GENETICS

HNF1B is a member of the homeodomain-containing superfamily of transcription factors, functioning either as a homodimer or as a heterodimer with HNF1A to regulate gene expression. The *HNF1B* gene is located on chromosome 17q12 and the protein has three distinct functional domains: the dimerization domain, the DNA binding domain, and the transactivation domain (Figure 1.2). Genetic changes comprise base substitutions or small insertions/deletions in 24/58 (41%) adult patients and 51/116 (44%) affected children or foetuses; deletions of the entire gene account for 34/58 (59%) adult patients and 65/116 (56%) affected children or foetuses.[1-3, 13, 18-24]

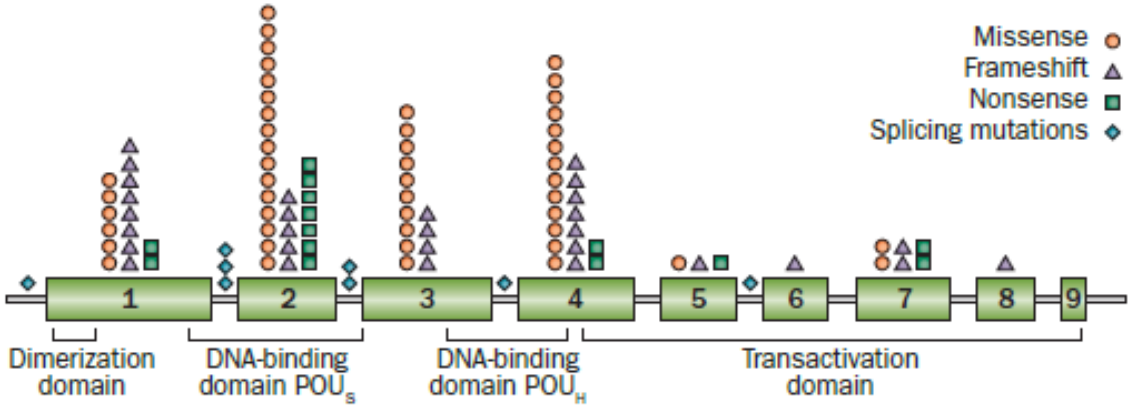

Figure 1.2 Distribution of mutations within the gene that encodes hepatocyte nuclear factor 1β.
The mutations shown in the schematic are documented in the Human Gene Mutation Database (accessed on 25 March 2014); here, they are grouped by mutation type within each of the nine exons and splice sites of the transcript. The numbered boxes refer to the exons. The functional domains of the HNF1B protein are shown beneath the gene transcript. Each symbol represents a separate mutation rather than an individual patient. Approximately 50% of patients have a whole-gene deletion (not pictured) although break points might not have been mapped or reported. Approved cDNA and protein level mutation names are shown in Appendix A.
Abbreviations: HNF1B, hepatocyte nuclear factor 1β; POU_H, Pit-1Oct-1/2Unc-86 homeodomain; POU_S, Pit-1Oct-1/2Unc-86 specific.

More than 50 different *HNF1B* mutations have been reported, including missense, nonsense, frame-shift and splicing mutations (Figure 1.2). The majority of identified mutations are clustered in the first four exons of the gene, with exons 2 and 4, and the

intron 2 splice site being mutation site hotspots.[25] There does not appear to be a correlation between the type or position of the mutation with particular clinical features.[26] Expression of the phenotype can vary considerably between families harbouring the same *HNF1B* mutation, as well as between affected members of individual families, which suggests that additional genetic and/or environmental modifiers might influence the HNF1B phenotype. Stochastic variation in temporal *HNF1B* gene expression during early development could also increase phenotypic diversity.

Whole-gene deletions of *HNF1B* were reported eight years following the first coding mutation.[27] The region of chromosome 17 that encompasses the *HNF1B* gene is susceptible to genomic rearrangement, which is mediated by non-allelic homologous recombination between segmental duplications flanking a 1.5-Mb region.[28] This type of genomic rearrangement is not detected by conventional direct sequencing techniques and instead requires gene dosage analysis; however, this will be facilitated by the increasing use of next generation sequencing technology.[29] Partial gene deletions have also been reported.[27]

There is no evidence to suggest that patients with a whole-gene deletion have a different phenotype to those with coding or splice site mutations. This is consistent with haploinsufficiency as the underlying disease mechanism.[20, 27] HNF1B-associated disease is generally considered to be inherited in an autosomal dominant manner. Nevertheless, whole-gene deletions, as well as coding and splice site mutations, can also arise spontaneously;[19, 26] the prevalence of spontaneous *HNF1B* deletion is reported to be as high as 50%.[18, 20] This explains why there is often no family history of renal disease or diabetes mellitus. The high frequency of *de novo* deletions is explained by the presence of flanking segmental duplications, and the increased rate of spontaneous mutations probably arises as a result of the decreased biological fitness of affected individuals.[28] Reduced fertility, as a result of genital tract malformations, has been reported among patients with HNF1B-associated disease; furthermore, the wide phenotypic variability might not be compatible with life, as is seen in cases of severe

congenital abnormalities of the kidneys and urinary tract (CAKUT) detected on prenatal ultrasonography and leading to termination of pregnancy.[11, 30]

FUNCTIONAL STUDIES

Organ development can be a highly conserved process between different species. The zebra fish shows high conservation of renal organogenesis with mammals and is therefore a convenient model system for functional studies. Three mutant alleles of *vhnf1*, the zebra fish homologue of *HNF1B,* were isolated in a zebra fish insertional mutagenesis screen. These mutants formed renal cysts and exhibited an underdeveloped pancreas and liver.[31]

The *Xenopus laevis* (frog) model system can also be used to study the development of the pronephros, the functional kidney throughout larval development, which is favourable owing to its simplicity and ease of accessibility. Nine different *HNF1B* mutations, associated with various renal phenotypes in humans, have been evaluated in *Xenopus*. *In vitro* analysis showed that seven of the subsequent mutant proteins failed to bind DNA, whereas two retained an intact DNA-binding domain and could bind DNA efficiently. Intact DNA binding correlated with the ability of the mutant protein to form dimers with wild-type protein and transactivate target genes. These modelled mutations all interfered with pronephros development to differing degrees when introduced into *Xenopus* embryos. The pattern of pronephros development seen in the developing embryo does not strictly correlate with the properties observed *in vitro* or in transfected cell lines, suggesting that functional studies in *Xenopus* may define features of the HNF1B transcription factor that are not detected in cells grown in culture.[32]

The transactivation potential of HNF1B depends on the synergistic action of the histone-acetyltransferases CREB-binding protein (CBP) and p300/CBP-associated factor (PCAF).

The transcriptional impairment of HNF1B mutants with normal DNA-binding activity correlated with the loss of association with one of these coactivators in co-immunoprecipitation studies. The activity of these mutant proteins in cell lines was not increased by the synergistic action of CBP and PCAF. Thus HNF1B-associated disease may result not only from defective DNA binding but also from diminished transactivation function through impaired recruitment of coactivator proteins.[33]

Investigation into the effect of *HNF1B* mutations within the intron 2 splice site was initially challenging owing to difficulties in accessing tissues with high levels of endogenous HNF1B expression.[14] These studies have been facilitated by the use of ectopic *HNF1B* transcription in Epstein-Barr virus-transformed lymphoblastoid cell lines; that is, making use of the presence of very low quantities of correctly spliced tissue-specific mRNAs in non-expressing tissues. This technique has been used to demonstrate how two mutations of the intron 2 splice donor site result in the deletion of exon 2 and are predicted to cause premature termination of the HNF1B protein.[34] Comparable levels of mRNA have been demonstrated in both renal tubule cells isolated from overnight urine and lymphoblastoid cells collected from the same patient with a *HNF1B* mutation.[35] Subsequent work has confirmed that measuring mRNA expression in urinary sediment can be a useful approach to assess the renal epithelial transcriptome in HNF1B-associated renal disease.[36]

THE ROLE OF HNF1B IN RENAL DEVELOPMENT

HNF1B is widely expressed in multiple foetal tissues and is required for visceral endoderm specification.[37] HNF1A is expressed later than HNF1B and is activated only during organogenesis.[38] In adult mice and rats, these transcription factors are expressed in the liver, kidneys, pancreatic islets, stomach and intestine. HNF1B is predominantly expressed in the kidneys, but is also expressed in the gonads, thymus and lungs. HNF1A is most highly expressed in the liver.[39] Despite the high degree of homology between these two transcription factors and the shared DNA binding site,[17] mutations in *HNF1B* usually

result in a multi-system disease, whereas mutations in *HNF1A* have to date solely been reported to cause MODY. This phenotypic variation is probably a reflection of the differential timing and localisation of expression of HNF1A and HNF1B during development.

Accurate development of the kidneys depends on the appropriate interaction between the ureteric bud and metanephric mesenchyme. The ureteric bud gives rise to the ureter, renal pelvis, and collecting duct, whereas the metanephric mesenchyme gives rise to the nephron.[40] Renal development starts with the induction of the ureteric bud from the nephric duct following glial-derived neurotrophic factor (GDNF) secretion from the adjacent metanephric mesenchyme, which activates the receptor tyrosine kinase Ret via the co-receptor GDNF-family receptor α1. The renal collecting system is formed by the invasion of the ureteric bud into the metanephric mesenchyme, with subsequent elongation and branching. Groups of mesenchymal cells proximal to the ureteric bud tips form pretubular aggregates, which differentiate first into comma-shaped and S-shaped bodies, and finally into the Bowman's capsule and tubules.[41] The exact role of HNF1B during this complex process remains to be fully determined. *In situ* hybridisation studies using human tissue samples have shown that *HNF1B* mRNA is highly expressed in the foetal collecting ducts, with lower levels of expression in the metanephric mesenchyme.[42] Several mouse studies have suggested that Hnf1b has an important function during the early stages of urogenital development.[43, 44] Absence of Hnf1b expression in the ureteric bud can result in abnormal ureteric bud branching and a failure of surrounding mesenchymal cells to transition into epithelia, a key step in early nephrogenesis.[43] Hnf1b seems to act upstream of *Wnt9b* and alters Wnt signalling, a pathway known to be crucial in early renal development.[43, 44]

Various animal models of *Hnf1b* deficiency have been used to examine the role of HNF1B during development. Germline inactivation of *Hnf1b* in mouse embryos is lethal, with death at day 6.5–7.0 postconception.[37] Renal-specific inactivation of *Hnf1b* in mice results in polycystic kidneys. This phenotype is associated with a marked reduction in the

transcriptional activation of the cystic disease genes *Umod*, *Pkhd1* and *Pkd2*. Hnf1b binds to DNA elements in these genes.[45] Further work also suggests a link between *Hnf1b* and *Pkhd1* during kidney development. Transgenic mice expressing a dominant-negative *Hnf1b* mutant gene under the control of a kidney-specific promoter, develop renal cysts. The cells lining these cysts lack *Pkhd1* mRNA; however, this transcript is present in surrounding morphologically normal tubules.[46]

HNF1B is thought to have a role in tubular development within the nephron. In mice, inactivation of *Hnf1b* in the metanephric mesenchyme leads to the formation of aberrant nephrons. These nephrons are characterised by glomeruli with a dilated Bowman's space directly connected to collecting ducts via a primitive tubule, owing to the absence of the proximal and distal tubules and loop of Henle. Lack of Hnf1b protein expression also results in deformed S-shaped bodies that lack the typical bulge of epithelial cells in the mid-limb, which usually gives rise to the proximal tubule and the loop of Henle. This phenotype might be associated with defective *Ixr1*, *Osr2* and *Pou3f3* gene expression plus abnormal Notch signalling activation.[47] The Notch signalling pathway is known to be important in tubular segmentation and glomerular formation.[48]

RENAL PHENOTYPE

Prevalence

CAKUT is common and a frequent cause of chronic kidney disease and end-stage renal disease; it accounts for >40% of cases in children in the United States. [49, 50] The term covers a broad range of disorders and several genes have been implicated in the pathogenesis of renal malformations, including *HNF1B*. Table 1.1 summarises the prevalence of HNF1B-associated renal disease in several study cohorts of ≥50 individuals, where both mutation and deletion screening of *HNF1B* was performed. The mean overall detection rate of *HNF1B* gene anomalies was 19%, ranging from 5–31% depending on the

phenotypic selection of the cohort. By contrast, *HNF1B* mutations and deletions are an infrequent cause of MODY and are likely to account for <1% of cases.[51]

Study	Cohort	Detection rate
Ulinski *et al.* (2006)[18]	Children with one of the following features: renal cysts; hyperechogenicity; hypoplasia; or a single kidney (n=80)	25 (31%)
Weber *et al.* (2006)[3]	Unrelated European children with renal hypodysplasia and chronic renal insufficiency, defined by an eGFR 15–75 ml/min/1.73 m^2 (n=99)	8 (8%)
Decramer *et al.* (2007)[19]	Foetuses with either cortical microcysts or isolated hyperechogenicity and/or bilateral foetal hyperechogenic kidneys (n=62)	18 (29%)
Edghill *et al.* (2008 and 2006)[20,26]	Unrelated white subjects with unexplained renal disease categorised as follows: renal cysts and cystic dysplasia; glomerulocystic kidney disease; atypical familial juvenile hyperuricaemic nephropathy; renal dysplasia; renal malformations; other (n=160)	38 (24%)
Adalat *et al.* (2009)[13]	Children with one of the following features: renal cysts and diabetes mellitus; undiagnosed renal cystic disease; or index patient with kidney malformations and a family history of renal disease, diabetes mellitus or gout (n=91)	21 (23%)
Nakayama *et al.* (2010)[21]	Japanese subjects with one of the following features: renal hypodysplasia; unilateral multicystic dysplastic kidney; a single kidney; or cystic kidneys (n=50)	5 (10%)
Heidet *et al.* (2010)[4]	Unrelated subjects with one of the following features: hyperechogenic kidneys with a size ≤3 SD; multicystic kidney disease; renal agenesis; renal hypoplasia; cystic dysplasia; or hyperuricaemic tubulointerstitial nephropathy not associated with *UMOD* mutation (n=377)	75 (20%)
Thomas *et al.* (2011)[2]	North American children ≤16 years with renal aplasia or hypoplasia enrolled in the Chronic Kidney Disease in Children study (eGFR 30–90 ml/min/1.73 m^2) (n=73)	4 (5%)
Madariaga *et al.* (2013)[1]	Foetuses with severe congenital abnormalities of the kidneys and urinary tract, that appeared isolated by foetal ultrasonography and led to termination of the pregnancy (n=103)	12 (12%)
Study cohorts of ≥50 subjects with renal disease were analysed. Both mutation and deletion screening of the *HNF1B* gene was performed on all subjects included in these cohorts. Abbreviation: eGFR, estimated glomerular filtration rate.		

Table 1.1 Detection rate of *HNF1B* genetic abnormalities

Morphology

Considerable variation is seen in the phenotype of HNF1B-associated renal abnormalities, despite the single genetic aetiology. Morphological renal abnormalities are commonly identified by ultrasound. Magnetic resonance imaging (MRI) can be used in selected cases and might be useful in the detection of extra-renal clinical features, such as pancreatic structural abnormalities.[52] Prenatal ultrasound can often detect clinical features of HNF1B-associated renal disease in the developing foetus, where the most frequently observed phenotype is isolated bilateral hyperechogenic kidneys of normal or slightly increased size.[19] In the postnatal period, the majority of these affected individuals have normal-sized or small kidneys with hyperechogenicity and/or renal cysts, which suggests a retardation of renal growth following birth.[4]

Cystic disease, including cystic dysplasia, is the main renal phenotype in both paediatric and adult populations. In the largest case series described to date, cystic disease was present in 73% of patients with HNF1B-associated kidney disease.[4] The major caveat when determining the prevalence of different renal characteristics is that no population-based data currently exists and the majority of the cohorts previously described were pre-selected for particular kidney abnormalities (see Table 1.1 for examples). Cysts are usually small in size,[18] often arising within the renal cortex, and are not reported to progressively increase in number over time.[22] Single kidneys were reported among five of 24 adults carrying *HNF1B* mutations/deletions, the largest series so far of adults with HNF1B-associated disease.[22] Single kidneys were initially hypothesised to be the result of involution of multicystic dysplastic kidneys over time; however, unilateral renal agenesis has also been identified by prenatal foetal imaging in 4/56 affected cases.[4, 18]

Other reported structural abnormalities include renal hypoplasia, horseshoe kidney (fusion of the two kidneys during embryonic development into a horseshoe-shaped structure) and duplex kidney.[3, 18, 20] Collecting system abnormalities, such as pelviureteric junction obstruction, have also been identified, but usually occur in conjunction with other renal structural abnormalities.[25] Isolated bilateral hydronephrosis and hydroureter have also

been reported.[13] *HNF1B* molecular defects were found in 2/34 individuals with prune-belly syndrome, which is characterised by a triad of dilatation of the urinary tract, deficiency or absence of the abdominal wall musculature and bilateral undescended testes.[53-55]

In the minority of cases, renal imaging can appear normal;[9, 22, 51] however, it is unclear how often this occurs as the majority of cohorts with HNF1B-associated disease that have been studied were pre-selected for kidney abnormalities. In view of the intra-familial variability in clinical features that is seen, it will be important to systematically collect phenotypic information from all affected family members as well as the proband.

Histology

Renal biopsies are not performed in many cases of HNF1B-associated disease, because renal cysts, or other structural anomalies, are often visualised by imaging. 19 histology results have been reported in the literature. The majority of these biopsies were performed as part of explorative clinical investigation of unexplained renal impairment before a genetic diagnosis being established; others have resulted from post-mortem examination following termination of pregnancy. Considerable variation was observed in the histological diagnosis, including hypoplastic glomerulocystic kidney disease (cortical glomerular cysts with dilatation of the Bowman spaces and primitive glomerular tufts in ≥5% of the cysts) among six patients;[3-5, 56] oligomeganephronia (reduced number of enlarged nephrons) in three patients;[9, 11, 57] and cystic renal dysplasia in two patients.[30, 58] All of these different renal phenotypes probably arise from abnormal nephron development. Other non-specific features include interstitial fibrosis, enlarged glomeruli/nephrons and glomerular cysts.[9, 57]

Malignancy

Imaging to screen for chromophobe renal cell carcinoma (RCC), a rare subtype of kidney cancer, should be considered for individuals with *HNF1B* gene anomalies. Following the

observation of chromophobe RCC in a patient with a known *HNF1B* mutation,[9] a series of 34 randomly selected renal neoplasms were screened for *HNF1B* gene inactivation. Biallelic inactivation was identified in 1/11 tissue samples owing to the development of a somatic *HNF1B* gene deletion in addition to a germline mutation.[59] Overexpression of *HNF1B* is common in clear cell ovarian cancer.[60] Several genome-wide association studies have also linked genetic variation in the *HNF1B* region with a risk of endometrial and prostate cancer.[61-64]

Biochemical abnormalities

Hypomagnesaemia: Hypomagnesaemia is a common feature of HNF1B-associated disease.[4, 13, 22] This condition was detected in 8/18 (44%) children with *HNF1B* mutation/deletion under follow-up for renal malformation; hypomagnesaemia was accompanied by hypermagnesuria and hypocalciuria. With the exception of one patient who presented with tetany, symptoms attributable to hypomagnesaemia were not reported in the other cases. HNF1B regulates the transcription of *FXYD2*, a gene that encodes the γ subunit of the Na^+/K^+-ATPase and is involved in the reabsorption of magnesium in the distal convoluted tubule.[13, 65] A mutation in *FXYD2* has been reported in one family to date with autosomal dominant hypomagnesaemia and hypocalciuria.[66] This finding suggests an additional role for HNF1B in the maintenance of tubular function.

Hyperuricaemia: Hyperuricaemia has been associated with disorders resulting from genetic abnormalities in *HNF1B*,[14] although serum urate levels have not yet been systematically measured in a large cohort. Patients might additionally present with early-onset gout, and some affected individuals with hyperuricaemia, early-onset gout and renal disease meet established criteria for familial juvenile hyperuricaemic nephropathy, a condition usually caused by mutations in *UMOD*, which encodes uromodulin.[67] The cause of hyperuricaemia in HNF1B-associated disease probably reflects both altered urate transport in the kidney and an early manifestation of renal impairment. Mice with renal-specific inactivation of *Hnf1b* develop polycystic disease, and exhibit markedly reduced transcriptional activation of *Umod.*[45] The majority of causative mutations in *UMOD* that

result in familial hyperuricaemic nephropathy are considered to exert dominant negative effects; therefore, it remains unclear how the same phenotype is associated with *HNF1B* haploinsufficiency.[4]

Renal function

Renal function is usually impaired in HNF1B-associated disease but can range from normal to end-stage renal disease (ESRD). A slowly progressive deterioration of renal function throughout adulthood has previously been described; a median yearly decline in the estimated glomerular filtration rate (eGFR) of 2.45 ml/min per 1.73 m^2 was observed in a study of 27 adults with an *HNF1B* mutation/deletion and a wide variety of renal phenotypes.[22] Four patients (15%) in this series progressed to ESRD, which is consistent with the frequency reported in a systematic review (12.8%).[25] The age at diagnosis of ESRD remains unpredictable, and has even been reported in early childhood.[20] The impact of causative mutations in *HNF1B* on renal function in the paediatric population is difficult to interpret owing to the young age of the patients and the lack of long-term follow-up. *HNF1B* gene anomalies can also be associated with severe prenatal renal anomalies, which may result in oligohydramnios, pulmonary hypoplasia and renal failure. This situation can in turn lead to perinatal death, the requirement for early renal replacement therapy or parental request for termination of the pregnancy.[1, 4]

Individuals with HNF1B-associated disease who are likely to require renal replacement therapy should be considered for renal transplantation. This patient group is at increased risk of developing new-onset diabetes after transplantation (NODAT), and an immunosuppressive regimen that avoids tacrolimus and reduces corticosteroid exposure may be beneficial.[68] Simultaneous pancreas and kidney (SPK) transplantation, a procedure usually reserved for patients with type 1 diabetes mellitus and ESRD, might be an option for patients with HNF1B-associated disease who present with both diabetes mellitus and ESRD. Three individuals with HNF1B-associated disease were successfully treated with either SPK or pancreas-after-kidney transplantation, and remained free of the need for insulin therapy 1 year after the procedure.[69, 70]

Age group	Differential diagnosis	Key distinguishing features	Diagnostic tests
Paediatric (≤16 years)	Early-onset ADPKD	Family history; diffuse cortical cysts	Renal ultrasound; *PKD1* and *PKD2* genetic testing in selected cases
	ARPKD	Medullary cysts; oligohydramnios with Potter's phenotype, absent urine from the foetal bladder and pulmonary hypoplasia in severe cases; congenital hepatic fibrosis	Renal and abdominal ultrasound; *PKHD1* genetic testing in selected cases
	Cystic dysplasia (idiopathic)	Echobright kidneys with cysts and decreased corticomedullary differentiation; absence of extra-renal features	Renal ultrasound and ^{99m}Tc-DMSA* renography
	Multicystic dysplastic kidney (idiopathic)	Unilateral; multiple unconnected cysts of varying size; absent renal pelvis and renal parenchyma; absence of extra-renal features	Renal ultrasound and ^{99m}Tc-DMSA* renography
	Nephronophthisis	Small kidneys with corticomedullary junction cysts; associated with several extra-renal features, including retinitis pigmentosa and ocular motor apraxia	*NPHP* genetic testing; renal biopsy in selected cases
	Obstructive dysplasia	Dilated upper tract	Renal ultrasound
	Tuberous sclerosis	Cysts and angiomyolipomas; skin fibromas; central nervous system involvement	Dermatological and ophthalmic evaluation; cranial MRI; renal ultrasound; *TSC1* and *TSC2* genetic testing in selected cases
Adult (>16 years)	Acquired cysts	Long duration of renal impairment and/or need for dialysis; shrunken kidneys; no family history	Renal ultrasound
	Autosomal dominant polycystic kidney disease	Enlarged kidneys with progressive increase in cyst burden over time; extra-renal cysts in liver, pancreas and spleen; intracerebral aneurysms; cardiac valvular abnormalities	Renal and abdominal ultrasound; cranial magnetic resonance angiography in selected cases; *PKD1* and *PKD2* genetic testing in selected cases
	Autosomal recessive polycystic kidney disease	Medullary cysts; hepatic periportal fibrosis; portal hypertension	Renal and abdominal ultrasound; *PKHD1* genetic testing in selected cases
	Medullary sponge kidney	Normal-sized kidneys or renal hypertrophy with echogenic medullary pyramids and calcification; usually asymptomatic but might be associated with urinary tract infection and nephrolithiasis	No diagnostic tests recommended as this is a benign condition with no specific treatment
	Simple cysts	Cortical cysts; normal-sized kidneys; absence of extra-renal features	Renal ultrasound
	Von Hippel–Lindau syndrome	Multiple tumours in the central nervous system, retina, adrenal glands, pancreas	Renal ultrasound +/- CT and/or MRI; 24 hour urine collection for

	and kidneys; renal cell carcinomas	catecholamines and metanephrines; *VHL* genetic testing

*Cystic dysplasia and multicystic dysplastic kidney are usually idiopathic but may also be seen in HNF1B-associated disease; renal ultrasound and ^{99m}Tc-DMSA will not distinguish between idiopathic cases and those with a known genetic cause. Abbreviations: HNF1B, hepatocyte nuclear factor 1β; NPHP, nephronophthisis; PKD1, polycystic kidney disease 1; PKD2, PKD2, polycystic kidney disease 2; PKHD1, polycystic kidney and hepatic disease 1; ^{99m}Tc-DMSA renography, technetium-99m-labelled dimercaptosuccinic acid; TSC1, tuberous sclerosis 1; TSC2, tuberous sclerosis 2; US, ultrasound; VHL, Von Hippel–Lindau tumour suppressor, E3 ubiquitin protein ligase.

Table 1.2 Differential diagnosis of renal cysts by age group at presentation[52, 71, 72]

Differential diagnosis

The differential diagnosis of HNF1B-associated kidney disease is wide given the considerable variation in renal phenotypes. Here we discuss the most common presentations of HNF1B-associated kidney disease. Moderately enlarged bilateral hyperechogenic kidneys are often identified following prenatal ultrasound scanning; autosomal recessive and autosomal dominant polycystic kidney diseases are the main differential diagnoses in such cases.[52] To facilitate diagnosis, ultrasonography should also assess for any associated extra-renal abnormalities and a complete family history is useful, with particular emphasis placed on renal disease and diabetes mellitus. Renal cysts are usually visualised after birth; Table 1.2 summarises the main differential diagnoses to consider depending on the age at presentation. Individuals with HNF1B-associated kidney disease might present with other forms of renal tract malformation, including renal agenesis or hypoplasia. The differential diagnosis in these cases can include other multi-organ syndromes that are not covered in this Review, such as branchio-oto-renal syndrome and renal coloboma syndrome.

EXTRA-RENAL PHENOTYPES

Diabetes mellitus

HNF1B has an important function in the early development and differentiation of the pancreas.[73, 74] *HNF1B* gene anomalies can, therefore, result in both reduced endocrine

and exocrine function. Diabetes mellitus is the most frequent extra-renal phenotype detected and it usually presents following renal disease in patients with an HNF1B-associated disorder that was identified in childhood. The mean age at diagnosis of diabetes mellitus is 24 years,[25] but can vary from the neonatal period[75, 76] to late middle age.[26] In a cohort of 27 adult *HNF1B* mutation/deletion carriers with a median age of 35 years, diabetes was present in 13 (48%); none of the patients in this series developed diabetic retinopathy or neuropathy before the close of the study.[22] Diabetes mellitus might manifest as NODAT and analysis of the *HNF1B* gene should be considered among individuals with unexplained CAKUT undergoing transplantation, to improve post-transplant management.[68] Presentation with diabetic ketoacidosis has also been described.[39] The majority of patients with HNF1B-associated diabetes mellitus respond poorly to sulphonylureas and so require treatment with insulin.[25, 77] By contrast, individuals with HNF1A-associated diabetes mellitus respond extremely well when treated with sulphonylureas.

The pathophysiology of diabetes mellitus reflects a combination of β-cell dysfunction and insulin resistance. Dysfunction of β-cells results in reduced insulin secretion, which is likely to be a consequence of pancreatic hypoplasia.[10] Decreased insulin secretion *in utero* leads to intrauterine growth retardation and low birth weight.[76] Patients with *HNF1B* mutations/deletions have reduced insulin sensitivity to endogenous glucose production but peripheral insulin sensitivity is normal.[78] This situation results in hyperinsulinaemia and associated dyslipidaemia, with raised levels of triglycerides and reduced levels of high-density lipoprotein.[77]

Exocrine pancreatic dysfunction

Pancreatic hypoplasia has been described in several individuals with HNF1B-associated disease.[9] Imaging studies have shown a reduced pancreatic structure, with less tissue corresponding to the body and tail of the pancreas and a slightly atrophic pancreatic head.[10] These characteristics are consistent with agenesis of the dorsal pancreas, the embryonic structure that gives rise to the pancreatic body, tail, and a small region of the

head. Studies in mice looking at pancreas development revealed that embryos completely deficient in Hnf1b had a significantly reduced dorsal pancreatic bud that was only transiently present, whereas the ventral bud was undetectable.[74] Two cases of complete pancreatic agenesis have been described in foetuses with severe renal abnormalities, which were found to have an *HNF1B* genetic abnormality following induced termination of the pregnancy.[1, 79]

The majority of patients with pancreatic hypoplasia also have subclinical pancreatic exocrine dysfunction, as evidenced by faecal elastase deficiency.[9, 10] Detailed assessments of pancreatic function, using rapid endoscopic secretin stimulation tests and secretin-stimulated MRI, have confirmed this co-morbidity among patients with HNF1B-associated disease.[80] Pancreatic exocrine hypersecretion has also been observed in affected individuals, and could be a compensatory mechanism for diminished pancreatic volume. These data suggest that the small pancreas observed in individuals with *HNF1B* mutations/deletions might be to the result of hypoplasia rather than atrophy.

Genital tract malformations

Genital malformations were described in some of the first cases of HNF1B-associated disease, providing an early clue that it is a multi-system disorder.[11] Such malformations are most common among females, and are usually caused by abnormalities in uterine development.[25] In a cohort of 108 females with congenital uterine abnormalities, heterozygous mutation or deletion of *HNF1B* was found in 9/ 50 patients (18%) who had both uterine and renal abnormalities, but in none of the 58 cases with isolated uterine abnormalities.[24] In the female embryo, the Müllerian ducts develop into the uterus, fallopian tubes, cervix, and the upper part of the vagina. Müllerian duct aplasia results in the formation of an underdeveloped, rudimentary uterus and vaginal aplasia, and affected individuals are infertile.[11] The corpus and cervix of the uterus and the upper third of the vagina are formed by fusion of the caudal parts of the Müllerian ducts. Failure of fusion can result in a bicornuate uterus, uterus didelphys or a double vagina.[58, 81] *HNF1B* is also a candidate gene for Mayer-Rokitansky-Küster-Hauser syndrome, which involves

congenital aplasia of the uterus, cervix and upper vagina with primary amenorrhoea and infertility.[82, 83]

Various genital tract malformations have been reported among males, including cryptorchidism, agenesis of the vas deferens, hypospadias, epididymal cysts and asthenospermia[9, 22, 58]; however, the potential implication of this association with *HNF1B* molecular defects is unclear owing to the small number of reported cases.

Abnormal liver function

Liver dysfunction in association with *HNF1B* gene mutations has been clinically documented since some of the original publications describing HNF1B-associated disease.[7] Liver dysfunction is a common clinical finding[9, 22] that usually manifests as an asymptomatic rise in the levels of liver enzymes, particularly alanine aminotransferase and γ-glutamyl transferase.[25] Four patients have been reported with neonatal cholestasis, with the results of liver biopsy indicating a reduction in the number of intrahepatic bile ducts.[19, 23, 84, 85] This infrequent phenotype is consistent with the paucity of bile ducts seen in mice with a liver-targeted deletion of *Hnf1b*.[86] Electron microscopy has demonstrated a reduction of normal primary cilia on the epithelial cells of the bile duct among patients with *HNF1B* genetic abnormalities, which could also contribute to cholestasis.[87]

Other clinical features

Increasing interest has focused on whether *HNF1B* gene anomalies might be associated with neurodevelopmental disorders. A 1.4 Mb deletion at chromosome 17q12, which includes the *HNF1B* gene, was found in 18/15,749 patients referred for clinical genetic testing because of autism spectrum disorders, developmental delay or cognitive impairment.[88] Seizures, structural brain abnormalities, mild facial dysmorphic features and macrocephaly have also been reported.[88, 89] The deleted stretch of DNA contains 15 genes; therefore, it is not clear what genetic mechanism gives rise to these observed

neurodevelopmental phenotypes. In a cohort of 53 children with whole gene deletion of *HNF1B* and cystic kidney disease, three had a diagnosis of autism.[90] This was more common than the 1/150–1/300 prevalence of autism recorded in the general paediatric population and suggests further work is needed in this area to ascertain the exact incidence in this group of patients. Nephrologists should therefore be aware of this potential association to ensure that referral to appropriate psychiatric services can be made where applicable.

Early development of hyperparathyroidism may be a previously unrecognised feature of HNF1B-associated disease. Relatively high levels of parathyroid hormone (PTH) ranging from 6.6-16.4 pmol/L, given the degree of renal function decline, have been reported in 6/11 unselected patients with known *HNF1B* gene anomalies undergoing follow-up at a single centre. Five of these six patients had hypomagnesaemia, which usually inhibits the release of PTH, whereas their plasma levels of calcium and phosphate were within the normal range. *In vitro* studies demonstrated that wild-type HNF1B can inhibit transcription of *PTH*; mutant *HNF1B* lacked this property.[91]

Other clinical features, including hearing loss and pyloric stenosis, have been reported in a small number of individuals with an *HNF1B* mutation/deletion.[25] Nevertheless, a causal link with *HNF1B* gene anomalies remains to be established.

AREAS FOR FUTURE RESEARCH

HNF1B-associated disease was first described in 1997, and despite the subsequent identification of many affected patients, research questions still remain regarding its functional and pathological consequences. The prevalence of *HNF1B* gene anomalies in the general population is unknown and it is likely that many cases remain undetected owing to the variable phenotype and frequency of *de novo* gene deletions. One study

identified three individuals with an *HNF1B* deletion from a group of 258 patients who met the clinical criteria for MODY and were not known to have renal disease.[51] Gene mutations were not assessed in this study so it is possible that other patients in this cohort had HNF1B-associated disease that was not identified. This observation highlights the requirement for an improved method of patient selection for genetic testing so that HNF1B-associated disease can be recognised and treated appropriately. Faguer *et al.* described an HNF1B score to be used as part of an algorithm for diagnosing HNF1B-associated disease. This score was created using a weighted combination of clinical features based on the frequency and specificity in HNF1B-associated disease reported in the published literature. The most discriminative characteristics included renal hyperechogenicity, cystic kidneys, MODY, pancreatic hypoplasia or exocrine insufficiency and genital tract abnormalities. 15 different clinical features are assessed in the score, which reflects the wide heterogeneity of organ involvement seen in this disease. It should, therefore, prove a useful tool for selecting patients for HNF1B testing but first requires validation in prospective studies in different populations.[92]

Studies showing a link between large deletions at chromosome 17q12 and neurodevelopmental disorders has led to speculation about the underlying mechanism and whether deletion of *HNF1B* within this region may be involved.[88, 89] *HNF1B* gene anomalies have not previously been associated with abnormalities of neural development and function. Further investigation in a large cohort of individuals with both *HNF1B* mutations and deletions is therefore required, and would have important implications for patient management.

The reasons for phenotypic variation in HNF1B-associated disease remain poorly understood. It is uncertain if such variation reflects the functional effects of different gene anomalies; stochastic variation resulting from minor differences in temporal expression in early development; genetic modifiers; or the contribution of additional neighbouring deleted genes in those patients with the 1.5-Mb deletion that includes *HNF1B*. Comparing large groups of patients, for example those with extreme phenotypic variation and those with

whole-gene deletions versus coding or splice site mutations, could help to identify some of the determinants of this varied phenotype. It will also be important to establish a prospective paediatric cohort, as the follow-up of affected children and adolescents will allow the development and progression of different clinical features to be studied.

Discoveries in zebra fish and mouse models have helped to identify how Hnf1b functions during different stages of renal development; however there is still much to be learned about the complex molecular pathways that are involved. The increasing use of next generation sequencing, which can allow sequencing of the entire human genome within several days, is predicted to identify novel genes involved in CAKUT over the next few years.[93] These gene products may act upstream or downstream of HNF1B and will therefore help to elucidate further the role of HNF1B in nephrogenesis.

CONCLUSIONS

HNF1B first generated interest in 1997 as a potential candidate gene for MODY; it is now known to be the most frequent monogenic cause of developmental renal disease. Molecular defects in *HNF1B* result in a multi-system disorder and some work has suggested that neurodevelopmental features, such as autism spectrum disorders, might be part of the phenotype among individuals with whole-gene deletions. HNF1B-associated disease is characterised by marked clinical heterogeneity, and a positive family history is often lacking; as a result, many patients are likely to have been missed. Further work is required to improve the identification of appropriate patients for genetic testing and to understand the phenotypic variation. *HNF1B* genetic testing should be considered for all patients with developmental renal disease, particularly if renal cysts or hyperechogenicity are detected or other extra-renal clinical features are present.

REVIEW CRITERIA

PubMed was searched using the following terms: “maturity onset diabetes mellitus of the young type 5”, “renal cysts and diabetes mellitus syndrome”, “hepatocyte nuclear factor 1 beta”, “transcription factor 2”, “HNF1beta”, “HNF1B”, “TCF2”, “MODY5” and “RCAD”. The search was restricted to articles published in English between January 1997 and June 2014. Other references and relevant articles published before 1997 were derived from the authors’ knowledge of the published literature.

CHAPTER 2

Assessment of the HNF1B score as a tool to select patients for *HNF1B* genetic testing

Rhian Clissold[a], Beverley Shields[b], Sian Ellard[a], Andrew Hattersley[b], Coralie Bingham[c]

[a] Institute of Biomedical and Clinical Science, University of Exeter Medical School, Exeter, UK

[b] NIHR Exeter Clinical Research Facility, University of Exeter Medical School, Exeter, UK

[c] Exeter Kidney Unit, Royal Devon and Exeter Hospital, Exeter, UK

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Authors' contributions

Andrew Hattersley, Coralie Bingham and I conceived the idea for the study. I collected and analysed the data under the supervision of Beverley Shields. All co-authors contributed to discussions regarding the results. I wrote the manuscript; all co-authors read and approved the final manuscript.

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INTRODUCTION

Heterozygous mutations and deletions in the gene encoding the transcription factor hepatocyte nuclear factor 1β (HNF1B) result in a multi-system disorder. They are the most common known monogenic cause of developmental kidney disease, which is present in the majority of cases.[1-3] The renal phenotype is very variable; cysts are the most frequent feature but single kidneys, hypoplasia, horseshoe kidneys, duplex kidneys, collecting system abnormalities, bilateral hydronephrosis and hyperuricaemic nephropathy may also be seen.[3, 4, 13, 14, 18, 20, 22, 25] HNF1B-related disease is often detected on prenatal ultrasound scanning, where bilateral hyperechogenic kidneys with normal or slightly increased size are commonly found.[19] Other clinical features include young-onset diabetes, pancreatic hypoplasia, genital tract malformations, deranged liver function tests, hypomagnesaemia, hyperuricaemia and early-onset gout.[5, 7, 9-14] Genetic changes comprise whole-gene deletions in approximately 50% of patients and base substitutions or small insertions-deletions in the remainder.[20, 27] Both may arise spontaneously; *de novo* whole-gene deletions are seen in about 50% of cases.[18, 19, 26] This means there is often no family history of renal disease or diabetes.

Given the marked clinical heterogeneity of HNF1B-related disease and frequent absence of a relevant family history, diagnosis is often challenging and it is likely that many cases remain undetected. Faguer and colleagues have recently developed a HNF1B score as a tool to help healthcare professionals select appropriate patients for genetic testing (Table 2.1).[92] It is calculated using 17 items, which include family history, antenatal discovery and organ involvement. The score performed well when tested in a cohort of 433 patients referred to the University Hospital of Toulouse in France for *HNF1B* gene analysis, with negative predictive value >99% and sensitivity 98.2% using a cut-off score of 8. We aimed to replicate this study by testing the clinical utility of the HNF1B score in a cohort of 686 patients who had undergone genetic testing for *HNF1B* molecular defects at Exeter Molecular Genetics Laboratory, which provides the UK national diagnostic testing service for the *HNF1B* gene.

Characteristics	Item	Value
Family history		+2
Antenatal renal abnormalities	Uni/bilateral abnormality by prenatal renal ultrasound scanning	+2
Kidneys and urinary tract		
Left kidney	Hyperechogenicity	+4
	Renal cysts	+4
	Hypoplasia	+2
	Multicystic and dysplastic kidney	+2
	Urinary tract malformation	+1
	Solitary kidney	+1
Right kidney	Hyperechogenicity	+4
	Renal cysts	+4
	Hypoplasia	+2
	Multicystic and dysplastic kidney	+2
	Urinary tract malformation	+1
	Solitary kidney	+1
Electrolyte or uric acid disorders	Low serum Mg^{2+} (<0.7 mmol/l)	+2
	Low serum K^{+} (<3.5 mmol/l)	+1
	Early-onset gout (>30 years of age)	+2
Pathological findings	Oligomeganephronia or glomerular cysts	+1
Pancreas[a]	MODY or hypoplasia of tail and neck of the pancreas or pancreatic exocrine insufficiency	+4
Genital tract	Genital tract abnormality[b]	+4
Liver	Liver test abnormalities of unknown origin[c]	+2

Abbreviations: HNF1B, hepatocyte nuclear factor 1β; MODY, maturity-onset diabetes of the young.
This score should be assessed after ruling out easily recognisable inherited renal diseases e.g. autosomal dominant or recessive polycystic kidney disease and renal coloboma syndrome.
[a]Maximal value of the item pancreas is 4.
[b]Bicornuate uterus, hemiuterus, uterus and upper vagina aplasia, epididymal cysts, bilateral absence of vas deferens.
[c]After exclusion of autoimmune, toxic or viral hepatitis.

Table 2.1 HNF1B score created by Faguer and colleagues (taken from reference 92)

CONCISE METHODS

Probands with renal disease referred for *HNF1B* genetic testing to Exeter Molecular Genetics Laboratory from 1998 to 2012 were included; the criterion for referral was suspicion of HNF1B-related disease by the referring clinician. Informed consent was obtained from individuals to perform genetic testing as part of their clinical care and the study was conducted in agreement with the Declaration of Helsinki Principles. Mutation screening was performed by sequencing of coding exons and exon-intron boundaries together with gene dosage assessment by multiplex ligation-dependent probe amplification as previously described.[20, 26] Clinical details were obtained from referral

information and used to assign an HNF1B score as described by Faguer *et al*.[92]

The characteristics of interest were renal structural anomalies not due to other recognised causes (including antenatal renal abnormalities, hyperechogenicity, cysts, hypoplasia, multicystic dysplastic kidney, urinary tract malformations, solitary kidney and glomerular cysts/oligomeganephronia on biopsy), young-onset diabetes (defined by age at diagnosis ≤35 years), pancreatic hypoplasia or evidence of pancreatic exocrine failure (either reduced faecal elastase or requirement for enzyme replacement therapy), a positive family history of either renal disease or diabetes in parent/child, genital tract malformations (including aplasia of the uterus and upper vagina, bicornuate uterus, hemiuterus, absence of vas deferens and epididymal cysts), liver test abnormalities of unknown aetiology, hypomagnesaemia (serum Mg^{2+} <0.7 mmol/l) and early-onset gout (defined by age at diagnosis <30 years).

The discriminatory ability of clinical features was determined by comparing proportions in patients with and without an *HNF1B* gene mutation/deletion. Pooled odds ratios (OR) were estimated for different characteristics using both this UK dataset and published data in the recent paper by Faguer *et al*. from a cohort of 433 patients referred to the University Hospital of Toulouse in France for *HNF1B* gene analysis.[92]

Performance of the HNF1B score in the UK cohort was evaluated by receiver-operating characteristic (ROC) curve analysis. Negative predictive value (NPV), positive predictive value (PPV), sensitivity and specificity were calculated using the recommended cut-off score of 8.

Statistical analyses

Differences in the frequencies of clinical features and HNF1B score were assessed using the Fisher's exact test for categorical variables and the Mann-

Whitney *U*-test for continuous variables. Effect size estimates were summarised using OR with 95% confidence intervals (CI). Pooled OR were estimated using the Mantel-Haenszel method with calculation of the 95% CI using the Robins, Breslow and Greenland variance formula. Discrimination between patients with and without an *HNF1B* mutation/deletion was assessed by determining the area under the curve of the ROC curve derived from the score. A *P*-value of <0.05 was considered to be statistically significant. All analyses were carried out using SPSS (version 22) and StatsDirect (version 2.7.8) statistical software.

RESULTS

UK cohort description

The cohort included 686 unrelated patients, with a male:female ratio of 1:1. 416 individuals (60.6%) were aged ≤16 years. The majority of the cohort had a congenital anomaly of the kidneys or urinary tract (CAKUT): 408 children (98.1%) and 246 adults (91.1%). A total of 177 patients (25.8%) from the 686 referred for genetic testing were found to have a heterozygous *HNF1B* gene anomaly: 78 (44.1%) had base substitutions or small insertions-deletions, 92 (52.0%) had whole-gene deletions and 7 had partial-gene deletions (4.0%).

The characteristics of the cohort are summarised in Table 2.2. In the paediatric population, detection of antenatal renal abnormalities, renal hyperechogenicity and renal cysts were all more common in *HNF1B* mutation/deletion carriers (*P*=0.0003, *P*=0.0008 and *P*=0.001, respectively). This is in contrast to the adult population where hypoplasia was the only discriminatory renal characteristic (*P* <0.0001). Young-onset diabetes was the only clinical feature to discriminate between patients with and without an *HNF1B* mutation/deletion in both the paediatric and adult cohorts (*P*=0.0002 and *P* <0.0001, respectively). The median age at diagnosis of diabetes was also younger in those with HNF1B-related disease at 16.5 years (IQR 12-26.8) compared to those without (median age 32.5 years, IQR 15.3-49.8), *P* <0.0001. Pancreatic hypoplasia and/or

	Total			Children (≤16 years)			Adults (>16 years)		
	HNF1B status			HNF1B status			HNF1B status		
	Mutation/deletion *n*=177	Normal *n*=509	*P* OR (95% CI)	Mutation/deletion *n*=116	Normal *n*=300	*P* OR (95% CI)	Mutation/deletion *n*=61	Normal *n*=209	*P* OR (95% CI)
Renal phenotype									
Antenatal renal abnormalities	54 (30.5%)	83 (16.3%)	***<0.0001*** 2.3 (1.5-3.4)	53 (45.7%)	80 (26.7%)	***0.0003*** 2.3 (1.5-3.6)	1 (1.6%)	3 (1.4%)	*1* 1.1 (0.1-11.2)
Hyperechogenicity	23 (13.0%)	22 (4.3%)	***0.0002*** 3.3 (1.8-6.1)	21 (18.1%)	20 (6.7%)	***0.0008*** 3.1 (1.6-6.0)	2 (3.3%)	2 (1.0%)	*0.2* 3.5 (0.5-25.4)
Renal cysts	136 (76.8%)	314 (61.7%)	***0.0002*** 2.1 (1.4-3.0)	93 (80.2%)	190 (63.3%)	***0.001*** 2.3 (1.4-3.9)	43 (70.5%)	124 (59.3%)	*0.1* 1.6 (0.9-3.0)
Hypoplasia	21 (11.9%)	24 (4.7%)	***0.002*** 2.7 (1.5-5.0)	6 (5.2%)	16 (5.3%)	*1* 1.0 (0.4-2.5)	15 (24.6%)	8 (3.8%)	***<0.0001*** 8.2 (3.3-20.5)
Multicystic and dysplastic kidney	6 (3.4%)	26 (5.1%)	*0.4* 0.7 (0.3-1.6)	6 (5.2%)	25 (8.3%)	*0.3* 0.6 (0.2-1.5)	0	1 (0.5%)	*1* 0 (0-65.1)
Urinary tract malformations	19 (10.7%)	52 (10.2%)	*0.9* 1.1 (0.6-1.8)	13 (11.2%)	29 (9.7%)	*0.7* 1.2 (0.6-2.4)	6 (9.8%)	23 (11.0%)	*1* 0.9 (0.3-2.3)
Solitary kidney	14 (7.9%)	54 (10.6%)	*0.4* 0.7 (0.4-1.3)	6 (5.2%)	21 (7%)	*0.7* 0.7 (0.3-1.8)	8 (13.1%)	33 (15.8%)	*1* 0.8 (0.4-1.8)
Glomerular cysts or oligomeganephronia on biopsy	4 (2.3%)	10 (2.0%)	*0.8* 1.2 (0.4-3.7)	2 (1.7%)	8 (2.7%)	*0.7* 0.6 (0.1-3.1)	2 (3.3%)	2 (1.0%)	*0.2* 3.5 (0.5-25.4)
Pancreas phenotype									
Diabetes with age of onset ≤35 years	60 (33.9%)	59 (11.6%)	***<0.0001*** 3.9 (2.6-5.9)	26 (22.4%)	25 (8.3%)	***0.0002*** 3.2 (1.7-5.8)	34 (55.7%)	34 (16.3%)	***<0.0001*** 6.5 (3.5-12.1)
Hypoplasia or exocrine failure	7 (4.0%)	1 (0.2%)	***0.0004*** 20.7 (2.5-169)	1 (0.9%)	0	*0.3* -	6 (9.8%)	1 (0.5%)	***0.0006*** 22.7 (2.7-192)
Other features									
Family history	64 (36.2%)	184 (36.1%)	*1* 1.0 (0.7-1.4)	36 (31.0%)	76 (25.3%)	*0.3* 1.3 (0.8-2.1)	28 (45.9%)	108 (51.7%)	*0.5* 0.8 (0.4-1.4)
Genital tract malformations	9 (5.1%)	16 (3.1%)	*0.2* 1.7 (0.7-3.8)	1 (0.9%)	8 (2.7%)	*0.5* 0.3 (0.04-2.6)	8 (13.1%)	8 (3.8%)	***0.01*** 3.8 (1.4-10.6)
Liver test abnormalities	15 (8.5%)	5 (1.0%)	***<0.0001*** 9.3 (3.3-26.1)	0	2 (0.7%)	1 0 (0-9.0)	15 (24.6%)	3 (1.4%)	***<0.0001*** 22.4 (6.2-80.5)
Hypomagnesaemia	11 (6.2%)	7 (1.4%)	***0.001*** 4.8 (1.8-12.5)	3 (2.6%)	6 (2%)	*0.7* 1.3 (0.3-5.3)	8 (13.1%)	1 (0.5%)	***<0.0001*** 31.4 (3.8-257)
Early-onset gout	3 (1.7%)	13 (2.6%)	*0.8* 0.7 (0.2-2.3)	1 (0.9%)	6 (2%)	*0.7* 0.4 (0.05-3.6)	2 (3.3%)	7(3.3%)	*1* 1.0 (0.2-4.8)
Abbreviations: CI, confidence interval; HNF1B, hepatocyte nuclear factor 1β; OR, odds ratio.									

Table 2.2 Characteristics of 686 patients tested for an *HNF1B* genetic abnormality at Exeter Molecular Genetics Laboratory

exocrine failure was significantly associated with *HNF1B* mutations/deletions in adults (*P*=0.0006) but not children; however, there was only 1 affected patient in the paediatric cohort so the numbers were too small to draw any conclusions. In the paediatric population, the frequency of other clinical features and a positive family history of renal disease or diabetes did not vary between patients with and without a diagnosis of HNF1B-related disease. However, in the adult population genital tract malformations, liver test abnormalities and hypomagnesaemia were all discriminatory (*P*=0.01, *P* <0.0001 and *P* <0.0001, respectively).

Estimation of effect size in UK and French cohorts

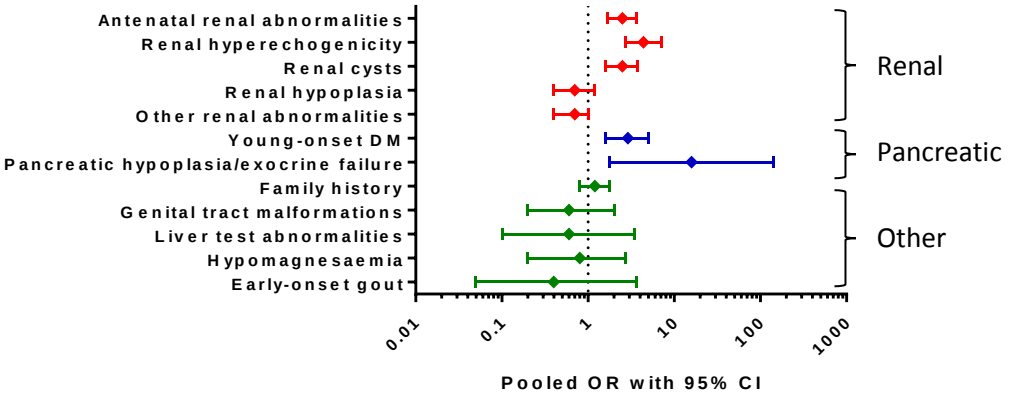
We then used pooled data from UK and French cohorts to see if the same clinical features were discriminatory; pooled odds ratios (OR) were used to estimate effect size for the different characteristics (Figure 2.1). In the paediatric referrals, antenatal renal abnormalities, renal hyperechogenicity and cysts were the renal characteristics with the largest OR of 2.5 (95% CI 1.7-3.7), 4.4 (2.7-7.2) and 2.5 (1.6-3.8), respectively. OR for young-onset diabetes and pancreatic hypoplasia/exocrine failure were 2.9 (1.6-5.1) and 15.9 (1.8-143). In the adult referrals, hypoplasia and cysts were the renal structural anomalies with the highest OR (3.6, 95% CI 1.9-7.1, and 1.9, 95% CI 1.1-3.2, respectively). OR for the pancreatic phenotype were similar to those seen in children. Large OR were seen for genital tract malformations (2.5, 1.1-5.2), liver test abnormalities (10.1, 4.5-23) and hypomagnesaemia (15.5, 4.6-52). This is in contrast to the paediatric population where the OR for these other clinical features were all <1. The 95% CI for some of these characteristics, such as pancreatic hypoplasia or exocrine failure, are very wide and this reflects the small number of patients affected.

Evaluation of HNF1B score in UK cohort

The median HNF1B score was higher in patients with an *HNF1B* mutation/deletion as compared with those without (10 [IQR 8-13.5] *versus* 8 [IQR 4-10], *P* <0.0001). There was no significant difference in score between those with whole-gene deletions and those with base substitutions or small insertions-deletions. The ROC curve, with *HNF1B* genetic test result as the dependent variable, is shown in Figure 2.2; area under the curve = 0.72 (95%

CI 0.67-0.76). Using the suggested cut-off score of 8 gave a sensitivity of 80%, specificity of 38%, NPV of 85% and PPV of 31%. The statistical performance of the HNF1B score using different cut-off scores is shown in Table 2.3.

A) Paediatric referrals



B) Adult referrals

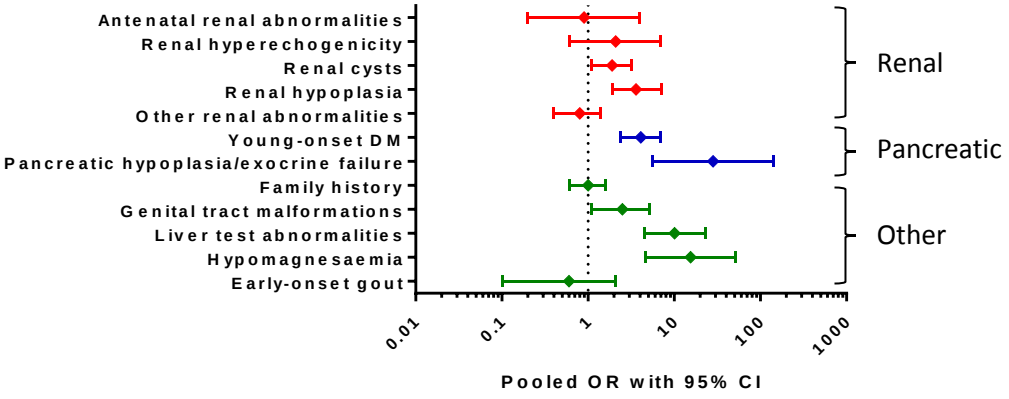


Figure 2.1 Forest plot showing the pooled odds ratios for making a genetic diagnosis of HNF1B-related disease for different clinical features in A) the paediatric and B) the adult cohorts of the combined referrals for *HNF1B* genetic testing to both Exeter, United Kingdom and Toulouse, France (n=1,119).
Other renal abnormalities include multicystic dysplastic kidney, urinary tract malformations, single kidney and glomerular cysts/oligomeganephronia on biopsy. Abbreviations: CI, confidence interval; DM, diabetes mellitus; HNF1B, hepatocyte nuclear factor 1β; OR, odds ratio.

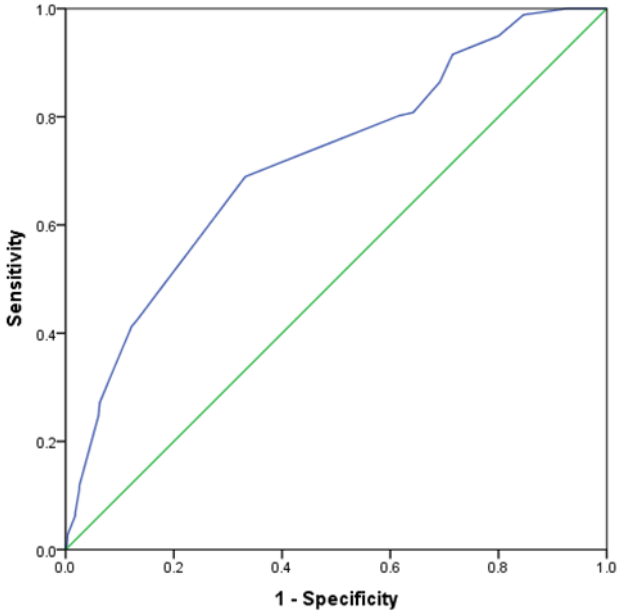


Figure 2.2 Receiver-operating characteristic curve showing the discriminative ability of the HNF1B score for all referrals for *HNF1B* genetic testing to Exeter Molecular Genetics Laboratory.
c-statistic = 0.72 (95% confidence interval 0.67 – 0.76). c-statistic was 0.71 (95% confidence interval 0.65-0.76) in the paediatric cohort and 0.75 (95% confidence interval 0.69-0.82) in the adult cohort.

HNF1B score	Sensitivity (%)	Specificity (%)	Negative predictive value (%)	Positive predictive value (%)	Missed cases (*n*)
≥2	100	7	100	27	0/177
≥4	95	20	92	29	9/177
≥5	92	28	91	31	15/177
≥6	86	31	87	30	24/177
≥7	81	36	84	30	34/177
≥8	***80***	***38***	***85***	***31***	***35/177***
≥9	70	65	86	41	54/177
≥10	69	67	86	42	55/177
≥12	41	88	81	54	104/177

Abbreviations: HNF1B, hepatocyte nuclear factor 1β.
Figures for the suggested cut-off score of 8 are given in bold italics. For comparison, sensitivity was 98%, specificity 41%, negative predictive value 99% and positive predictive value 20% using a threshold of 8 in the work published by Faguer and colleagues and only 1 of the 56 confirmed cases would have been missed.[20]

Table 2.3 Sensitivity, specificity, negative predictive value and positive predictive value of HNF1B score using different cut-off scores

DISCUSSION

In this study, we retrospectively generated an HNF1B score for 686 referrals for *HNF1B* genetic testing to one UK centre and found it discriminated between patients with and without a mutation/deletion reasonably well with an area under the curve of 0.72. This provides further evidence that this clinical scoring system may be a useful screening tool to select individuals for *HNF1B* genetic testing. Applying the suggested cut-off score of 8 gave a sensitivity of 80% and a NPV of 85% so this threshold cannot be reliably used to exclude individuals with a lower score from genetic testing.

This work has limitations which may explain the lower NPV of the HNF1B score in this cohort (85% *versus* 99.4% in the original French cohort). 35 confirmed *HNF1B* mutation/deletion carriers in our referrals had a score below 8 so would not have been initially considered for *HNF1B* genetic testing according to the diagnostic strategy suggested by Faguer and colleagues.[92] Some of these false negative results may be the result of the score being calculated retrospectively using clinical details available at the time of referral. These were based on routinely collected clinical information so not all characteristics were systematically assessed for. We also included all patients who underwent *HNF1B* genetic testing at our centre from 1998 to 2012 and some of the clinical features, such as hypomagnesaemia, have only been associated with HNF1B-related disease in recent years. Use of the HNF1B score is suggested as part of a diagnostic algorithm where genetic testing should be reconsidered in individuals with a score <8 if new features suggestive of HNF1B-related disease occur. Many of the patients in our dataset may have scored ≥8 with more complete data during follow-up. It will therefore be important to assess the performance of the score in a prospective study.

There are differences between the UK and French datasets. In the UK cohort, many of the renal structural anomalies were less common in individuals regardless of their *HNF1B* status compared to a large group of patients referred to a centre in France for *HNF1B* gene analysis; antenatal renal abnormalities were seen in 137/686 (20.0%) UK referrals

but 153/433 (35.3%) French referrals.[92] In contrast, young-onset diabetes was more prevalent in the UK cohort with 33.9% of *HNF1B* mutation/deletion carriers affected compared to only 5.4% in the French dataset. These differences are likely to reflect the fact that the Exeter Molecular Genetics Laboratory has a particular interest in maturity-onset diabetes of the young (MODY) whereas the University Hospital of Toulouse specialises in inherited renal disease. However, it also highlights the importance of appropriate counselling and monitoring for diabetes in affected individuals and their families. In both UK and French datasets, patients underwent genetic testing based on clinician suspicion of HNF1B-related renal disease and the majority had CAKUT. This has led to a selection bias that limits the applicability of the study results. This is in keeping with the literature to date, where the majority of cohorts with HNF1B-related disease that have been described were pre-selected for particular kidney abnormalities.[1-4, 6, 7, 9, 11, 19]

In the absence of any current population-based data, these two large datasets provide an important source of information on HNF1B-related disease. Similar clinical features discriminated between patients with and without an *HNF1B* mutation/deletion in both UK and pooled datasets, with both showing differences between paediatric and adult cohorts. Antenatal renal abnormalities, renal hyperechogenicity and cysts were discriminatory in children, whereas renal hypoplasia and cysts were discriminatory in adults. Pancreatic abnormalities were discriminatory in both age groups whereas genital tract malformations, liver test abnormalities and hypomagnesaemia all had a large effect size in adults only. Due to the small numbers, some of the OR have very large CI so the true effect size is difficult to estimate. However, the data suggests that the antenatal detection of renal hyperechogenicity or cysts plus the presence of either diabetes or pancreatic hypoplasia in children should prompt clinicians to consider a diagnosis of HNF1B-related disease. In adults, the renal phenotype seems to be less discriminatory so extra-renal features are worth testing for in order to decide whether genetic testing may be required. The collection of systematic data on clinical features and biomarkers in a large cohort of *HNF1B* mutation/deletion carriers unselected for phenotype will allow more accurate modelling in

different age groups and may lead to the evolution of a simpler score based only on the most discriminative features.

In summary, we have replicated the discriminative power of the recently-described HNF1B score in a large cohort of individuals referred for *HNF1B* genetic testing to one UK centre. The lower NPV and sensitivity using the suggested cut-off of 8 would have led to missed cases of HNF1B-related disease in this dataset. This highlights the need for validation in a prospective cohort and recalculation of the score if a new feature of HNF1B-related disease occurs.

CHAPTER 3

17q12 microdeletions but not intragenic *HNF1B* mutations are associated with a neurodevelopmental phenotype

Rhian L. Clissold[a,b]; Charles Shaw-Smith[c]; Peter Turnpenny[c]; Benjamin Bunce[d]; Detlef Bockenhauer[e]; Larissa Kerecuk[f]; Simon Waller[g]; Pamela Bowman[a]; Tamsin Ford[a]; Sian Ellard[a,d]; Andrew T. Hattersley[a,b,h]; Coralie Bingham[a,i]

[a] University of Exeter Medical School, Exeter, UK

[b] NIHR Exeter Clinical Research Facility, Royal Devon and Exeter NHS Foundation Trust, Exeter, UK

[c] Clinical Genetics Department, Royal Devon and Exeter NHS Foundation Trust

[d] Department of Molecular Genetics, Royal Devon and Exeter NHS Foundation Trust

[e] Great Ormond Street Hospital for Children NHS Foundation Trust, London, UK and University College London Centre for Nephrology, London, UK

[f] Birmingham Children's Hospital, Birmingham, UK

[g] Evelina London Children's Hospital, St Thomas' Hospital, London, UK

[h] Macleod Diabetes and Endocrine Centre, Royal Devon and Exeter NHS Foundation Trust

[i] Exeter Kidney Unit, Royal Devon and Exeter NHS Foundation Trust

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Authors' contributions

Coralie Bingham, Andrew Hattersley, Sian Ellard and I conceived the idea for the study. I collected and analysed all the data. Charles Shaw-Smith and Peter Turnpenny assessed participants for facial dysmorphic features via photographs. Benjamin Bunce performed the droplet digital PCR. Detlef Bockenhauer, Larissa Kerecuk and Simon Waller assisted with recruitment in their respective paediatric nephrology departments. Pamela Bowman and Tamsin Ford helped with the selection of tools used to assess neurodevelopmental phenotype. All co-authors contributed to discussions regarding the results. I wrote the manuscript; all co-authors read and approved the final manuscript.

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INTRODUCTION

Heterozygous mutations and deletions in the gene encoding the transcription factor hepatocyte nuclear factor 1β (HNF1B) are the commonest known monogenic cause of developmental kidney disease.[1-3] The phenotype of HNF1B-associated renal disease is very variable despite this single genetic aetiology. Abnormalities are often detected on prenatal ultrasonography, where bilateral hyperechogenic kidneys with normal or slightly increased size are commonly found.[19] Cystic disease, including cystic dysplasia, is usually seen in both paediatric and adult populations but other developmental kidney disease has been reported, including single kidneys, hypoplasia, horseshoe kidneys, duplex kidneys, collecting system abnormalities, bilateral hydronephrosis and hyperuricaemic nephropathy.[3, 4, 13, 14, 18, 20, 22, 25] Biochemical abnormalities, including hypomagnesaemia and hyperuricaemia, are common.[13, 14] HNF1B-associated disease is a multi-system disorder; extra-renal phenotypic features include early-onset diabetes mellitus, pancreatic hypoplasia, genital tract malformations and abnormal liver function tests.[5, 7, 9-12] Genetic changes in the *HNF1B* gene comprise either whole-gene deletions (approximately half of patients) or intragenic mutations (base substitutions or small insertions/deletions within the *HNF1B* gene).[20, 27] Both may arise spontaneously; 50% of whole-gene deletions are *de novo*.[18, 19, 26] This means there is frequently no family history of renal disease or diabetes.

The majority of patients with a whole-gene deletion have an approximate 1.3 Mb deletion at chromosome 17q12, which includes the entire *HNF1B* gene.[94] These recurrent microdeletions of 17q12 are mediated by flanking segmental duplications via nonallelic homologous recombination.[28] Unlike most genomic disorders, the 17q12 deletion was not initially thought to be associated with developmental delay or intellectual disability. More recent work has shown that neurodevelopmental disorders, including autism spectrum disorders (ASD), are part of the phenotype in patients referred for testing via clinical genetics rather than renal services.[88, 95-98] A study by Laffargue *et al.* suggests that the neuropsychological phenotype is less severe than that previously reported when the 17q12 deletion is identified secondary to renal abnormalities.[94] Comparison of 26

children with *HNF1B* deletions and 13 with point mutations under the care of paediatric nephrologists showed no significant differences in relation to learning abilities and schooling, although the deletion group tended to have lower intelligence quotients (IQs) and more educational difficulties at school than those with a mutation. However, formal neuropsychological evaluation was only carried out in a small subset of the cohort (11/39) and several of the children included in the study were too young to evaluate for schooling difficulties and ASD.

The 1.3 Mb deleted region contains 14 genes in addition to *HNF1B* and it is not clear what genetic mechanism gives rise to this neurodevelopmental phenotype. One hypothesis is haploinsufficiency of one of these 15 genes. HNF1B is involved in hindbrain development in both zebra fish and mice and so is a good candidate to be the aetiological gene.[99, 100] There have been rare reports of learning difficulties and epilepsy in five patients with *HNF1B* gene mutations, which would support this.[5, 22, 101] Another candidate is *LHX1*, which is also expressed in the brain during early development; a mouse model with a targeted mutation of *Lhx1* confirms its role as a key regulator of the vertebrate head organiser.[102, 103] A study investigating new hotspots of copy-number variation associated with ASD has implicated *ACACA* within the 17q12 deletion.[104] However, no single gene deletions or mutations resulting in haploinsufficiency and neurological disease in humans have been detected in either of these genes to date. An alternative hypothesis would involve more complex interactions between genes within the deleted 17q12 region and other transcription factors giving rise to an increased risk of neurodevelopmental disorders.[94] In this study, we systematically compared the neurodevelopmental phenotype of patients with either an *HNF1B* intragenic mutation or 17q12 deletion to determine whether haploinsufficiency of the *HNF1B* gene is responsible for this aspect of the phenotype.

METHODS

Recruitment and HNF1B genetic analysis

Participants were recruited from January 2013 to October 2015 from four sites in the UK (adult renal and diabetes units at the Royal Devon and Exeter Hospital; paediatric renal units at Great Ormond Street Hospital for Children, Evelina London Children's Hospital and Birmingham Children's Hospital). Inclusion criteria included the presence of either an *HNF1B* intragenic mutation or whole-gene deletion on genetic testing performed due to underlying renal abnormalities or diabetes and current age ≥4 years. All eligible patients were invited to participate. Informed written consent was obtained from all adult participants and parents of child participants, with assent from those aged <16 years. The study was conducted in agreement with the Declaration of Helsinki Principles and approved by a regional ethics committee (National Research Ethics Service Committee South West – Frenchay). A total of 38 patients from 28 unrelated families with HNF1B-associated disease agreed to participate.

Initial mutation screening was performed by sequencing of coding exons and exon-intron boundaries together with gene dosage assessment by multiplex ligation-dependent probe amplification as previously described.[20, 26] Droplet digital PCR was used to confirm the presence of an approximate 1.3 Mb deletion at chromosome 17q12 in the 20 patients with an *HNF1B* whole-gene deletion. This assay measured gene dosage for *ZNHIT3* and *HNF1B*, the most 5' and 3' genes within the recurrent 1.3 Mb 17q12 deletion. Droplet digital PCR was performed using the Bio-Rad QX200 (Bio-Rad Laboratories, Hercules, CA) and following standard protocols. Briefly, a reaction mix containing 22 ng genomic DNA, primers and QX200 ddPCR EvaGreen supermix (Bio-Rad Laboratories) was subjected to the automated QX200 Droplet Generator (Bio-Rad Laboratories) to produce emulsions according to the manufacturer's instructions. After PCR using a standard thermocycler (Bio-Rad Laboratories), sample fluorescence was assessed by the QX200 Droplet Reader (Bio-Rad Laboratories) and absolute quantification of amplified DNA product was determined by Poisson distribution using QuantaSoft software (Bio-Rad Laboratories). A full methodology, including primer sequences, is available in Appendix B.

Clinical evaluation

Renal and extra-renal involvement in participants, including neurodevelopmental disorders, was documented using a standardised assessment of medical records and participant/parent interview in all cases plus educational psychology reports where available. An Index of Multiple Deprivation 2007 score was derived for each participant using their postcode and was used as an overall measure of deprivation. Imaging results from ultrasonography, computed tomography or magnetic resonance imaging were reviewed to look for kidney, pancreas and genital tract abnormalities. Glomerular filtration rate (GFR) was estimated using the (i) Schwartz-Haycock formula in children,[105] optimised for children with renal malformations assessed in each individual paediatric renal unit where possible, and (ii) simplified Modification of Diet in Renal Disease formula in adults.[106] GFR was set at 0 for patients on renal replacement therapy. Proteinuria was defined as albumin:creatinine ratio >30 mg/mmol or protein:creatinine ratio >50 mg/mmol. Hypomagnesaemia was defined as serum magnesium <0.7 mmol/L and hyperuricaemia as a serum urate level above the upper limit of the normal reference range for age and sex from the analysing laboratory. Diabetes was diagnosed either according to World Health Organisation guidelines or on the basis of established treatment with oral hypoglycaemic agents/insulin. Abnormal liver function tests were defined as serum alanine aminotransferase, aspartate transaminase, gamma-glutamyl transferase or alkaline phosphatase levels above the upper limit of the normal reference range for age and sex from the analysing laboratory.

Brief behavioural screening was carried out in 4-16 year olds using the Strengths and Difficulties Questionnaire (SDQ).[107] The questionnaire was completed by parents and included 25 items on psychological attributes covering 5 areas: 1) emotional symptoms, 2) conduct problems, 3) hyperactivity/inattention, 4) peer relationship problems and 5) prosocial behaviour. Scores from areas 1-4 were added together to generate a total difficulties score. An impact supplement was also administered, which provided further information on chronicity, distress, social impairment and burden to others if the child was felt to have a problem. A similar questionnaire with slightly different wording was given to

adolescents for self-completion. This self-report version is suitable for young people aged around 11-16 years, depending on their level of understanding and literacy.[108] The questionnaire templates can be found in Appendix B.

Autistic traits were assessed using the Autism Spectrum Quotient (AQ) in participants of normal intelligence (defined in this study as IQ ≥70). Three different versions of this questionnaire were available from the Autism Research Centre depending on participant age: child (completed by the parent of each child participant aged 4-11 years), adolescent (completed by the parent of each child participant aged 12-15 years) and adult (completed by each participant aged ≥16 years); see Appendix B.[109-111] AQ scores were converted to percentages for standardisation between the different age groups. Cognitive ability was assessed in all participants using the Kaufman Brief Intelligence Test, Second Edition.[112] This is an individually administered measure of verbal and nonverbal intelligence, which yields an overall score known as the IQ composite (an age-based standard score with a mean of 100 and a standard deviation of 15).

Facial photographs of participants were taken and assessed by two experienced clinical geneticists for dysmorphic features previously associated with the 17q12 deletion. The assessors were blinded to the genetic status of each participant. Head circumference was measured and converted to a percentile using British 1990 (UK90) growth reference charts for children and separate centile charts for adults.[113] Macrocephaly was defined as head circumference >90th percentile.

Statistical analysis

Qualitative variables were described with percentages and quantitative variables with median and interquartile range (IQR). Differences between *HNF1B* gene mutation and deletion groups were assessed using the Fisher's exact test for categorical variables and the Mann-Whitney *U*-test for continuous variables. A *P*-value of <0.05 was considered to be statistically significant. The Bonferroni method was used to correct for multiple comparisons when evaluating dysmorphic features and inter-rater agreement between the

two independent assessors was quantified using Cohen's kappa coefficient. All analyses were carried out using StataSE (version 13.1) and GraphPad statistical software.

RESULTS

General characteristics are similar in both HNF1B mutation and deletion groups

38 individuals participated in the study; 18 (47%) had a known intragenic *HNF1B* mutation and 20 (53%) had a whole-gene deletion. The intragenic mutations included four nonsense, 13 insertions/deletions and one missense change (Table 3.1). The presence of the common 1.3 Mb 17q12 deletion was confirmed by dosage analysis of *ZNHIT3* and *HNF1B*, the most 5' and 3' of the 15 genes within the interval, by droplet digital PCR in the deletion group.

Patient study number	Nucleotide change	Amino acid change	Functional effect	Inheritance
2	c.982_986delCCTCT	p.P328fs	Frameshift	*De novo*
4	c.982_986delCCTCT	p.P328fs	Frameshift	Maternal (son of patient 2)
6	c.1138delG	p.V380fs	Frameshift	*De novo*
10	c.544+3_544+4insT	p.?	Splice site	Maternal (daughter of patient 13)
11	c.544+3_544+4insT	p.?	Splice site	Maternal (daughter of patient 10)
12	c.544+3_544+4insT	p.?	Splice site	Maternal (son of patient 10)
13	c.544+3_544+4insT	p.?	Splice site	Unknown
14	c.544+3_544+4insT	p.?	Splice site	Unknown (half-sister of patient 13)
20	c.1235dupC	p.V413fs	Frameshift	Maternal (son of patient 23)
21	c.1006dup	p.H336fs	Frameshift	Unknown
22	c.1006dup	p.H336fs	Frameshift	Paternal (son of patient 21)
23	c.1235dupC	p.V413fs	Frameshift	Unknown
24	c.541C>T	p.R181*	Nonsense	Paternal
25	c.541C>T	p.R181*	Nonsense	Paternal
26	c.541C>T	p.R181*	Nonsense	Paternal
31	c.398A>G	p.N133S	Missense	*De novo*
38	c.544C>T	p.Q182*	Nonsense	*De novo*
39	c.1048dup	p.V350fs	Frameshift	Unknown

Table 3.1 Details of intragenic *HNF1B* mutations

Both mutation and deletion groups were similar in terms of general characteristics (Table 3.2). Median age at study inclusion was similar between the groups, as was gender

breakdown. Participants were predominantly of White British origin, reflecting the fact that 61% were recruited from South West England. Levels of deprivation were similar in both mutation and deletion groups as measured using the median Index of Multiple Deprivation 2007 score. Cysts or cystic dysplasia was the renal phenotype seen most commonly in both groups, similar to cohorts with HNF1B-associated kidney disease previously

	***HNF1B* mutation** ***n*=18**	**17q12 microdeletion** ***n*=20**	***P***
Median age, years (IQR)	19 (13-45)	15.5 (11-35)	*0.3*
Sex, *n* (%)	M 8 (44), F 10 (56)	M 8 (40), F 12 (60)	*1*
Ethnicity, *n* (%)	White 18 (100)	White 19 (95), mixed 1 (5)	*1*
Median Indices of Deprivation 2007 score (IQR)	25 (16-46)	21 (12-30)	*0.4*
RENAL PHENOTYPE			
Renal abnormality, *n* (%)			
Cysts/cystic dysplasia	12 (67)	17 (85)	*0.3*
Other[a]	4 (22)	3 (15)	
(Unknown)	2 (11)	0	
Median age at diagnosis of renal disease, years (IQR)	0 (0-20)	0 (0-24)	*0.7*
Renal replacement therapy, *n* (%)	3 (17)	1 (5)	*0.3*
Median GFR, mL/min/1.73 m^2 (IQR)	42.6 (31-60)	81.4 (56-91)	***0.002***
Proteinuria[b*]**, *n* (%)**	2 (13)	2 (11)	*1*
(Unknown)	0	4 (21)	
Median serum magnesium*, mmol/L (IQR)	0.7 (0.67-0.75)	0.58 (0.53-0.69)	***0.01***
Hypomagnesaemia[c*]**, *n* (%)**	6 (40)	12 (63)	*0.3*
(Unknown)	1 (7)	4 (21)	
Hyperuricaemia[d*]**, *n* (%)**	10 (67)	3 (16)	***0.004***
(Unknown)	2 (13)	7 (37)	
Gout, *n* (%)	6 (33)	2 (10)	*0.1*
EXTRA-RENAL PHENOTYPE			
Pancreas			
Diabetes, *n* (%)	7 (39)	8 (40)	*1*
Median age at diagnosis of diabetes (IQR)	19 (18-37)	29 (17-32)	*1*
Pancreatic hypoplasia[e†]**, *n* (%)**	1 (6)	5 (25)	*0.2*
Median faecal elastase, *μ*g/g stool (IQR)	402.5 (170-500)	280 (167-433)	*0.8*
Genital tract			
Genital tract malformation[f†]**, *n*(%)**	1 (6)	2 (10)	*1*
Liver			
Abnormal liver function tests[†]**, *n* (%)**	5 (28)	6 (30)	*1*

Abbreviations: F, female; GFR, glomerular filtration rate; IQR, interquartile range; M, male.
[a]Other renal structural abnormalities included single kidney, collecting system abnormalities and bilateral hydronephrosis.
[b]Proteinuria defined as albumin:creatinine ratio >30 mg/mmol or protein:creatinine ratio >50 mg/mmol.
[c]Hypomagnesaemia defined as serum magnesium <0.7 mmol/L. [d]Hyperuricaemia defined as serum urate level above upper limit of normal reference range for age and sex from analysing laboratory. [e]Hypoplasia of body and/or tail of pancreas.
[f]Genital tract malformations included (i) unilateral undescended testicle and blind-ending epididymis, (ii) bilateral undescended testicles and (iii) bicornuate uterus. *Only assessed in individuals with native renal function. [†]Not systematically assessed for.

Table 3.2 Characteristics of study patients with either an *HNF1B* intragenic mutation or 17q12 microdeletion

described in the literature.[4] Renal function was worse in the mutation group with a median estimated GFR of 42.6 mL/min/1.73 m^2 (IQR 31-60) compared to 81.4 (IQR 56-91) in the deletion group, *P*=0.002. Serum magnesium levels were lower in the deletion group whereas hyperuricaemia was more common in the mutation group; both of these findings may be explained by the greater degree of renal impairment seen in the mutation group. Diabetes was present in approximately 40% of patients in both groups. Other extra-renal phenotypes were also similar between mutation and deletion groups.

The 38 patients included in this study represented 45% of those with HNF1B-associated disease who were eligible to take part from the four different sites. Table 3.3 compares the general characteristics of participants and non-participants. Briefly, the two groups were similar in terms of genetic abnormality, age, gender, levels of deprivation and renal phenotype. The only difference was in ethnicity, with other ethnic groups besides White being more commonly represented amongst non-participants. However, the data available for non-participants was incomplete with 13/47 (28%) having no information on ethnicity recorded.

	Participants ***n*=38**	**Non-participants[ŧ]** ***n*=47**	***P***
Genetic abnormality, *n* (%)			
Mutation	18 (47)	17 (36)	*0.4*
Whole-gene deletion	20 (53)	30 (64)	
Median age, years (IQR)	17 (12-38)	14 (11-20)	*0.06*
Sex, *n* (%)	M 16 (42), F 22 (58)	M 24 (51), F 23 (49)	*0.5*
Ethnicity, *n* (%)			
White	37 (97)	20 (43)	***<0.001***
Mixed	1 (3)	2 (4)	
Asian		9 (19)	
Black/African/Caribbean		3 (6)	
Unknown		13 (28)	
Median Indices of Deprivation 2007 score (IQR)	23 (13-36)	25 (15-36)	*0.9*
Renal abnormality, *n* (%)			
Cysts/cystic dysplasia	29 (81)	37 (82)	*1*
Other*	7 (19)	8 (19)	

Abbreviations: F, female; IQR, interquartile range; M, male.
[ŧ]All had either an *HNF1B* point mutation or whole-gene deletion on previous genetic testing and current age ≥4 years.
*Other renal structural abnormalities included single kidney, collecting system abnormalities and bilateral hydronephrosis; in 5 cases the imaging results were not known.

Table 3.3 General characteristics of participants and non-participants who were eligible to take part in the study

Brief behavioural screening shows higher levels of psychopathology and impact in children with a deletion

Use of the parent-report SDQ revealed more patient difficulties in the deletion group with a median total difficulties score of 15.5 (IQR 10-20) compared to 7 in the mutation group (IQR 3.5-7.5, *P*=0.048; Figure 3.1). This is also higher than the mean total difficulties score of 8 (standard deviation 5.8) obtained in a normative sample of 10,438 British school-aged children.[114] When analysing the four subsections of the total difficulties score, conduct problems and peer relationship problems were more common in the deletion group: median scores were 2.5 (IQR 2-5) and 4.5 (IQR 1-6), respectively vs. 0.5 (IQR 0-1) and 0 (IQR 0-0.5) in the mutation group, *P*=0.04 and 0.02, respectively (Figure 3.2). Five of the ten children with a deletion scored above the suggested clinical cut-point of 15; all of these children apart from one had already been referred for further psychological evaluation.

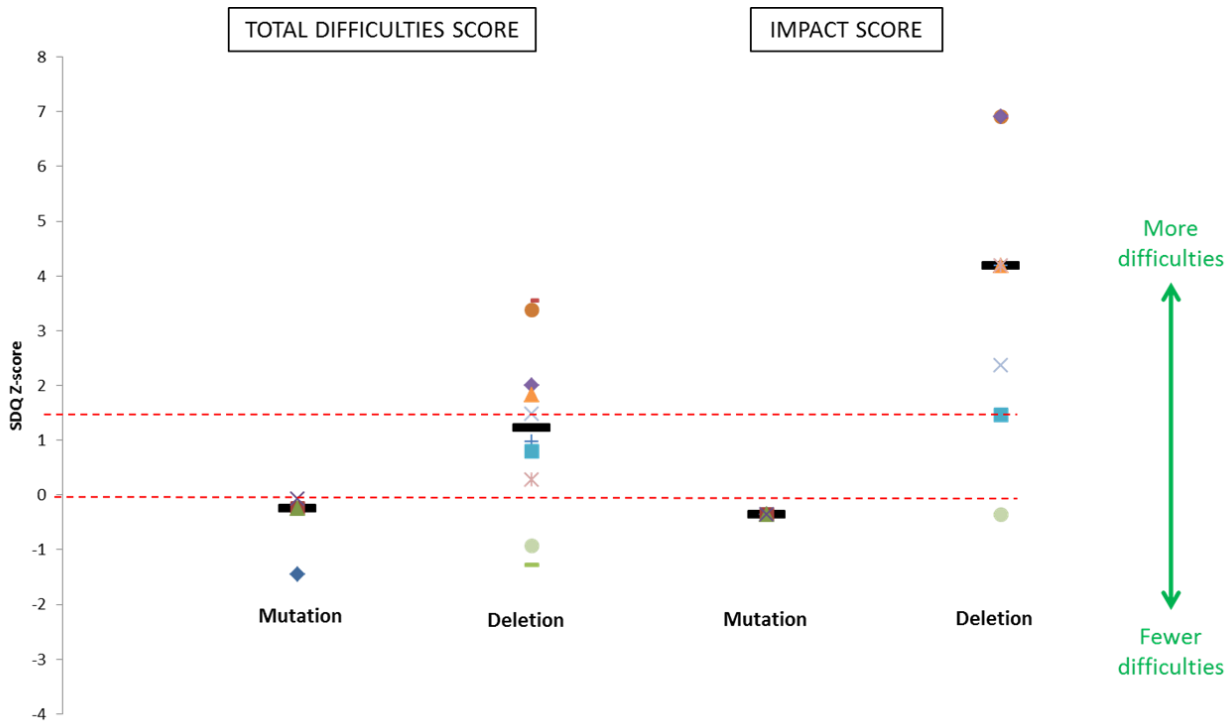

Figure 3.1 Patient difficulties as shown by parent-report Strengths and Difficulties Questionnaire (SDQ) scores (presented as Z-scores) for individuals <18 years with both *HNF1B* gene mutations (*n*=4) and 17q12 microdeletions (*n*=10).
Individual scores are represented as different-shaped points and group medians as black bold horizontal lines. X axis represents school-age population mean, red dashed horizontal line above represents suggested clinical cut-point (90th percentile).

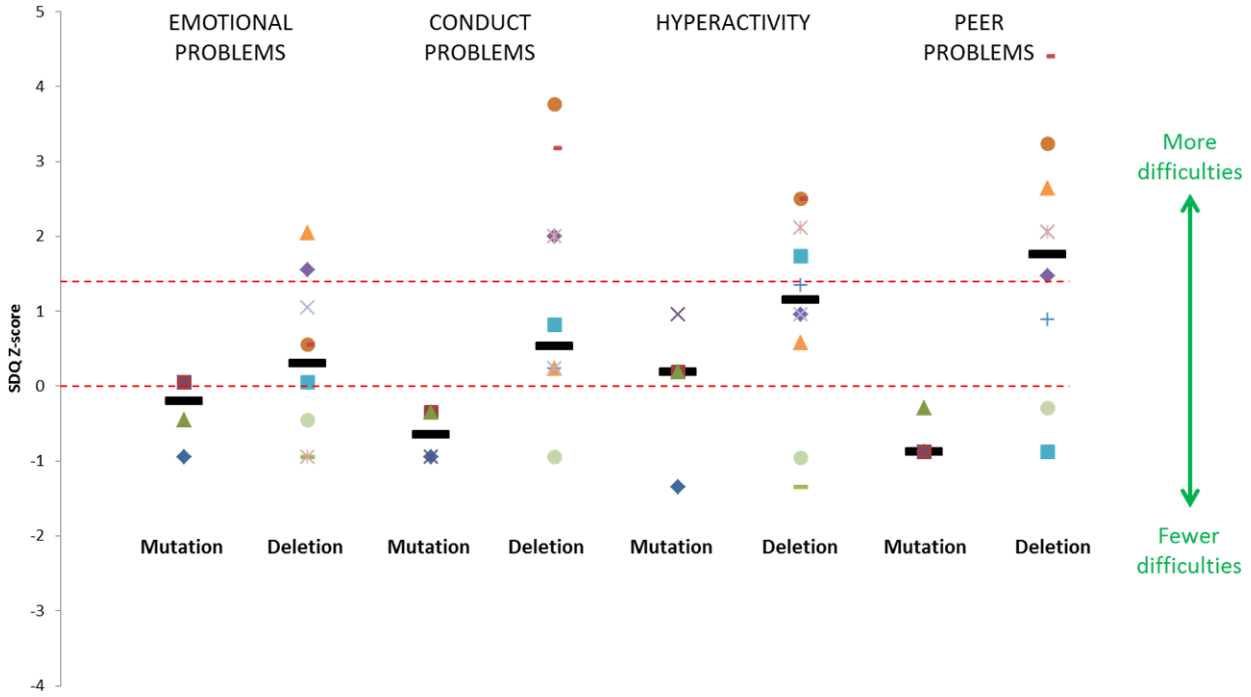

Figure 3.2 Patient difficulties as shown by subsections of the parent-report Strengths and Difficulties Questionnaire (SDQ) scores (presented as Z-scores) for individuals <18 years with both *HNF1B* gene mutations (*n*=4) and 17q12 microdeletions (*n*=10).
Individual scores are represented as different-shaped points and group medians as black bold horizontal lines. X axis represents school-age population mean, red dashed horizontal line above represents suggested clinical cut-point (90th percentile).

Trait	Median SDQ score (IQR)		*P*	British means (standard deviation) for 5-15 year olds
	HNF1B mutation	17q12 microdeletion		
	n=4	*n*=3		*n*=4,228
Total difficulties	10.5 (6-17)	13 (4-30)	0.5	10.3 (5.2)
Impact	0 (0-1)	1 (0-7)	0.3	0.2 (0.8)
Emotional problems	1.5 (1-3.5)	5 (0-6)	0.6	2.8 (2.1)
Conduct problems	3 (1.5-4.5)	0 (0-7)	0.7	2.2 (1.7)
Hyperactivity	5 (3-7)	6 (2-8)	0.7	3.8 (2.2)
Peer problems	1 (0.5-2)	2 (2-9)	0.1	1.5 (1.4)
Abbreviations: IQR, interquartile range; SDQ, Strengths and Difficulties Questionnaire				

Table 3.4 Patient difficulties as shown by self-report Strengths and Difficulties Questionnaire scores for individuals aged 11-17 years with both *HNF1B* gene mutations (n=4) and 17q12 microdeletions (n=3).
Normative British school-age data from a large national survey of child and adolescent mental health is shown as a comparison.[114]

Parental scores for the impact of these difficulties on the child's life were similarly high in the deletion group with a median score of 5 (IQR 2-8). This was compared to a median score of 0 in the mutation group (IQR 0-0, *P*=0.02) and a mean score of 0.4 (standard deviation 1.1) in the large normative sample mentioned previously.[114] Table 3.4 shows that use of the self-report SDQ in those aged 11-17 years yielded similar total difficulties and impact scores between mutation and deletion groups; however, the numbers in these groups were too small to draw any meaningful conclusions (*n*=7 in total).

Clinical diagnosis of neurodevelopmental disease in patients with a deletion

8/20 (40%) participants with a deletion had a clinical diagnosis of either an ASD, attention deficit hyperactivity disorder (ADHD) and/or learning difficulties requiring a Statement of Special Educational Needs or attendance at a special school compared to 0/18 with a mutation, *P*=0.004 (Figure 3.3A). Of these eight patients, four had co-morbidity with learning difficulties accompanying a diagnosis of ASD and/or ADHD (Figure 3.3B and Table 3.5). According to the second national survey of children's mental health and well-being carried out in 2004, the prevalence of ASD in British children was 0.9% and hyperkinetic disorder/ADHD was 1.5%.[115] Therefore, the frequency of ASD and ADHD found in participants with a deletion in this study far exceeds the baseline population rates.

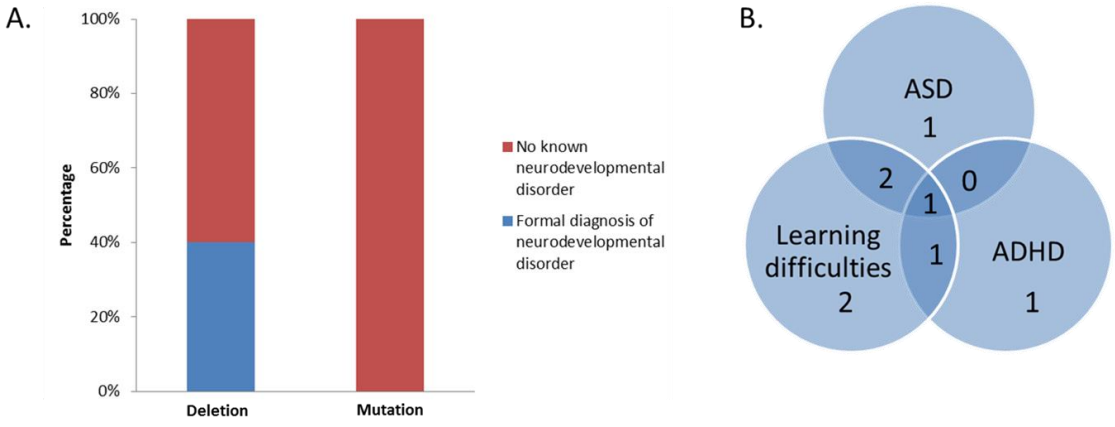

Figure 3.3 (A) Stacked bar chart showing percentage of patients within both 17q12 microdeletion (*n*=20) and *HNF1B* mutation (*n*=18) groups with a known neurodevelopmental disorder including autism spectrum disorder (ASD), attention deficit hyperactivity disorder (ADHD) and/or learning difficulties requiring a Statement of Special Educational Needs or attendance at a special school. (B) Venn diagram illustrating the breakdown and overlap of diagnoses in the eight patients with a deletion and neurodevelopmental disorder.

Patient study number	Age (years)	Sex	*HNF1B* genetic abnormality	Details of neurodevelopmental disorder	SDQ scores		AQ	IQ
					Total difficulties score	Impact score		
5	14	Male	Whole-gene deletion (c.1-?_1674+?del)	ADHD and dyspraxia diagnosed aged 5 years; treatment with methylphenidate; attendance at mainstream school with Statement of Special Educational Needs.			68%	114
8	33	Male	Whole-gene deletion (c.1-?_1674+?del)	Learning difficulties with attendance at special school; Asperger's syndrome diagnosed in early twenties.			63%	69
9	8	Female	Whole-gene deletion (c.1-?_1674+?del)	ASD diagnosed aged 8 years.	17	3	91%	107
16	9	Female	Whole-gene deletion (c.1-?_1674+?del)	Difficulties with literacy and numeracy skills, attention and concentration plus speech and language skills; attendance at mainstream school with Statement of Special Educational Needs recommending 9.25 hours of additional specialist teaching support per week.	10	5	41%	76
27	12	Male	Whole-gene deletion (c.1-?_1674+?del)	Extreme delayed speech with no words, requiring speech and language input; ASD diagnosed aged 5 years with attendance at special school for children with autism since age 8 years.	19	5	84%	76
33	14	Male	Whole-gene deletion (c.1-?_1674+?del)	ASD and ADHD diagnosed aged 11 years after long history of challenging behaviours at home and school; attendance at special school for children with emotional and behavioural difficulties.	29	8		
34	16	Female	Whole-gene deletion (c.1-?_1674+?del)	Specific learning difficulties (dyslexic), coordination difficulties (dyspraxia) plus receptive and expressive language delay diagnosed aged 8 years; attendance at mainstream school with Statement of Special	14	5	34%	50

				Educational Needs recommending 15 hours of additional specialist teaching support per week.				
35	13	Male	Whole-gene deletion (c.1-?1674+?del)	Attention Deficit Disorder diagnosed aged 6 years; treatment with methylphenidate.	28	8	28%	96
Abbreviations: ADHD, attention deficit hyperactivity disorder; ASD, autism spectrum disorder; AQ, Autism Spectrum Quotient; IQ, intelligence quotient; SDQ, Strengths and Difficulties Questionnaire.								

Table 3.5 Details of study patients with HNF1B-associated disease and a clinically diagnosed neurodevelopmental disorder

17q12 deletions are associated with more autistic traits

Patients with a deletion had a higher median AQ (43% [IQR 28-68] vs. 29% [IQR 16-42] in the mutation group, *P*=0.02), indicating a greater number of autistic traits (Figure 3.4A). Although the AQ is not a diagnostic tool, cut-offs have been described for identifying individuals who may have clinically significant levels of autistic traits. However, referral for a full diagnostic assessment is only warranted if the individual is also suffering a degree of distress as a result of these traits.[109-111] 6/38 (16%) participants scored above the suggested cut-off; of these, all had a deletion and 3/6 had a confirmed diagnosis of ASD. To see if the AQ results were being skewed by a small number of individuals with a high number of autistic traits, the analysis was repeated after excluding those with a known ASD. Although there was a trend towards a higher AQ in the deletion group (median AQ 36% [IQR 28-52] vs. 29% [IQR 16-42] in the mutation group), this did not reach statistical significance (*P*=0.08; Figure 3.4B) but may have done so in a larger sample (*n*=64).

Cognitive ability is similar in both HNF1B mutation and deletion groups

The median IQ composite was similar in both mutation and deletion groups (97 [IQR 83-104] vs. 91 [IQR 76-107], *P*=0.6; Figure 3.5). Two participants with a deletion scored in the lower extreme category with IQ <70.

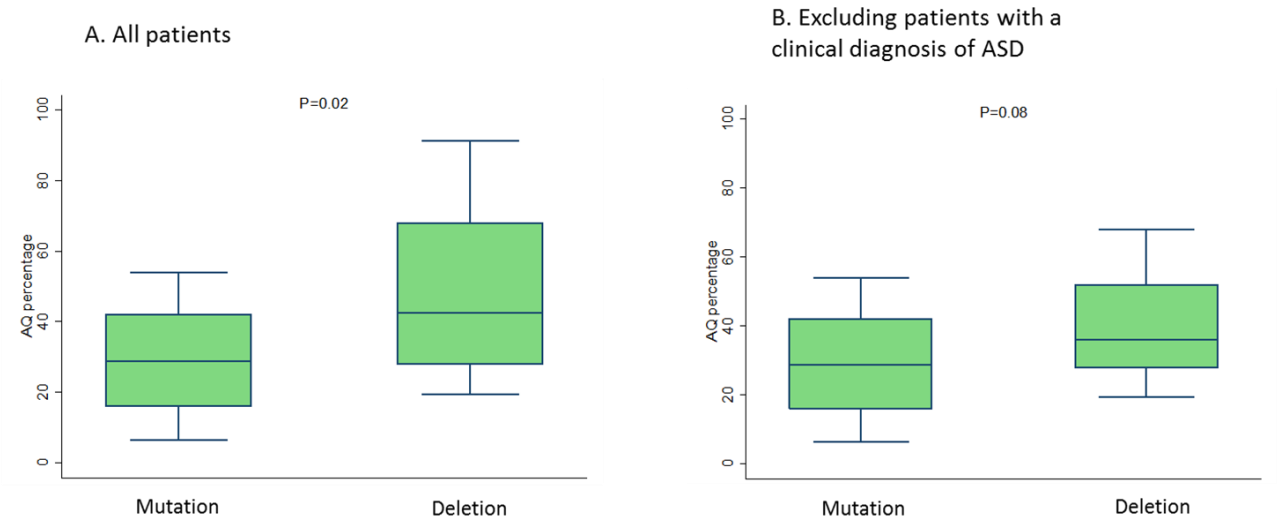

Figure 3.4 Quantification of autistic traits using the Autism Spectrum Quotient (AQ) in individuals with HNF1B-associated disease of normal intelligence (defined as IQ ≥70).
(A) Inclusion of all study patients with IQ ≥70 (*n*=36). (B) Exclusion of patients with a clinical diagnosis of an autism spectrum disorder (ASD; *n*=33).

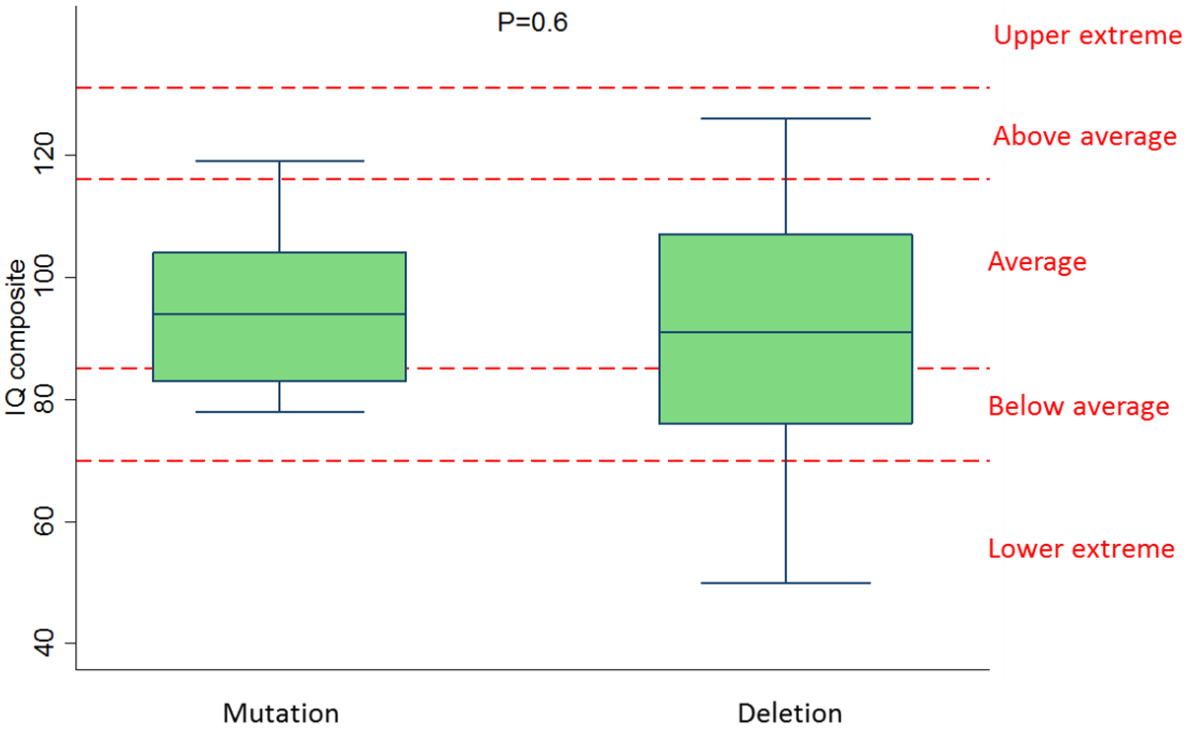

Figure 3.5 Intelligence quotient (IQ) composite scores in individuals with HNF1B-associated disease.
Different IQ classifications are shown by the red dashed horizontal lines.

Facial dysmorphic features considered as a whole may be predictive of the presence of a 17q12 deletion

Facial photographs were analysed in 33 participants (18 with an intragenic *HNF1B* mutation, 15 with a deletion). None of the facial dysmorphic features previously described in association with the 17q12 deletion differed in frequency between the mutation and deletion groups (Table 3.6). Variation in results between the two assessors was seen although overall inter-rater agreement was fair with a kappa coefficient of 0.4 (95% confidence interval 0.3-0.5). A previously undescribed feature of anteverted nares was noted in 13/15 (87%) patients with a deletion compared to only 6/18 (33%) with a mutation (*P*=0.004). When facial dysmorphic features were considered as a whole by both assessors to predict whether an individual had a deletion, sensitivity was 83% and specificity was 79% (Figure 3.6). 9/37 (24%) patients had a head circumference >90th percentile but there was no difference in macrocephaly between the two groups (5/19 [26%] in deletion group vs. 4/18 [22%] in mutation group, *P*=1).

Figure 3.6 Photographs of two study patients with a known *HNF1B* whole-gene deletion demonstrating the high forehead, high arched eyebrows, long philtrum, long face and anteverted nares that, taken as a whole, suggest the presence of a deletion.

Feature[†]	*HNF1B* mutation (*n*=18)	17q12 microdeletion (*n*=15)	*P**	Inter-rater agreement	
				Cohen's kappa coefficient (95% confidence interval)	Strength of agreement
High forehead	10 (56%)	13 (87%)	0.07	0.2	Fair
Arched & high eyebrows	8 (44%)	8 (53%)	0.7	0.6	Moderate
Epicanthal folds	0	0	1	-	-
Downslanting palpebral fissures	2 (11%)	4 (27%)	0.4	0.6	Good
Deep set eyes	4 (22%)	3 (20%)	1	-0.1	Worse than expected by chance alone
Ptosis	5 (28%)	7 (47%)	0.3	0.4	Fair
Depressed nasal bridge	0	3 (20%)	0.08	-0.04	Worse than expected by chance alone
Long philtrum	5 (28%)	9 (60%)	0.09	0.2	Poor
Malar flattening	6 (33%)	10 (67%)	0.08	0.4	Fair
Full cheeks	4 (22%)	5 (33%)	0.7	0.4	Moderate
Long face	12 (67%)	12 (80%)	0.5	0.3	Fair
Facial asymmetry	1 (6%)	3 (20%)	0.3	-0.07	Worse than expected by chance alone
Anteverted nares	6 (33%)	13 (87%)	0.004	0.2	Fair

[†]The presence of a clinical feature was determined using the results of one or both assessors; inter-rater agreement between the two assessors was quantified using Cohen's kappa coefficient

*The Bonferroni method was used to correct for multiple comparisons; statistical significance was re-set at $P < 0.05/13 = 0.004$

Table 3.6 Assessment of facial dysmorphic features in study patients with either *HNF1B* mutation or 17q12 microdeletion by two independent clinical geneticists

DISCUSSION

The results of this study demonstrate that a neurodevelopmental phenotype is only seen in individuals with a 17q12 deletion. Compared to patients with an intragenic mutation, patients with a deletion had a greater number of autistic traits using the AQ and children displayed higher levels of psychopathology and impact on brief behavioural screening using the parent-report SDQ. Indeed, 40% of participants with a deletion had been clinically diagnosed with a neurodevelopmental disorder; ASD and ADHD were seen much more commonly in the deletion group than predicted from population prevalence rates. Most (17/18) of the patients with intragenic mutations had a nonsense or insertion/deletion loss of function mutation, predicted to result in reduced protein expression. The discrepancy in neurodevelopmental phenotype between the intragenic mutation and deletion groups suggests it is not simply haploinsufficiency of the *HNF1B* gene that is responsible for this aspect of the phenotype in individuals with a 17q12 deletion.

Our findings highlight the importance for nephrologists to be aware of this association between 17q12 microdeletion and neurodevelopmental disease to ensure referral to psychiatric services where appropriate. The features of conditions such as ASD can range from mild to severe and can also fluctuate over time and in response to different life events; this variable expression adds to the diagnostic challenges posed by these disorders.[116] Individuals with a deletion and their families should be informed of the increased risk of a neurodevelopmental disorder so they can report any concerning symptoms if they arise to allow prompt investigation.

The results of this study contrast with recent work concluding that when children are diagnosed with a 17q12 deletion secondary to renal abnormalities, the neurodevelopmental phenotype is less severe than previously suggested in the literature.[94] In this French cohort, only 1/26 patients with an *HNF1B* whole-gene deletion were diagnosed with autism as compared to 0/13 in the mutation group. However, the percentage of children with normal school progression requiring no educational support

was lower in the deletion group (62.5% vs. 82%). It is possible that the lack of statistical difference between the two groups in terms of psychomotor development, school progression and educational support may be explained by the younger age at study inclusion. Although both studies included a similar number of participants and all had HNF1B-associated disease identified secondary to renal disease or diabetes, the median age at inclusion was only 5.5 years (range 0.8–17) compared to 17 years (range 4–65) in our UK cohort. Schooling difficulties cannot be assessed in the very young and the features of neurodevelopmental disease may be more apparent as children become older; the median age at diagnosis of ASD, ADHD and learning difficulties in our cohort was 8 years (IQR 5.5-9.5). Earlier work from another French cohort of 53 children with hyperechogenic or cystic kidneys and a 17q12 deletion reported autism in 3 cases (5.7%), a greater proportion than predicted from the prevalence of ASD in the paediatric population.[90] This is in keeping with the increased frequency of ASD in deletion patients we described in our study.

When considered in isolation, none of the facial dysmorphic features previously described in association with a 17q12 deletion was statistically more common in the deletion group in this study. This contrasts with findings by Laffargue and colleagues, who reported that a high forehead, deep set eyes and chubby cheeks were more frequently seen in the presence of a deletion rather than a mutation.[94] However, when the craniofacial characteristics in our series were assessed as a whole, two independent clinical geneticists were able to predict the presence of a deletion with a sensitivity of 83% and specificity of 79%. This supports the prior hypothesis that the 17q12 deletion is associated with a mild but characteristic facial phenotype[88] and that another genetic mechanism besides *HNF1B* haploinsufficiency is causative.

Interestingly, we found that patients with an intragenic *HNF1B* mutation had a significantly lower median eGFR than patients with a deletion, although this is unlikely to be related to the neurodevelopmental differences between the two groups. Ulinski *et al.* described the phenotype of 25 children with HNF1B-associated renal disease and found no difference in

renal function between individuals with an *HNF1B* whole-gene deletion and those with point mutations.[18] A later series that included 75 patients with HNF1B-associated renal disease showed that the proportion of individuals with renal impairment was significantly higher in those with a truncating mutation (nonsense, frameshift or splice site) than in those with a deletion (P=0.01).[4] The authors hypothesised that the older age of the patients with truncating mutations may partly explain the difference in renal function between the two groups; however, the mutation and deletion groups in our study were similarly matched in terms of median age. 17/18 intragenic mutations described in our series were truncating.

The results from this study provide the first detailed description of the neurodevelopmental phenotype of both children and adults diagnosed with HNF1B-associated disease. Both mutation and deletion groups were similarly matched in terms of general characteristics and participants were systematically assessed for neurodevelopmental features using validated screening tools. However, several limitations were associated with this work. Despite inviting all eligible patients from the four different sites to take part, the study cohort represented only 45% of the total due to either inability to contact individuals despite several attempts or a negative response to participation. Therefore, the exact prevalence and spectrum of neurodevelopmental disorders in HNF1B-associated renal disease and diabetes remains unknown. Although individuals were systematically assessed using a combination of screening tools, participant/parent interview and review of medical records, comprehensive screening tools and diagnostic tests for ASD and ADHD were not used. This means less severe disease may have been missed. Finally, genetic screening for other known causes of neurodevelopmental disease (e.g. Fragile X, other copy number variants) was not undertaken.

None of the patients with an intragenic *HNF1B* mutation in our study had a diagnosis of ASD, ADHD or significant learning difficulties. Five individuals with HNF1B-associated disease secondary to gene mutation and either learning difficulties and/or epilepsy have been described, although other genetic causes were not excluded.[5, 22, 101] To date,

there have been no reports of *HNF1B* intragenic mutation and either ASD or ADHD presented in the literature. This supports our hypothesis that it is not haploinsufficiency of the *HNF1B* gene that is responsible for this aspect of the phenotype in individuals with a 17q12 deletion. It also highlights that further work is needed in this area to determine the cause of the phenotypic variability seen in these patients.

In summary, 17q12 microdeletions but not intragenic mutations are associated with a neurodevelopmental phenotype. All affected families should be informed of this risk and referred for appropriate psychiatric assessment if concerning symptoms arise.

CHAPTER 4

Exocrine pancreatic dysfunction is common in HNF1B-associated renal disease and can be symptomatic

Rhian Clissold[a,b]; Jon Fulford[a,b]; Michelle Hudson[b]; Beverley Shields[a]; Timothy McDonald[a]; Sian Ellard[a]; Andrew Hattersley[a,b]; Coralie Bingham[c]

[a] University of Exeter Medical School, Exeter, UK

[b] National Institute for Health Research Exeter Clinical Research Facility, Royal Devon and Exeter National Health Service Foundation Trust, Exeter, UK

[c] Exeter Kidney Unit, Royal Devon and Exeter National Health Service Foundation Trust, Exeter, UK

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Authors' contributions

Andrew Hattersley, Coralie Bingham and I conceived the idea for the study. I collected the data for the individuals with HNF1B-associated disease; Michelle Hudson coordinated data collection for the healthy controls. Jon Fulford carried out pancreatic magnetic resonance imaging in a subset of the HNF1B cohort. I analysed the data. All co-authors contributed to discussions regarding the results. I wrote the manuscript; all co-authors read and approved the final manuscript.

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INTRODUCTION

Hepatocyte nuclear factor 1β (HNF1B) is a transcription factor with important roles in the development of the kidney, pancreas, liver and genital tract.[117] Heterozygous mutations and deletions of the *HNF1B* gene are the most common known monogenic cause of developmental kidney disease.[1-3] Despite this single genetic aetiology, the phenotype of HNF1B-associated renal disease is very variable (Box 1). Biochemical abnormalities, including hypomagnesemia and hyperuricemia, are also frequently seen.[13, 14] HNF1B-associated disease is a multisystem disorder and extra-renal phenotypic features include young-onset diabetes mellitus, pancreatic hypoplasia, abnormal liver function tests and genital tract malformations.[5, 7, 9-12] Genetic changes comprise either *HNF1B* intragenic mutations (one-half of patients) or an approximate 1.3 Mb deletion at chromosome 17q12, which includes the entire *HNF1B* gene.[20, 27] Both may arise spontaneously, which means there is often no family history of renal disease or diabetes.[18, 19, 26] In view of the clinical heterogeneity of the condition and frequent absence of a family history, diagnosis can be challenging and it is likely that many cases remain undetected.

- Bilateral hyperechogenic kidneys with normal or slightly increased size on antenatal ultrasonography
- Renal cysts (including cystic dysplasia and multicystic dysplastic kidney)
- Single kidney
- Renal hypoplasia
- Horseshoe kidney
- Duplex kidney
- Isolated bilateral hydronephrosis and hydroureter
- Collecting system abnormality (usually in conjunction with other renal structural abnormality)

Box 4.1 The variable phenotype of HNF1B-associated renal disease

Imaging of the pancreas in HNF1B-associated disease with either computed tomography (CT) or magnetic resonance imaging (MRI) has shown less tissue corresponding to the body and tail of the pancreas, with a slightly atrophic head.[9, 10] This is consistent with

agenesis of the dorsal pancreas, the embryonic structure that gives rise to the pancreatic body, tail and a small section of the head. Pancreatic exocrine hypersecretion has been observed in patients with HNF1B-associated disease using secretin-stimulated MRI and rapid endoscopic secretin stimulation tests; this is likely to be a compensatory mechanism for reduced pancreatic volume and provides further evidence that the small pancreas seen on imaging is due to hypoplasia rather than atrophy.[80] Complete pancreatic agenesis has been reported in two foetuses that also had severe renal abnormalities and were subsequently found to have an *HNF1B* gene mutation following induced termination of the pregnancy.[79]

Pancreatic hypoplasia in HNF1B-associated disease has been associated with subclinical pancreatic exocrine insufficiency. This has mainly been studied in small series of patients with *HNF1B* molecular abnormalities and diabetes using indirect tests of pancreatic function, usually faecal elastase-1 measurement in stool.[9, 10, 23] Lower exocrine pancreatic function involving both ductal and acinar cells has been confirmed in direct testing with rapid endoscopic secretin tests and secretin-stimulated MRI in seven individuals with *HNF1B* mutations or deletions.[80] To our knowledge, only one case of symptomatic pancreatic exocrine insufficiency requiring treatment in HNF1B-associated disease has been described in the literature to date.[85] In this study, our aims were to measure faecal elastase-1 in patients with HNF1B-associated disease regardless of diabetes status and assess the degree of symptoms associated with pancreatic exocrine deficiency.

MATERIALS AND METHODS

Recruitment and genetic analysis

Participants with HNF1B-associated disease were recruited from January 31, 2013 to October 10, 2015 from three sites in the United Kingdom (adult renal and diabetes units at the Royal Devon and Exeter Hospital; paediatric renal units at Great Ormond Street

Hospital for Children and Evelina London Children's Hospital), as previously described.[118] Inclusion criteria included the presence of either an *HNF1B* intragenic mutation or whole-gene deletion on genetic testing performed due to underlying renal abnormalities or diabetes and current age ≥4 years. Informed written consent was obtained from all adult participants and parents of child participants, with assent from those aged <16 years. The study was conducted in agreement with the Declaration of Helsinki principles and approved by a regional ethics committee (National Research Ethics Service Committee South West—Frenchay). A total of 29 patients from 20 unrelated families with HNF1B-associated disease participated. Mutation screening was performed by sequencing of coding exons and exon-intron boundaries together with gene dosage assessment by multiplex ligation-dependent probe amplification as previously described.[20, 26]

Faecal elastase-1 was also measured in a cohort of healthy controls in order to define a low faecal elastase-1 concentration based on the 2.5th percentile. Healthy controls were recruited from March 4, 2015 to August 19, 2016 from two sites in the United Kingdom (NIHR Exeter Clinical Research Facility at the Royal Devon and Exeter Hospital; Oxford Centre for Diabetes, Endocrinology and Metabolism at the Oxford University Hospitals NHS Foundation Trust). Inclusion criteria included age 16 to 75 years, ethnicity reflective of local demographic and capacity to consent. Informed written consent was obtained from all participants. The study was conducted in agreement with the Declaration of Helsinki principles and approved by a regional ethics committee (South West - Frenchay Research Ethics Committee). A total of 99 individuals participated. The median age of this cohort was 61.7 years (interquartile range [IQR] 52.8-66.3). 39/99 (39%) were male and all were of White ethnicity. The median faecal elastase-1 concentration was 1580 mcg/g stool (IQR 1000-2000). The 2.5th percentile for faecal elastase-1 in this cohort was a concentration of 410 mcg/g stool (Figure 4.1). There was only a weak association between increasing age and lower faecal elastase-1 concentrations with Spearman's ρ -0.2, P=0.02 (Figure 4.2).

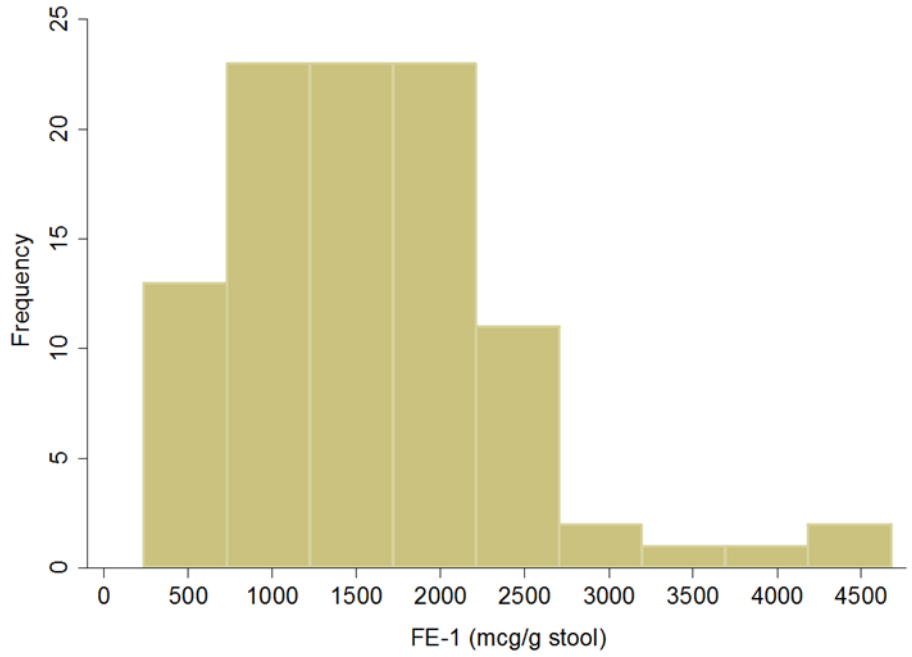

Figure 4.1 Histogram of faecal elastase-1 (FE-1) concentrations in a cohort of healthy controls (*n*=99)

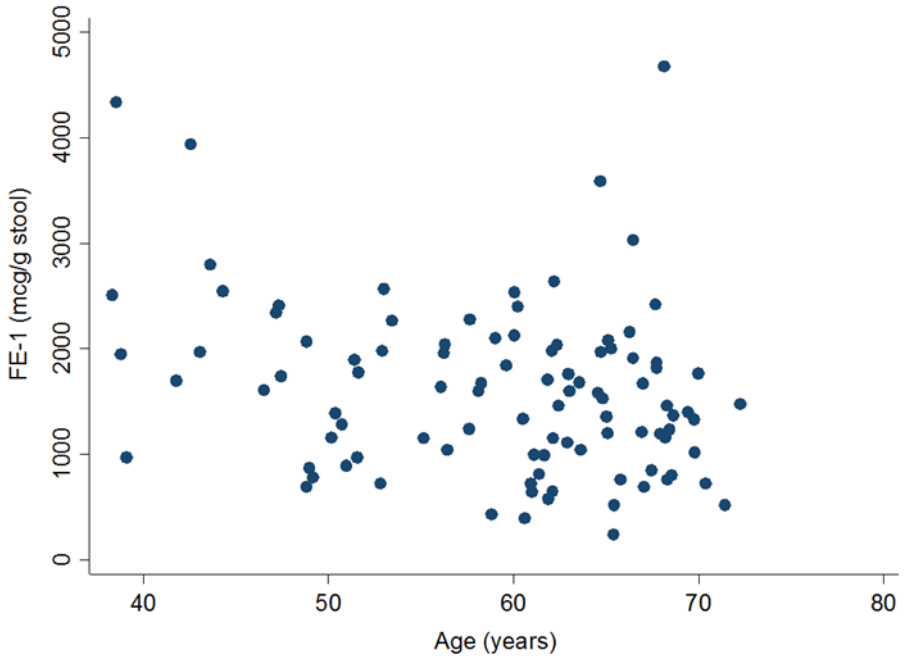

Figure 4.2 Scatter plot of age versus faecal elastase-1 (FE-1) concentration in a cohort of healthy controls (*n*=99)

Clinical evaluation

Relevant medical details, including symptoms related to pancreatic exocrine dysfunction (abdominal pain, loose stools and unintentional weight loss), were documented using a standardised assessment of medical records and participant/parent interview. Diabetes was diagnosed either according to World Health Organization guidelines or on the basis of established treatment with oral hypoglycaemic agents/insulin. In order to measure endogenous insulin production, urinary C-peptide/creatinine ratio (UCPCR) was measured on a post-prandial urine sample taken approximately two hours after a meal stimulus.[119]

Faecal elastase-1 concentration was assessed by enzyme-linked immunosorbent assay on a single spot stool sample at the Royal Cornwall Hospital; healthy control samples were tested using a x10 dilution to obtain an absolute value for faecal elastase-1 as the majority of samples were expected to have a result greater than the 500 mcg/g detection limit of the assay. Faecal elastase-1 <200 mcg/g is considered abnormal, with measurements of 100-200 mcg/g suggestive of moderate to mild pancreatic exocrine insufficiency and measurements <100 mcg/g suggestive of severe insufficiency.[120] Previous imaging results from CT or MRI were reviewed to look for pancreas abnormalities. All patients with HNF1B-associated disease were also invited to undergo pancreatic MRI using a 1.5-T Philips Intera system utilising three-dimensional gradient echo and spin echo sequences, with and without fat suppression, at a range of orientations. Images were subsequently reviewed and reported by a consultant radiologist in order to assess pancreatic structure and ensure there were no incidental findings of clinical concern.

Statistical analysis

Qualitative variables were described with percentages and quantitative variables with median and IQR. Differences between groups were assessed using the Fisher exact test for categorical variables and the Mann-Whitney *U* test for continuous variables. Correlations were tested by Spearman's ρ. A *P*-value of <0.05 was considered to be statistically significant. All analyses were carried out using StataSE (version 14, StataCorp, College Station, TX) and GraphPad statistical software (GraphPad Software, La Jolla, CA).

RESULTS

Participant characteristics

The median age of individuals with HNF1B-associated renal disease was 25 years (IQR 14-44) and 13/29 (45%) were male. The majority of the cohort were White with just 1/29 (3%) being of Mixed ethnicity. 14/29 (48%) had diabetes.

Exocrine pancreatic deficiency is common in HNF1B-associated disease and can be symptomatic

Faecal elastase-1 was low (below the 2.5th percentile of the control cohort) in 18/29 (62%) patients with HNF1B-associated renal disease. 8/29 (28%) had a faecal elastase-1 concentration suggestive of exocrine pancreatic insufficiency at <200 mcg/g stool (Figure 4.3); in 4/29 (14%) the measurement was below 100 mcg/g stool, in keeping with severe deficiency.

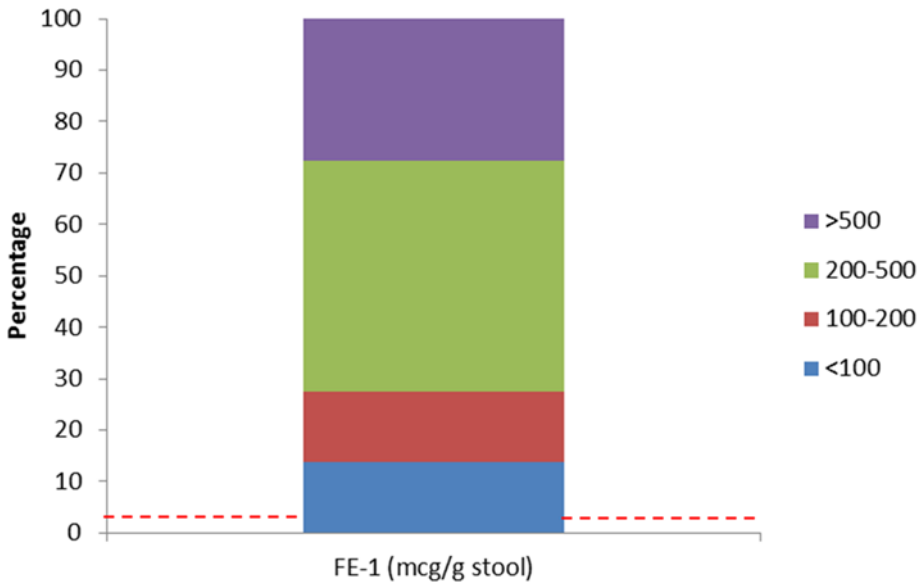

Figure 4.3 Bar chart showing percentage of individuals with HNF1B-associated disease with faecal elastase-1 (FE-1) measurements <100 mcg/g stool (suggestive of severe pancreatic exocrine insufficiency), 100-200 mcg/g stool (moderate to mild insufficiency), 200-500 and >500 mcg/g stool.
The dotted red line indicates that 3/99 (3%) of healthy controls had a FE-1 measurement of 200-500 mcg/g stool, whereas in the remainder it was >500.

3/8 individuals with a faecal elastase-1 measurement <200 mcg/g stool suffered with abdominal pain, loose stools and/or unintentional weight loss. All reported symptomatic improvement and weight gain after commencing pancreatic enzyme replacement therapy. In all three cases it had taken several months for symptoms to be attributed to faecal elastase deficiency and treatment to be commenced (Table 4.1).

Patient	Age at study entry (years)	Sex	Genetic abnormality	Diabetes status	FE-1, mcg/g stool	Pancreatic insufficiency	
						Age at diagnosis (years)	Details
2	36	F	Mutation (c.982_986delCCTCT)	Diagnosed aged 20 years, on insulin	90	39	FE-1 measured by renal team after several months of abdominal pain and diarrhoea; resolution of symptoms and weight gain after treatment commenced.
3	61	F	Deletion (c.1-?_1674+?del)	HbA1c of 56 mmol/mol identified at age 59 years but no treatment commenced	31	63	Known pancreatic atrophy and calcification from previous imaging for abdominal pain under surgical team. FE-1 measured by gastroenterology after referral with several months of abdominal pain, loose stools and weight loss; improvement in symptoms and 6 kg weight gain after pancreatic enzyme replacement therapy commenced.
18	43	F	Deletion (c.1-?_1674+?del)	Diagnosed aged 32 years, on insulin	107	43	Referred to gastroenterology with abdominal pain, diarrhoea (with occasional blood mixed in) and weight loss; colonoscopy performed and found to be normal. FE-1 measured by research team; commenced on pancreatic enzyme replacement therapy with symptomatic improvement.

Abbreviations: FE-1, faecal elastase-1; F, female.

Table 4.1 Details of symptomatic faecal elastase deficiency in three individuals with HNF1B-associated renal disease

Individuals with low faecal elastase-1 levels have radiological evidence of pancreatic hypoplasia

6/29 participants underwent pancreatic imaging with either CT or MRI. 4/6 had abnormalities detected: one was reported to show diffuse pancreatic atrophy with calcification of the head and body plus common bile duct dilatation, whereas the other three demonstrated absence or atrophy of the body and tail of the pancreas only. All four patients had been diagnosed with diabetes and faecal elastase-1 measurements ranged from 31-280 mcg/g stool. 2/6 individuals had scans reported within normal limits. One of these patients was a 20 year old male without evidence of diabetes and a normal faecal elastase-1 result of 432 mcg/g stool. The other patient was a 65 year old female who had been diagnosed with new-onset diabetes after transplantation at the age of 62 years and had a faecal elastase-1 result >500 mcg/g stool.

Low faecal elastase-1 concentrations are seen in HNF1B-associated disease both with and without diabetes

Faecal elastase-1 measurements were compared in individuals with HNF1B-associated disease according to diabetes status. Faecal elastase-1 levels were low in 7/15 (47%) HNF1B patients without diabetes compared to 11/14 (79%) of those with diabetes, P=0.1 (Figure 4.4A). However, only 1/15 (7%) of those without diabetes had a faecal elastase-1 measurement <200 mcg/g stool compared to 7/14 (50%) of those with diabetes, P=0.01 (Figure 4.4B). There was only a weak association between increasing duration of diabetes and lower faecal elastase-1 concentrations with Spearman's ρ -0.3, P=0.4 (Figure 4.5).

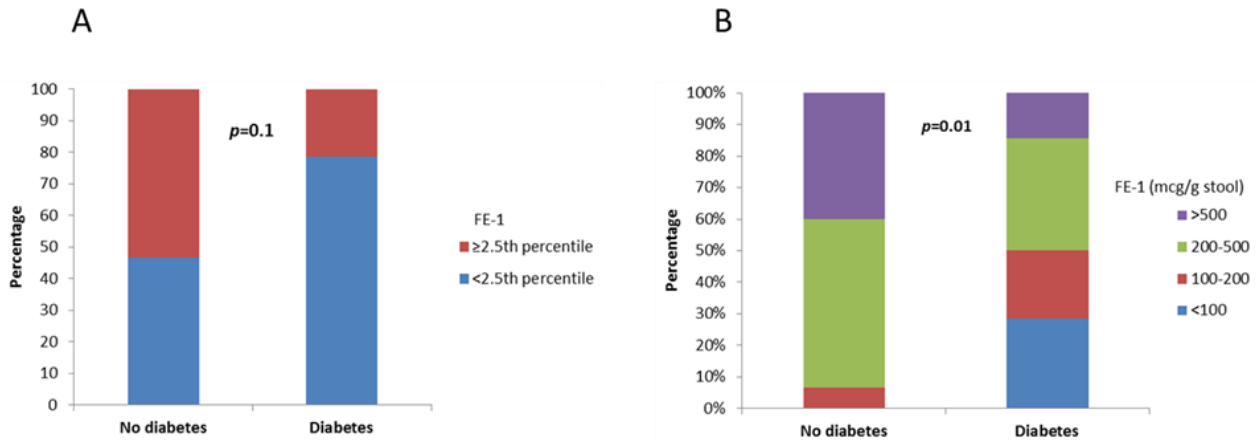

Figure 4.4 Bar charts showing percentage of individuals with HNF1B-associated disease according to diabetes status with faecal elastase-1 (FE-1) measurements (A) either below or above the 2.5th percentile of a healthy control cohort and (B) <100 mcg/g stool (suggestive of severe pancreatic exocrine insufficiency), 100-200 mcg/g stool (moderate to mild insufficiency), 200-500 and >500 mcg/g stool.

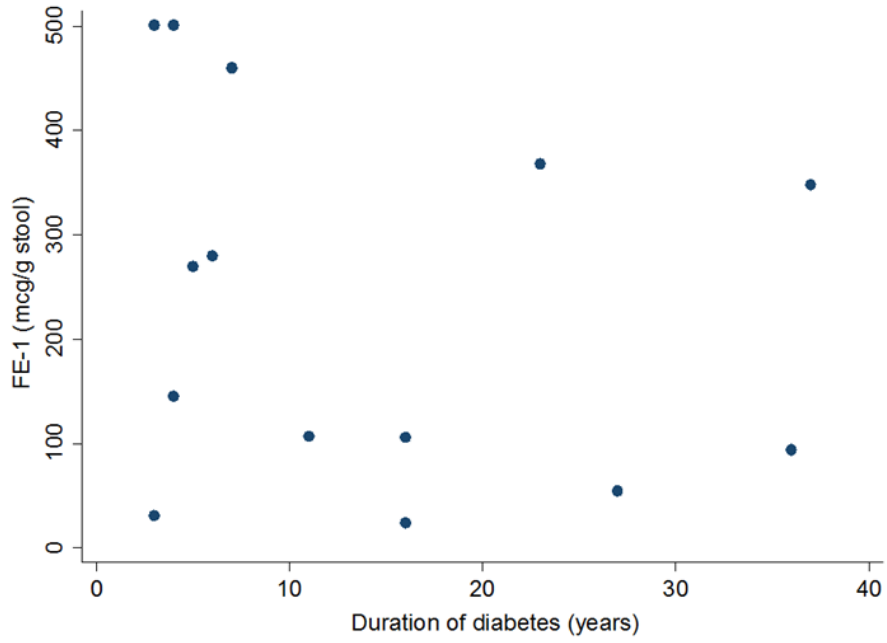

Figure 4.5 Scatter plot of duration of diabetes versus faecal elastase-1 (FE-1) concentration in a cohort of patients with HNF1B-associated disease and diabetes (*n*=14).
A value of 501 mcg/g stool was assigned to the two individuals with a faecal elastase-1 result of >500 mcg/g stool, although the actual value may have been higher than this.

Feature	HNF1B-associated disease, no DM (*n*=15)	HNF1B-associated disease with DM (*n*=14)	*P*
Median age, years (IQR)	14 (9-19)	43.5 (34-55)	***0.0005***
Sex, *n* (%)	M 9 (60), F 6 (40)	M 4 (29), F 10 (71)	*0.1*
Genetic abnormality, *n* (%)	Mut 9 (60), del 6 (40)	Mut 7 (50), del 7 (50)	*0.7*
Median BMI, kg/m^2 (IQR)	20 (18-27)	23 (20-25)	*0.1*
Median creatinine, μmol/L (IQR)	79 (55-115)	116 (90-144)	***0.03***
Median HbA1c, mmol/mol (IQR)	39 (37-42)	60 (55-74)	***0.0005***
Median UCPCR, nmol/mmol (IQR)	2.1 (1.4-5.6)	1.1 (0.6-1.5)	***0.04***
Abbreviations: BMI, body mass index; del, deletion; DM, diabetes mellitus; HNF1B, hepatocyte nuclear factor 1B; IQR, interquartile range; mut, mutation; UCPCR, urinary C-peptide creatinine ratio.			

Table 4.2 Characteristics of individuals with HNF1B-associated disease according to diabetes status

The two groups were different with respect to many clinical characteristics (Table 4.2). The median age of the cohort without diabetes was 14 years (IQR 9-19) compared to 43 years (IQR 33-55) in the group with diabetes, *P*=0.0004. In those patients with more severe pancreatic disease, endogenous insulin secretion assessed by UCPCR was lower than in those without diabetes (median UCPCR 1.1 nmol/mmol [IQR 0.6-1.5] compared to 2.1 [1.4-5.6], respectively; *P*=0.04).

DISCUSSION

We have demonstrated that faecal elastase-1 deficiency is common in HNF1B-associated renal disease and exocrine pancreatic dysfunction may be more symptomatic than previously published. This has important implications for the screening and treatment of these patients. 3/8 individuals in this study with a faecal elastase-1 measurement <200 mcg/g stool had abdominal pain, loose stools and weight loss. The first series of patients described in 2004 reported faecal elastase <200 mcg/g in 6/7 asymptomatic individuals

with diabetes secondary to *HNF1B* gene mutation.[9] The only published report of symptomatic pancreatic insufficiency in HNF1B-associated disease involved the identification of diabetes and a small pancreas on imaging in an individual aged 5 years; pancreatic enzyme replacement therapy became necessary from the age of 16 years and lead to a normalisation of body mass index.[85] There was a significant delay in attributing symptoms to pancreatic insufficiency in the three cases identified in this paper and one of the patients even underwent a colonoscopy before the correct diagnosis was made. All showed symptomatic improvement with weight gain once treatment with pancreatic enzyme replacement therapy was commenced. This highlights how difficult it can be to diagnose pancreatic insufficiency and how prompt treatment benefits patients. Clinicians should have a low threshold for arranging pancreatic function testing for individuals with known HNF1B-associated disease, even when subtle symptoms such as mild abdominal discomfort and bloating are present.

This is the largest series of faecal elastase-1 measurements in individuals with HNF1B-associated renal disease recruited irrespective of diabetes status; previous reports of faecal elastase deficiency in association with an *HNF1B* mutation or deletion have usually been from smaller series of patients with diabetes or prediabetes.[9, 10, 23, 80] Tjora *et al.* included one patient with an *HNF1B* mutation and normal glucose tolerance in their study of exocrine pancreatic function using direct testing; this individual was 38 years old with normal pancreas anatomy on imaging and a faecal elastase-1 measurement of 312 mcg/g stool.[80] An earlier study from the same group recruited an affected 6 year old girl with no pancreatic body and tail identified on imaging and a faecal elastase-1 concentration of 131 mcg/g stool; she had developed impaired glucose tolerance when studied again at the age of 8 years.[10, 80] We included 15 HNF1B patients without diabetes in this study; 7/15 (47%) had a low faecal elastase-1 measurement but only 1/15 (7%) had a measurement <200 mcg/g stool. We would hypothesise that pancreatic insufficiency and diabetes in HNF1B-associated disease are associated as they are secondary to reduced exocrine and endocrine cells as a result of pancreatic hypoplasia. However, caution must be applied when interpreting the results between the HNF1B cohorts with and without diabetes as any differences may reflect the discrepancy in age between the two groups. It would be very

interesting in future work to follow a cohort of paediatric patients with *HNF1B* mutations and deletions over time using serial indirect pancreatic function testing and imaging to see if these non-invasive tests can be used to predict who will develop diabetes and exocrine insufficiency and at what age.

Several limitations were associated with this work. The cohort of healthy controls used to define the lower limit of the normal range for faecal elastase-1 were older; the median age was 61.7 years (IQR 52.8-66.3) compared to 24.5 years (IQR 14-44) in the individuals with HNF1B-associated renal disease. However, faecal elastase concentrations decline with age so defining a cut-off for faecal elastase-1 using the 2.5th percentile of a younger control cohort may have yielded an even higher value than the 410 mcg/g stool used in this study.[121] The median faecal elastase-1 measurement of 1580 mcg/g stool (IQR 1000-2000) in our local control cohort is higher than values reported for healthy controls in other studies.[121-123] This may reflect assay differences between laboratories but, as we have defined low faecal elastase-1 as measurements that fall below the 2.5th percentile of a local healthy control group, this make our results generalisable. Furthermore, we found that our local cut-off of 410 mcg/g stool correlated with the presence or absence of pancreatic hypoplasia on radiological imaging. Finally, our small sample size of *n*=29 individuals with HNF1B-associated disease means we may have been underpowered to make definitive comments on the comparison of patients with and without diabetes. Trends seem to suggest that low faecal elastase-1 levels are more common when diabetes is present and that there may be an association between increasing duration of diabetes and lower faecal elastase-1 concentrations; however, these findings need further exploration.

There is no consensus as to when *HNF1B* genetic testing should be performed. Two tools have been developed in recent years to help select individuals who would benefit from screening: (i) the *HNF1B* score designed by Faguer *et al.* and (ii) adapted criteria for *HNF1B* analysis proposed by Raaijmakers and colleagues.[92, 124] The *HNF1B* score assigns a value of 4 points if pancreatic exocrine insufficiency is present; a cut-off score of 8 is suggested for consideration of *HNF1B* gene analysis. However, faecal elastase was

not systematically assessed in either of these studies. It is cheap and easy to measure, requiring only a single spot stool sample. Given that low faecal elastase-1 concentrations were seen in 18/29 (62%) patients with HNF1B-associated renal disease in this study, it would be interesting to test the role of faecal elastase-1 as a biomarker for HNF1B-associated disease in a large cohort of individuals with congenital anomalies of kidneys and urinary tract. In the interim, we suggest that the finding of a low faecal elastase measurement in individuals with developmental kidney disease of uncertain cause should prompt referral for *HNF1B* genetic testing.

Conclusions

In summary, faecal elastase-1 deficiency is an important feature of HNF1B-associated renal disease even when diabetes is not present. Faecal elastase-1 should be measured in all individuals with an *HNF1B* mutation or deletion complaining of abdominal pain, loose stools or unintentional weight loss. The role of faecal elastase-1 as a biomarker for HNF1B-associated disease requires further investigation.

CHAPTER 5

Serum and urine electrolytes as biomarkers for HNF1B-associated renal disease

INTRODUCTION

Hepatocyte nuclear factor 1β (HNF1B) is a transcription factor with important roles in the development of the kidney, pancreas, liver and genital tract.[117] Heterozygous mutations and deletions of the *HNF1B* gene are the most common known monogenic cause of developmental kidney disease.[1-3] Despite this single genetic aetiology, the phenotype of HNF1B-associated renal disease is very variable (Box 5.1). Electrolyte abnormalities, including hypomagnesemia, are also frequently seen.[13] HNF1B-associated disease is a multisystem disorder and extra-renal phenotypic features include young-onset diabetes mellitus, pancreatic hypoplasia, abnormal liver function tests and genital tract malformations.[5, 7, 9-12] Genetic changes comprise either *HNF1B* intragenic mutations (one-half of patients) or an approximate 1.3 Mb deletion at chromosome 17q12, which includes the entire *HNF1B* gene.[20, 27] Both may arise spontaneously, which means there is often no family history of renal disease or diabetes.[18, 19, 26] In view of the clinical heterogeneity of the condition and frequent absence of a family history, diagnosis can be challenging and it is likely that many cases remain undetected.

- Bilateral hyperechogenic kidneys with normal or slightly increased size on antenatal ultrasonography
- Renal cysts (including cystic dysplasia and multicystic dysplastic kidney)
- Single kidney
- Renal hypoplasia
- Horseshoe kidney
- Duplex kidney
- Isolated bilateral hydronephrosis and hydroureter
- Collecting system abnormality (usually in conjunction with other renal structural abnormality)

Box 5.1 The variable phenotype of HNF1B-associated renal disease

Hypomagnesaemia is a common feature of HNF1B-associated disease. Since the association was first described in 2009, several studies have systematically assessed serum magnesium levels in individuals with *HNF1B* mutations and deletions: the

prevalence of hypomagnesaemia ranges from 8-44% in children and 50-63% in adults.[13, 22, 124, 125] This is accompanied by renal magnesium wasting; hypocalciuria is also frequently reported. HNF1B, along with its dimerisation cofactor pterin-4 alpha-carbinolamine dehydratase 1 (PCBD1), regulates the transcription of *FXYD2*.[13, 126] This gene encodes the *γ* subunit of the Na^+/K^+–ATPase, which sets up a voltage gradient to allow magnesium reabsorption in the distal convoluted tubule via transient receptor potential melastatin type 6 (TRPM6) channels.[127] Homozygous mutations in *PCBD1* also result in hypomagnesaemia and hypermagnesuria; heterozygous mutations in *FXYD2* cause an autosomal dominant renal hypomagnesaemia with hypocalciuria.[66, 128]

There is no consensus as to when *HNF1B* genetic testing should be carried out. Two tools have been developed in recent years to help select patients who would benefit from screening. The *HNF1B* score, designed by Faguer and colleagues, assigns a value of 2 points if a low serum magnesium (<0.7 mmol/L) is found; a cut-off score of 8 is suggested for consideration of *HNF1B* gene analysis.[92] In adapted criteria for *HNF1B* analysis proposed by Raaijmakers *et al.*, associated hypomagnesaemia in individuals with congenital anomalies of kidneys and urinary tract (CAKUT) increased the likelihood of finding a mutation by a factor of 4.2.[124] Magnesium is cheap and easy to measure so would make an attractive biomarker for HNF1B-associated disease; however, serum magnesium levels are within the normal reference range in approximately 50% of cases. Hypermagnesuria seems to be a consistent finding, although renal magnesium excretion is usually only reported in the context of hypomagnesaemia.[13, 22] It is unknown if renal magnesium wasting is also seen in patients with *HNF1B* mutations and deletions even when serum magnesium measurements fall within the normal reference range. In this pilot study, our aims were to measure both serum and urine magnesium and calcium levels in individuals with an *HNF1B* gene mutation/deletion and compare to a cohort of patients followed up in a general nephrology clinic in order to assess their potential as biomarkers for HNF1B-associated disease.

METHODS

Recruitment and genetic analysis

Participants with HNF1B-associated disease were recruited from January 31, 2013 to October 10, 2015 from three sites in the United Kingdom (adult renal and diabetes units at the Royal Devon and Exeter Hospital; paediatric renal units at Great Ormond Street Hospital for Children and Evelina London Children's Hospital), as previously described.[118] Inclusion criteria included the presence of either an *HNF1B* intragenic mutation or whole-gene deletion on genetic testing performed due to underlying renal abnormalities or diabetes, current age ≥4 years and estimated glomerular filtration rate (eGFR) ≥30 mL/min/1.73m^2. All eligible patients were invited to participate. Informed written consent was obtained from all adult participants and parents of child participants, with assent from those aged <16 years. The study was conducted in agreement with the Declaration of Helsinki principles and approved by a regional ethics committee (National Research Ethics Service Committee South West—Frenchay). A total of 21 patients from 15 unrelated families with HNF1B-associated disease agreed to participate. Mutation screening was performed by sequencing of coding exons and exon-intron boundaries together with gene dosage assessment by multiplex ligation-dependent probe amplification (MLPA) as previously described.[20, 26]

Control data from 24 individuals matched for age, sex and renal function was obtained from May 7th, 2014 to January 21st, 2016 from general nephrology clinics at the Royal Devon and Exeter Hospital. Inclusion criteria included current age ≥4 years and eGFR ≥30 mL/min/1.73m^2.

Clinical evaluation

Relevant medical details were documented using a standardised assessment of medical records and participant/parent interview. Imaging results from ultrasonography, computed tomography or magnetic resonance imaging were reviewed to look for kidney abnormalities. GFR was estimated using the (i) Schwartz-Haycock formula in children, optimized for children with renal malformations assessed in each individual paediatric

renal unit where possible, and (ii) simplified Modification of Diet in Renal Disease formula in adults.[105, 106] Magnesium, calcium and creatinine were measured in serum and spot urine samples. Hypomagnesaemia was defined as serum magnesium <0.7 mmol/L. Renal magnesium and calcium fractional excretions were assessed as follows: (urine [electrolyte] x serum [creatinine])/(serum [electrolyte] x urine [creatinine]) x 100. Hypermagnesuria was defined as a fractional excretion of magnesium (FEMg) >4% and hypocalciuria as a fractional excretion of calcium (FECa) <1%. Diabetes was diagnosed either according to World Health Organization guidelines or on the basis of established treatment with oral hypoglycaemic agents/insulin.

Statistical analysis

Qualitative variables were described with percentages and quantitative variables with median and interquartile range (IQR). Differences between groups were assessed using the Fisher exact test for categorical variables and the Mann-Whitney *U* test for continuous variables. Discrimination between patients with and without HNF1B-associated renal disease was assessed by determining the area under the curve (AUC) of the receiver-operating characteristic (ROC) curve. Correlations were tested by Spearman's ρ. A *P*-value of <0.05 was considered to be statistically significant. All analyses were carried out using StataSE (version 14, StataCorp, College Station, TX) and GraphPad statistical software (GraphPad Software, La Jolla, CA).

RESULTS

Age, sex and renal function are similar between HNF1B and clinic cohorts

Age and sex were similar in both individuals with HNF1B-associated disease and those under follow up in a general nephrology clinic (Table 5.1). Renal function tended to be better in the clinic patients with a median eGFR of 84 ml/min/1.73m^2 (IQR 54-95), compared to 53.5 (IQR 42.5-83) in the HNF1B cohort, but this did not reach statistical significance (*P*=0.2). The majority of patients (90%) with an *HNF1B* mutation/deletion had a renal diagnosis of either cysts or cystic dysplasia whereas there were a range of

diagnoses in the clinic cohort. Diabetes was present in 10/21 (48%) individuals with HNF1B-associated disease but only 2/24 (8%) clinic patients, *P*=0.006.

Feature	Patients from general nephrology clinic (*n*=24)	Patients with HNF1B-associated disease (*n*=21)	*P*
Median age, years (IQR)	23.5 (21.5-50.5)	25 (15-38)	0.2
Sex, *n* (%)	M 13 (54), F 11 (46)	M 9 (43), F 12 (57)	0.6
HNF1B genetic abnormality, *n* (%)	-	Mutation 12 (57), deletion 9 (43)	-
Renal diagnosis, *n* (%)	ADPKD 4 (17), other cystic renal disease 2 (8), PUV 2 (8), membranous nephropathy 2 (8), other* 14 (58)	Renal cysts 16 (76), cystic dysplasia 3 (14), single kidney 1 (5), collecting system abnormality 1 (5)	-
Diabetes, *n* (%)	2 (8)	10 (48)	**0.006**
Median creatinine, μmol/L (IQR)	78 (63.5-125.5)	112 (73-136)	0.4
Median GFR, mL/min/1.73m^2 (IQR)	84 (54-95)	53.5 (42.5-83)	0.2

Abbreviations: ADPKD, autosomal dominant polycystic kidney disease; F, female; GFR, glomerular filtration rate; HNF1B, hepatocyte nuclear factor 1B; IQR, interquartile range; M, male; PUV, posterior urethral valves.
*Other renal diagnoses included atypical haemolytic uraemic syndrome, cortical necrosis, focal segmental glomerulosclerosis, hyperoxaluria, mesangiocapillary glomerulonephritis, microscopic haematuria, nephrotic syndrome, neuropathic bladder, tacrolimus nephrotoxicity, tubulointerstitial nephritis, partial Wilms tumour-aniridia syndrome and chronic kidney disease of uncertain cause.

Table 5.1 Characteristics of study participants

One third of patients with HNF1B-associated disease have serum magnesium levels within the normal reference range

The median magnesium concentration was 0.66 mmol/L (IQR 0.56-0.7) in the HNF1B cohort compared to 0.87 (IQR 0.82-0.91) in the clinic cohort, *P*<0.0001 (Figure 5.1A). 14/21 (67%) patients with *HNF1B* mutations/deletions had hypomagnesaemia compared to 2/24 (8%) clinic patients, *P*<0.0001 (Figure 5.1B). The ROC curve for serum magnesium, with HNF1B-associated renal disease status as the dependent variable, is shown in figure 5.1C; AUC=0.95 (95% confidence interval [CI] 0.90-1). A cut-off for serum magnesium of 0.75 mmol/L was 100% sensitive and 87.5% specific for patients with HNF1B-associated disease.

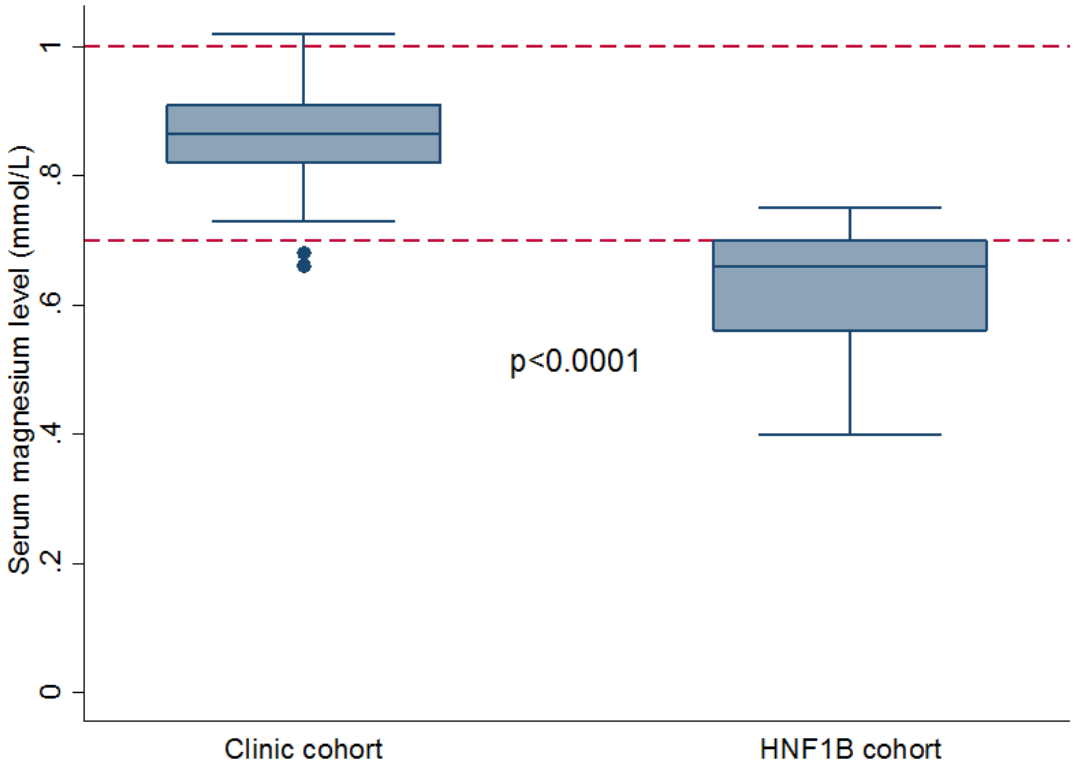

Figure 5.1A Box plots showing serum magnesium levels in both patients under follow up in a general nephrology clinic and individuals with HNF1B-associated renal disease.
The area between the dashed red lines represents the normal reference range for serum magnesium (0.7-1 mmol/L).

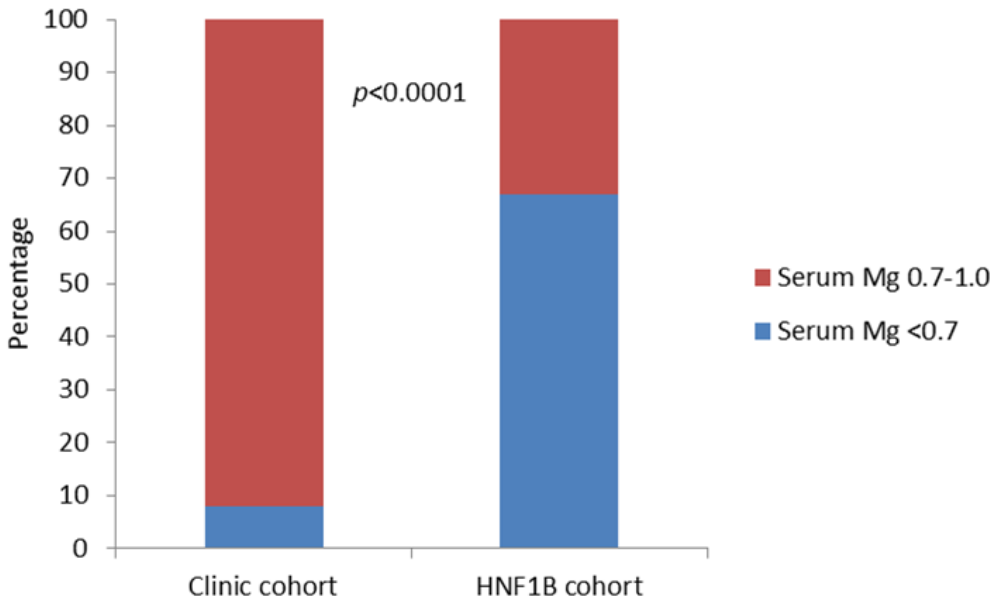

Figure 5.1B Stacked bar charts showing the percentage of patients with hypomagnesaemia in both clinic and HNF1B cohorts.
Abbreviations: HNF1B, hepatocyte nuclear factor 1B; Mg, magnesium.

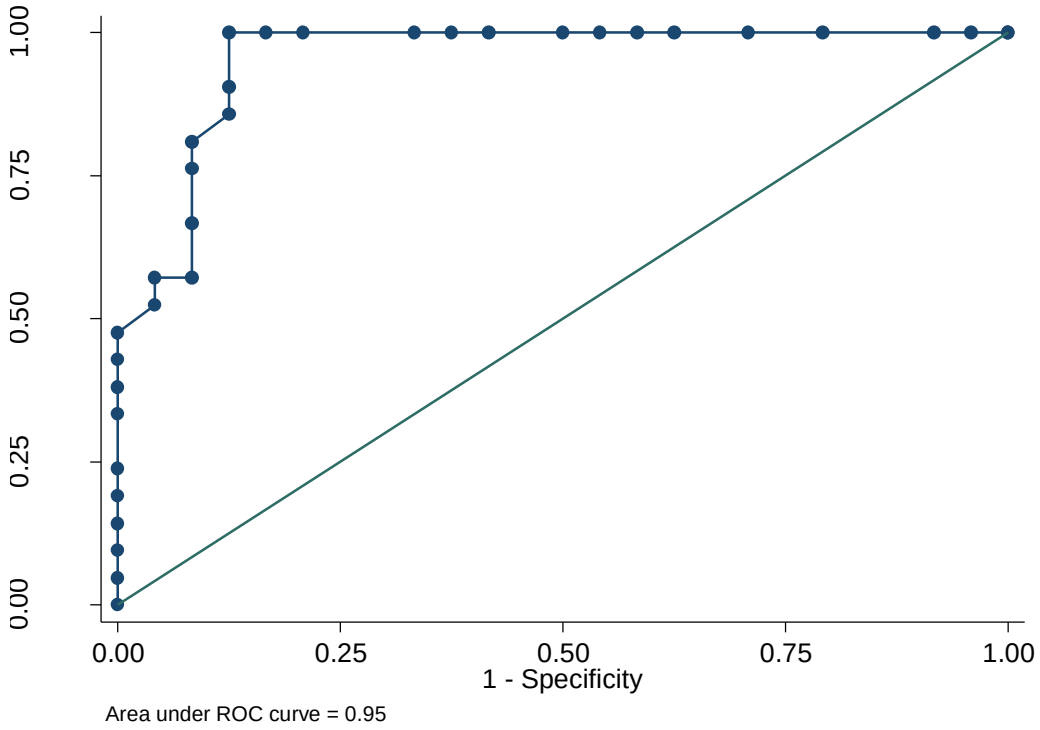

Figure 5.1C ROC curve for serum magnesium, with HNF1B-associated renal disease status as the dependent variable. Abbreviations: ROC, receiver-operating characteristic.

All individuals with HNF1B-associated disease have FEMg >4%

The median FEMg was 9.1% (IQR 6.5-13.5) in the HNF1B cohort compared to 2.6% (IQR 2.1-4.8) in the clinic cohort, *P*<0.0001 (Figure 5.2A). All patients with *HNF1B* mutations/deletions (*n*=21) had hypermagnesuria with FEMg >4% compared to only 8/24 (33%) clinic patients, *P*<0.0001 (Figure 5.2B). The ROC curve for FEMg, with HNF1B-associated renal disease status as the dependent variable, is shown in figure 5.2C; AUC=0.92 (95% CI 0.84-1). A cut-off for FEMg of 4.1% was 100% sensitive and 71% specific for patients with HNF1B-associated disease.

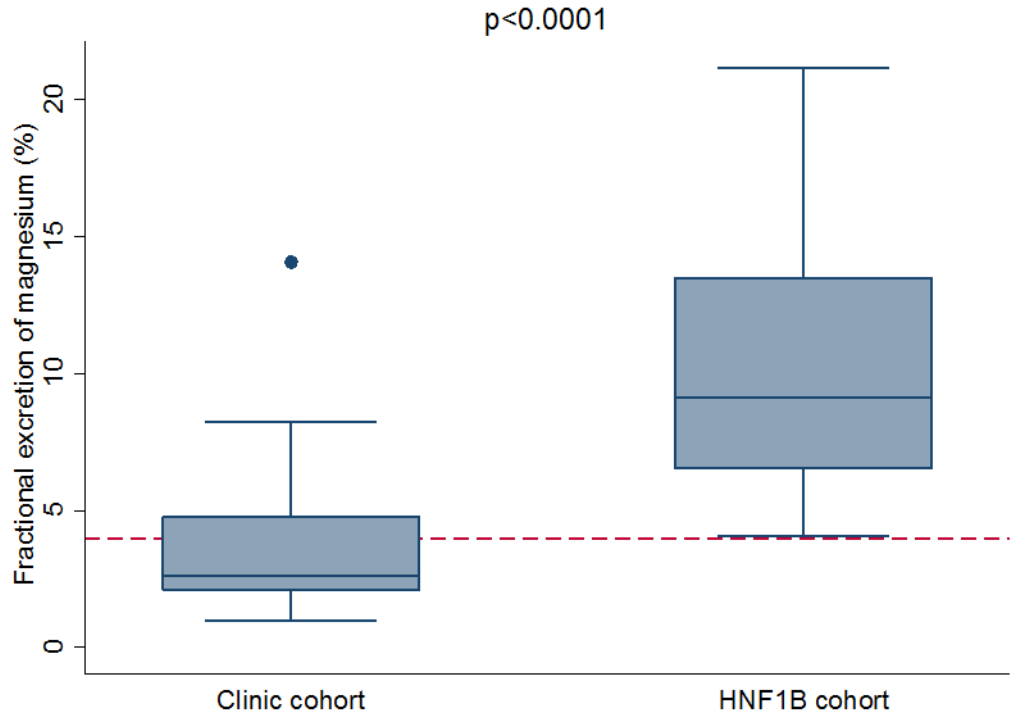

Figure 5.2A Box plots showing FEMg in both patients under follow up in a general nephrology clinic and individuals with HNF1B-associated renal disease.
The area above the red dashed line represents hypermagnesuria.

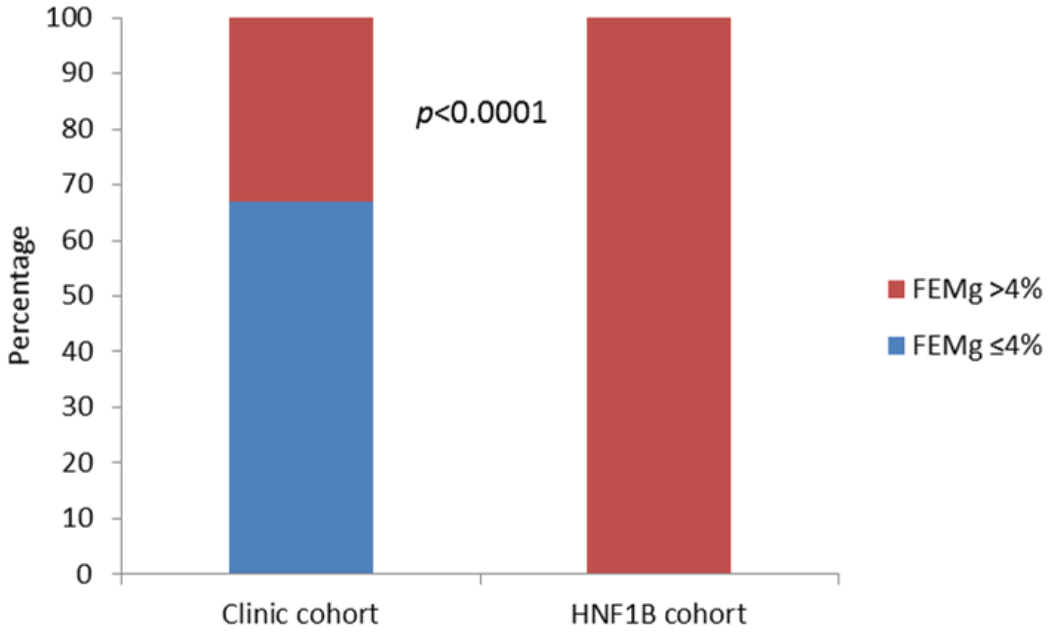

Figure 5.2B Stacked bar charts showing the percentage of patients with hypermagnesuria in both clinic and HNF1B cohorts.
Abbreviations: FEMg, fractional excretion of magnesium; HNF1B, hepatocyte nuclear factor 1B.

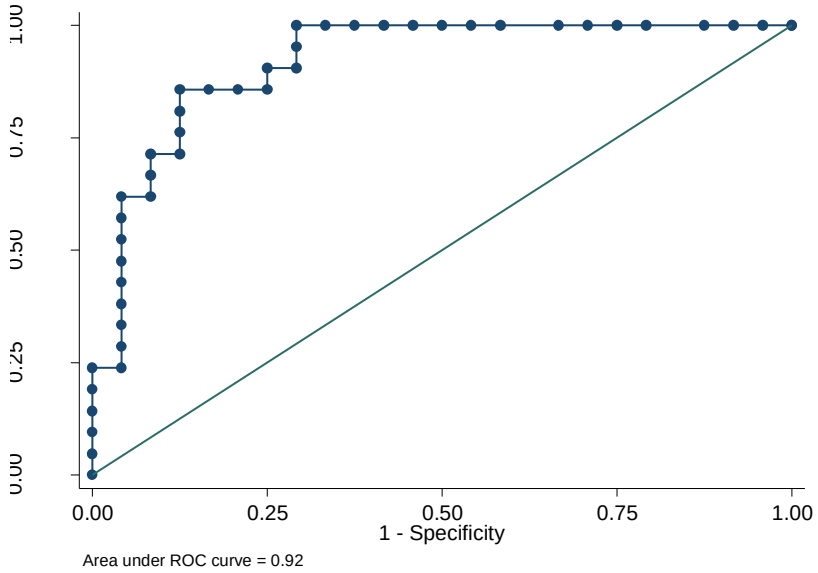

Figure 5.2C ROC curve for FEMg, with HNF1B-associated renal disease status as the dependent variable. Abbreviations: ROC, receiver-operating characteristic.

Serum calcium levels are similar between HNF1B and clinic cohorts

The median calcium concentration was 2.33 mmol/L (IQR 2.24-2.38) in the HNF1B cohort compared to 2.31 (IQR 2.27-2.36) in the clinic cohort, *P*=0.8 (Figure 5.3A). The ROC curve for serum calcium, with HNF1B-associated renal disease status as the dependent variable, is shown in figure 5.3B; AUC=0.48 (95% CI 0.30-0.66).

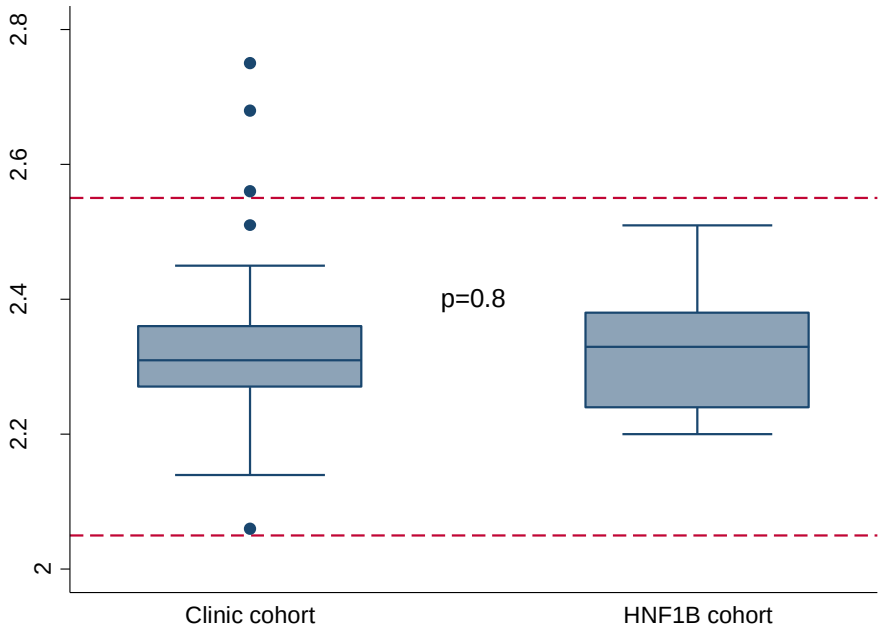

Figure 5.3A Box plots showing serum calcium levels in both patients under follow up in a general nephrology clinic and individuals with HNF1B-associated renal disease.
The area between the dashed red lines represents the normal adult reference range for serum calcium (2.05-2.55 mmol/L); the paediatric reference range for serum calcium is 2.2-2.7 mmol/L.

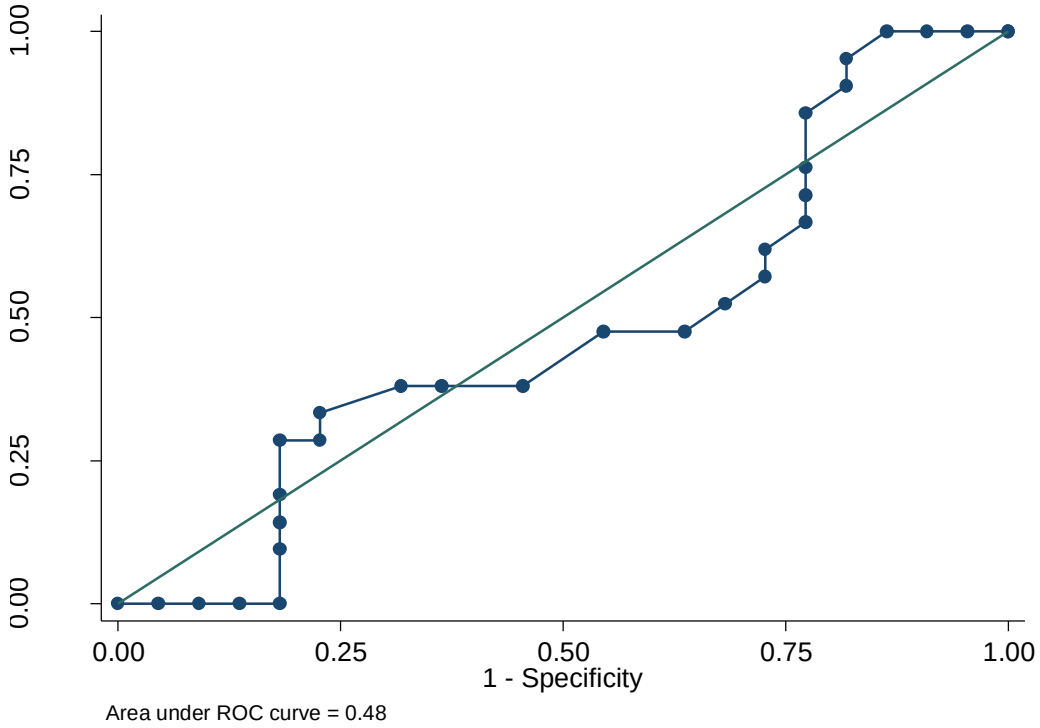

Figure 5.3B ROC curve for serum calcium, with HNF1B-associated renal disease status as the dependent variable.
Abbreviations: ROC, receiver-operating characteristic.

Three quarters of patients with HNF1B-associated disease also have hypocalciuria

Median FECa was 0.5% (IQR 0.3-1.0) in individuals with an *HNF1B* mutation/deletion compared to 1.4% (IQR 0.3-1.8) in clinic patients, although this was not statistically significant with *P*=0.2 (Figure 5.4A). However, 15/20 (75%) of those with HNF1B-associated disease had hypocalciuria with FECa <1% compared to only 8/22 (36%) clinic patients, *P*=0.02 (Figure 5.4B). The ROC curve for FECa, with HNF1B-associated renal disease status as the dependent variable, is shown in figure 5.4C; AUC=0.63 (95% CI 0.44-0.81). A cut-off for FECa of 1.29% was 85% sensitive and 55% specific for patients with HNF1B-associated disease.

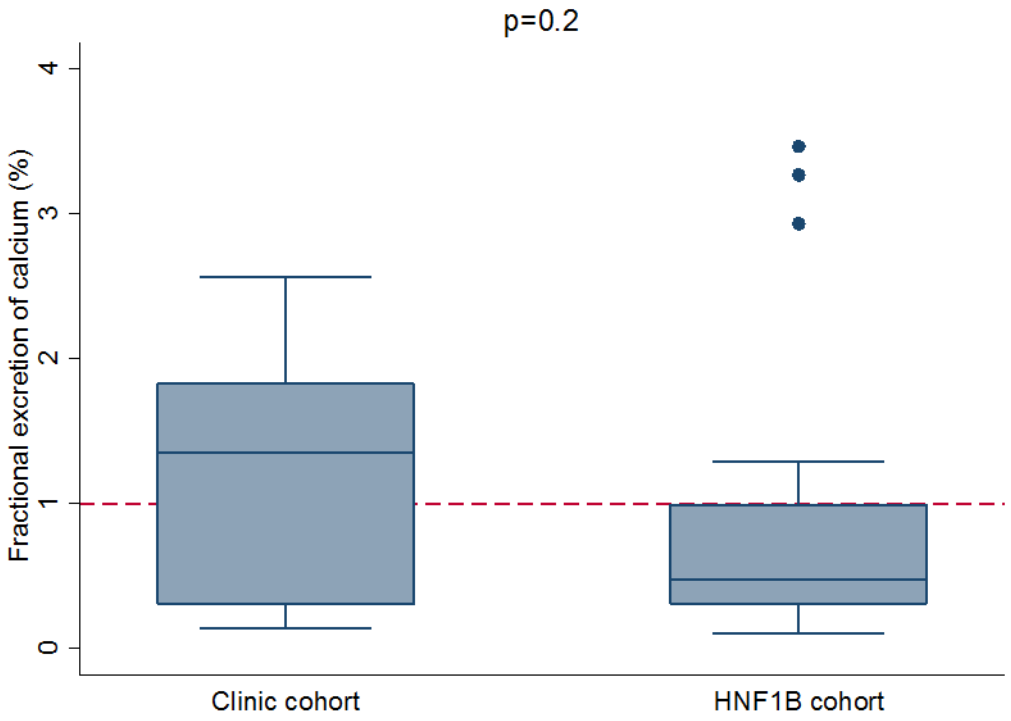

Figure 5.4A Box plots showing FECa in both patients under follow up in a general nephrology clinic and individuals with HNF1B-associated renal disease.
The area below the red dashed line represents hypocalciuria.

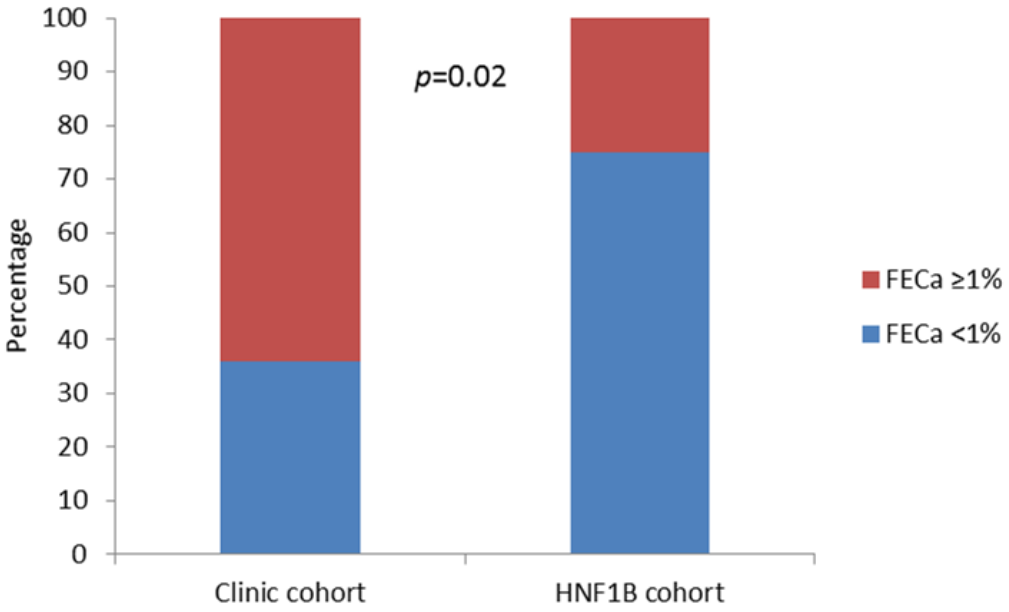

Figure 5.4B Stacked bar charts showing the percentage of patients with hypocalciuria in both clinic and HNF1B cohorts. Abbreviations: FECa, fractional excretion of calcium; HNF1B, hepatocyte nuclear factor 1B.

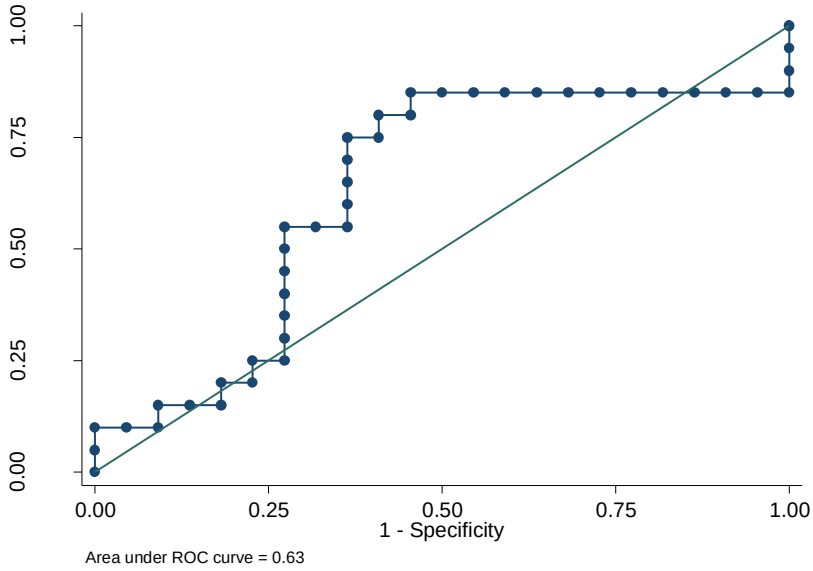

Figure 5.4C ROC curve for FECa, with HNF1B-associated renal disease status as the dependent variable. Abbreviations: ROC, receiver-operating characteristic.

In the patients with HNF1B-associated disease, comparison of those with a mutation versus those with a deletion showed results similar to those presented in Chapter 3: renal function was worse in those with a mutation (median eGFR 44.5 ml/min/1.73m^2 [IQR 39.5-

62] vs. 83 [IQR 53.5-95] in those with a deletion, *P*=0.03) and serum magnesium was higher (median magnesium level 0.70 mmol/L [IQR 0.67-0.74] vs. 0.56 [IQR 0.53-0.6 in the deletion group, *P*=0.01]. There were no significant differences in FEMg, serum calcium and FECa between mutation and deletion groups.

Hypomagnesaemia and hypermagnesuria are more marked in patients with HNF1B-associated disease when diabetes is also present

Median serum magnesium concentrations were lower and FEMg higher in individuals with an *HNF1B* mutation/deletion when diabetes was also present (Table 5.2). However, this group were older and renal function tended to be worse (median eGFR 43 ml/min/1.73 m^2 [IQR 39-71] compared to 62 [IQR 50-95] in those without diabetes; *P*=0.07). Simple correlation analysis showed moderate associations between (i) age and both serum magnesium and FEMg in patients with HNF1B-associated disease and (ii) eGFR and FEMg in all patients (Figure 5.5).

	No diabetes (*n*=11)	**Diabetes (*n*=10)**	***p***
Median serum Mg, mmol/L (IQR)	0.69 (0.64-0.74)	0.56 (0.52-0.69)	**0.03**
Median FEMg, % (IQR)	6.5 (4.9-10.5)	13.9 (9.1-15.5)	**0.003**
Median FECa, % (IQR)	0.4 (0.2-0.8)	0.6 (0.4-2.9)	0.1
Median age, years (IQR)	15 (9-18)	40 (34-45)	**0.0004**
Median eGFR, mL/min/1.73 m^2 (IQR)	62 (50-95)	43 (39-71)	0.07

Abbreviations: eGFR, estimated glomerular filtration rate; FECa, fractional excretion of calcium; FEMg, fractional excretion of magnesium; IQR, interquartile range; Mg, magnesium.

Table 5.2 Comparison of individuals with HNF1B-associated disease depending on diabetes status

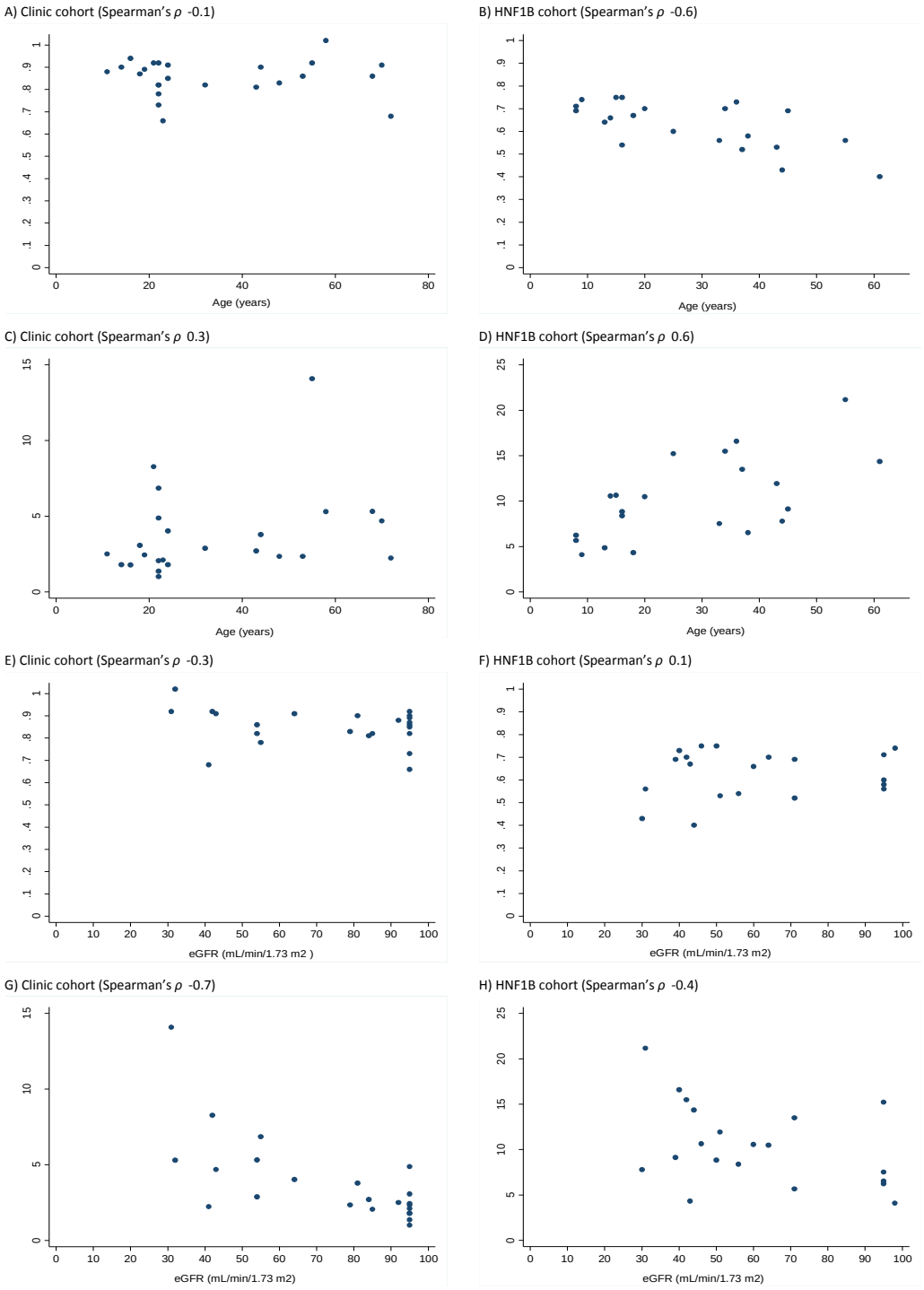

Figure 5.5 Scatter plots for A&B) age and serum magnesium level, C&D) age and fractional excretion of magnesium, E&F) eGFR and serum magnesium level and G&H) eGFR and fractional excretion of magnesium.
Spearman's ρ was classified as follows: <4, weak association; 0.4-0.7, moderate association; ≥0.7, strong association.
Abbreviations: eGFR, estimated glomerular filtration rate.

DISCUSSION

The results of this pilot study show that using a cut-off for serum magnesium of ≤0.75 mmol/L was 100% sensitive and 87.5% specific for the presence of an *HNF1B* mutation/deletion, even though one third of patients with HNF1B-associated disease had a serum magnesium level within the normal reference range. All individuals in the HNF1B cohort had hypermagnesuria with FEMg >4% and a cut-off of ≥4.1% was 100% sensitive and 71% specific.

The high sensitivity we found when using serum magnesium and/or FEMg as diagnostic criteria for HNF1B-associated renal disease in this cohort makes them useful rule-out tests. *HNF1B* genetic testing is expensive; the current National Health Service cost for *HNF1B* sequencing and dosage analysis by MLPA in the United Kingdom is £350. Our results suggest serum magnesium and FEMg could be highly sensitive biomarkers for HNF1B-associated renal disease, which are both cheap (approximately £0.52) and easy to measure. If these results are confirmed in a larger study of individuals with CAKUT, they should be implemented as screening tests in routine clinical care. In the interim we suggest that serum magnesium measurements ≤0.75 mmol/L and FEMg ≥4.1% should lead to referral for *HNF1B* genetic testing in individuals with developmental kidney disease of uncertain cause, even if serum magnesium levels are within the normal reference range.

This is the largest series of patients with HNF1B-associated disease where renal magnesium wasting has been reported and confirms that hypermagnesuria seems to be a universal finding. Adalat *et al.* described eight children in their HNF1B cohort with hypomagnesaemia and hypermagnesuria; FEMg ranged from 4.5-14.3% with a median value of 6.5%.[13] FEMg ranged from 4-22% in seven adults with an *HNF1B* mutation/deletion in a study by Faguer and colleagues; 2/7 had a serum magnesium level >0.75 mmol/L.[22] Hypomagnesemia was also common in the HNF1B cohort of our series;

14/21 (67%) individuals had a serum magnesium concentration <0.7 mmol/L, which confirms the findings of previous work.[13, 22, 124]

Several limitations were associated with this study. Firstly, our control cohort included patients followed up in a general nephrology clinic where the prevalence of HNF1B-associated disease is expected to be very low. It will be important to assess serum magnesium, FEMg and FECa as diagnostic criteria for HNF1B disease in a cohort of individuals with CAKUT. Secondly, only 6/21 (29%) HNF1B patients in this study were children and the youngest participant was 8 years old; it is unknown if these results are translatable to a younger cohort. This will be important to ascertain as *HNF1B* genetic testing is often indicated in early life if developmental kidney disease is identified during prenatal ultrasonography. Unpublished observations presented by Shazia Adalat and colleagues at the British Renal Society and Renal Association conference in 2014 suggest that biochemical abnormalities during early childhood are an unreliable indicator for *HNF1B* testing. Simple correlation analysis in our pilot study showed that serum magnesium levels tended to decrease with age in the HNF1B cohort, whilst FEMg increased with age. Finally, in order to make the cohorts in this study as representative of routine clinical practice as possible we analysed data from all individuals even if they were prescribed medications that could alter serum or urine magnesium measurements or where blood and urine samples were not taken on the same day. The latter mainly applied to paediatric patients where samples were not as easy to obtain; Appendix C shows the stability of serum magnesium levels over time to support the use of these samples. We repeated the analyses after excluding these individuals to confirm that the main results remain very similar (Appendix C).

Hypocalciuria was also a frequent finding in those with an *HNF1B* mutation/deletion; 15/20 (75%) had a FECa <1%, although the median FECa of 0.5% (IQR 0.3-1.0) was not significantly lower than the clinic cohort at 1.4% (IQR 0.3-1.8). There is limited data on renal calcium excretion in HNF1B-associated disease. Faguer and colleagues reported FECa in eight adults with HNF1B nephropathy: results ranged from 0.2-4% and 4/8 (50%)

had hypocalciuria.[22] Unpublished observations presented by Adalat *et al.* at the British Renal Society and Renal Association conference in 2014 showed that hypocalciuria was present in 14 children with *HNF1B* mutation where data was available on urinary calcium excretion and FECa was calculated to be <0.5% in all of these patients. Thus it seems that hypocalciuria becomes less common in older individuals with HNF1B-associated disease. Previous work has demonstrated an increase in daily urine calcium output in the first two decades of life in 336 healthy subjects aged 3-89 years, which may explain this finding.[129]

Median serum magnesium levels were lower and FEMg higher in individuals with an *HNF1B* mutation/deletion who also had diabetes. Hypomagnesaemia is often seen in patients with type 2 diabetes.[130] Osmotic diuresis and inappropriate magnesuria seems to be the most important underlying mechanism but the causes are likely to be multifactorial and include poor dietary intake, altered insulin metabolism, glomerular hyperfiltration, diuretic administration and recurrent metabolic acidosis.[131] We also found that increasing age was moderately associated with lower serum magnesium measurements and higher FEMg in the HNF1B cohort; declining eGFR was moderately associated with higher FEMg in both clinic and HNF1B cohorts. Therefore, the finding of worse magnesium biochemistry in patients with both HNF1B-associated disease and diabetes may be related instead to the older age and tendency to a lower eGFR seen in this group rather the presence of diabetes.

CONCLUSIONS

In summary, this pilot study suggests serum magnesium and FEMg are highly sensitive biomarkers for HNF1B-associated renal disease; hypermagnesuria was seen in all patients with an *HNF1B* mutation/deletion even if serum magnesium measurements were within the normal reference range. If these results are confirmed in a larger study of

patients with CAKUT, they could be implemented as cheap screening tests for *HNF1B* genetic testing in routine clinical care.

CHAPTER 6

Discussion

This thesis addresses several of the issues surrounding the recognition of HNF1B-associated disease, including assessing the clinical utility of an existing HNF1B scoring system and examining the neurodevelopmental phenotype of patients with either an *HNF1B* intragenic mutation or 17q12 deletion. Measurements of serum and urine magnesium and calcium levels, plus faecal elastase-1, are proposed as potential biomarkers; two pilot studies assessing these in HNF1B-associated disease are presented.

This chapter provides an overview of the main findings of the thesis, the impact of the results, limitations of the work and potential areas for future research.

CHAPTER 2: ASSESSMENT OF THE HNF1B SCORE AS A TOOL TO SELECT PATIENTS FOR *HNF1B* GENETIC TESTING

Summary and impact of findings

This chapter tests the clinical utility of the HNF1B score, developed by Faguer and colleagues in 2014 to help select appropriate patients for genetic testing, in a large number of referrals for *HNF1B* gene analysis to the UK diagnostic testing service for the *HNF1B* gene. An HNF1B score was assigned for 686 UK referrals for *HNF1B* genetic testing using clinical information available at the time of referral. Performance of the score was evaluated by receiver-operating characteristic (ROC) curve analysis. The relative discriminatory ability of different clinical features for making a genetic diagnosis of HNF1B-associated disease were estimated in the UK dataset alone and pooled with French data.[92]

The HNF1B score discriminated between patients with and without a mutation or deletion reasonably well with an area under the curve (AUC) of 0.72 (95% confidence interval [CI] 0.67-0.76). Applying the suggested cut-off score of ≥8 gave a negative predictive value of 85%. Although this suggests that this clinical scoring system may be a useful screening tool to select individuals for *HNF1B* gene analysis, it cannot be reliably used to exclude individuals with a lower score from genetic testing.

In a pooled analysis using data from UK and French cohorts, antenatal renal abnormalities, renal hyperechogenicity and cysts were discriminatory in children, whereas renal hypoplasia and cysts were discriminatory in adults. Pancreatic abnormalities were discriminatory in both whereas other extra-renal characteristics, including genital tract malformations, liver test abnormalities and hypomagnesaemia, only had a large effect size in adults. In terms of clinical practice, determining the presence of renal structural and pancreatic abnormalities alone is likely to be sufficient in order to decide whether a child should be referred for *HNF1B* genetic testing, whereas in adults the assessment of extra-renal features is also useful.

Limitations

One of the main limitations of this work was that the HNF1B score was calculated retrospectively using clinical details available at the time of referral; not all clinical features were systematically assessed. We also included data from all referrals for *HNF1B* genetic testing to our centre from 1998 to 2012 but some of the clinical features, such as hypomagnesaemia, have only been recognised as being part of the phenotype later on in this time period. In both UK and French datasets, patients underwent genetic testing based on referrer suspicion of HNF1B-associated disease and the majority had a congenital anomaly of the kidneys or urinary tract (CAKUT). This results in a selection bias that limits the applicability of the study findings but is in keeping with the literature to date, where the majority of cohorts with HNF1B-associated disease that have been described were pre-selected for particular kidney abnormalities (see Table 1.1 in Chapter 1: Introduction for overview of these study cohorts).

Future research

Validation of the HNF1B score in a prospective cohort is required. Since publication of this scoring system in 2014, further criteria for *HNF1B* gene analysis in patients with CAKUT have also been published by Raaijmakers *et al*.[124] They propose restricting *HNF1B* genetic testing to only those patients with bilateral major renal anomalies and in particular, renal cysts of unknown origin in combination with hypomagnesaemia; major renal anomalies were defined as foetal bilateral hyperechogenic kidneys, multicystic dysplastic kidney, renal agenesis, hypoplastic or dysplastic kidneys and cysts of uncertain origin. In order to collect prospective data, all new referrals for *HNF1B* genetic testing to Exeter Molecular Genetics Laboratory (approximately 100-120 referrals/year) would include completion of a request form that records information on all the clinical features seen in HNF1B-associated disease. This would then allow the clinical utility of both Faguer's HNF1B score and Raaijmaker's criteria for *HNF1B* analysis to be compared and a more refined model to be developed.

CHAPTER 3: 17Q12 MICRODELETIONS BUT NOT INTRAGENIC *HNF1B* MUTATIONS ARE ASSOCIATED WITH A NEURODEVELOPMENTAL PHENOTYPE

Summary and impact of findings

This chapter systematically compares the neurodevelopmental phenotype of patients with either an *HNF1B* intragenic mutation or 17q12 deletion. Both children and adults with HNF1B-associated disease were recruited from four sites in the UK and underwent brief behavioural screening using the Strengths and Difficulties Questionnaire (SDQ) plus assessment of autistic traits, cognitive ability and dysmorphic features.

Use of the parent-reported SDQ in children demonstrated more behavioural difficulties in the deletion group with a median total difficulties score of 15.5 (interquartile range [IQR] 10-20) compared with 7 in the mutation group (IQR 3.5-7.5), *P*=0.048. Parental scores for the impact of these difficulties on the child's life were similarly high in the deletion group. Eight of 20 participants (40%) with a deletion had a clinical diagnosis of either an autism spectrum disorder (ASD), attention deficit hyperactivity disorder (ADHD) and/or learning difficulties requiring a Statement of Special Educational Needs or current attendance at a special school compared with 0 of 18 with a mutation, *P*=0.004. The 17q12 deletion was associated with more autistic traits but the median IQ composite was similar in both mutation and deletion groups (97 [IQR 83-104] *versus* 91 [IQR 76-107] respectively; *P*=0.6). Two independent clinical geneticists were able to predict the presence of a deletion with a sensitivity of 83% and specificity of 79% when assessing facial dysmorphic features as a whole. Taken together, these results demonstrate that a neurodevelopmental phenotype is only seen in individuals with a 17q12 deletion and support the hypothesis that it is not simply haploinsufficiency of the *HNF1B* gene that is responsible for this clinical feature. These findings should allow more tailored counselling of patients with HNF1B-associated renal disease and their families plus ensure worrying symptoms are promptly investigated and referred for appropriate psychiatric assessment.

Limitations

Our study cohort of *n*=38 represented only 45% of eligible patients with HNF1B-associated renal disease or diabetes over the four recruitment sites due to either inability to contact individuals or patients declining to participate. Other ethnic groups besides White British are also likely to be underrepresented as only 43% of non-participants were White British compared to 97% of participants; however, there was a lot of missing ethnicity data for non-participants so this difference may not be so marked. Therefore, the exact prevalence and spectrum of neurodevelopmental disorders in HNF1B-associated renal disease and diabetes remains unknown. Diagnostic tests for ASD and ADHD were not used in this study so less severe disease may have been missed in both mutation and deletion groups. Genetic screening for other known causes of neurodevelopmental disease (e.g. Fragile X, other copy-number variants [CNVs]) was also not undertaken.

Future research

It would be interesting in further studies to perform more comprehensive neurocognitive assessment but this is logistically challenging in a rare genetic condition such as HNF1B-associated disease where individuals are geographically diverse. One way of addressing this would be to use the Development and Well-Being Assessment (DAWBA), which is a package of questionnaires, interviews and rating techniques designed to generate ICD-10 and DSM-IV psychiatric diagnoses in 5-17 year olds.[132] Information is collected via an interview with parents and 11-17 year olds themselves, as well as a questionnaire completed by teachers; one particular advantage is that the DAWBA interviews can be administered by computers to participants in their own home. Information from the different informants is drawn together by a computer program that predicts the likely diagnosis. Experienced clinical raters can then review all the data and decide whether to accept or overturn the computer-generated diagnosis.

It is unclear why only some individuals with a 17q12 deletion develop a neurodevelopmental phenotype whilst others do not. It will be important in future work to screen these patients for other genetic causes of neurodevelopmental disease, in particular the presence of other CNVs. Girirajan and colleagues used array comparative

genomic hybridisation to analyse the genomes of 2,312 children known to carry a CNV associated with intellectual disability and congenital abnormalities and found 10.1%, including cases with 17q12 deletion, carried a second large CNV in addition to the primary genetic lesion.[133] Interestingly, a recent study describing intellectual disability in individuals with HNF1B-associated diabetes excluded other large genomic deletions in all tested patients using single nucleotide polymorphism array analyses.[134]

CHAPTER 4: EXOCRINE PANCREATIC DYSFUNCTION IS COMMON IN HNF1B-ASSOCIATED RENAL DISEASE AND CAN BE SYMPTOMATIC

Summary and impact of findings

Faecal elastase-1 levels have only been reported in a small number of individuals with HNF1B-associated disease, the majority of which have diabetes. In this chapter, we measured faecal elastase-1 in patients with HNF1B-associated disease regardless of diabetes status and assessed the degree of symptoms associated with pancreatic exocrine deficiency. Faecal elastase-1 was measured by enzyme-linked immunosorbent assay on a single spot stool sample in 29 patients with a known *HNF1B* mutation or deletion. We defined a low faecal elastase-1 concentration based on the 2.5th percentile of 99 healthy control individuals without diabetes (410 mcg/g stool). Symptoms related to pancreatic exocrine dysfunction (abdominal pain, loose stools and unintentional weight loss) were assessed by direct questioning and a subset of the HNF1B cohort (*n*=6) underwent pancreatic imaging with either computerised tomography or magnetic resonance imaging scanning.

Faecal elastase-1 was below the 2.5th percentile of the control cohort in 18/29 (62%) patients with HNF1B-associated renal disease. All individuals with a low faecal elastase-1 level who underwent imaging (*n*=4) had radiological evidence of hypoplasia of the body and tail of the pancreas. 8/29 (28%) had a faecal elastase-1 measurement suggestive of exocrine pancreatic insufficiency at <200 mcg/g stool; of these, three suffered with abdominal pain, loose stools and/or unintentional weight loss. All three experienced symptomatic improvement and weight gain after commencing pancreatic enzyme replacement therapy. Faecal elastase-1 was low in 7/15 (47%) HNF1B patients without diabetes compared to 11/14 (79%) of those with diabetes, *P*=0.1. Therefore, faecal elastase-1 deficiency is a common feature of HNF1B-associated renal disease even when diabetes is not present and pancreatic exocrine deficiency may be more symptomatic than previously suggested. Faecal elastase-1 should be measured in all patients with known HNF1B-associated disease complaining of chronic abdominal pain, loose stools or unintentional weight loss. The discovery of a low faecal elastase-1 concentration in

individuals with developmental kidney disease of uncertain cause should prompt referral for *HNF1B* genetic testing.

Limitations

The small sample size of *n*=29 individuals with HNF1B-associated disease means we may have been underpowered to make definitive comments on the comparison of patients with and without diabetes. Trends seem to suggest that low faecal elastase-1 levels are more common when diabetes is present and that there may be an association between increasing duration of diabetes and lower faecal elastase-1 concentrations; however, these findings need further exploration. The cohort of healthy controls used to define the lower limit of the normal range for faecal elastase-1 were older; the median age was 61.7 years (IQR 52.8-66.3) compared to 24.5 years (IQR 14-44) in the individuals with HNF1B-associated renal disease. However, faecal elastase concentrations decline with age so defining a cut-off for faecal elastase-1 using the 2.5th percentile of a younger control cohort may have yielded an even higher value than the 410 mcg/g stool used in this study.[121] The median faecal elastase-1 measurement of 1580 mcg/g stool (IQR 1000-2000) in our local control cohort is higher than values reported for healthy controls in other studies.[121-123] This may reflect assay differences between laboratories but, as we have defined low faecal elastase-1 as measurements that fall below the 2.5th percentile of a local healthy control group, this make our results generalisable. Furthermore, we found that our local cut-off of 410 mcg/g stool correlated with the presence or absence of pancreatic hypoplasia on radiological imaging.

Future research

It would be interesting in future work to follow a cohort of paediatric patients with *HNF1B* mutations and deletions over time using serial indirect pancreatic function testing and imaging to see if these non-invasive tests can be used to predict who will develop diabetes and exocrine insufficiency and at what age. It would also be useful to test the role of faecal elastase-1 as a biomarker for HNF1B-associated disease in a large cohort of individuals with CAKUT; it is cheap compared to genetic testing and easy to measure, requiring only a single spot stool sample.

CHAPTER 5: SERUM AND URINE ELECTROLYTES AS BIOMARKERS FOR HNF1B-ASSOCIATED RENAL DISEASE

Summary and impact of findings

This chapter assesses the potential of serum and urine magnesium and calcium levels as biomarkers for HNF1B-associated disease. Magnesium, calcium and creatinine were measured in serum and spot urine samples in 21 individuals with an *HNF1B* molecular abnormality and 24 patients from a general nephrology clinic. Renal magnesium and calcium fractional excretions were assessed as follows: (urine [electrolyte] x serum [creatinine])/(serum [electrolyte] x urine [creatinine]) x 100.

Compared to the clinic cohort, individuals with an *HNF1B* mutation or deletion had a significantly lower serum magnesium level and higher fractional excretion of magnesium (FEMg). There was also a tendency towards a lower fractional excretion of calcium (FECa). The independent ROC curves for serum magnesium and FEMg, with HNF1B-associated renal disease status as the dependent variable, resulted in an AUC of 0.95 and 0.92, respectively.

This pilot work suggests serum magnesium and FEMg could be highly sensitive biomarkers for HNF1B-associated renal disease. If these results are confirmed in a larger study of patients with CAKUT, they could be implemented as very cheap (approximately £0.52) screening tests for *HNF1B* genetic testing in routine clinical care. In the interim we suggest that serum magnesium measurements ≤0.75 mmol/L and FEMg ≥4.1% should lead to referral for *HNF1B* genetic testing in individuals with developmental kidney disease of uncertain cause, even if serum magnesium levels are within the normal reference range.

Limitations

The main limitation of this pilot work was the use of patients followed up in a general nephrology clinic as the control cohort, where the prevalence of HNF1B-associated

disease is likely to be very low. Only small numbers of participants were included in each cohort and there was a tendency towards worse renal function in those with an *HNF1B* molecular abnormality. Therefore, it will be important to assess the role of serum magnesium, FEMg and FECa as biomarkers for HNF1B-associated disease in a larger cohort of individuals with CAKUT and better matching of renal function. We included data from all individuals, even if blood and urine samples were not taken on the same day or they had been prescribed medications that had the potential to alter serum or urine electrolyte measurements. Although this has the advantage of making the cohorts more representative of routine clinical practice, it may also introduce bias so we repeated the analyses after excluding these individuals to confirm that the main results remain very similar.

It is unknown if these results are translatable to a younger cohort as only 6/21 (29%) HNF1B patients in this study were children and the youngest participant was 8 years old. This will be important to ascertain as *HNF1B* genetic testing is often indicated in early life if developmental kidney disease is identified during prenatal ultrasonography. Unpublished observations presented by Shazia Adalat and colleagues at the British Renal Society and Renal Association conference in 2014 suggest that biochemical abnormalities during early childhood are an unreliable indicator for *HNF1B* testing. Simple correlation analysis in our pilot study supports this; serum magnesium levels tended to decrease with age in the HNF1B cohort, whilst FEMg increased with age. It will be interesting to see if serum and urine electrolytes remain discriminatory for HNF1B-associated disease in a younger age group; if so, different cut-offs may need to be defined.

Future research

Firstly, it would be interesting to expand on the current pilot study by recruiting a larger number of individuals with HNF1B-associated disease and comparing serum and urine electrolyte measurements to a control cohort of patients with CAKUT who are matched for age, sex and renal function. We are also planning a prospective cohort study to be offered to individuals attending paediatric nephrology clinics in Turkey (Figure 6.1). We will have a

target *n* of ≥100 participants and would expect to find an *HNF1B* molecular abnormality in approximately 10% of the cohort.[124]

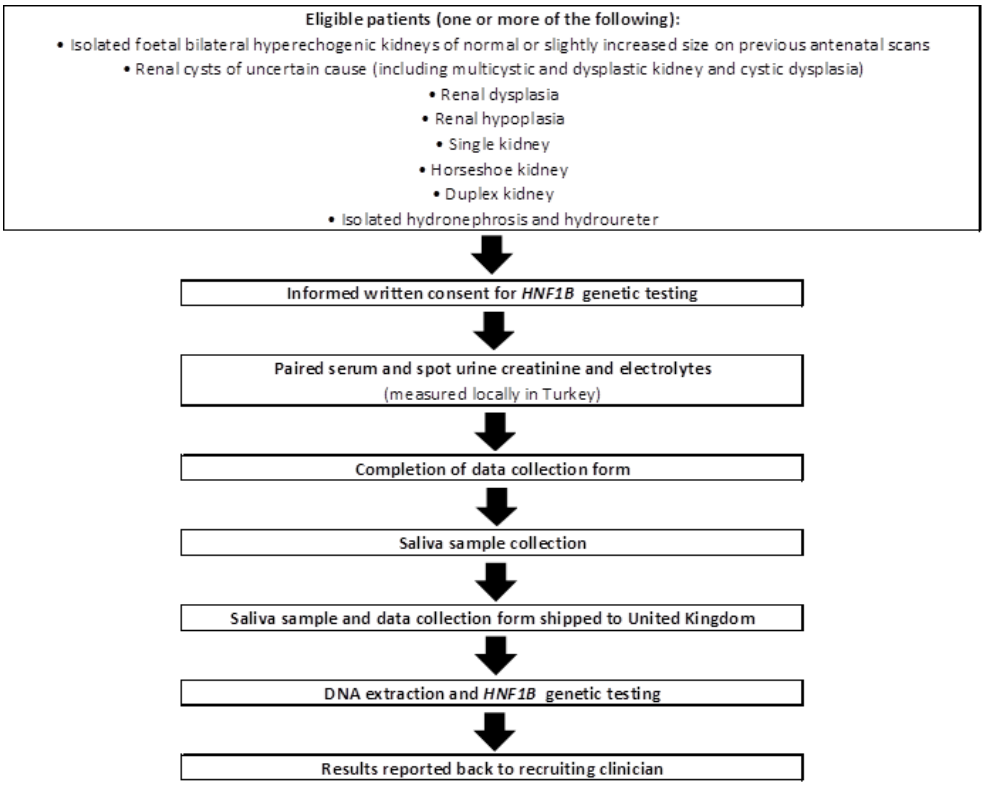

Figure 6.1 Flow chart outlining proposed study

Eligible patients will be identified via routine clinical practice. Inclusion criteria will include one or more of the following: (i) isolated foetal bilateral hyperechogenic kidneys of normal or slightly increased size on previous antenatal scans; (ii) renal cysts of uncertain cause (including multicystic and dysplastic kidney and cystic dysplasia); (iii) renal dysplasia; (iv) renal hypoplasia; (v) single kidney; (vi) horseshoe kidney; (vii) duplex kidney and (viii) isolated hydronephrosis and hydroureter. Patients will be excluded if they have renal cysts thought to be secondary to *PKD1/PKD2/PKHD1* mutations and/or are on renal replacement therapy. Informed written consent for genetic testing will be required from parents of child participants, preferably with assent from those aged <16 years. All participants will have measurement of creatinine, magnesium, calcium and potassium in

paired serum and spot urine samples; this will be performed by the recruiting clinician in their local hospital. Routine demographic and clinical data, including previous renal imaging results, will be entered onto a standardised data collection form. Participants will then be asked to provide a one-off saliva sample; this will be stored at room temperature and shipped to the UK together with the data collection form. All participants will undergo *HNF1B* genetic testing using DNA extracted from the saliva. The results of the genetic test will be reported back to the recruiting clinician. Serum and urine electrolyte measurements will then be compared between participants with newly identified HNF1B-associated disease and those with a negative genetic test result.

OVERALL CONCLUSIONS

The aim of this thesis was to explore some of the issues surrounding disease recognition in individuals with heterozygous mutations and deletions of the *HNF1B* gene. In brief, this work has examined the neurodevelopmental phenotype seen with *HNF1B* molecular abnormalities, tested the clinical utility of an HNF1B scoring system and assessed potential biomarkers for HNF1B-associated disease. The data presented in this thesis has been presented at national and European meetings and published in several medical journals.

We have shown that neurodevelopmental disorders, such as ASD and ADHD, are only seen in individuals with HNF1B-associated disease secondary to 17q12 deletion. It is important that nephrologists and diabetologists are aware of this association so they can counsel patients and their families appropriately and ensure that any behavioural problems are identified and referred for further investigation promptly. Despite the first work linking the 17q12 deletion with autism being published in 2010, very few of the families I met during the course of this study were aware of the association.[135] Parents with an affected child often experienced a lot of guilt surrounding their child's behavioural problems and found a sense of relief in learning that they may be related to their underlying genetic condition.

In our description of the neurodevelopmental phenotype of 38 patients with HNF1B-associated renal disease, we commented that the median estimated glomerular filtration rate (eGFR) was significantly higher in those with a 17q12 deletion compared to those with an *HNF1B* intragenic mutation. The mutation and deletion groups in this series were similarly matched in terms of age and 17/18 intragenic mutations described were truncating. This finding has now been confirmed in the largest series to date of individuals with HNF1B-associated disease; Dubois-Laforgue and colleagues have recently described the phenotype, long-term follow-up and genotype/phenotype correlations in 201 adults with *HNF1B* molecular abnormalities.[136] They report that patients with an *HNF1B* mutation

have a worse renal prognosis than those with a 17q12 deletion as they had a higher frequency of chronic kidney disease stages 3-4/end-stage renal disease and a lower eGFR at follow-up. Haploinsufficiency is accepted as the underlying disease mechanism in HNF1B-associated disease so it is unclear why there should be a difference in renal function between individuals with an *HNF1B* mutation and those with a 17q12 deletion.[27] One hypothesis already suggested by Dubois-Laforgue *et al.* is that some intragenic *HNF1B* mutations may exert a dominant-negative effect that results in a more severe phenotype. Although truncating mutations anywhere other than the terminal exon usually cause transcript degradation via the nonsense-mediated decay (NMD) pathway,[137] previous work has shown that *HNF1B* mutations have a varying degree of susceptibility to NMD and this could fit with a potential dominant-negative effect.[35] Another hypothesis involves the protective effect of one of the other genes lost in the 17q12 deletion. Further functional studies to explore some of these ideas will be important and may help to explain the phenotypic diversity that has remained unexplained in HNF1B-associated disease since the condition was first described.

Rapid advances in the field of molecular genetics over the last five years have seen the widespread introduction of next generation sequencing and it is expected that the costs associated with genetic testing will subsequently fall. Therefore it may be possible to perform gene analysis for all individuals identified as having CAKUT in the near future using a specific “CAKUTome”, which screens for mutations using targeted gene sequencing in a high throughput manner. Although this should lead to a genetic diagnosis for more patients, it will also lead to an increase in the number of variants of unknown significance. Scoring systems, such as the HNF1B score, and biomarkers will have an important role to play in helping to determine the pathogenicity of novel mutations and this highlights the importance of ongoing research that focuses on improving the identification of HNF1B-associated disease.

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APPENDIX A

HNF1B mutations and protein effects, as listed in the Human Gene Mutation Database (accessed on 25 March 2014)

Table 1 | *HNF1B* mutations and protein effects, as listed in the Human Gene Mutation Database*

Mutation type and location	Nucleotide change †NM_000.458.2	Protein effect †NM_000,458.2
Missense		
Exon 1	c.3G>T	p.Met1Ile
	c.107C>T	p.Ser36Phe
	c.182T>G	p.Val61Gly
	c.226G>T	p.Gly76Cys
	c.329T>G	p.Val110Gly
	c.335G>C	p.Arg112Pro
Exon 2	c.374T>C	p.Ile125Thr
	c.395A>C	p.His132Pro
	c.398A>G	p.Asn133Ser
	c.406C>G	p.Gln136Glu
	c.443C>T	p.Ser148Leu
	c.443C>G	p.Ser148Trp
	c.451T>C	p.Ser151Pro
	c.452C>G	p.Ser151Cys
	c.457C>A	p.His153Asn
	c.466A>G	p.Lys156Glu
	c.476C>T	p.Pro159Leu
	c.478A>G	p.Met160Val
	c.490A>C	p.Lys164Gln
	c.494G>A	p.Arg165His
	c.494G>C	p.Arg165Pro
	c.517G>A	p.Val173Ile
Exon 3	c.662A>T	p.Asp221Val
	c.698G>A	p.Arg233His
	c.704G>A	p.Arg235Gln
	c.715G>C	p.Gly239Arg
	c.721G>A	p.Ala241Thr
	c.728A>C	p.Gln243Pro
	c.758A>C	p.Gln253Pro
	c.766C>T	p.Pro256Ser
	c.780G>C	p.Glu260Asp
	c.791T>C	p.Leu264Ser
Exon 4	c.818G>A	p.Cys273Tyr
	c.826C>G	p.Arg276Gly
	c.827G>A	p.Arg276Gln
	c.854G>A	p.Gly285Asp
	c.856C>G	p.Leu286Val
	c.860G>T	p.Gly287Val
	c.865A>G	p.Asn289Asp
	c.883C>T	p.Arg295Cys
	c.884G>A	p.Arg295His
	c.884G>C	p.Arg295Pro
	c.895T>G	p.Trp299Gly
	c.906C>A	p.Asn302Lys
	c.1006C>G	p.His336Asp
Exon 5	c.1108G>A	p.Gly370Ser
Exon 7	c.1395C>G	p.Ser465Arg
	c.1474G>A	p.Gly492Ser
Nonsense		
Exon 1	c.232G>T	p.Glu78†
	c.301G>T	p.Glu101†
Exon 2	c.406C>T	p.Gln136†
	c.439C>T	p.Gln147†
	c.513G>A	p.Trp171†
	c.526C>T	p.Gln176†
	c.529C>T	p.Arg177†
	c.541C>T	p.Arg181†
	c.544C>T	p.Gln182†
Exon 4	c.826C>T	p.Arg276†
	c.931C>T	p.Gln311†

	c.1136C>A	p.Ser379*
Exon 7	c.1360C>T	p.Gln454*
	c.1408C>T	p.Gln470*
Frameshift		
Exon 1	c.18delG	p.Ser7fs
	c.46delC	p.Leu16fs
	c.143delT	p.Leu48fs
	c.206_207delAC	p.His69fs
	c.211_217delAAGGGCC	p.Lys71fs
	c.281_284dupAGCT	p.Gln96fs
	c.322delG	p.Ala108fs
	c.335_342delGGATGCTC	p.Arg112fs
Exon 2	c.353delC	p.Pro118fs
	c.477delT	p.Met160*
	c.487delC	p.Gln163fs
	c.499_504delGCTCTGinsCCCCT	p.Ala167fs
	c.534delG	p.Ile179fs
Exon 3	c.717delG	p.Ala241fs
	c.717dupG	p.Pro240fs
	c.727delC	p.Gln243fs
	c.786_787dupGG	p.Ala263fs
Exon 4	c.840delC	p.Ser281fs
	c.840dupC	p.Ser281fs
	c.949delG	p.Ala317fs
	c.972_973delCA	p.His324fs
	c.983delC	p.Pro328fs
	c.982_986delCCTCT	p.Pro328fs
	c.1006dupC	p.His336fs
Exon 5	c.1055dupA	p.Tyr352*
Exon 6	c.1302delC	p.Met435*
Exon 7	c.1363_1364delAG	p.Ser455fs
	c.1406_1413dupTGCAGCCC	p.Val472fs
Exon 8	c.1561dupC	p.Gln521fs
Splicing mutations		
Intron 1	c.345-1G>A	p.?
Intron 2	c.544+1G>A	p.?
	c.544+1G>C	p.?
	c.544+1G>T	p.?
Intron 3	c.809+1G>A	p.?
	c.810-2A>C	p.?
Intron 4	c.1046-2A>G	p.?
Intron 6	c.1339+1G>A	p.?
Deletions and duplications		
Exons 1–4	c.1-?_1045+?del	p.0?
Exons 1–8	c.1-?_1653+?del	p.0?
Exons 1–9	c.1-?_1674+?del	p.0?
Exon 2	c.409-?_483+?del	p.?
Exons 3–4	c.545-?_1045+?del	p.?
Exon 4	c.810-?_1045+?del	p.?
Exon 5	c.1118-?_1147del	p.?
Exon 5	c.1118-?_1147dup	p.?
Exons 5–9	c.1046-?_1674+?del	p.?

* http://www.hgmd.cf.ac.uk/ac/index.php *HNF1B* gene mutations are numbered with respect to GenBank cDNA sequence NM_000458.2, and described according to Human Genome Variation Society guidelines (http://www.hgvs.org). Numbering is based on +1 as the A of the major start codon of exon 1. Frameshift mutations have been predicted using Mutalyzer (https://mutalyzer.nl). The functional domains of the HNF1B protein are as follows: dimerisation domain p.1–32; POU_S DNA binding domain p.90–187; POU_H DNA binding domain p.235–311; transactivation domain p.311–557. Abbreviations: c., complementary; del, deletion; dup, duplication; HNF1B, hepatocyte nuclear factor 1β; ins, insertion; p., amino acid sequence level; POU_S, Pit-1Oct-1/2Unc-86 specific; POU_H, Pit-1Oct-1/2Unc-86 homeodomain; ?, protein has not been analysed.

APPENDIX B

Droplet digital PCR methodology and results

Droplet digital PCR was carried out on genomic DNA from participants with a known *HNF1B* whole-gene deletion (as determined by multiplex ligation-dependent probe amplification) to confirm the presence of a larger approximate 1.3 Mb deletion at chromosome 17q12; this was performed using the Bio-Rad QX200 system (Bio-Rad Laboratories, Hercules, CA) and following standard protocols. Four primer pairs were designed; two target genes (*ZNHIT3* and *HNF1B*) within the usual minimally deleted region containing 15 genes and two are hypothesised to target genes (*TAF15* and *SOCS7*) outside the deletion (Figure B.1). Primer sequences are shown in Table B.1.

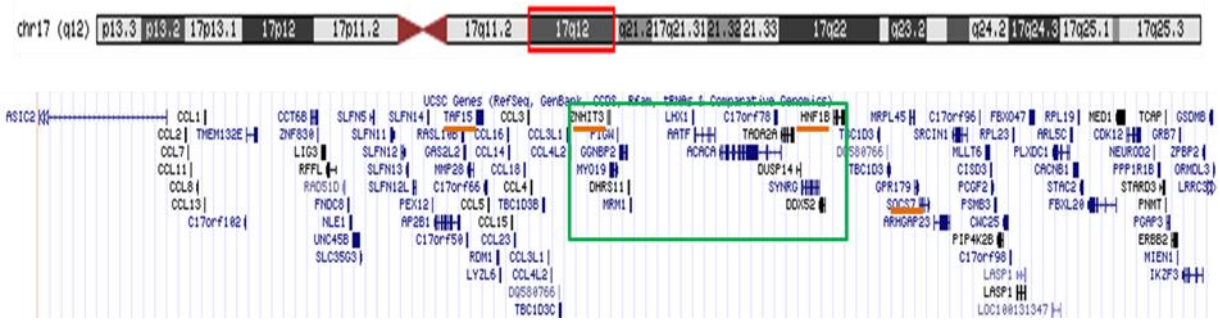

Figure B.1 Schematic of chromosome 17 adapted from the UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) Assembly (**http://genome.ucsc.edu/**).
17q12 is highlighted by the red box and blown up below to show the different genes within the region. The 15 genes involved in the common 1.3 Mb deletion seen at 17q12 are highlighted by the green box. The orange bars underline the position of the four primer pairs used in this experiment.

Gene	Forward primer	Reverse primer
***TAF15* (exon 6)**	TAGCCAAGGTGGAAGAGCAC	TGATAGGACTGCTGGTTTTGAC
***ZNHIT3* (exon 3)**	TACCCCAGACACTTGCTTCC	GTTTTCCACAGGCTTTACGG
***HNF1B* (exon 2)**	TTTTGCATAGACCATAGGTAGCAC	TCTGGGGGATGTTGTGTTG
***SOCS7* (exon 2)**	AATGCATATGTGTTCATTTCTCC	TGTTTCCCAACAGGTCTCAG

Table B.1 Primer sequences for droplet digital PCR

An assay mix containing 22 ng of genomic DNA, QX200 ddPCR EvaGreen supermix (Bio-Rad Laboratories) and gene assay at a final concentration of 100 nM per primer in a 22 μL final volume was prepared. 20 μL of assay mix and 70 μL of ddPCR droplet oil (Bio-Rad Laboratories) were subjected to the automated QX200 Droplet Generator (Bio-Rad Laboratories) to generate ~20,000 water-in-oil droplets. 40 μL of the oil and sample droplet emulsions were then transferred into a 96 well plate and thermocycled in a standard thermocycler (Bio-Rad Laboratories) at 95°C for 10 minutes, 94°C for 30 seconds and 57°C for 1 minute (repeated 39 times) and 98°C for 10 minutes. Sample fluorescence was measured by the QX200 Droplet Reader (Bio-Rad Laboratories) and absolute quantification of amplified DNA product was calculated by Poisson distribution using QuantaSoft software (Bio-Rad Laboratories). Copy number values for the assays were calculated as x/y, where "x" is the concentration of the target assay and "y" is the concentration of the reference assay using *SOCS7*; as all patients are predicted to have a heterozygous deletion of ~1.3 Mb at chromosome 17q12, we hypothesised that copy number values would be in the order of 0.5 for *ZNHIT3* and *HNF1B* (both genes that lie within the commonly deleted region) and 1.0 for *TAF15* and *SOCS7* (both genes that lie outside the commonly deleted region).

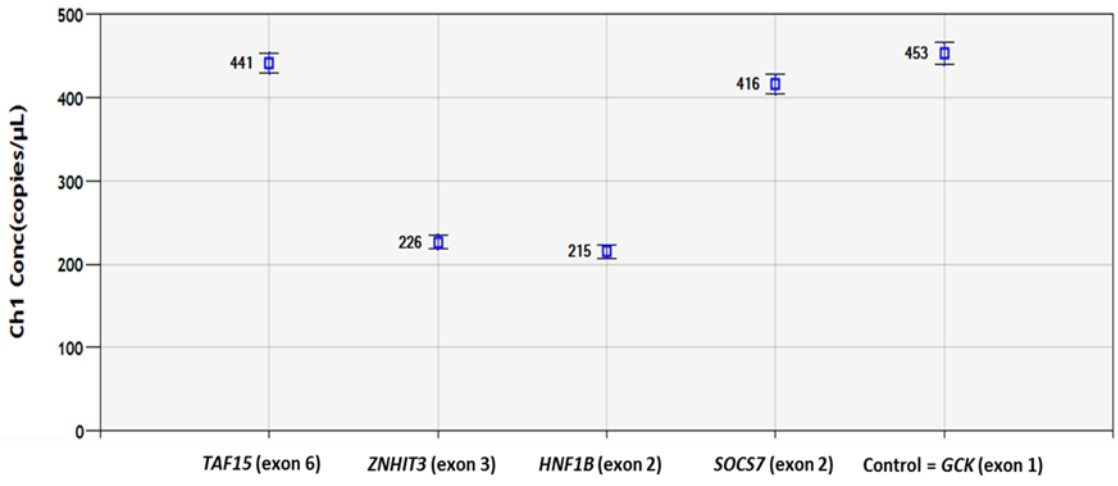

Figure B.2 Example of output using QuantaSoft software for patient 34.
Copies of DNA per μL are shown for each of the five genes. Copy number value could then be calculated by dividing the concentration of the target assay by the concentration of the reference assay (*SOCS7*).

Droplet digital PCR was carried out on genomic DNA from 20/20 participants with a known *HNF1B* whole-gene deletion (Figure B.2 and Table B.2). Copy number values ranged from 0.78-1.23 for *TAF15*, a gene that lies outside the region, whereas they were approximately 50% lower at 0.38-0.61 for *ZNHIT3* and 0.40-0.63 for *HNF1B*, both genes that are involved in the common 1.3 Mb deletion. These results suggest that all 20 patients with a *HNF1B* whole-gene deletion actually had a larger, approximate 1.3 Mb, deletion at chromosome 17q12 as hypothesised.

Patient study number	Copy number value = concentration of target assay/concentration of reference assay using *SOCS7* (95% confidence interval)			
	TAF15	***ZNHIT3***	***HNF1B***	***SOCS7***
1	0.67* (0.65-0.69)	0.38 (0.37-0.40)	0.40 (0.39-0.42)	1.00 (0.97-1.03)
3	1.04 (1.01-1.07)	0.52 (0.50-0.54)	-	1.00 (0.97-1.03)
5	0.96 (0.93-0.98)	0.46 (0.44-0.48)	0.49 (0.47-0.50)	1.00 (0.97-1.03)
7	0.97 (0.94-1.00)	0.51 (0.49-0.53)	0.55 (0.53-0.57)	1.00 (0.97-1.03)
8	0.78 (0.76-0.80)	0.41 (0.40-0.43)	0.49 (0.48-0.51)	1.00 (0.98-1.02)
9	0.95 (0.92-0.99)	0.58 (0.55-0.60)	0.56 (0.53-0.58)	1.00 (0.97-1.04)
16	1.23 (1.20-1.27)	0.50 (0.48-0.52)	0.61 (0.59-0.63)	1.00 (0.97-1.03)
17	1.02 (1.00-1.05)	0.45 (0.43-0.46)	0.47 (0.46-0.49)	1.00 (0.98-1.03)
18	0.99 (0.96-1.02)	0.55 (0.53-0.57)	0.63 (0.61-0.66)	1.00 (0.97-1.03)
19	0.87 (0.85-0.90)	0.42 (0.40-0.44)	0.42 (0.40-0.44)	1.00 (0.96-1.04)
27	0.97 (0.94-1.00)	0.61 (0.59-0.64)	0.54 (0.52-0.57)	1.00 (0.97-1.03)
28	0.96 (0.93-0.98)	0.48 (0.46-0.50)	0.42 (0.41-0.44)	1.00 (0.98-1.03)
29	1.03 (1.00-1.06)	0.44 (0.42-0.45)	0.51 (0.49-0.53)	1.00 (0.97-1.03)
30	1.06 (1.03-1.09)	0.55 (0.53-0.57)	0.57 (0.55-0.59)	1.00 (0.97-1.03)
32	1.01 (0.98-1.04)	0.55 (0.52-0.57)	0.54 (0.52-0.56)	1.00 (0.97-1.03)
33	0.94 (0.91-0.97)	0.51 (0.49-0.54)	0.54 (0.51-0.56)	1.00 (0.97-1.03)
34	1.06 (1.03-1.09)	0.54 (0.52-0.56)	0.52 (0.50-0.54)	1.00 (0.97-1.03)
35	1.10 (1.07-1.13)	0.60 (0.57-0.62)	0.61 (0.59-0.63)	1.00 (0.97-1.03)
37	0.97 (0.93-1.01)	0.52 (0.49-0.55)	0.49 (0.46-0.51)	1.00 (0.96-1.04)
*Repeat droplet digital PCR gave a result of 0.97 (0.93-1.00); - denotes no assay result				

Table B.2 Copy number values confirmed by droplet digital PCR for each patient with a known *HNF1B* whole-gene deletion previously determined by multiplex ligation-dependent probe amplification

Strengths and Difficulties Questionnaire

Version P [4-16] for completion by parents and version S [11-17] for self-completion.

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Autism Spectrum Quotient


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APPENDIX C

Stability of serum magnesium levels over time

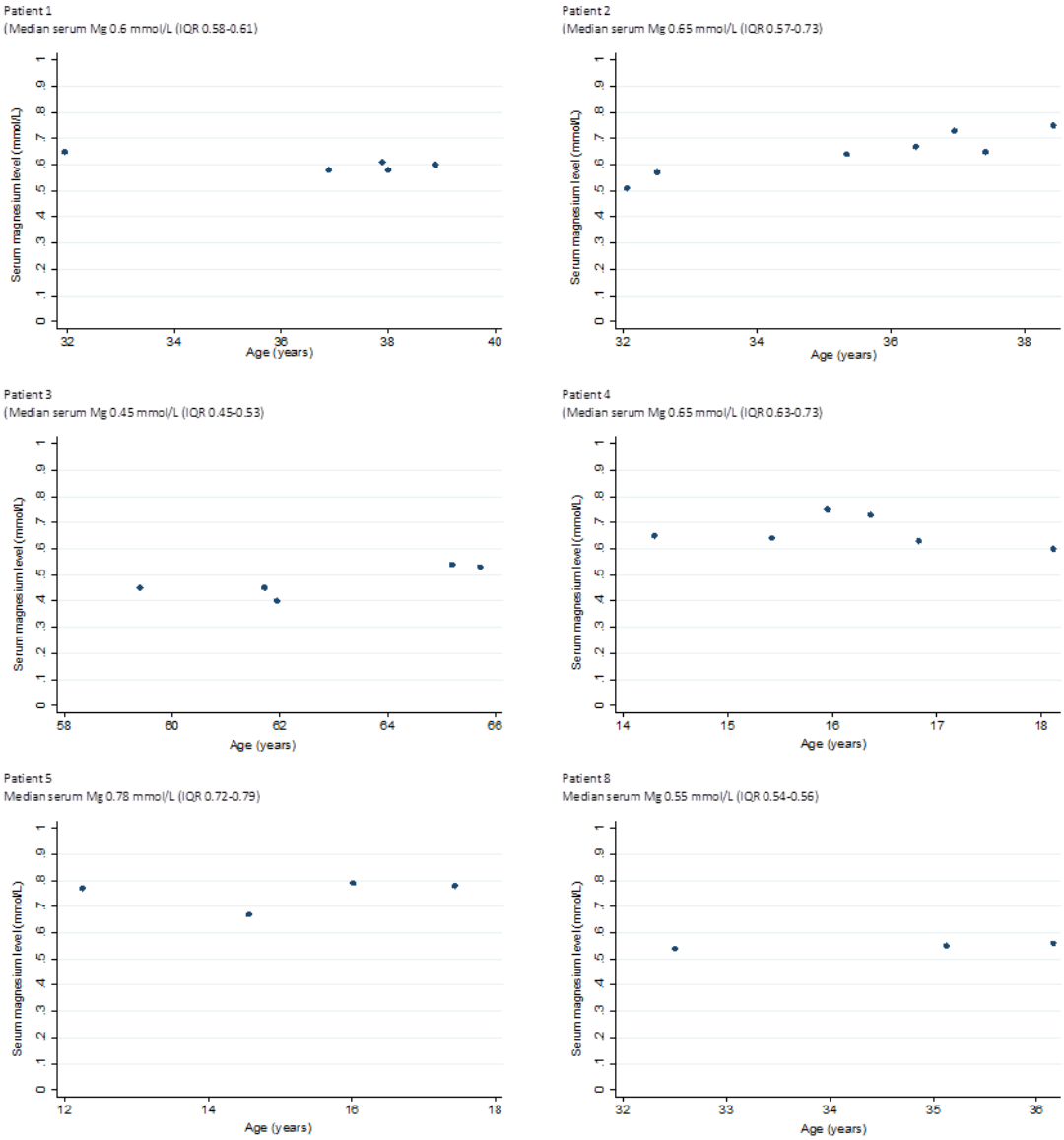

Figure C.1 Scatter plots for age and serum magnesium level in six individuals with HNF1B-associated renal disease. Abbreviations: IQR, interquartile range; Mg, magnesium.

Fractional excretion of electrolytes in the urine should be calculated using paired blood and urine samples. However, this is not always possible to achieve; patients are not always able to produce a urine sample at the same time as venepuncture and study participants, particularly children, often prefer previous results to be used rather than

undergo a further blood test. On reviewing previous laboratory results, we noticed that serum electrolyte concentrations were relatively stable over time; Figure C.1 shows serum magnesium measurements (taken in either general practice or out-patient departments) over time in six individuals with HNF1B-associated renal disease. Therefore, we decided to include results from non-paired blood and urine samples in our analysis where (i) samples were taken within 6 months of each other and (ii) previous serum measurements of electrolytes and creatinine, where available, were stable.

Reanalysis of data from chapter 5 after applying further exclusion criteria

This section presents the results from reanalysis of the data in chapter 5 after applying the following additional exclusion criteria: (i) use of medications that could alter serum or urine electrolyte measurements e.g. magnesium supplementation, diuretics, calcineurin inhibitors (CNIs) and proton pump inhibitors (PPIs) and (ii) use of unpaired blood and urine samples. This led to the exclusion of eight patients from the clinic cohort (diuretics, *n*=3; CNIs, *n*=3; PPIs, *n*=2) and seven patients from the HNF1B cohort (unpaired blood and urine samples, *n*=5; PPIs, *n*=2).

Age, sex and renal function are similar between HNF1B and clinic cohorts

Age and sex were similar in both individuals with HNF1B-associated disease and those under follow up in a general nephrology clinic (Table C.1). Renal function tended to be better in the clinic patients with a median estimated glomerular filtration rate (eGFR) of 92 ml/min/1.73m^2 (interquartile range [IQR] 55-95), compared to 58 (IQR 44-71) in the HNF1B cohort, but this did not quite reach statistical significance (*P*=0.07). The majority of patients (93%) with an *HNF1B* mutation/deletion had a renal diagnosis of either cysts or cystic dysplasia whereas there were a range of diagnoses in the clinic cohort. Diabetes was present in 8/14 (57%) individuals with HNF1B-associated disease but only 1/16 (6%) clinic patients, *P*=0.004.

Feature	Patients from general nephrology clinic (*n*=16)	Patients with HNF1B-associated disease (*n*=14)	*p*
Median age, years (IQR)	22 (18.5-37.5)	29 (15-37)	1.0
Sex, *n* (%)	M 8 (50), F 8 (50)	M 4 (29), F 10 (71)	0.3
HNF1B genetic abnormality, *n* (%)	-	Mutation 7 (50), deletion 7 (50)	-
Renal diagnosis, *n* (%)	ADPKD 4 (25), other cystic renal disease 2 (13), PUV 2 (13), other* 8 (50)	Renal cysts 12 (86), cystic dysplasia 1 (7), single kidney 1 (7)	-
Diabetes, *n* (%)	1 (6)	8 (57)	**0.004**
Median creatinine, μmol/L (IQR)	76 (59-124)	113 (90-136)	0.2
Median GFR, mL/min/1.73m^2 (IQR)	92 (55-95)	58 (44-71)	0.07

Abbreviations: ADPKD, autosomal dominant polycystic kidney disease; F, female; GFR, glomerular filtration rate; HNF1B, hepatocyte nuclear factor 1B; IQR, interquartile range; M, male; PUV, posterior urethral valves.
*Other renal diagnoses included atypical haemolytic uraemic syndrome, cortical necrosis, hyperoxaluria, microscopic haematuria, neuropathic bladder, partial Wilms tumour-aniridia syndrome and chronic kidney disease of uncertain cause.

Table C.1 Characteristics of study participants

5/14 (36%) patients with HNF1B-associated disease have serum magnesium levels within the normal reference range

The median magnesium concentration was 0.68 mmol/L (IQR 0.54-0.7) in the HNF1B cohort compared to 0.88 (IQR 0.82-0.91) in the clinic cohort, *P*<0.0001 (Figure C.2A). 9/14 (64%) patients with *HNF1B* mutations/deletions had hypomagnesaemia compared to 1/16 (6%) clinic patients, *P*=0.001 (Figure C.2B). The receiver-operating characteristic (ROC) curve for serum magnesium, with HNF1B-associated renal disease status as the dependent variable, is shown in figure C.2C; area under the curve (AUC)=0.96 (95% confidence interval [CI] 0.90-1). A cut-off for serum magnesium of 0.75 mmol/L was 100% sensitive and 87.5% specific for patients with HNF1B-associated disease.

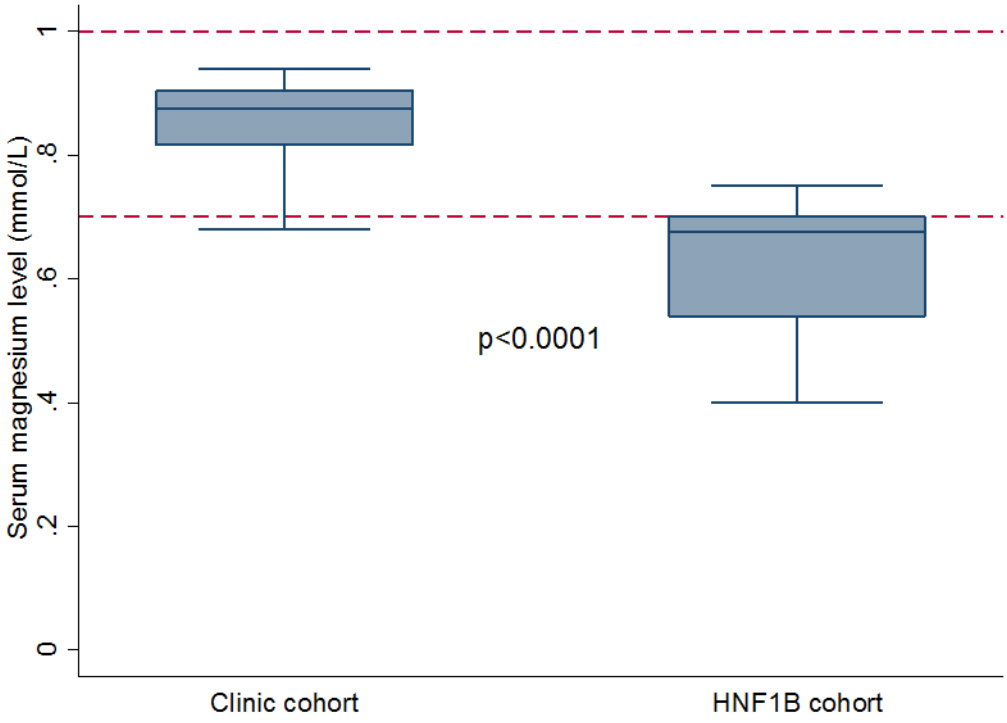

Figure C.2A Box plots showing serum magnesium levels in both patients under follow up in a general nephrology clinic and individuals with HNF1B-associated renal disease.
The area between the dashed red lines represents the normal reference range for serum magnesium (0.7-1 mmol/L).

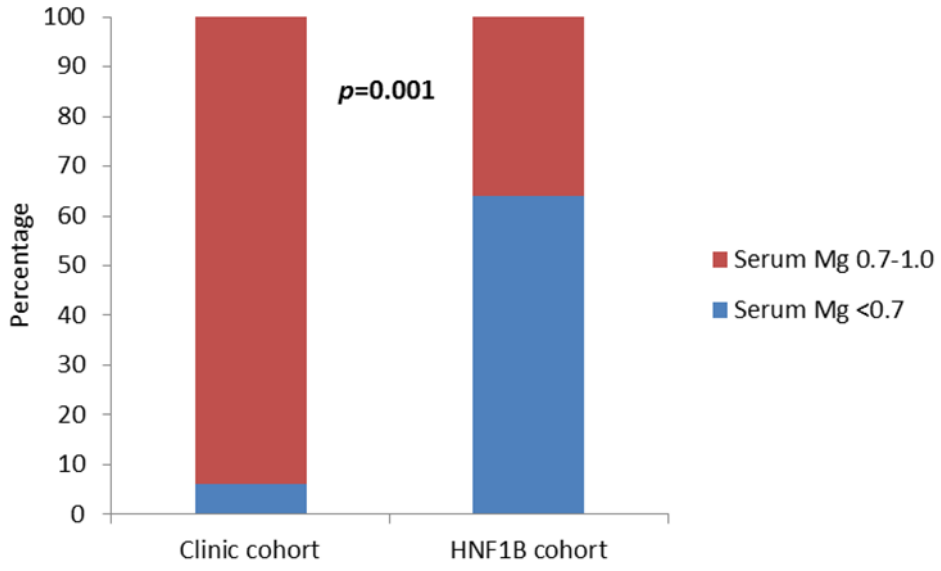

Figure C.2B Stacked bar charts showing the percentage of patients with hypomagnesaemia in both clinic and HNF1B cohorts.
Abbreviations: HNF1B, hepatocyte nuclear factor 1B; Mg, magnesium.

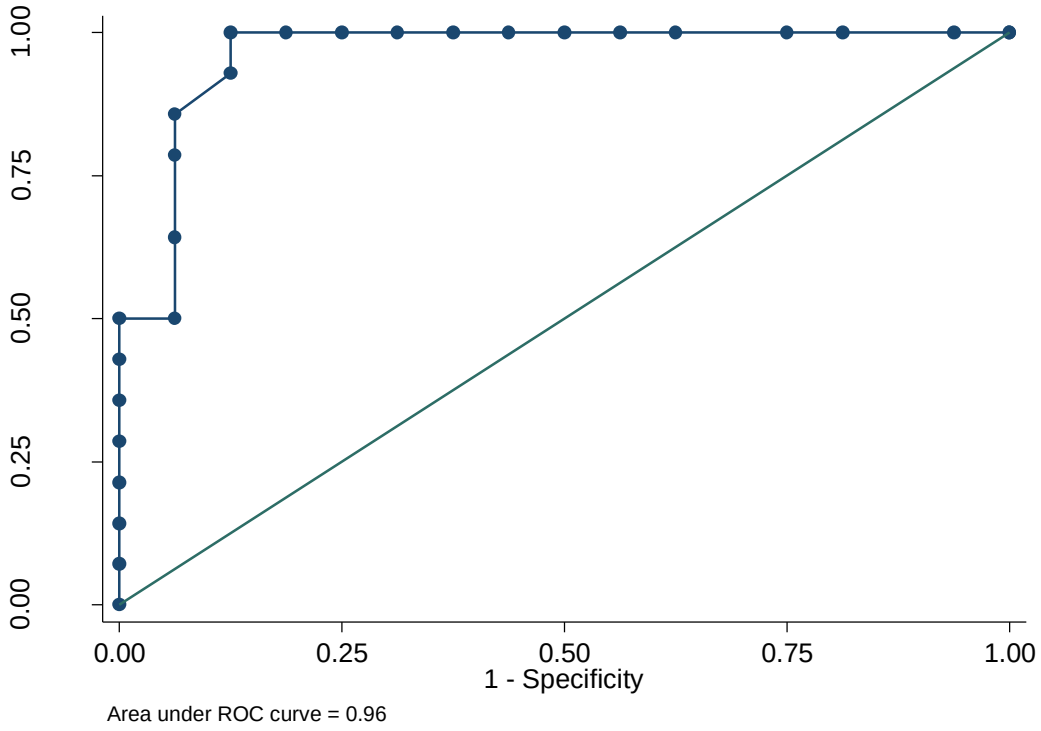

Figure C.2C ROC curve for serum magnesium, with HNF1B-associated renal disease status as the dependent variable. Abbreviations: ROC, receiver-operating characteristic.

All individuals with HNF1B-associated disease have fractional excretion of magnesium (FEMg) >4%

The median FEMg was 10.6% (IQR 8.4-14.4) in the HNF1B cohort compared to 2.6% (IQR 2.0-3.9) in the clinic cohort, *P*<0.0001 (Figure C.3A). All patients with *HNF1B* mutations/deletions (*n*=14) had hypermagnesuria with FEMg >4% compared to only 4/16 (25%) clinic patients, *P*<0.0001 (Figure C.3B). The ROC curve for FEMg, with HNF1B-associated renal disease status as the dependent variable, is shown in figure C.3C; AUC=0.98 (95% CI 0.94-1). A cut-off for FEMg of 5.65% was 100% sensitive and 87.5% specific for patients with HNF1B-associated disease.

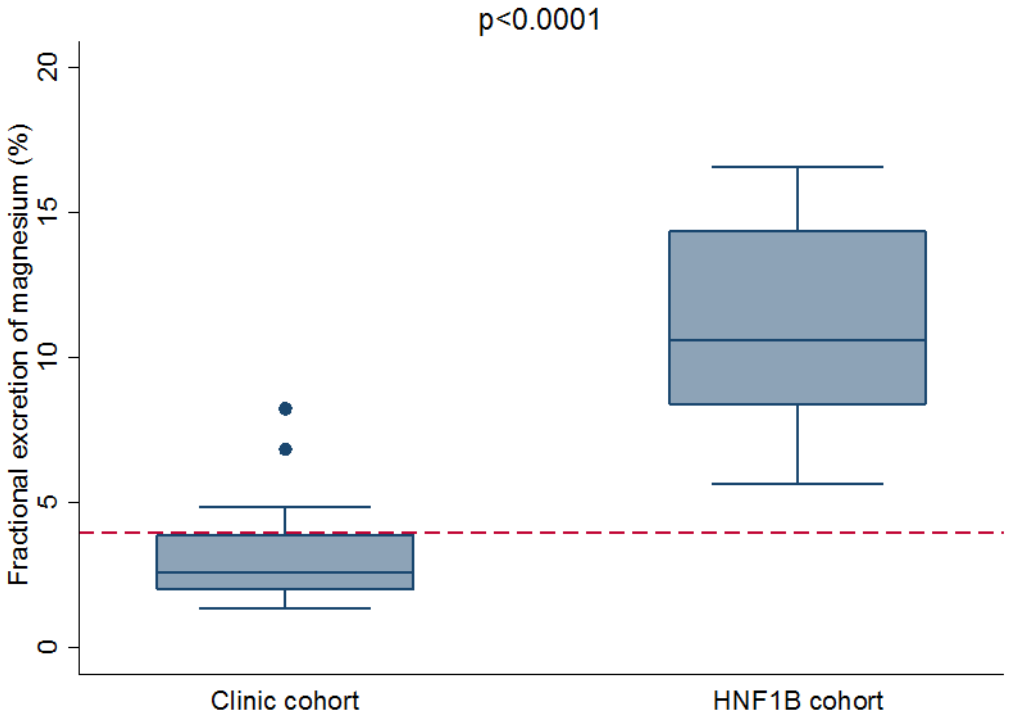

Figure C.3A Box plots showing FEMg in both patients under follow up in a general nephrology clinic and individuals with HNF1B-associated renal disease.
The area above the red dashed line represents hypermagnesuria.

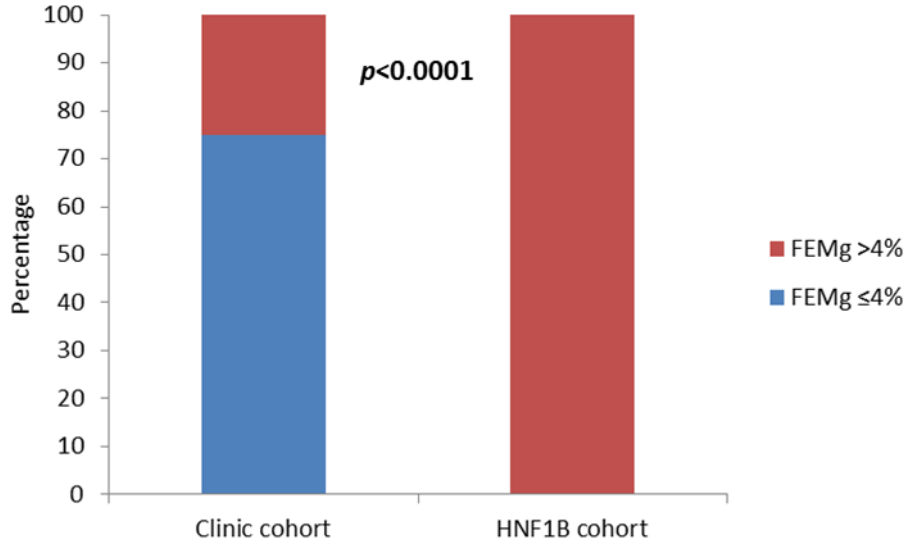

Figure C.3B Stacked bar charts showing the percentage of patients with hypermagnesuria in both clinic and HNF1B cohorts.
Abbreviations: FEMg, fractional excretion of magnesium; HNF1B, hepatocyte nuclear factor 1B.

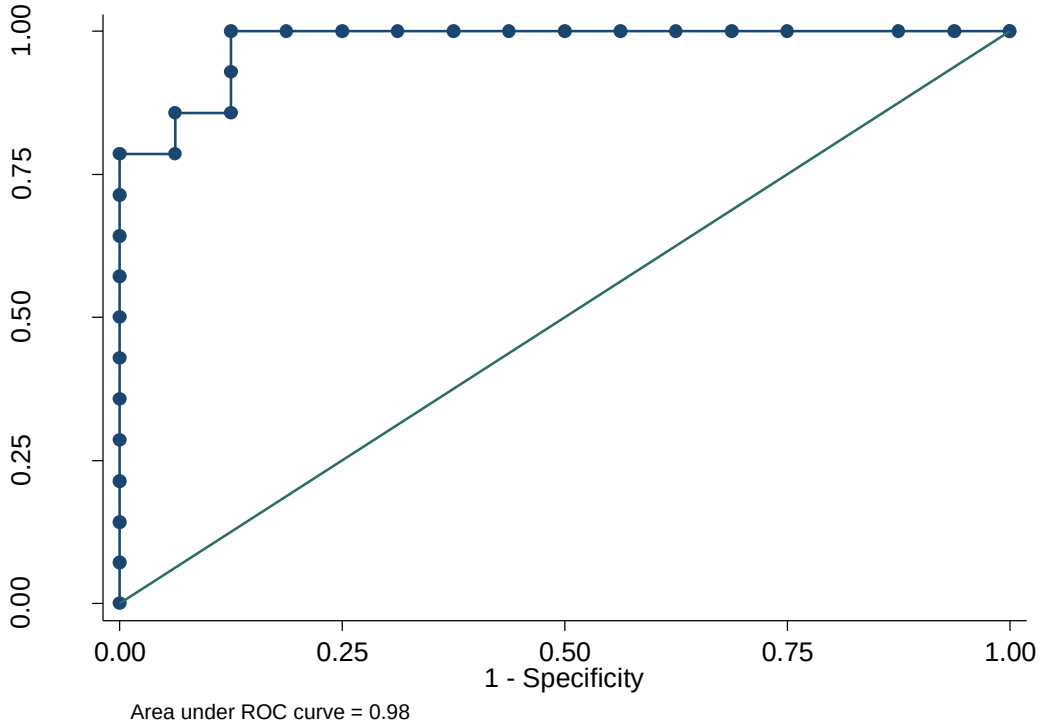

Figure C.3C ROC curve for FEMg, with HNF1B-associated renal disease status as the dependent variable. Abbreviations: ROC, receiver-operating characteristic.

Serum calcium levels are similar between HNF1B and clinic cohorts

The median calcium concentration was 2.29 mmol/L (IQR 2.24-2.37) in the HNF1B cohort compared to 2.28 (IQR 2.17-2.32) in the clinic cohort, *P*=0.4 (Figure C.4A). The ROC curve for serum calcium, with HNF1B-associated renal disease status as the dependent variable, is shown in figure C.4B; AUC=0.48 (95% CI 0.30-0.66).

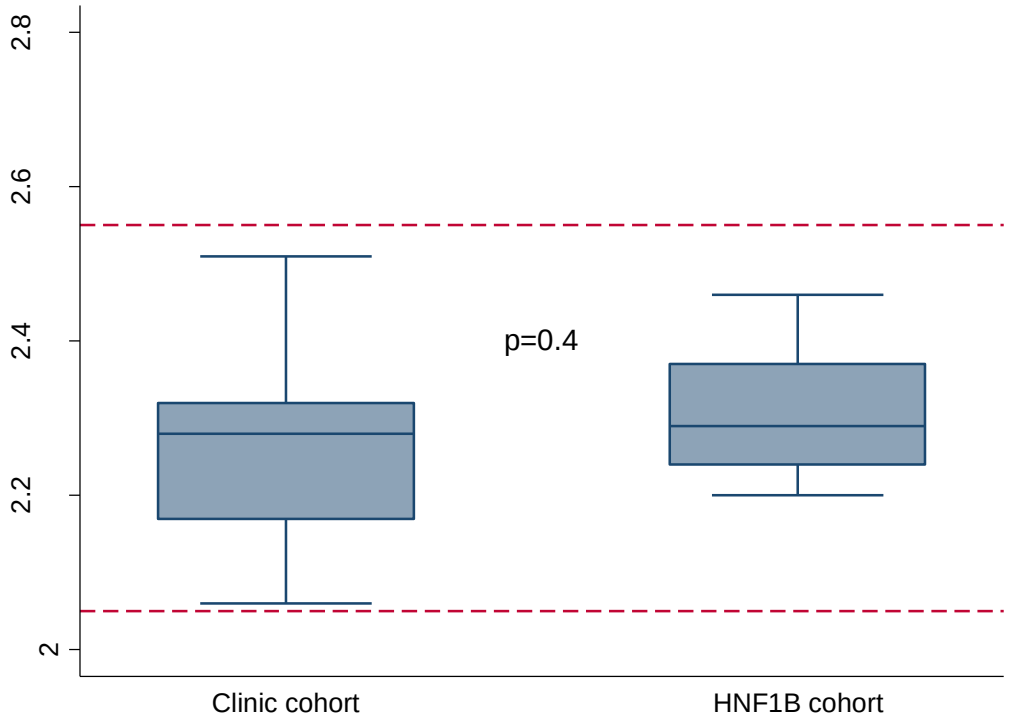

Figure C.4A Box plots showing serum calcium levels in both patients under follow up in a general nephrology clinic and individuals with HNF1B-associated renal disease.
The area between the dashed red lines represents the normal adult reference range for serum calcium (2.05-2.55 mmol/L); the paediatric reference range for serum calcium is 2.2-2.7 mmol/L.

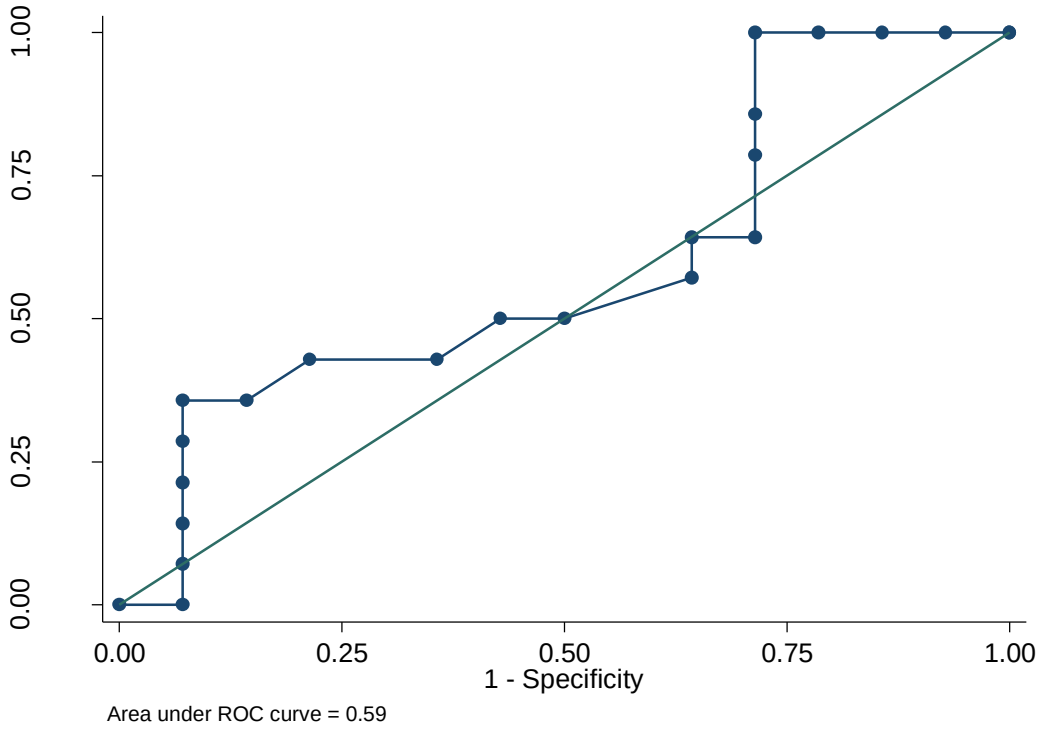

Figure C.4B ROC curve for serum calcium, with HNF1B-associated renal disease status as the dependent variable.
Abbreviations: ROC, receiver-operating characteristic.

9/13 (69%) patients with HNF1B-associated disease also have hypocalciuria

Median fractional excretion of calcium (FECa) was 0.6% (IQR 0.4-1.3) in individuals with an *HNF1B* mutation/deletion compared to 1.4% (IQR 0.6-1.7) in clinic patients, although this was not statistically significant with *P*=0.3 (Figure C.5A). However, 9/13 (69%) of those with HNF1B-associated disease had hypocalciuria with FECa <1% compared to only 4/14 (29%) clinic patients, *P*=0.06 (Figure C.5B). The ROC curve for FECa, with HNF1B-associated renal disease status as the dependent variable, is shown in figure C.5C; AUC=0.61 (95% CI 0.37-0.85).

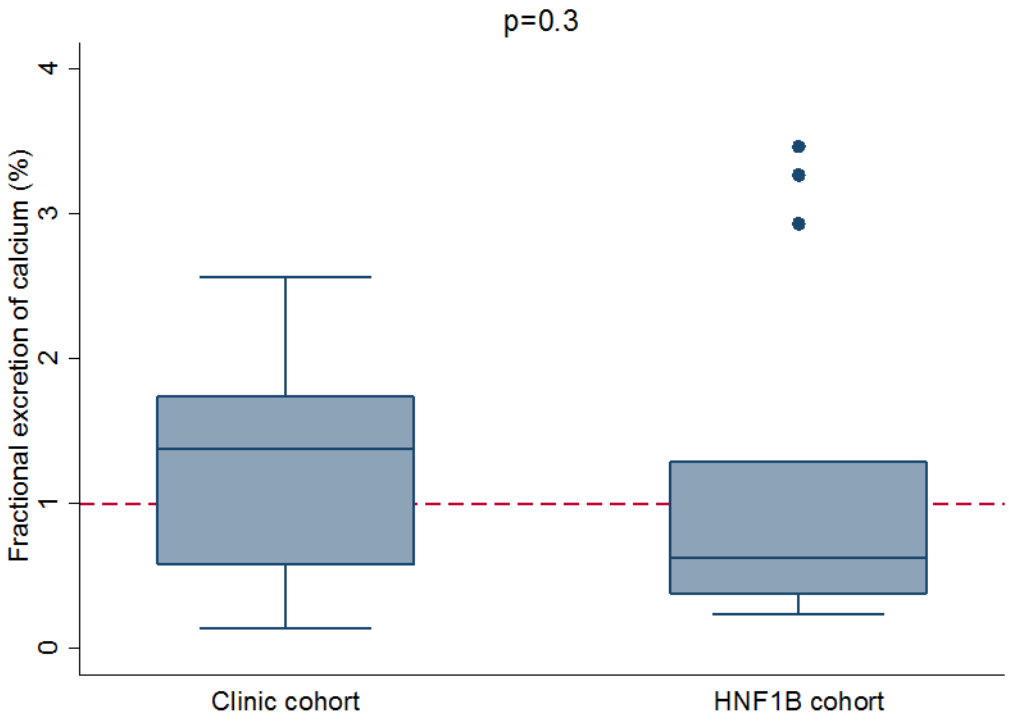

Figure C.5A Box plots showing FECa in both patients under follow up in a general nephrology clinic and individuals with HNF1B-associated renal disease.
The area below the red dashed line represents hypocalciuria.

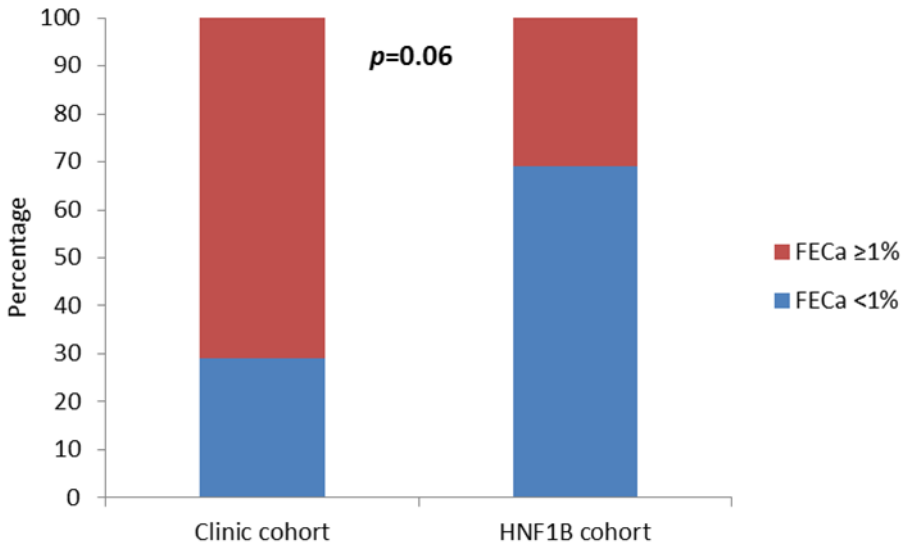

Figure C.5B Stacked bar charts showing the percentage of patients with hypocalciuria in both clinic and HNF1B cohorts.
Abbreviations: FECa, fractional excretion of calcium; HNF1B, hepatocyte nuclear factor 1B.

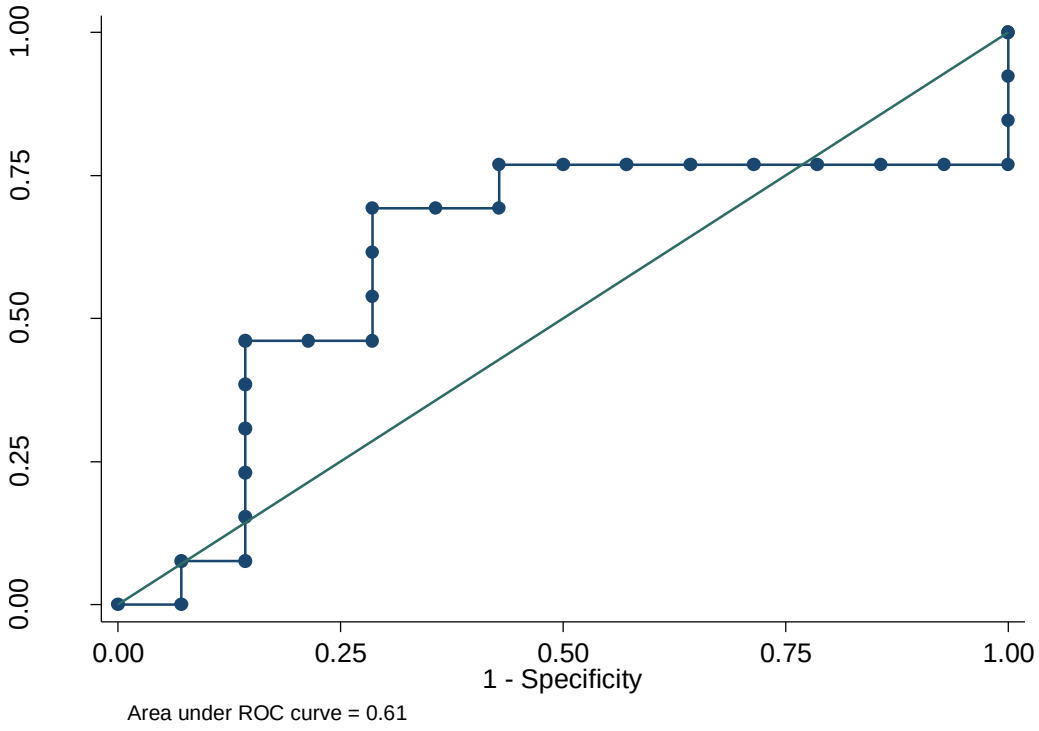

Figure C.5C ROC curve for FECa, with HNF1B-associated renal disease status as the dependent variable.
Abbreviations: ROC, receiver-operating characteristic.

Hypermagnesuria is more marked in patients with HNF1B-associated disease when diabetes is also present

Median FEMg was higher in individuals with an *HNF1B* mutation/deletion when diabetes was also present (Table C.2); there was also a tendency to a lower serum magnesium level. However, this group were older and renal function tended to be worse (median eGFR 47.5 ml/min/1.73 m^2 [IQR 41-83] compared to 62 [IQR 56-71] in those without diabetes; *P*=0.3). Simple correlation analysis showed moderate associations between (i) age and serum magnesium in all patients, (ii) age and FEMg in patients with HNF1B-associated disease and (ii) eGFR and FEMg in all patients (Figure C.6).

	No diabetes (*n*=6)	**Diabetes (*n*=8)**	***p***
Median serum Mg, mmol/L (IQR)	0.70 (0.66-0.71)	0.58 (0.53-0.70)	0.2
Median FEMg, % (IQR)	9.4 (6.3-10.6)	13.9 (10.5-15.3)	**0.03**
Median FECa, % (IQR)	0.6 (0.3-0.8)	0.9 (0.4-3.1)	0.2
Median age, years (IQR)	14.5 (8-16)	36.5 (33.5-44)	**0.002**
Median eGFR, mL/min/1.73 m^2 (IQR)	62 (56-71)	47.5 (41-83)	0.3
Abbreviations: eGFR, estimated glomerular filtration rate; FECa, fractional excretion of calcium; FEMg, fractional excretion of magnesium; IQR, interquartile range; Mg, magnesium.			

Table C.2 Comparison of individuals with HNF1B-associated disease depending on diabetes status

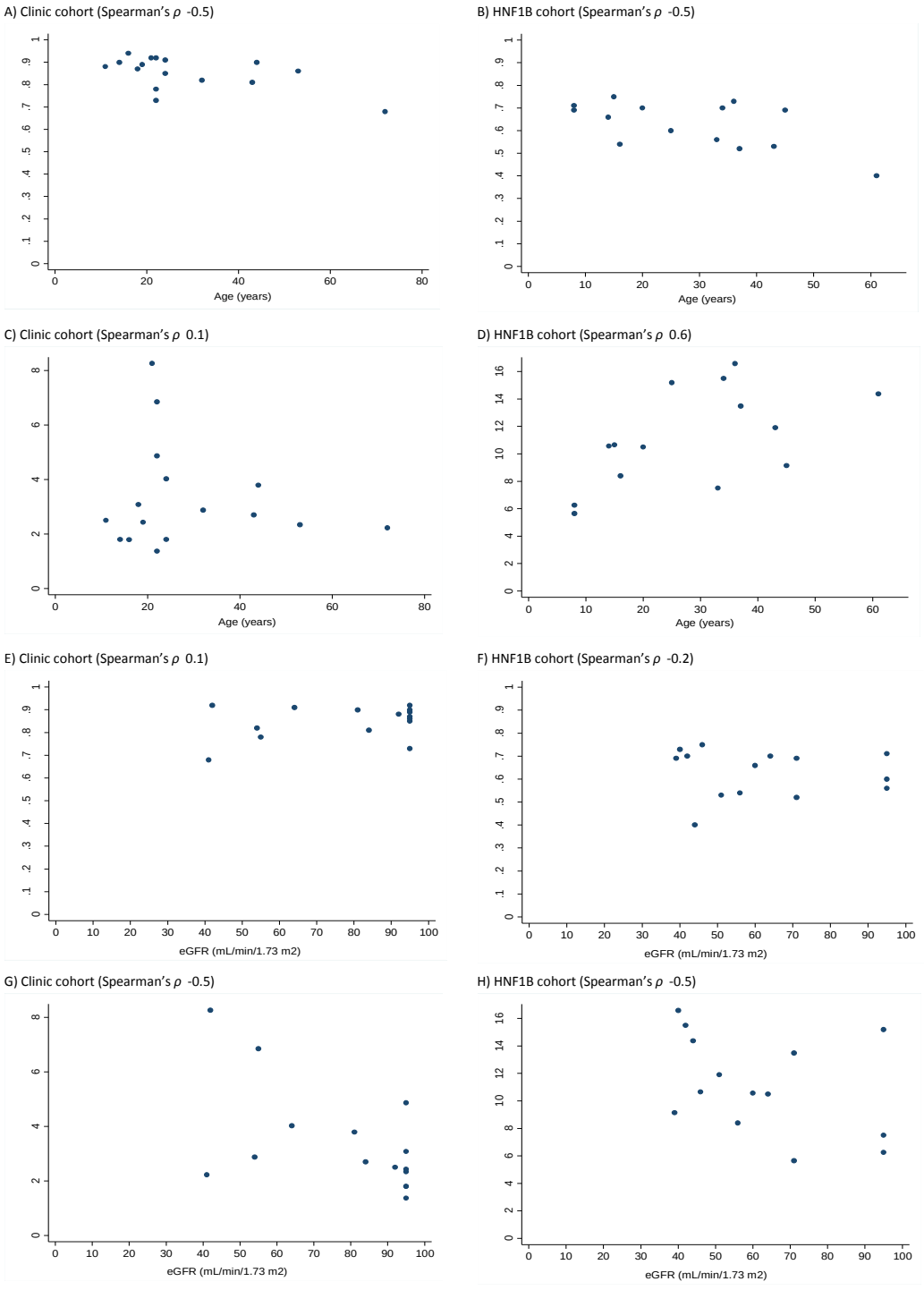

Figure C.6 Scatter plots for A&B) age and serum magnesium level, C&D) age and fractional excretion of magnesium, E&F) eGFR and serum magnesium level and G&H) eGFR and fractional excretion of magnesium.
Spearman's ρ was classified as follows: <4, weak association; 0.4-0.7, moderate association; ≥0.7, strong association.
Abbreviations: eGFR, estimated glomerular filtration rate.