Defining Genotype-Phenotype Correlations in Children with Congenital Hyperinsulinism

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Abbreviations

ABCC8	ATP- binding cassette, sub-family C
ANOVA	Analysis of variance
BWS	Beckwith-Wiedemann Syndrome
CHI	Congenital Hyperinsulinism
CPT-1	Carnitine palmitoyl transferase-1
DAG	Diacylglycerol
ECL	Enhanced chemiluminescence
EIHI	Exercise induced hyperinsulinism
ER	Endoplasmic Reticulum
18F-DOPA-PET	Fluorine-18 L-3,4-dihydroxyphenylalanine positron emission tomography
FFA	Free fatty acids
GCK	Glucokinase
GDH	Glutamate Dehydrogenase
GIP	Gastric inhibitory peptide
GLP-1	Glucagon-like peptide 1
GLUD1	Glutamate Dehydrogenase 1
GLUT	Glucose transporter
G6P	Glucose-6-phosphate
GPR40	G-protein coupled receptor 40
GRP	Gastrin releasing peptide

GSIS	Glucose stimulated insulin secretion
GTP	Guanosine triphosphate
HADH	Hydroxyacyl- Co A Dehydrogenase
HEK293	Human Embryonic Kidney cells 293
HH	Hyperinsulinaemic Hypoglycaemia
HI/HA syndrome	Hyperinsulinism/ Hyperammonaemia syndrome
HNF4A	Hepatocyte nuclear factor 4 alpha
HRP	Horseradish peroxidase
IUGR	Intra-uterine growth retardation
IGFBP-1	Insulin growth factor binding protein 1
IPF-1	Insulin Promoter Factor 1
K_{ATP}	ATP sensitive potassium channel
KCNJ11	Potassium inwardly-rectifying channel, subfamily J, member 11
Kir6.2	Inwardly rectifying potassium channel 6.2
Kv channels	Voltage-gated potassium channels
LB broth	Lucia-Bertani broth
LDH	Lactate Dehydrogenase
LC-CoA	Long-chain acyl-CoA
LSD	Least Significant Difference
MCT-1	Monocarboxylase transporter 1
MEM	Minimum Essential Medium
MODY	Maturity onset diabetes of the young

NBD	Nucleotide binding domain
NEUROD1	Neurogenic differentiation 1
ONS	Office for National Statistics
PACAP	Pituitary adenylate cyclase activating polypeptide
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PKC	Protein kinase C
PPARα	Peroxisome proliferator-activated receptor alpha
Rb^{86}	Rubidium
SDS	Standard deviation score
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLC16A1	solute carrier family 16, member 1
SSTR	Somatostatin receptor
SUR1	Sulfonylurea receptor 1
TCA	Tricarboxylic acid
TMD	Transmembrane domain
VIP	Vasoactive intestinal peptide
WT	Wild type

Abstract

Background: Congenital hyperinsulinism (CHI) is a clinically heterogeneous condition. Mutations in seven genes (*ABCC8, KCNJ11, GLUD1, GCK, HADH*, *SLC16A1* and *HNF4A*) are known to cause CHI, with mutations in *HNF4A* being the most recent identified genetic aetiology. Recessive mutations in *ABCC8/KCNJ11* cause severe medically unresponsive hyperinsulinaemic hypoglycaemia (HH). Recently, dominant mutations in these genes have been described that cause mild, medically responsive HH. Controversy exists on whether these dominant *ABCC8/KCNJ11* mutations predispose to diabetes mellitus in adulthood or not. The phenotype and prevalence of the genetic subgroups in a large cohort of patients has not been studied previously.

Aims: The aims of this thesis include:

1. To investigate genotype/phenotype correlations in a large cohort of patients with CHI by comparing different genetic aetiologies
2. To examine the prevalence and phenotype of patients with HH resulting from *HNF4A* gene mutations
3. To study the phenotype of dominantly inherited K_{ATP} channel mutations causing CHI and functionally characterize the novel dominant mutations identified

Methods: 300 patients with biochemically confirmed CHI were recruited. Detailed clinical information was collected prior to genotyping. *ABCC8* and *KCNJ11* genes were sequenced in all patients with CHI that were unresponsive to diazoxide, the mainstay of medical treatment in CHI. Mutations in the *GCK, GLUD1* and *HADH* genes were sought in patients with diazoxide responsive CHI with hyperammonaemia (HI/HA) (or leucine sensitivity (*GLUD1*)), raised 3-hydroxybutyryl-carnitine (*HADH*) or positive family history and/or delayed presentation (*GCK*). If no mutations were identified and in all other patients with diazoxide responsive CHI (and where diazoxide responsiveness was not known); *ABCC8*, *KCNJ11* and *HNF4A* genes were sequenced. The clinical characteristics of patients with the different genetic aetiologies identified were collated and the phenotypic characteristics of the patients found to have a *HNF4A* mutation were compared with the phenotypic characteristics of patients with transient and/ or diazoxide responsive CHI and a K_{ATP} mutation (n= 27), *GLUD1* mutation (n= 13) or a *HADH* mutation (n=3). The one-way

analysis of variance (ANOVA) test was used to compare the phenotypic data followed by LSD post-test to test for statistical significance.

Protein sensitivity was investigated in patients with a *HADH* mutation (n=3). Upon confirmation of protein sensitivity, leucine tolerance test were conducted in these patients to understand the mechanism of protein sensitivity.

The phenotype of ten families with dominant *ABCC8/KCNJ11* mutations and the prevalence of diabetes mellitus in the adult mutation carriers were also studied in detail. Functional consequences of six novel dominant K_{ATP} channel mutations (five *ABCC8* and one *KCNJ11*) were examined by reconstituting the K_{ATP} channel in HEK293 cells and evaluating the effect of drugs (diazoxide, glibenclamide) and metabolic poisoning on the channels using ^{86}Rb flux assay.

Results: Mutations were identified in 146/300 patients (48.6%). Mutations in the *ABCC8/KCNJ11* were the commonest genetic cause identified (n=117, 39%). Among diazoxide unresponsive patients (n=105), mutations in these two genes were identified in 92 (87.6%); of whom 63 patients had recessively inherited mutations while four patients had three novel dominantly inherited *ABCC8* mutations (G1485E, D1506E and M1514K). Among the diazoxide responsive patients (n=183), mutations were identified in 51 patients. These include mutations in the *ABCC8*(n=25), *KCNJ11*(n=3), *HNF4A*(n=7), *GLUD1*(n=16) and *HADH*(n=3). No mutations were identified in 132 (72%) patients in this group.

Heterozygous missense mutations were detected in 15 patients with HI/HA, two of which are novel (N410D, D451V). In addition, a patient with a normal serum ammonia concentration (21µmol/l) was heterozygous for a novel missense mutation P436L. Functional analysis of this mutation confirmed that it is associated with a loss of GTP inhibition. Seizure disorder was common (43%) in our cohort of patients with a *GLUD1* mutation.

The study identified a novel homozygous missense mutation (M188V) in the *HADH* gene in a patient with normal acylcarnitines and urine organic acids. Hydroxyacyl-Coenzyme A dehydrogenase activity was significantly decreased compared with controls (index patient mean 26.8 ± SEM 4.8mU/mg protein vs. controls 48.0 ± 8.1; p=0.029) in skin fibroblasts.

This patient and two other children with CHI due to *HADH* gene mutations were severely protein sensitive. The three children also demonstrated marked leucine sensitivity.

HNF4A mutations were identified to cause persistent CHI in addition to transient CHI, reported previously. 3/8 children with an *HNF4A* mutation did not have a diabetic parent. Children with *HNF4A* mutations had increased birth weight (median +2.4 SDS) and presented early (median of day 1). Patients with a K_{ATP} channel mutation were also large at birth (birth weight SDS ranged between -1.96 to +4.66) with an early age of presentation (ranging from 1 day to 365 days). In contrast, patients with a *GLUD1/ HADH* mutation were diagnosed later (mean of 157 and 125 days respectively) and were of normal birth weight (mean birth wt SDS of -0.11 and -1.09 respectively).

Study of the phenotype of the dominant *ABCC8/KCNJ11* mutations identified an increased prevalence (57%) of diabetes in the adult mutation carriers. Functional studies on the novel *ABCC8/KCNJ11* mutations showed no ^{86}Rb efflux when the mutant channels were activated, thus confirming the pathogenicity of the mutations.

Conclusions: A genetic diagnosis was possible in only 48.6% of patients with mutations in the *ABCC8* gene being the commonest cause.

Recessively inherited mutations in the *ABCC8/ KCNJ11* are associated with diazoxide unresponsive disease. However, the phenotype associated with dominant *ABCC8/ KCNJ11* mutations is variable, ranging from mild medically responsive CHI to severe early onset CHI requiring a near total pancreatectomy. In adults, dominant *ABCC8/ KCNJ11* mutations may also be an important cause of dominantly inherited early onset diabetes mellitus.

Patients with hyperinsulinism due to mutations in the *GLUD1* gene have a high risk of epilepsy and may have normal serum ammonia concentrations. Hence *GLUD1* mutational analysis may be indicated in patients with leucine sensitivity; even in the absence of hyperammonaemia.

Mutations in the *HADH* gene are associated with protein induced HH due to leucine sensitivity, suggesting a novel biochemical pathway by which *HADH* regulates leucine

induced insulin secretion. Patients with CHI due *HADH* gene mutations may have normal acylcarnitines and urine organic acids. Hence, sequencing of *HADH* must be considered in patients with diazoxide responsive HH from consanguineous families, even in the absence of these features.

In this large series, *HNF4A* mutations were the third common cause of diazoxide responsive CHI causing both transient and persistent HH, even in the absence of a family history of diabetes. *HNF4A* sequence analysis must hence be considered in all patients diagnosed with HH in the first week of life, irrespective of a family history of diabetes mellitus.

Future Work: The vast majority of patients with diazoxide responsive CHI had no mutations identified suggesting other novel mechanisms of insulin secretion. Understanding the genetic aetiology of CHI in this large cohort of patients will provide novel insights into pancreatic beta-cell physiology and have implications for hypoglycaemia and diabetes mellitus.

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Chapter 1- Introduction

The Physiology of Blood Glucose Control and the Role of Insulin

Glucose is the principal fuel for most mammalian cells and their metabolic needs. The brain, under normal conditions, uses glucose as an obligatory substrate. Since the brain has a limited supply of endogenous glucose and glycogen, it relies on a sustained and continuous supply of glucose from the blood (Jones *et al.,* 1975). Hence the maintenance of normoglycaemia (fasting blood glucose concentration of 3.5-5.5mmol/L) is important for the normal functioning of the brain; especially during active periods of brain development and growth in infancy and childhood. Conditions that cause hypoglycaemia in childhood can therefore cause permanent damage to the brain with long term neuro-developmental deficits and have enormous implications for children and their carers.

Blood glucose concentration is tightly regulated by maintaining a balance between glucose production and glucose utilisation. In normal individuals blood glucose remains in a narrow range despite periods of feeding and fasting. This equilibrium is maintained by a complex interplay of glucose, insulin and counter-regulatory hormones that include glucagon, catecholamines, growth hormone and cortisol. This chapter describes the pathways involved in glucose production and utilisation, the role of insulin in regulating the balance between them and details the complex regulation of insulin secretion.

1.1 Glucose production

Ingested carbohydrates are broken down to form monosaccharides which are then transported to liver and converted into glucose. Apart from ingested carbohydrates, glucose is also produced by the following processes:

Glycogenolysis: This involves the breakdown of stored glycogen to release glucose. Glycogenolysis occurs in liver and muscle (where glycogen is stored) in response to glucagon and/ or adrenaline secretion. This maintains the blood glucose concentration during fasting and provides the brain with a ready source of glucose. The first step involves activation of glycogen phosphorylase that phosphorylates glucose molecules in the glycogen chain to form glucose 1-phosphate, which is converted to glucose 6-phosphate. This is then converted to glucose by glucose-6-phosphatase. Glucagon and insulin control glycogenolysis by their opposing actions on these two enzymes.

Gluconeogenesis: This involves the synthesis of glucose from non-carbohydrate sources such as lactate, pyruvate, glycerol and amino acids (especially alanine). This process mainly occurs in the liver and to some extent in the kidneys. Glycogenolysis begins within 2–3 h after a meal in humans while gluconeogenesis assumes a much greater importance with prolonged fasting. The rate of gluconeogenesis is controlled principally by the activities of certain unidirectional enzymes, such as phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-bisphosphatase and glucose-6-phosphatase (G6Pase). The genes encoding these proteins are tightly controlled at the transcriptional level by key hormones, particularly insulin, glucagon and glucocorticoids. In the fasted state, insulin levels drop while glucagon secretion goes up resulting in increased glycogenolysis and gluconeogenesis. In the fed state; insulin increases, suppressing glycogenolysis and hepatic glucose production.

1.2 Glucose utilisation

Glucose is utilised by nearly all peripheral tissues. It enters the cells via two different types of membrane associated carrier proteins, the sodium-coupled glucose transporters (SGLT) and glucose transporter facilitators (GLUT). The distribution of these transporters varies in different tissues and depends upon the kinetic properties of the transporters and the requirement of the tissues. It is beyond the scope of this thesis to discuss each of the transporters and the complex mechanism occurring at the cell level that leads to glucose entry in the cell. A few important aspects of glucose transport are discussed instead. GLUT1 and GLUT3 transporters facilitate the uptake of glucose in the brain. GLUT1 is mainly expressed in the microvasculature of the brain and is responsible for transfer of glucose across the blood brain barrier, while GLUT3 is predominantly expressed in the neurons of the brain. In rats, hypoglycaemia has been shown to increase the expression of GLUT1 mRNA (Simpson *et al.,* 1999).

GLUT2, a low-affinity glucose transporter is predominantly expressed in the pancreatic ß-cells, liver, kidney, and small intestine. This transporter plays an important role in maintaining blood glucose concentrations because of its role in glucose absorption from the intestinal lumen, reabsorption of glucose in kidneys, glucose sensing in the pancreatic ß-cells and glucose uptake and release in the hepatocytes. The kinetic properties of GLUT2 allow transport of glucose within these tissues even at high blood glucose concentrations. Hence in these tissues glucose utilisation is not dependent on the number and activity of the glucose transporters but on the blood glucose concentration (Brown 2000).

GLUT4 is an insulin-sensitive glucose transporter, most abundantly found in the skeletal and cardiac muscle and adipose tissue (James *et al.,* 1989) and is the main glucose transporter in these tissues. Insulin stimulation transports GLUT4 present in the cytoplasmic vesicles to the cell membrane, increasing the glucose uptake in these tissues (James *et al.,* 1989).

After entry into the cells, glucose undergoes three metabolic fates:

1) Glycolysis- catabolism of glucose in the cells to produce adenosine triphosphate (ATP)
2) Glycogenesis- storage as glycogen (in the liver and muscle)
3) Conversion to fatty acids and storage as triglycerides (in adipose tissue)

These fates are dependent upon the blood glucose concentration, length of fasting and the hormonal milieu. Among the hormones that play a role, the principal hormone is insulin. The following section gives a brief description about the synthesis, actions and regulation of insulin secretion and its role in glucose physiology.

1.3 Insulin: Structure and Synthesis

Insulin is a hormone produced in the ß-cells of the islets of Langerhans in the pancreas. It is a 51 amino acid polypeptide consisting of two chains (A & B) that are connected by disulphide bridges and has a molecular weight of 5.8 kDa.

Preproinsulin is the primary precursor molecule that consists of a signal peptide, the A and B chains of insulin and a connecting peptide (or C-peptide) (Steiner *et al.*, 1967). It is processed in the endoplasmic reticulum to proinsulin by removal of the signal peptide. Proinsulin is then folded for the disulfide bonds to be formed between the A and B chains. It is then transported to the Golgi apparatus where it is packaged into secretory vesicles.

These vesicles contain endopeptidases that catalyse the conversion of proinsulin to form mature insulin. The C-peptide is liberated from the centre of the proinsulin sequence and the ends (the A chain and B chain) are connected by disulfide bonds. Insulin and c-peptide are stored together in the secretory vesicles and are released by exocytosis on appropriate stimulation.

1.4 Physiological actions of insulin

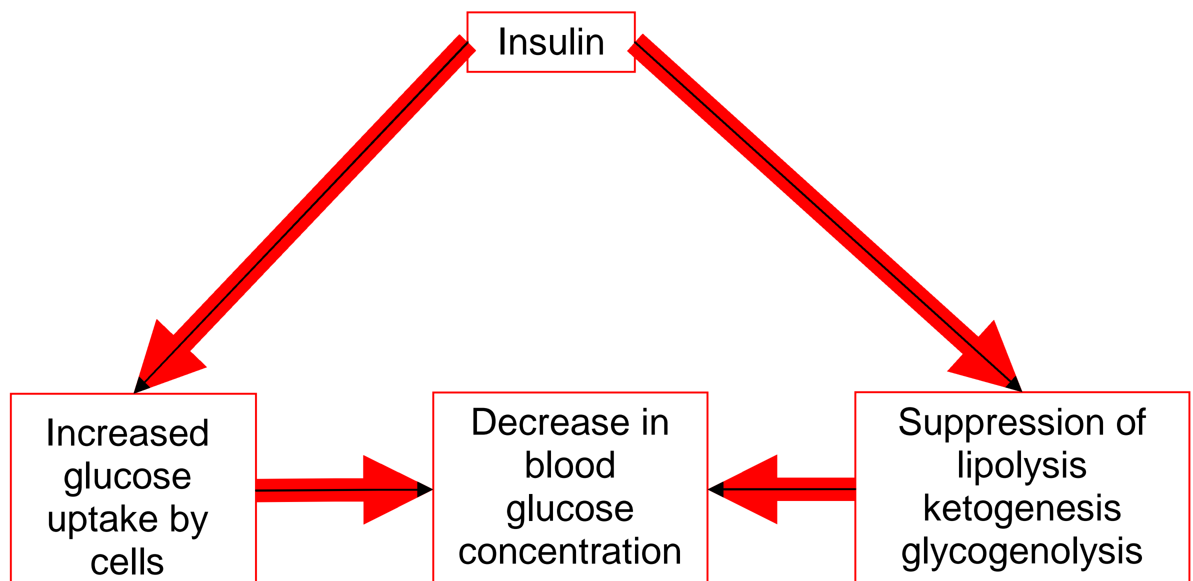
Insulin is an anabolic hormone. It decreases glucose production and increases glucose utilisation by the following effects on carbohydrate, fat and protein metabolism (figure 1.1):

i. Effect on carbohydrate metabolism: Insulin inhibits the release of glucose from stored glycogen in the liver (glycogenolysis). It also inhibits gluconeogenesis directly by inhibiting the key gluconeogenic enzyme PEPCK, and indirectly by inhibiting the release of free fatty acids (FFA) from adipose tissue. It activates enzymes for glycogenesis (conversion of glucose to glycogen) and glucose utilisation (glycolysis) (Radziuk and Pye, 2001). Insulin also facilitates glucose uptake in skeletal muscle and adipose tissue by its action on the glucose transporter (GLUT-4) (Scheepers *et al.,* 2004). In the absence of insulin, GLUT-4 glucose transporters are removed from the cell membrane and stored in cytoplasmic vesicles. Finally, insulin activates hexokinase, which phosphorylates glucose to form glucose-6-phosphate. This facilitates glucose transport through the GLUT-2 transporter in the hepatocytes and keeps the intracellular concentrations of glucose low.

ii. Effect on lipid metabolism: Insulin inhibits the mobilisation of FFA/ glycerol from adipose tissue. It stimulates free fatty acid synthesis (lipogenesis) and its conversion to triglycerides in the liver. Insulin has also been shown to increase cholesterol synthesis by increasing the key enzyme involved in cholesterol synthesis, ß-hydroxy-ß-methylglutaryl coenzyme- A (HMG-CoA) reductase (Lakshmanan *et al.,* 1973).

iii. Effect on protein metabolism: Insulin reduces the release of amino acids from muscle by inhibiting proteolysis. It also increases the uptake of amino acids into liver and muscle and stimulates their conversion to protein.

Figure 1.1: Summary of the effects of insulin. Insulin reduces blood glucose concentration by inhibiting glycogenolysis, gluconeogenesis, lipolysis and ketogenesis and by enhancing the uptake of glucose by insulin sensitive tissues.



1.5 The regulation of insulin secretion

As insulin is the principal hormone that controls blood glucose concentration, the regulation of insulin secretion plays a key role in maintaining normoglycaemia. Insulin secretion in turn is controlled by a number of stimuli which include nutrients, hormones and neurotransmitters. Glucose is the major stimulus for insulin secretion such that a rise in blood glucose concentration leads to an increase in insulin secretion and vice-versa. After a meal, when the blood glucose concentration increases, insulin secretion is stimulated leading to a drop in blood glucose by mechanisms described above (figure 1.1). In between meals, when there is no dietary glucose intake, there is less insulin and more glucagon secreted. This stimulates glycogenolysis, gluconeogenesis and stops glycogenesis to maintain the blood glucose concentration.

1.5.1 Nutrient induced insulin secretion

1.5.1.1 Glucose stimulated insulin secretion (GSIS):

Glucose induces a biphasic pattern of insulin release. First-phase insulin release occurs within the first few minutes after exposure to an elevated glucose concentration; this is followed by a more sustained second phase of insulin release.

In the pancreatic ß-cells, glucose enters the cytoplasm through facilitative glucose transporters, especially the glucose transporter 2 (GLUT2) (Johnson *et al.,* 1990; Scheepers *et al.,* 2004). GLUT2 has a high Km for glucose (approximately 40mmol/L) and is expressed predominantly in the liver, kidney, ß-cells of the pancreas and intestinal mucosal cells. The high Km for glucose of GLUT2 allows glucose transport by pancreatic ß-cells and hepatocytes in proportion to the blood glucose concentration (Gould *et al.,* 1991; Scheepers *et al.,* 2004). This, along with the glucose sensing abilities of the enzyme

glucokinase, leads to the control of insulin secretion by the pancreas and the uptake or release of glucose by hepatocytes as required, to regulate blood glucose concentration.

Glucose entry into the cells is followed by phosphorylation by the highly glucose specific enzyme, glucokinase. Glucokinase acts as a glucose sensor and due to its high Km, it does not saturate under physiological basal glucose levels. This allows the pancreatic ß-cells to adjust the rate of insulin secretion and hence this is thought to be the rate limiting step for insulin secretion (Matschinsky, 2002). Following phosphorylation, glucose is metabolized to pyruvate (glycolysis) which then enters the Kreb's cycle to produce ATP. ATP triggers closure of the adenosine triphosphate-sensitive potassium channels (K_{ATP}) channels located on the ß-cell membrane which leads to insulin release. The structure of the K_{ATP} channels and the mechanism by which the channels couple metabolic signals generated from glucose metabolism to insulin secretion is discussed below.

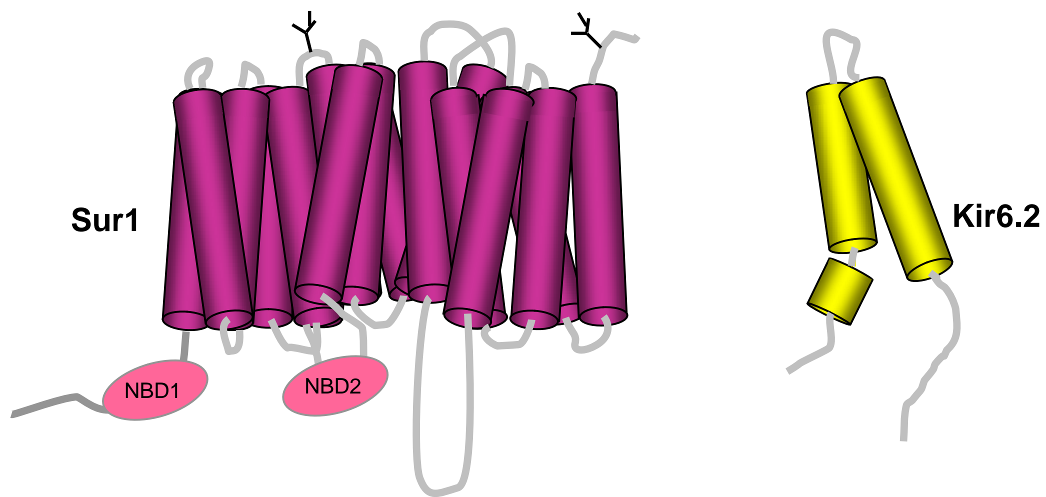
1.5.1.1.1 Overview of K_{ATP} channels and their role in GSIS:

The K_{ATP} channels are hetero-octameric complexes comprising of four inwardly rectifying potassium channels (Kir6.2) and four sulphonylurea receptor 1 (SUR1) subunits. The Kir6.2 forms the pore of the channel and the SUR1 (an ATP Binding Cassette Transporter) acts as a regulatory subunit (Inagaki *et al.,* 1995) (figure 1.2 b). K_{ATP} channels are regulated by adenine nucleotides to convert changes in cellular metabolic levels into membrane excitability. Each subunit of the K_{ATP} channel is known to be differentially regulated. The Kir6.2 subunit determines the biophysical properties of the channel complex including K^+ selectivity, rectification, inhibition by ATP and activation by acyl-CoAs (Tucker *et al.,* 1998). The sulfonylurea receptors endow K_{ATP} channels with sensitivity to the stimulatory actions of magnesium-nucleotides and K_{ATP} channel openers (e.g.

diazoxide, nicorandil) and the inhibitory effects of sulphonylureas and glinides (Aguilar-Bryan L *et al.,* 1999).

The molecular topology of SUR1 consists of three transmembrane domains- TMD0, TMD1 andTMD2; each of which consist of five, five, and six membrane spanning regions respectively (Conti *et al.,* 2001). SUR1 also has two nucleotide binding domains (NBD-1 and NBD-2) on the cytoplasmic side (figure 1.2 a) with which it senses changes in the intracellular ATP/ADP ratio and transmits the signal to the pore. NBD1 appears to be the principal site for ATP binding, whereas NBD2 binds MgADP (Conti *et al.,* 2001). NBD-1 and NBD-2 are located in the loop between TMD1 and TMD2 and in the C-terminus, respectively. These binding domains cooperate with each other in mediating the nucleotide regulation of the pore function (Ueda *et al.,* 1999). NBDs of SUR contain highly conserved motifs among ABC proteins: Walker A motif, Walker B motif, ABC signature motif (also known as linker sequence or LSGGQ motif), and an invariant glutamine and histidine residue (also known as the Q-loop and H-loop, respectively). Walker A and Walker B motifs are directly involved in nucleotide binding (Matsuo *et al.,* 2005).

1.2 A



1.2 B

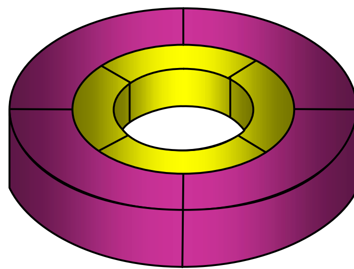


Figure 1.2A: Schematic outline of the components of the ß-cell K_{ATP} channel**.** The K_{ATP} channel is composed of two proteins: SUR1 which consists of 17 transmembrane domains with two intracellular nucleotide binding (NBD) motifs. Two N-linked glycosylation sites are present on amino acids 10 and 1049. Kir6.2 has 3 transmembrane segments.

Figure 1.2B: illustrating the hetero-octameric arrangement of the K_{ATP} channel.

(Figure adapted from James *et al.,* 2009.)

K_{ATP} channels can only function if they are assembled and correctly transported to the cell membrane surface (trafficking). The assembly and trafficking of K_{ATP} channels are intricately linked processes. Only octameric K_{ATP} channel complexes are capable of expressing on the cell membrane surface. For example both Kir6.2 and SUR1 possess an endoplasmic reticulum (ER) retention signal (RKR) that prevents the trafficking of each subunit to the plasma membrane in the absence of the other subunit (Zerangue 1999). Coexpression of two subunits masks these retention signals, allowing them to move to the plasma membrane. The retention signal is present in the C terminal region of Kir6.2 and in an intracellular loop between TMD1 and NBD-1 in SUR1. Truncation of the C-terminus of Kir6.2 deletes its retention signal, allowing functional expression of Kir6.2 in the absence of SUR1 subunit (Tucker *et al.,* 1997). In addition to these retrograde signals, the C terminus of SUR1 has an anterograde signal, composed in part of a dileucine motif and downstream phenylalanine, which is required for K_{ATP} channels to exit the ER/cis-Golgi compartments and transit to the cell surface (Sharma *et al.,* 1999). Deletion of as few as seven amino acids, including the phenylalanine, from SUR1 markedly reduces surface expression of K_{ATP} channels (Babenko *et al.,* 1998). Thus, one function of SUR is as a chaperone protein, to facilitate the surface expression of Kir6.2.

There is also some evidence that Kir6.2 provides a reciprocal service for SUR1 (Clement *et al.,* 1997).The SUR1 protein shows a high-affinity binding capacity to the sulfonylurea glibenclamide, indicating that SUR1 confers sulfonylurea binding (Inagaki *et al.,* 1996). The sulfonylurea drugs (glibenclamide and tolbutamide) inhibit the channels and are used in the treatment of non-insulin-dependent (type II) diabetes mellitus. The other class of drugs, known as potassium channel openers (*e.g.* diazoxide), activate the channel and are used to suppress insulin secretion.

In euglycaemic conditions, the K_{ATP} channels are open which allows potassium efflux from the pancreatic ß-cell. This keeps the ß-cell membrane at a negative potential at which voltage-gated calcium (Ca^{2+}) channels are closed. An increase in the blood glucose concentration leads to production of ATP. The increase in the ATP/ ADP ratio triggers closure of the K_{ATP} channels leading to the depolarisation of the ß-cell membrane. This in turn leads to the opening of the voltage gated Ca^{2+} channels and Ca^{2+} ion influx. The entry of calcium ions triggers exocytosis of insulin (figure 1.3).

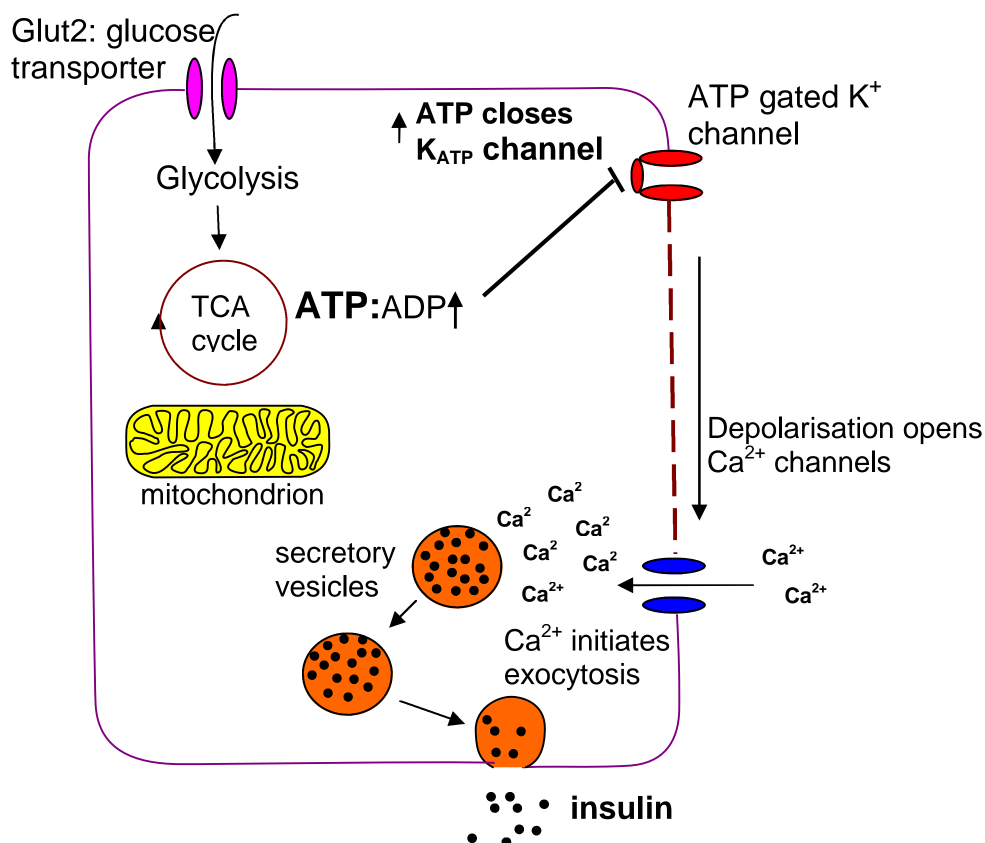


Figure 1.3: Outline of the pancreatic ß-cell showing the role of K_{ATP} channels in regulating insulin secretion. ß-cell K_{ATP} channels play a key role in transducing the metabolic signals generated from glucose metabolism to changes in plasma membrane electrical activity and insulin secretion. (Figure adapted from Kapoor *et al.*, 2009d)

Apart from the above described K_{ATP} channel-dependent pathway, glucose also stimulates insulin secretion via K_{ATP} channel independent pathways (Gembal *et al.,* 1992). The K_{ATP} channel-independent pathways act synergistically to augment the secretory responses to increased intracellular calcium and lead to the second and more sustained phase of glucose-stimulated insulin secretion. However the mechanisms of these pathways are poorly defined. The two widely proposed theories are:

1. High glucose concentrations lead to increased citrate levels and increased malonyl-CoA. This inhibits carnitine palmitoyl transferase-1 (CPT1) activity and leads to suppression of fatty acid oxidation (Corkey *et al.,* 1989, Prentki *et al.,* 1992). It has been proposed that the consequent accumulation of long chain acyl-CoAs in the extracellular space enhances the release of insulin by unknown mechanisms (Prentki *et al.,* 1992).

2. Glucose generates glutamate in the pancreatic ß-cell mitochondria which acts as a signalling molecule for insulin secretion (Maechler and Wollheim, 1999). In support of this theory, Maechler and Wollheim showed that glucose causes elevated intracellular glutamate concentration and direct addition of glutamate to INS-1 cells led to insulin secretion. However, these studies were performed on the INS-1 cell line that does not have a K_{ATP} channel independent pathway. Furthermore, other studies performed on pancreatic ß-cell islets did not show an elevation in intracellular glutamate levels following exposure to glucose or that increased intracellular glutamate (following exposure to glutamine) leads to insulin secretion (MacDonald and Fahein, 2000). In defence of the theory, it has been pointed out that a) the alkalinising effect of glutamine may adversely effect the cell and the affect of glutamate on insulin secretion and b) the findings in isolated islets may not represent what actually happens in the pancreatic ß-cell (Rutter, 2001).

It has also been proposed that adenine and guanine nucleotides, that control the K_{ATP} channel dependent pathway, also control the K_{ATP} channel-independent pathway (Detimary *et al.,* 1996). The control of both pathways by the intracellular nucleotide concentrations would explain the co-ordination between the two pathways. However this has not been supported by a report that studied the effect of chronic leucine exposure on islets. The study showed reduced glucose stimulated insulin secretion associated with a reduction in ATP/ADP ratio on chronic exposure to leucine. However the K_{ATP} independent insulin release remained unchanged despite the associated changes in the intracellular nucleotides (Anello *et al.* 2001).

In summary, although it is widely accepted that the second phase of glucose-stimulated insulin secretion is due to the K_{ATP} channel-independent pathways, the mechanisms involved in this pathway are poorly understood.

Finally, glucose also stimulates translation of preformed mRNA encoding insulin and increases the transcription of the insulin gene (Melloul *et al.,* 1993).

1.5.1.2 Amino acid stimulated insulin secretion

Amino acids can, under appropriate conditions, enhance insulin secretion from primary islet ß-cells and ß-cell lines. However, surprisingly little data exist on the molecular mechanisms through which metabolism of amino acids impact on changes of ionic flux and other processes leading to exocytosis of insulin. There are three fundamental mechanisms by which amino acids may stimulate insulin secretion *1*) direct depolarisation of the plasma membrane by transport of cationic amino acids such as L-arginine, resulting in opening of voltage-activated L-type Ca^{2+} channels; *2*) co-transport with Na^{+}, resulting in opening of voltage-activated L-type Ca^{2+} channels; and *3*) metabolism of the amino acid (e.g, L-

leucine), resulting in an elevation of the ATP/ADP ratio, which results in membrane depolarisation and Ca^{2+} influx following closure of the K_{ATP} channels.

The cationically charged amino acid, L-arginine has been shown to stimulate insulin secretion by direct depolarisation of the plasma membrane and resultant rise in intracellular Ca^{2+} concentrations (Smith *et al.,* 1997; Herchuelz *et al.,* 1984). However, as L-arginine is found at a very low physiological concentration in the plasma, this mechanism is probably not relevant *in vivo*. Other amino acids, which are co-transported with Na^{+}, can also depolarize the cell membrane as a consequence of Na^{+} transport and thus induce insulin secretion by activating voltage-dependent calcium channels.

L-alanine is consumed at high rates in rat islets and in BRIN-BD11 cell lines and this is enhanced in the presence of glucose (Dixon *et al*., 2003). L-alanine has been shown to have an insulinotropic effect in cell lines and in rat islets (Brennan *et al*., 2002) which may be due to co-transport with Na^{+} resulting in membrane depolarisation and increased intracellular calcium (Dunne *et al.,* 1990). L-alanine is also metabolized by BRIN-BD11 cells to give end products such as glutamate, aspartate, and lactate. (Brennan *et al.,* 2002). Hence insulinotropic action of L-alanine may be secondary to a combination of co-transport with Na^{+} and ATP generation via metabolism.

L-Glutamine has also been shown to be consumed at high rates by both islets and BRIN-BD11 ß-cells (Dixon *et al.,* 2003). Although L-glutamine is rapidly taken up and metabolized by islets, it alone does not initiate or potentiate insulin secretion. However, it stimulates L-leucine stimulated insulin secretion via the GDH (glutamate dehydrogenase) pathway. GDH is a highly allosteric enzyme and L-leucine is a potent activator of GDH. In the presence of L-leucine, GDH activation leads to increased entry of glutamine into the

TCA cycle and subsequent oxidation (Sener and Malaisse, 1980). It is known that glucose inhibits glutaminolysis in ß-cells presumably via GTP-dependent allosteric inhibition of GDH, resulting in accumulation of L-glutamate and thus product-dependent inhibition of glutaminase, and as a result blocks leucine-stimulated insulin secretion (Gao *et al.,* 1999).

The action of intracellular L-glutamate on insulin secretion has been the subject of considerable debate (Newsholme *et al.,* 2007). As discussed in section 1.5.1.1, confusion over the importance of glutamate in glucose-stimulated insulin secretion has arisen as a result of opposing findings: total cellular glutamate concentrations have been reported to increase in response to elevated glucose, in human, mouse and rat islets as well as in clonal ß-cells by some groups, causing increased insulin secretion (Maechler and Wollheim, 1999; Rubi *et al.,* 2001), whereas others reported no such change (Bertrand *et al.,* 2002).

L-Leucine stimulates insulin release in pancreatic ß-cells by a process that involves: (i) increased mitochondrial metabolism by activation of GDH and (ii) an increase in ATP production by transamination of leucine to *α*-ketoisocaproate and subsequent entry into the TCA cycle via acetyl-CoA (Sener and Malaisse, 1980). It has also been reported that leucine and *α*-ketoisocaproate stimulate insulin release via distinct mechanisms (Gao *et al.,* 2003).

In summary, although the exact mechanisms are not completely understood, it is clear that amino acids play an important role in regulation of insulin secretion and stimulate or synergistically increase insulin secretion by various mechanisms.

1.5.1.3 The role of fatty acid metabolism and insulin secretion

Free fatty acids are important to the pancreatic ß-cell for its normal function, its capacity to compensate for insulin resistance, and its failure in type 2 diabetes mellitus (McGarry 2002; Prentki *et al.,* 2002). Both stimulatory and detrimental effects of free fatty acids on pancreatic ß-cells have long been recognized (Haber *et al.,* 2003; Yaney and Corkey, 2002). Acute exposure of the pancreatic ß-cell to both high glucose concentrations and saturated free fatty acids results in a substantial increase of insulin release, whereas a chronic exposure results in desensitisation and suppression of secretion. Reduction of plasma free fatty acids levels in fasted rats or humans severely impairs glucose-induced insulin release. Physiological plasma levels of free fatty acids are therefore important for regulation of normal ß-cell function. However the key regulatory molecular mechanisms controlling the interplay between glucose and fatty acid metabolism and thus insulin secretion are not well understood. Several different mechanisms have been proposed by which fatty acids or their metabolites regulate insulin secretion.

One mechanism involves the AMP-activated protein kinase/malonyl-CoA/long-chain acyl-CoA (LC-CoA) signalling network in which glucose, together with other anaplerotic fuels, increases cytosolic malonyl-CoA, which inhibits fatty acid partitioning into oxidation, thus increasing the availability of LC-CoA for signalling purposes (Corkey *et al.,* 2000). Another mechanism involves glucose-responsive triglyceride (TG)/free fatty acid (FFA) cycling. In this pathway, glucose promotes LC-CoA esterification to complex lipids such as TG and diacylglycerol, concomitant with glucose stimulation of lipolysis of the esterification products, with renewal of the intracellular FFA pool for reactivation to LC-CoA. Diacylglycerol and LC-CoA may regulate increase insulin secretion by K_{ATP} channel dependent and independent mechanisms (Corkey *et al.,* 2000). The elevation of cytosolic LC-CoA could potentiate insulin secretion by direct acylation of regulatory proteins.

Alternatively, LC-CoAs may be converted to other bioactive metabolites, for example diacylglycerol (DAG), which activate downstream effectors such as protein kinase C (Prentki *et al.,* 1992).

Finally, FFAs have been shown to stimulate insulin secretion via activation of the G-protein coupled receptor GPR40 on the pancreatic ß-cell membrane (Itoh *et al.,* 2003). GPR40 is abundantly expressed in the ß-cells. In the presence of glucose, activation of GPR40 has been shown to cause an increase in the the intracellular levels of Ca^{2+}, leading to insulin secretion (Shapiro *et al.,* 2005). It is thought that this is mediated via increased phospholipase C activity which leads to the production of inositol 1,4,5-trisphosphate (IP_3) and DAG. DAG can activate protein kinase C with subsequent insulin secretion while IP3 can cause the release of stored Ca^{2+} (Morgan and Dhayal, 2009). Activation of GPR40 also has an effect on the voltage-gated potassium channels (Kv channels). The Kv channels play a role in membrane repolaristion and facilitate the closure of voltage sensitive Ca^{2+} channels. Activation of GPR40 receptors have been shown to inhibit these channels, enhancing membrane depolarization and increasing Ca^{2+} influx (Feng *et al.,* 2006).

1.5.1.3.1 The role of HADH in pancreatic ß-cell physiology

Mitochondrial Short-chain L-3-Hydroxyacyl-CoA dehydrogenase (HADH) is involved in the second dehydrogenation step of the ß-oxidation pathway (figure 1.4). It catalyses the conversion of L3-hydroxyacyl CoAs to 3-Ketoacyl CoAs, this reaction also utilises NAD^+ as cofactor producing NADH (He *et al.,* 1999; Vredendaal *et al.,* 1996). HADH is very highly expressed in pancreatic ß-cells suggesting it has an important role in insulin secretion (Agren *et al.,* 1977). ß-cells express relatively low levels of other ß-oxidation enzymes

such as acyl-CoA dehydrogenase short, medium, and long chain and acetyl-coenzyme A acyltransferase 2.

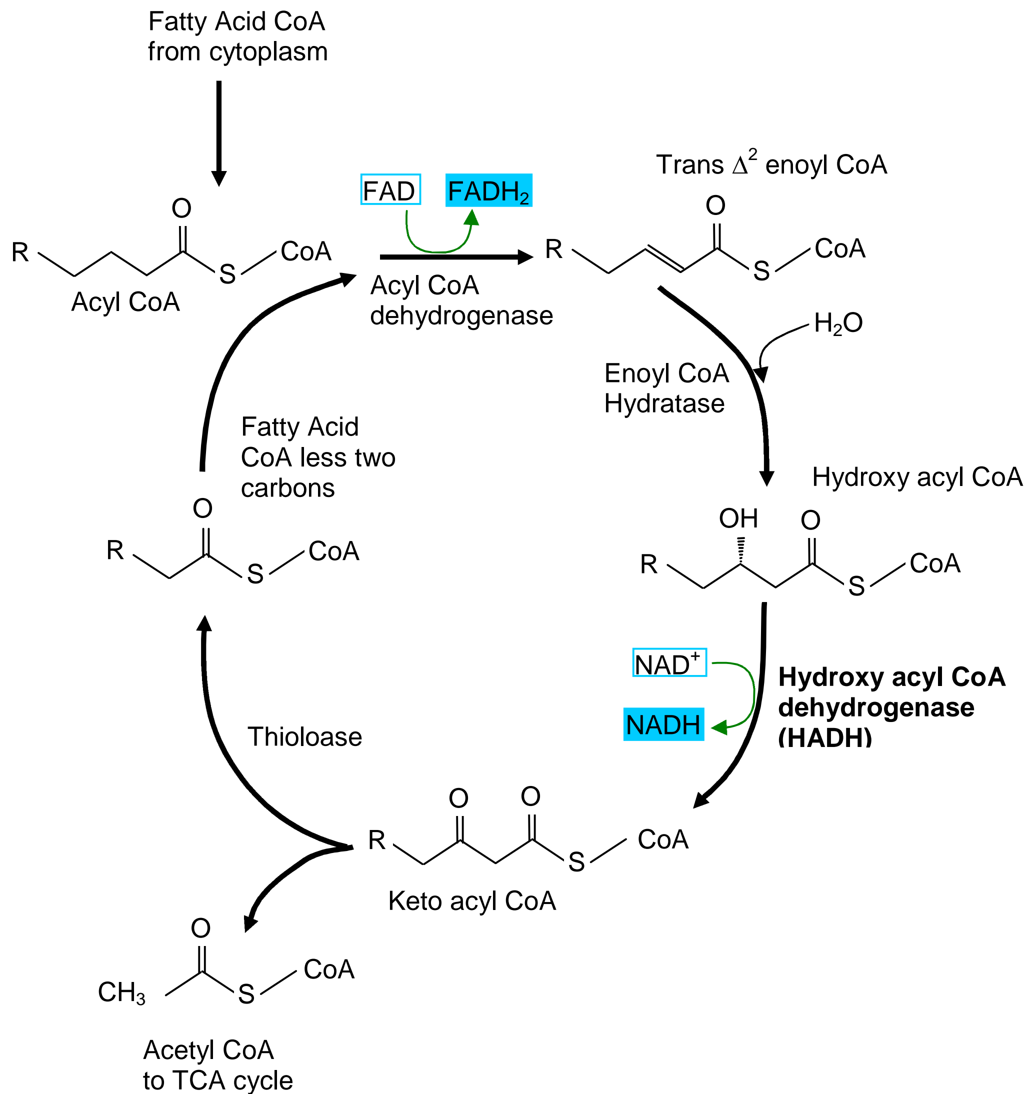


Figure 1.4: The role of HADH in fatty acid oxidation pathway. HADH is involved in the second step and catalyses the conversion of Hydroxy-acyl CoA to Keto acyl CoA, in the presence of NAD^+.

Several studies have now shown that HADH has a pivotal role in regulating insulin secretion (Hardy *et al.,* 2007; Martens *et al.,* 2007) and interacting with other genes which are known to be important for ß-cell development and function (Lantz *et al.,* 2004; Sund *et al.,* 2001). In one study suppression of HADH activity using siRNA caused a significant increase in basal insulin secretion compared to untreated cells (Hardy *et al.,* 2007). This demonstrates for the first time that HADH is required directly in ß-cells for the regulation of basal insulin release. The addition of diazoxide did not alter the enhanced basal insulin secretion caused by suppression of *HADH*, indicating that HADH functions directly in ß-cells to regulate a K_{ATP} channel independent pathway to insulin secretion.

In another study (Martens *et al.,* 2007) based on rat ß-cells and in the ß-cell line INS1 832-13, *HADH* silencing resulted in elevated insulin release at low and at high glucose concentrations, which appeared not to be caused by increased rates of glucose metabolism or an inhibition of fatty acid oxidation. These data indicated that the normal ß-cell phenotype is characterized by a high expression of *HADH* and a low expression of other ß-oxidation enzymes. Down-regulation of *HADH* causes an elevated secretory activity suggesting that this enzyme protects against inappropriately high insulin levels and hypoglycaemia (Martens *et al.,* 2007).

HADH also interacts with key genes involved in pancreatic ß-cell function. For example *Foxa2* is able to bind and trans-activate the *HADH* gene (Lantz *et al.,* 2004). ß-cell specific *Foxa2* knockout mice show a three fold down regulation of HADH (Lantz *et al.,* 2004). These mice have severe hyperinsulinaemic hypoglycaemia due to unregulated insulin secretion. *Foxa2* encodes a transcription factor (forkhead box A2) that has been postulated to play a central role in ß-cell development due to its ability to bind to and

transactivate *pancreatic duodenal homeobox 1* (*Pdx1*) *cis*-regulatory elements in vitro (Ben-Shushan *et al.,* 2001).

Hence it is clear from the limited studies above that HADH has an extremely important but unknown role in regulating insulin secretion. Understanding the molecular mechanisms of how mutations in the *HADH* gene lead to unregulated insulin secretion will provide novel insights into pancreatic ß-cell physiology.

1.5.2 Non-nutrient regulation of insulin secretion:

In addition to the regulation of insulin secretion by nutrients, a variety of hormones and neurotransmitters also regulate insulin secretion and are thought to potentiate the nutrient led insulin secretion.

1.5.2.1 Hormones

Hormones secreted from the other islet cells are known to regulate insulin secretion. For example, somatostatin secreted by the delta (δ) cells inhibits insulin release. Somatostatin may exert an inhibitory effect on insulin secretion via various mechanisms. It has been reported to inhibit insulin gene transcription (Redmon *et al.,* 1994) and adenylate cyclase (Seaquist *et al.,* 1992) and may also act by activation of K_{ATP} channels. In contrast, glucagon released by the alpha (α) cells of the islets is thought to stimulate insulin release. In a study on digested islets that caused α-cell loss, glucose induced insulin secretion was significantly reduced in comparison to normal islets (Li *et al.,* 2002). Insulin secretion was then shown to improve on application of exogenous glucagon, suggesting a paracrine regulation of insulin secretion by glucagon. Insulin itself is thought to inhibit glucagon secretion, supported by the decrease in glucagon response to hypoglycaemia in the

presence of intraislet hyperinsulinaemia (Banarer *et al.*, 2002). The actual decrease in insulin secretion during hypoglycaemia is thought to be an important signal in glucagon secretion (Israelian *et al.,* 2005). Insulin may inhibit glucagon secretion via K_{ATP} channel activation (Franklin *et al.,* 2005). Gamma aminobutyric acid (GABA), a neurotransmitter is a known inhibitor of glucagon secretion and insulin is also thought to exert its inhibitory action on secretion of glucagon by stimulation of Type A GABA receptors in the α-cells (Xu *et al.*, 2006).

In addition, the gastrointestinal hormones (incretins), glucagon-like peptide 1 (GLP-1) and gastric inhibitory peptide (GIP) are known to stimulate insulin secretion. These gut hormones account for the greater insulin secretion seen in response to an oral glucose load in comparison with an intravenous load (Fehmann HC *et al.*, 1995); called the incretin effect. GLP-1 is secreted from the intestinal endocrine L-cells, mainly located in the distal ileum and colon; while GIP is released from intestinal K-cells that are located more proximally in the small intestine. The main stimulus for the secretion of the gut hormones is a meal. GLP-1 and GIP have analogous effects on the pancreatic ß-cells. They exert their insulotropic effect via various mechanisms that include- increase in cAMP, direct inhibition of the K_{ATP} channels, direct effect on the Ca^{2+} channels and Ca^{2+} stores in the cells leading to raised intracellular Ca^{2+} levels, effect on the voltage dependent K^+ channels (Kv) and prevention of ß-cell repolarisation and stimulation of exocytosis (Baggio and Drucker, 2007).

1.5.2.2 Neurotransmitters and neuropeptides

The islets of Langerhans are highly innervated by sympathetic, parasympathetic and sensory nerves which store neurotransmitters and neuropeptides. The classical neurotransmitters include acetylcholine (parasympathetic) and noradrenaline

(sympathetic). The neuropeptides that are expressed in the islets and influence insulin secretion include vasoactive intestinal peptide (VIP), pituitary adenylate cyclase activating polypeptide (PACAP) and gastrin-releasing peptide (GRP) at the parasympathetic nerve terminals and galanine and neuropeptide Y at the sympathetic nerve terminals. Parasympathetic stimulation is known to stimulate insulin secretion and sympathetic stimulation inhibits insulin secretion.

Acetylcholine and the parasympathetic neuropeptides (VIP, PACAP and GRP) are all stimulated by vagal activation and are known to stimulate secretion of glucagon, pancreatic polypeptide and somatostatin along with insulin (Ahren B, 2000). Acetylcholine directly stimulates insulin secretion from the ß-cells via the muscarinic receptors. Binding of an agonist to the muscarinic acid receptors activates the phosphoinositide pathway which leads to insulin secretion via an increase in cytosolic Ca^{2+} levels and via activation of protein kinase C (PKC) (Ahren, 2000). VIP and PACAP stimulate insulin secretion by activation of adenylate cyclase and formation of cyclic AMP. cAMP has a stimulatory effect on extracellular Ca^{2+} intake and may also have an insulotropic effect by its action on the exocytosis machinery (Winzell and Ahren, 2007).

Noradrenaline inhibits insulin secretion via its effect on the α-2 adrenoceptors on the islet ß-cells. Activation of the α-2 adrenoceptors have been shown to decrease intracellular Ca^{2+} levels, decrease cAMP levels and also inhibit the exocytotic machinery, all inhibiting insulin secretion. However, catecholamines could also stimulate insulin secretion by activating the ß-2 adrenoceptors and by stimulating glucagon secretion (Ahren, 2000). Adrenaline also suppresses insulin release and may exert its effect on the presynaptic adrenoceptors. A study on isolated mouse pancreatic islets showed that the suppressive effect of adrenaline on insulin release requires intact sympathetic nerve terminals;

suggesting that adrenaline probably acts on the presynaptic adrenocoeptors and that its action on insulin release may be mediated via the release of an inhibitory neurotransmitter which may be noradrenaline or a pancreatic neuropeptide (Karlsson *et al.,* 1997).

1.6 Summary

Insulin release is regulated by a complex interplay of nutritional, hormonal and neural factors. Glucose is the major trigger for insulin secretion and along with the other nutrients and gastrointestinal hormones provides the signal for insulin release in the prandial phase.

Chapter 2- Introduction

Hyperinsulinaemic Hypoglycaemia

Hypoglycaemia or low blood glucose concentration is a common endocrine and metabolic problem encountered in childhood. Its particular importance in childhood stems from the deleterious neurodevelopmental sequelae of reduced blood glucose supply to the brain. The neurological outcome not only depends on the level and duration of hypoglycaemia; but it also depends on the cause of the hypoglycaemia. The cause of hypoglycaemia also determines the long term management of the child. The commonest cause of severe and persistent hypoglycaemia is hyperinsulinaemic hypoglycaemia.

2.1 Hyperinsulinaemic Hypoglycaemia

Under normal physiology, the pancreatic ß-cells secrete the appropriate amount of insulin to maintain normoglycaemia. The inappropriate secretion of insulin from pancreatic ß-cells in relation to the blood glucose concentration leads to hyperinsulinaemic hypoglycaemia (HH). This is a major cause of persistent and recurrent hypoglycaemia in the neonatal and infancy period (Aynsley-Green *et al.,* 2000). The inappropriate insulin secretion drives glucose into insulin sensitive tissues (such as skeletal muscle, adipose tissue and the liver) and simultaneously inhibits glucose production (via glycolysis and gluconeogenesis), as well as suppressing fatty acid release and ketone body synthesis (inhibition of lipolysis and ketogenesis). This metabolic “footprint” of insulin action (hyperinsulinaemic hypoglycaemia, with inappropriately low fatty acid and ketone body formation) makes the brain highly susceptible to hypoglycaemic brain injury. The brain is not only deprived of its most important substrate (namely glucose) but also ketone bodies which form an

alternative source of fuel. HH therefore is associated with a high risk of neurological handicap, cerebral palsy and epilepsy (Tyrrell VJ *et al.,* 2001, Meissner T *et al.,* 2003).

Any patient with recurrent or persistent hypoglycaemia can potentially have hyperinsulinism. A powerful clue to the dysregulated insulin secretion is the calculation of the intravenous glucose infusion rate required to maintain normoglycaemia. An intravenous glucose infusion rate of >8mg/kg/min (normal is 4-6mg/kg/min) is virtually diagnostic of hyperinsulinism. Biochemically, HH is diagnosed by the demonstration of an inappropriate concentration of serum insulin (and/or c-peptide) for the concentration of blood glucose (spontaneous or provoked) with inappropriately low concentrations of serum ketone bodies and fatty acids during the hypoglycaemic episode. A 'normal' concentration of insulin is abnormal in the context of a low blood glucose concentration (Aynsley-Green *et al.,* 2000). There is no correlation between the serum insulin concentration and the severity of the hypoglycaemia. In adults, ß-cells are exquisitely sensitive to the prevailing blood glucose concentration such that insulin secretion is suppressed when the blood glucose concentration reaches around 4mmol/L (Bolli and Fanelli, 1999). In the neonatal period, the serum insulin concentrations have been reported to be higher and not as closely linked to the blood glucose concentrations as in older children and adults (Hawdon *et al.,* 1993). Hence in some difficult cases the diagnosis of HH is not based on an isolated serum insulin concentration but on the clinical presentation and the biochemical profile of insulin action (low ß-hydroxybutyrate and fatty acid concentrations).

The counter-regulatory hormonal response to hypoglycaemia is blunted in some infants with HH with inappropriately low serum cortisol and glucagon concentration (Hussain *et al.,* 2003; Hussain *et al.,* 2005a). In some patients a positive glycaemic response (rise in the blood glucose concentration of >1.5mmol/L) following an intramuscular/intravenous injection of glucagon at the time of hypoglycaemia provides supportive evidence (Finegold

et al., 1980). A glycaemic response to a subcutaneous dose of octreotide may also aid diagnosis along with decreased serum concentrations of insulin growth factor binding protein 1 (IGFBP-1) as insulin suppresses the transcription of the *IGFBP-1* gene (Levitt Katz *et al.,* 1997). The diagnostic criteria for HH are summarized in table 2.1.

Table 2.1: Diagnostic biochemical features of hyperinsulinaemic hypoglycaemia (published Kapoor *et al.,* 2009c):

Glucose infusion rate >8 mg/kg/min
Laboratory blood glucose <3 mmol/L with -detectable serum insulin/C-peptide -suppressed/low serum ketone bodies -suppressed/low serum fatty acids -suppressed branch chain amino acids
Supportive evidence (when diagnosis is in doubt): Positive glycaemic (>1.5mmol/L) response to intramuscular/ intravenous glucagon Positive glycaemic response to a subcutaneous/intravenous dose of octreotide Low levels of serum IGFBP1

2.1.1 Causes of Hyperinsulinaemic Hypoglycaemia

HH may be congenital (congenital hyperinsulinism CHI) due to mutations in genes involved in regulating insulin secretion. Mutations in seven different genes (*ABCC8, KCNJ11, GLUD1, GCK, HADH*, *SLC16A1* and *HNF4A*) have been reported which lead to unregulated insulin secretion. The next section in this chapter gives a detailed overview of the mechanisms known to cause CHI.

HH may also develop secondary to certain risk factors (such as maternal diabetes mellitus, birth asphyxia and intra-uterine growth retardation) (Collins *et al.,* 1984) The HH observed

in these groups is sometimes referred to as “transient”. Most newborns with these risk factors will have HH that tends to last for a few days and then resolves. However a subgroup of newborns with intra-uterine growth retardation (IUGR) and perinatal asphyxia can have a protracted or prolonged form of HH that requires treatment with diazoxide, persists for several months, and then resolves spontaneously (Fafoula O *et al.,* 2006; Hoe *et al.,* 2006). At present it is not clear why some infants with perinatal asphyxia and IUGR have protracted HH.

HH may occur in association with developmental syndromes (such as Beckwith-Weidemann syndrome (BWS)) and rare metabolic conditions such as congenital disorders of glycosylation (CDG syndromes). The most common syndrome associated with HH is BWS. This syndrome is characterised by prenatal and/or postnatal overgrowth, macroglossia, anterior abdominal wall defects, organomegaly, hemihypertrophy, ear lobe creases, helical pits, renal tract abnormalities and a propensity to develop tumours. HH is observed in about 50% of patients with BWS (Munns *et al.,* 2001). In the vast majority of patients with BWS the HH is usually transient and resolves spontaneously. However a small number of patients (5% of cases), have persistent HH requiring medical therapy or even sub-total pancreatectomy (Hussain *et al.,* 2005b).

‘’Dumping syndrome’’ is a rare cause of HH, classically observed in infants in the post-prandial phase following gastro-oesophageal surgery (Bufler *et al.,* 2001). Another rare cause of post prandial HH is due to mutations in the insulin receptor gene (Højlund *et al.,* 2004). An insulinoma, isolated or as a part of multiple endocrine neoplasia syndrome type 1 (MEN1), may present with HH (Grant *et al.,* 2005). Finally, Munchausen by proxy can present as factitious HH due to administration of insulin or anti diabetic drugs such as

sulphonylureas. In some cases, this has led to misdiagnosis and consequent pancreatectomy (Giurgea *et al.*, 2005). The causes of HH are summarized in table 2.2.

Table 2.2: Summary of the known causes of hyperinsulinaemic hypoglycaemia (published Kapoor *et al.*, 2009c):

Causes of Hyperinsulinaemic Hypoglycaemia
Congenital Hyperinsulinism (Mode of inheritance) *ABCC8* (Autosomal recessive and dominant) *KCNJ11* (Autosomal recessive and dominant) *GLUD1* (Dominant) *GCK* (Dominant) *HADH* (Recessive) *HNF4A* (Dominant) *SLC16A1* (Exercise induced) (Dominant)
Secondary to (usually transient) Maternal diabetes mellitus (gestational and insulin dependent) Intrauterine growth retardation (IUGR) Perinatal asphyxia Rhesus isoimmunisation
Metabolic conditions Congenital disorders of glycosylation (CDG), Type 1a/b/d Tyrosinaemia type I
Associated with Syndromes Beckwith-Wiedemann Soto Kabuki Usher Timothy Costello Trisomy 13 Mosaic Turner Central Hypoventilation Syndrome
Other causes Dumping syndrome Insulinoma (sporadic or associated with Multiple Endocrine Neoplasia (MEN) Type 1) Insulin gene receptor mutations Factitious HH (Munchausen-by-proxy)

2.2 Congenital Hyperinsulinism

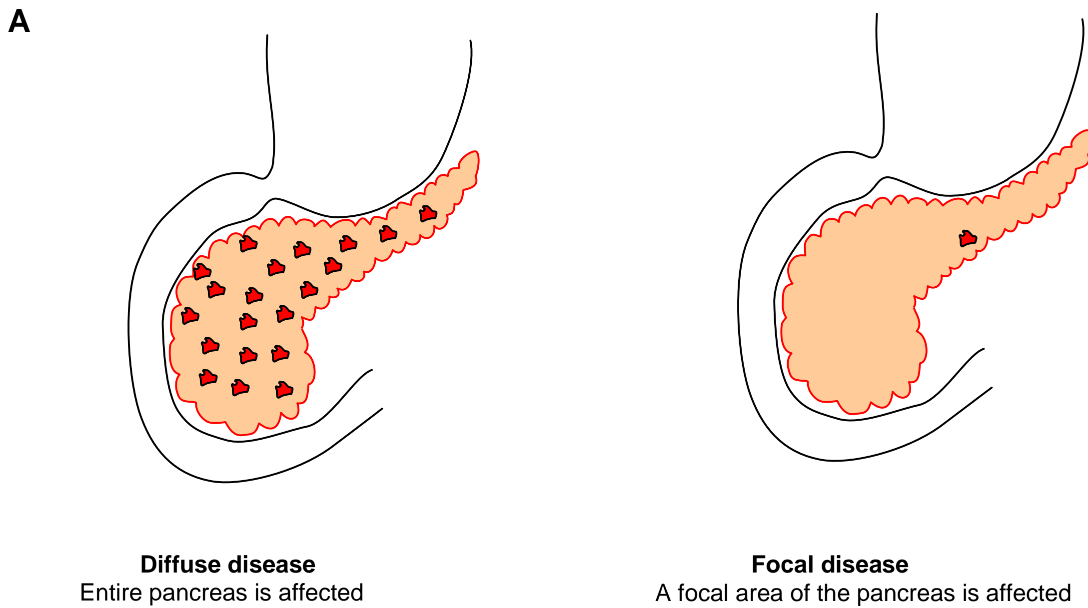
Congenital Hyperinsulinism (CHI) is a clinically, genetically, morphologically and functionally heterogeneous condition. Children with CHI may present with non-specific symptoms of hypoglycaemia such as poor feeding, lethargy and irritability or severe symptoms such as apnoea, seizures or coma. The age of presentation is variable with the severe forms typically presenting in the neonatal period and the milder forms presenting later in infancy or childhood with recurrent symptoms of hypoglycaemia. Symptoms generally develop after a period of fasting or when the child is unwell. However, some forms of CHI demonstrate protein and/ or leucine sensitivity and symptoms are manifested or aggravated in the post-prandial period following a protein rich meal rather than a fast (Hsu *et al.,* 2001). Macrosomia is a common feature in infants, reflecting fetal hyperinsulinaemia; however not all babies with CHI are macrosomic.

CHI occurs due to defects in key genes involved in regulating the secretion of insulin from ß-cells. So far, mutations in seven different genes (*ABCC8, KCNJ11, GLUD1, GCK, HADH*, *SLC16A1* and *HNF4A*) that lead to dysregulated secretion of insulin have been described (Hussain, 2008a). An understanding of the genetic mechanisms leading to CHI has helped in understanding the heterogeneity and the clinical management of this condition. However, mutations in these seven genes account for only 50% of the cases with CHI and the genetic basis of the remaining 50% is still unknown. Both sporadic and familial forms of CHI have been recognised. Sporadic CHI has an estimated incidence of 1 in 27,000 in Ireland and 1 in 50,000 in Finland (Otonkoski *et al.,* 1999). However, in communities where consanguinity is a common factor, the incidence can be as high as 1 in 3,000 (Otonkoski *et al.,* 1999).

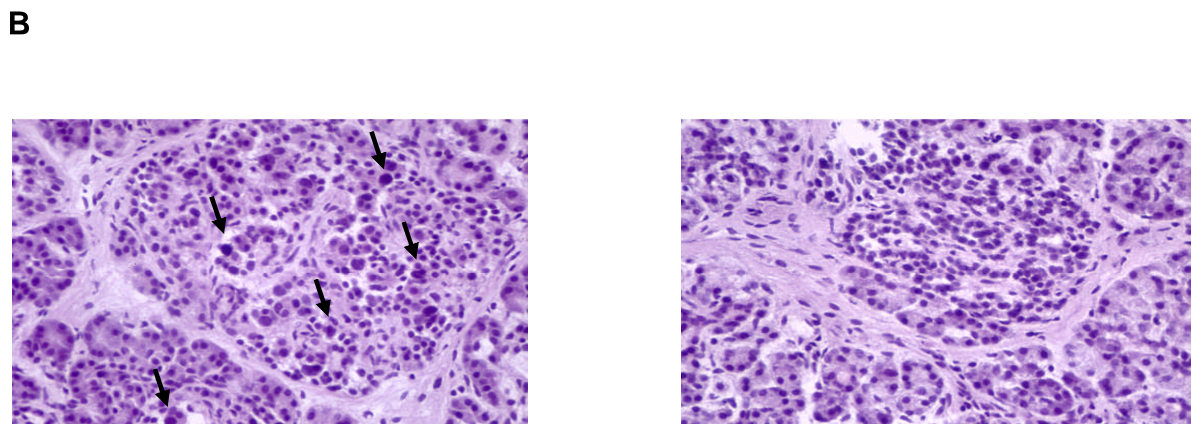
CHI is not only clinically and genetically heterogeneous; but is also varied in its histopathological forms. Histologically, it has been classified into a diffuse form where the pancreatic ß-cells show increased nuclear size throughout the pancreas on haematoxylin and eosin (H&E) staining; and a focal form characterized by the presence of a focal adenomatous hyperplasia with no nucleomegaly confined to a single region of the pancreas (figure 2.1). Recently, atypical forms of the disease have also been described where the histology cannot be classified into the diffuse or focal forms (Hussain *et al.,* 2008b).

Figure 2.1: The two major histological subgroups of CHI. A: Diffuse CHI affects the entire pancreas while the focal form is localised to a single region of the pancreas. (Figure adapted from Kapoor *et al.,* 2009c). B: Histological appearances of diffuse CHI vs focal CHI. In diffuse CHI, enlarged ß-cell nuclei (indicated by arrows) are visualised on H&E staining throughout the pancreas.

A



B



2.2.1 The genetic mechanisms causing CHI

As discussed in section 1.1.5.1.1, the pancreatic ß-cell adenosine triphosphate-sensitive potassium channels (K_{ATP} channels) play a pivotal role in glucose stimulated insulin secretion (GSIS). The channels couple glucose metabolism to membrane electrical activity and insulin release in pancreatic ß-cells. The SUR1 and Kir6.2 proteins are encoded by the *ABCC8* and *KCNJ11* genes, respectively. Activating mutations in these genes are known to cause neonatal diabetes mellitus (Flanagan *et al.,* 2007). Therefore, it is not surprising that inactivating mutations in these two genes lead to CHI. The molecular mechanisms causing CHI due to mutations in these genes and other genes known to cause CHI are discussed below.

2.2.1.1 *ABCC8* and *KCNJ11* gene mutations

A. Recessive inactivating mutations in *ABCC8* and *KCNJ11*

The *ABCC8* gene consists of 39 exons and spans more than 100kb of genomic DNA (Aguilar-Bryan *et al.*, 1995). The human SUR1 cDNA contains a single open reading frame that encodes for 1,582 amino acids with a molecular weight of 177kDa (GenBank NM_000352.2). Kir6.2 consists of a single exon encoding for a protein of 390 amino acids (GenBank NM_000525.2). The *ABCC8* and *KCNJ11* genes are both located on chromosome 11p15.1, separated by only a small stretch of 4.5 kb of DNA (Aguilar-Bryan *et al.*, 1995). Missense, frameshift, nonsense, insertions/deletions (macrodeletion), splice site and regulatory mutations have been reported in *ABCC8* and *KCNJ11*. So far more than 150 mutations have been reported in *ABCC8* and 25 in *KCNJ11* (Flanagan *et al.*, 2009). In the Ashkenazi Jewish population two common mutations (F1388del and c.3992–

9G>A) account for 90% of all cases of CHI (Nestorowicz *et al.,* 1996) whereas in the Finnish population, two founder mutations have been reported (V187D and E1507K) (Otonkoski *et al.,* 1999; Huopio *et al*., 2000). Recessive inactivating mutations in *ABCC8* and *KCNJ11* usually cause severe CHI which in the vast majority of patients is unresponsive to medical therapy (with diazoxide- the mainstay of medical treatment in CHI). However some recessively inherited mutations may be milder and may respond to treatment with diazoxide (Dekel *et al.,* 2002). The molecular basis of recessive inactivating *ABCC8* and *KCNJ11* mutations involves multiple defects in K_{ATP} channel biogenesis and turnover, in channel trafficking from the endoplasmic recticulum and Golgi apparatus to the plasma membrane and alterations of channels in response to both nucleotide regulation and open-state frequency. Compound heterozygous mutations may result in complex interactions (Muzyumba *et al.,* 2007) and the response to treatment is likely to be dependent on the combined effect of the two mutations.

i. Defects in channel biogenesis and turnover

The mechanisms that control the maturation and assembly of K_{ATP} channels are not well understood. Pulse-labeling studies have shown that when Kir6.2 is expressed individually, it is either degraded rapidly (about 60% is lost with a half life of 36 min) or it assembles very slowly into a long-lived species (half life of 26 h) (Crane *et al*., 2004). Expressed alone SUR1 has a long half life of 25.5 h. When Kir6.2 and SUR1 are co-expressed, they associate rapidly and the fast degradation of Kir6.2 is eliminated (Crane *et al*., 2004). Two mutations, *KCNJ11* (W91R) and *ABCC8* (F1388del), identified in patients with the severe form of CHI, profoundly alter the rate of Kir6.2 and SUR1 turnover, respectively (Crane *et al*., 2004). Both mutant subunits associate with their respective partners but dissociate freely and degrade rapidly suggesting that the mutations alter channel biogenesis and turnover.

ii. Defects in channel trafficking

Some mutations in *ABCC8* (such as R1437Q(23)X, F1388del and R1394H) have been shown to cause loss of channel activity due to a defect in the trafficking of the channel subunits from the endoplasmic recticulum compartment to the cell surface (Dunne *et al.,* 1997; Cartier *et al,* 2001; Partridge *et al.,* 2001 and Shyng *et al.,* 1998). Trafficking of K_{ATP} channels requires that the endoplasmic reticulum-retention signal, RKR, present in both SUR1 and Kir6.2 is shielded during channel assembly. Mutations such as L1544P may cause a trafficking defect by improper shielding of this RKR signal (Taschenberger *et al.,* 2002). In addition, an anterograde signal on the C-terminus has also been identified to play an important role in the surface expression of the K_{ATP} channels (Sharma *et al.,* 1999). Hence mutations in *ABCC8* that lead to C-terminal truncation may cause CHI by preventing normal trafficking.

Mutations in *KCNJ11* can also cause defective trafficking and truncated non-functional proteins. For example the Kir6.2 mutation (Y12X) causes the synthesis of a truncated nonfunctional protein (Nestorowicz *et al*., 1997) whereas another mutation (W91R) leads to defective channel assembly with a rapid degradation in the endoplasmic reticulum (ER) (Crane *et al*., 2004). Recently a new homozygous mutation H259R (*KCNJ11)* has been shown to lead to a nonfunctional K_{ATP} channel with impaired trafficking to the cell membrane (Marthinet *et al*., 2005).

iii. Defects of channel regulation

The SUR1 subunit plays a key role in determining the pharmacological regulation of K_{ATP} channels with SUR1 acting as a conductance regulator of Kir6.2. The sensitivity of K_{ATP} channels to changes in ATP, ADP, and guanosine (GTP, GDP) nucleotides involves both subunits. The functional regulation of K_{ATP} channels is induced by changes in the ATP/ADP

ratio. This involves cooperative interactions of nucleotides at both subunits with the actions of ATP-induced inhibition of Kir6.2 being countered by the activation of ADP at SUR1. Hence mutations that affect the regulation of the K_{ATP} channels by altering its sensitivity to changes in ADP/ATP will lead to unregulated insulin secretion. Several mutations in *ABCC8* (for example R1420C, T1139M and R1215Q) have now been described that result in the loss of ADP-dependent gating properties of the channel (Shyng *et al.,* 1998; Matsuo *et al.*, 2000 and Nichols *et al.*, 1996). Loss of ADP-dependent gating results in the constitutive inhibition of K_{ATP} channels by ATP.

B) Dominant inactivating *ABCC8* and *KCNJ11* mutations

Dominant inactivating mutations in *ABCC8* and *KCNJ11* have been described which lead to CHI (Huopio *et al.*, 2000; Thornton *et al.*, 2003; Lin *et al.*, 2006 and Pinney *et al.*, 2008). However the phenotype of patients with dominant inactivating mutations in *ABCC8* and *KCNJ11* seems to be much milder to that of patients with recessive inactivating *ABCC8* and *KCNJ11* mutations. Patients with dominant mutations seem to be responsive to medical therapy with diazoxide, may present later than those with recessive mutations and do not require a pancreatectomy (Pinney *et al.*, 2008). In one large study of 16 families with dominant CHI caused by mutations in *ABCC8* or *KCNJ11* all of the mutations were conservative single–amino acid changes, allowing for normal channel formation at the plasma membrane (Pinney *et al.*, 2008). Whereas recessive mutations cause near absence of K_{ATP} channel activity (have defects in channel biogenesis or trafficking of mature functional channels to the plasma membrane) dominant mutations demonstrate normal channel assembly with their respective wild type partner and normal trafficking of assembled channels to the plasma membrane when expressed in vitro.

A dominant missense CHI causing mutation F55L (*KCNJ11)* has also been shown to greatly reduce the open probability of K_{ATP} channels in intact cells without affecting channel expression (Lin *et al.*, 2006). It was shown that the low channel activity was due to reduced channel response to membrane phosphoinositides and/or long-chain acyl-CoAs, as application of exogenous phosphatidylinositol-4, 5-bisphosphate (PIP_2) or oleoyl-CoA restored channel activity similar to that seen in wild type channels.

C) The genetic basis of focal CHI

CHI presents as two different morphological forms: a diffuse form with functional abnormality of islets throughout the pancreas and a focal form with focal islet cell adenomatous hyperplasia, which can be cured by partial pancreatectomy (Rahier *et al.*, 2000). Focal CHI is characterized by nodular hyperplasia of islet-like cell clusters, including ductuloinsular complexes and hypertrophied insulin cells with giant nuclei (Rahier *et al.*, 2000; Goossens et al., 1989). The genetic aetiology of focal CHI is distinct from that of diffuse CHI. Focal adenomatous hyperplasia involves the specific loss of the maternal chromosome 11p15 region (loss of heterozygosity or LOH) and a constitutional mutation of a paternally inherited allele of the genes *ABCC8/KCNJ11* encoding the K_{ATP} channel (Verkarre *et al.*,1998; de Lonlay *et al.*, 1997; Fournet *et al.*, 2001; Fournet *et al.*, 2000). It has been recently demonstrated that the LOH results from paternal uniparental disomy (UPD) of chromosome 11p15.5-11p15.1 (Damaj *et al.*, 2008). The UPD unmasks the paternally inherited K_{ATP} channel mutation at 11p15.1 (figure 2.2) which leads to uncontrolled secretion of insulin.

LOH studies have shown that the affected region encompasses two regions of interest: the 11p15.5 region, subject to imprinting, and the 11p15.1 region containing the *ABCC8/*

KCNJ11 genes, which are not imprinted (de Lonlay *et al.*, 1997). The 11p15.5 chromosome region involved contains an imprinted domain, including several imprinted genes characterized by mono-allelic expression (Giannoukakis *et al.,* 1993; Lee *et al.,* 1997; Matsuoka *et al.,* 1995 and Zhang *et al.*, 1992). These include four maternally expressed genes (*H19* a candidate tumour suppressor gene, *P57KIP2* a negative regulator of cell proliferation, *KVLQT1* the gene coding for the potassium channel involved in the long QT syndrome, HASH2 a transcription factor and one paternally expressed gene the insulin-like growth factor 2 (*IGF2*) (Giannoukakis *et al.,* 1993; Zhang *et al.*, 1992; Hao *et al.*, 1993; Hatada *et al.*, 1996 and Guillemot *et al.*, 1995). The imbalance between imprinted genes (increased *IGF2* and diminished *H19* and *P57KIP2*) gives rise to the increase in the proliferation of the affected ß-cell, a striking feature of focal adenomatous hyperplasia not observed in the diffuse form (Fournet *et al.*, 2001). *H19* seems directly or indirectly to modulate cytoplasmic levels of the product of the *IGF2* allele and thus the *H19* gene seems to be an antagonist to *IGF2* in trans (Fournet *et al.*, 2001).

The probability for this somatic chromosomal event to occur in a foetus carrying a heterozygous mutation of *ABCC8/KCNJ11* of paternal origin is < 1% (Fournet *et al.*, 2001). Recently it was demonstrated that individual patients with focal CHI may have more than one focal pancreatic lesion due to separate somatic loss of the maternal allele encompassing the 11p15 region (Giurgea *et al.*, 2006).

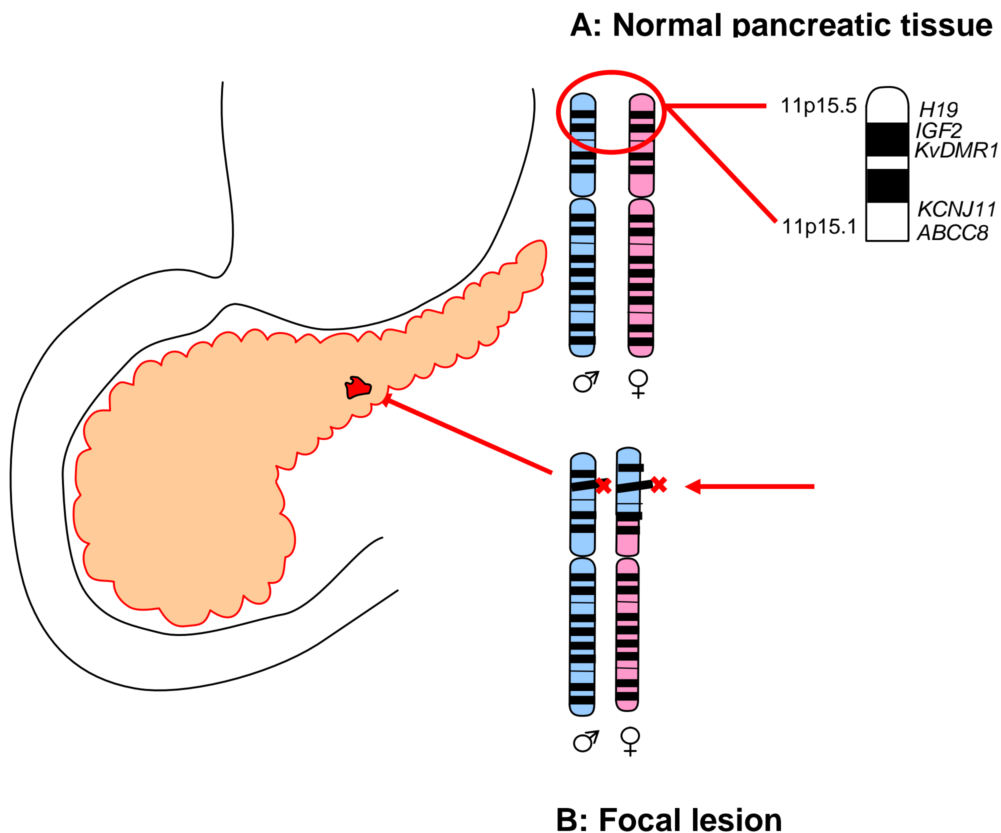


Fig 2.2: Genetic aetiology of a focal lesion (figure adapted from James *et al.*, 2009).

Fig 2.2A shows normal parental chromosomes 11 with the distal region of the short arm harboring the *ABCC8* and *KCNJ11* channel genes, and imprinted genes that influence cellular proliferation.

Fig 2.2B explains the genetic basis of a focal lesion caused by paternal uniparental disomy (UPD) of chromosome 11p15.5-11p15.1 within a single pancreatic ß-cell. The UPD unmasks the paternally inherited K_{ATP} channel mutation at 11p15.1 and causes altered expression of imprinted genes that include the maternally expressed tumour suppressor genes *H19* and *CDKN1C*, and the paternally expressed growth factor *IGF2*, at 11p15.5. This disruption in the expression of key cell cycle genes results in clonal expansion of the single cell and dysregulated insulin secretion from the resulting focal lesion.

2.2.1.2 Activating mutations in G*LUD1* (Glutamate Dehydrogenase) gene: the cause of Hyperinsulinism/ Hyperammonaemia (HI/HA) syndrome

Hyperinsulinism hyperammonaemia (HI/HA) syndrome (OMIM 606762) describes a form of CHI whereby patients have recurrent symptomatic HH associated with a raised serum ammonia concentration. HI/HA is the second most common form of CHI. Patients typically present with postprandial HH following the ingestion of a protein meal (due to leucine hypersensitivity) (Stanley *et al.,* 1998) though they can have fasting hypoglycaemia as well. Zammarchi *et al.* (1996) first described a family with leucine sensitive hypoglycaemia associated with a raised serum ammonia level. Then Weinzimer *et al.* (1997) described two more unrelated patients with a similar clinical and biochemical phenotype. Later, Stanley *et al.* (1998) were able to show that mutations in the *GLUD1* gene (which encodes for the intra-mitochondrial enzyme glutamate dehydrogenase, GDH) form the molecular and biochemical basis of the HI/HA syndrome and explain the link between leucine hypersensitivity and the hyperammonaemia.

Glutamate dehydrogenase (GDH) is highly expressed in the pancreas, liver, kidney and in the brain (Hudson *et al.,* 1993). It plays a key role in intermediary metabolism regulating protein synthesis and degradation. By an oxidative deamination reaction GDH converts glutamate to α-ketoglutarate and ammonia using NAD^+ (nicotinamide adenine dinucleotide) and $NADP^+$ as co-factors (Li *et al.*, 2009). The α-ketoglutarate is channelled into the citric acid cycle as a substrate to increase the ATP/ADP (adenosine triphosphate/ adenosine diphosphate) ratio. The increased ATP/ADP ratio causes the closure of the pancreatic ß-cell K_{ATP} channels with depolarisation of the ß-cell membrane, increased calcium entry and subsequent insulin exocytosis. The activity of GDH is under complex allosteric regulation by effectors such as ADP (adenosine diphosphate), GTP (guanine

triphosphate) and leucine (Fang *et al.*, 2002). In the HI/HA syndrome activating (heterozygous missense single amino acid substitutions) mutations in the *GLUD1* gene generally make GDH insensitive to repression by high energy phosphate compounds like GTP and in rare cases increase basal GDH activity (Yorifuji *et al.*, 1999 and Fujioka *et al.*, 2001). The loss of inhibition by GTP increases the rate of oxidation of glutamate in the presence of leucine, thereby increasing insulin secretion. The clinical picture is hence characterized by postprandial hypoglycaemia following a protein meal (fasting hypoglycaemia may also occur).

Human GDH is a homohexamer, with two trimeric subunits. Each subunit further is composed of three domains; the glutamate binding domain, the NAD binding domain, and the antenna domain (Smith *et al.*, 2001). The NAD^+ and glutamate-binding domains of bovine GDH are similar to those of the bacterial forms of the enzyme, while the antenna domain is unique to mammals (Rajagopalan *et al.*, 1999). The GTP binding site lies between the antenna and NAD binding domain while the ADP binding site lies behind the glutamate binding domain and under the pivot helix (Smith *et al.*, 2002). Missense mutations causing HI/HA were first described to occur in exons 11 and 12 that form the hinge and antenna regions of GDH and lie close to the binding site for GTP (Stanley *et al.,* 1998 and Stanley *et al.,* 2000). Mutations were then described in exons 6 and 7 that lie in the GTP binding region (Miki *et al.,* 2000; Santer *et al.,* 2001). Finally, mutations in exon 10 were described located in the α-helix of the antenna like structure of the GDH (Fujioka *et al.,* 2001; Yorifuji *et al.,* 1999). Most cases of HI/HA syndrome occur sporadically (Stanley *et al.,* 2000); though familial cases of dominantly inherited mutations are also described (Santer *et al.,*2001). These locations and the mutations described so far are depicted in figure 2.3A/B.

Figure 2.3A/ 2.3B: 2.3A: Cartoon showing location of known mutations along a representation of the *GLUD1* gene, the different colours signify the subunits of the protein. 2.3B. Representation of the structure of the GDH monomer, six of these make up the functional hexamer. The blue region interacts with GTP and ADP, the yellow structure is the pivot helix, the green region represents the antenna and the purple region represents the glutamate binding domain.

Figure 2.3A:

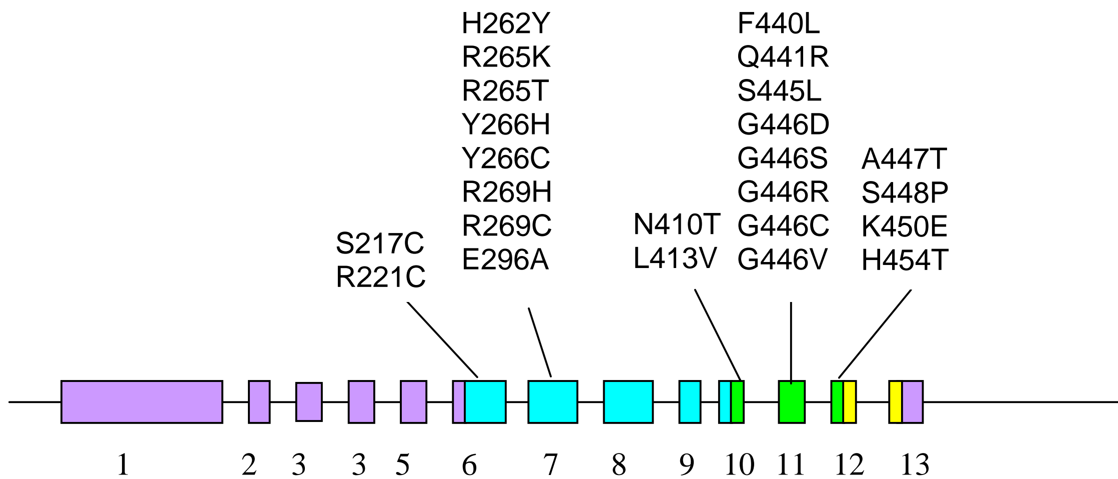
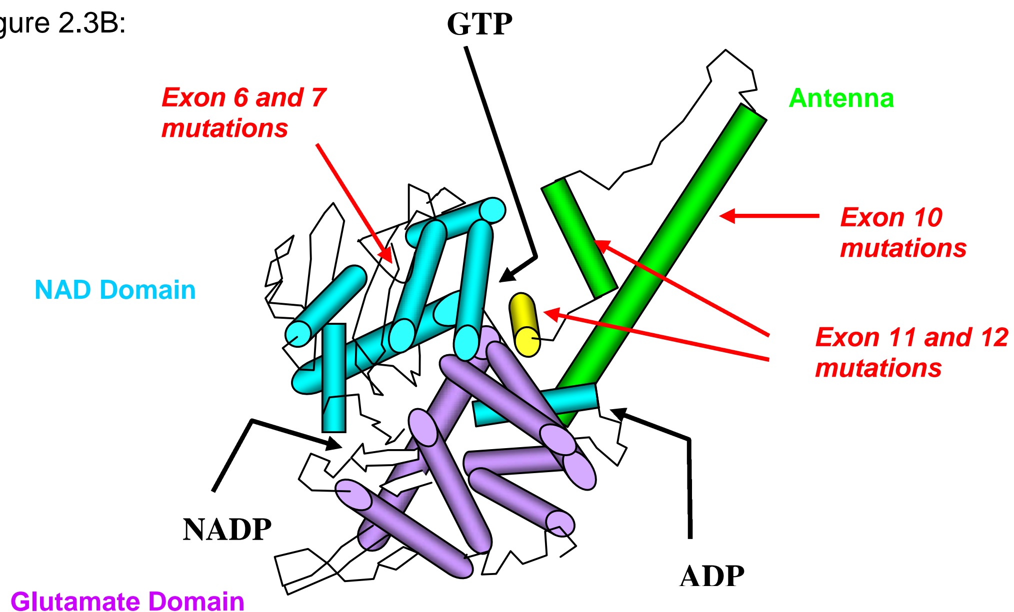


Figure 2.3B:



Most of the described mutations are at residues that directly bind with GTP, impairing the inhibition of GDH by GTP. However mutations occurring in residues that do not directly bind with GTP are also known to cause impaired GTP inhibition. It is postulated that these mutations either impair the communication between the subunits that is essential for GTP inhibition or have a deleterious effect on the complex motion of the protein causing loss of GTP binding (Smith *et al.,* 2008). Islets isolated from mice that express the mutant GDH in pancreatic ß-cells show an increased rate of glutaminolysis, increased insulin release in response to glutamine, and increased sensitivity to leucine-stimulated insulin secretion (Li *et al.,* 2006). Transgenic GDH mice expressing the H454Y mutation have a similar phenotype to humans with fasting hypoglycaemia as well as demonstrating leucine and glutamine stimulated insulin secretion (Li *et al.,* 2006).

The exact mechanism causing the hyperammonaemia remains unknown. It is possible that the hyperammonaemia is the result of increased GDH activity in the hepatocytes leading to either an increase in the production of ammonia from glutamate and/ or a depletion of glutamate with reduction in the production of N-acetylglutamate, an allosteric activator essential for the first step of ammonia detoxification (Kelly and Stanley 2001). N-acetylglutamate regulates the activity of carbamyl phosphate synthetase 1 (CPS) which produces urea from ammonia. These high levels of ammonia are resistant to treatment with drugs such as sodium benzoate, N-carbamyl glutamate or to protein restriction. The hyperammonaemia observed in these patients seems to be asymptomatic compared to patients who have hyperammonaemia due to other metabolic disorders such as the urea cycle disorders (Kelly and Stanley 2001). Figure 2.4 shows the postulated biochemical pathways leading to hyperinsulinaemia in the pancreas and hyperammonaemia in the liver.

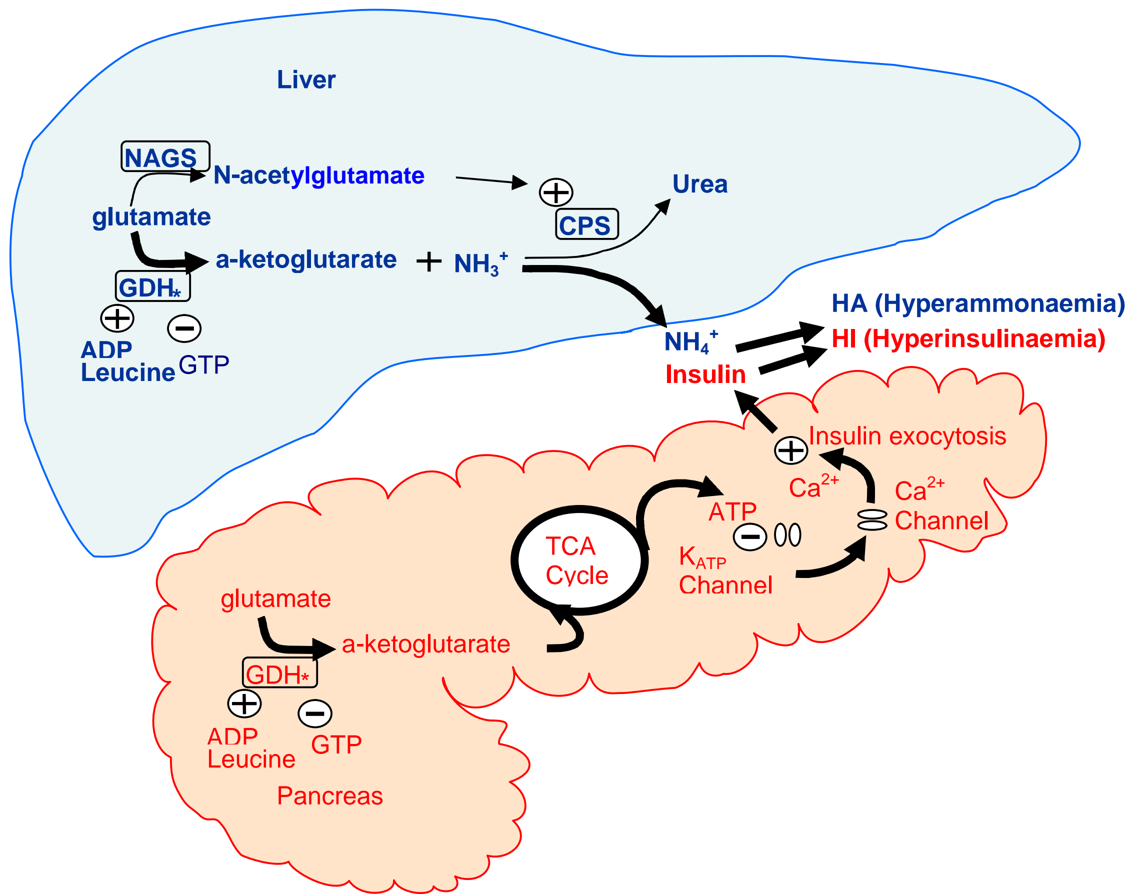


Figure 2.4: Postulated biochemical pathways leading to hyperinsulinaemia in the pancreas and hyperammonemia in the liver. In the pancreatic ß-cell increased activity of GDH supplies α- ketoglutarate to the TCA cycle generating ATP, which closes the ATP-gated potassium channel (K_{ATP}) depolarising the cell and opening calcium channels. Increased intracellular calcium promotes exocytosis of insulin vesicles. In the liver increased activity of GDH generates ammonia, and depleted glutamate results in reduced substrate for n-acetylglutamate synthase (NAGS). N-acetylglutamate is a co-factor for carbamoyl-phosphate synthetase 1 (CPS) which produces urea from ammonia.

Patients with HI/HA develop seizures and other neurological complications more frequently than other patients with CHI (Raizen *et al.,* 2005; Bahi- Buisson *et al.,* 2008a; Bahi-Buisson *et al.,* 2008b). In a cohort of patients with HI/HA, 77% of patients were reported to have a learning disability and 50% developed epilepsy (Bahi- Buisson *et al.,* 2008b) in comparison with a prevalence of 26-44% of developmental delay and 25% of epilepsy in all forms of CHI (Menni *et al.,* 2001; Meissner *et al.*, 2003). The frequency of seizures at presentation may be due to the effect of hypoglycaemic brain injury or the hyperammonaemia, or may be due to increased GDH activity in the brain (Bahi-Buisson *et al.,* 2008a). A study done on patients with HI/HA who developed epilepsy showed that they had structurally normal brain MRI scans, except for one patient who had frontal atrophy (Bahi- Buisson *et al.,* 2008b). Mutations in exon 6 and 7 of the *GLUD1* gene have been reported to be associated with the development of epilepsy in childhood in patients with HI/HA compared with mutations in exons 11 and 12 (Bahi- Buisson *et al.,* 2008b). Treatment of seizures with anticonvulsants such as carbamezapine or oxcarbezapine can worsen seizure frequency and therefore should be used with caution (Raizen *et al.,* 2005).

HI/HA is characterised by having mild to moderate hypoglycaemia which is usually responsive to diazoxide treatment at minimal doses (5-15mg/kg/day) in the majority of cases. In some patients protein restriction will also help in the management of the hypoglycaemia. It also has been shown that carbohydrate ingestion prior to ingestion of protein may reduce the leucine hypersensitivity of these patients (Kelly *et al.,* 2001). However there has been a case reported of severe hypoglycaemia in a patient with a *GLUD1* gene mutation who was resistant to medical treatment and required a pancreatectomy (MacMullen *et al.,* 2001).

In summary the HI/HA syndrome occurs due to activating mutations in the *GLUD1* gene. These mutations remove the inhibitory effect of GTP on the enzyme GDH and in turn increase enzyme activity by leucine. Patients typically present with HH following the ingestion of a protein meal (leucine hypersensitivity). The characteristic biochemical finding is hyperammonaemia, which is unaffected by feeding, fasting or pharmacological intervention. Patients with HI/HA syndrome have a high risk of developing epilepsy. Dietary protein restriction and pharmacological intervention with diazoxide is the mainstay of treatment.

2.2.1.3 Activating mutations in Glucokinase (*GCK*) gene

Glucokinase (hexokinase IV or D) is one member of the hexokinase family of enzymes. The name, glucokinase, is derived from its relative specificity for glucose under physiologic conditions. Glucokinase is a key regulatory enzyme in the pancreatic ß-cells. It operates as a monomer and phosphorylates glucose on carbon 6 with MgATP as a second substrate to form glucose-6-phosphate (G6P) as a first step in the glycolytic pathway. It plays a crucial role in the regulation of insulin secretion and has been termed the pancreatic ß-cell glucose sensor on account of its kinetics, which allows pancreatic ß-cells to change glucose phosphorylation rate over a range of physiological glucose concentrations (4–15mmol/L) (Matschinsky, 2002). These kinetic characteristics are the enzyme's low affinity for glucose (concentration of glucose at which the enzyme is half maximally activated, 8–10mmol/L), positive cooperativity for glucose, and lack of inhibition by its product G6P (Matschinsky, 2002). The enzyme has at least two clefts, one for the active site, binding glucose and MgATP and the other for a putative allosteric activator. Glucokinase activity is closely linked to the K_{ATP} and calcium channels of the ß-cell membrane, resulting in a threshold for glucose-stimulated insulin release of approximately 5mmol/L, which is the set point of glucose homoeostasis (Zelent *et al.*, 2005).

Heterozygous inactivating mutations in *GCK* cause maturity-onset diabetes of the young (MODY), homozygous inactivating *GCK* mutations result in permanent neonatal diabetes whereas heterozygous activating *GCK* mutations cause CHI (Glaser *et al.*, 1998; Gloyn *et al.*, 2003; Cuesta-Munoz *et al.*, 2004). A number of heterozygous *GCK* mutations have been reported in sporadic cases (*de novo* mutations) or families with dominantly inherited CHI. Activating *GCK* mutations increase the affinity of GCK for glucose and alter (reset) the threshold for glucose stimulated insulin secretion. All reported activating mutations

cluster in a region of the enzyme which has been termed the allosteric activator site and is remote to the substrate-binding site. The allosteric site of GCK is where small molecule activators bind, suggesting a critical role of the allosteric site in the regulation of GCK activity (Ralph *et al.*, 2008). Both GCK activators and activating mutations increase enzyme activity by enhancing the affinity for glucose (Heredia *et al.*, 2006). There is no evidence to suggest that over expression of GCK (increased gene dosage effect) is a likely cause of CHI (Van de Bunt 2008).

The clinical symptoms and course of patients with *GCK* mutations cover a broad spectrum even within the same family with the same mutation, implicating a complex mechanism for GCK regulation. Most of the *GCK* mutations reported to date cause mild diazoxide responsive CHI. However a “de novo” mutation in *GCK* (Y214C) was described in a patient with medically unresponsive CHI (Cuesta-Munoz *et al.*, 2004). This mutation was located in the putative allosteric activator domain of the protein and functional studies of purified recombinant glutathionyl S transferase fusion protein of GK-Y214C showed a sixfold increase in its affinity for glucose with the threshold for GSIS predicted to be 0.8mmol/L, as compared with 5mmol/L in the wild-type enzyme. Recently, three patients were described where diazoxide therapy was effective in the patient with the M197I mutation, ineffective in the patient with the ins454A mutation and only partially effective in the patient with the W99L mutation (Sayed *et al.,* 2009). The threshold for GSIS was estimated to be 1.1mmol/L for the ins454A mutation, 2.2mmol/L for the W99L mutation and 3.5mmol/L for the M197I mutation, accounting for the heterogeneity in the phenotype. In the largest study performed to date on a pool of patients (n=108) who were negative for mutations in the *ABCC8* and *KCNJ11* genes the prevalence of CHI due to mutations in *GCK* was estimated to be about 7% (Christesen *et al.*, 2008).

2.2.1.4 Loss of function mutations in Hydroxyacyl-CoA Dehydrogenase (*HADH)* gene

HADH encodes for the enzyme L-3-hydroxyacyl-Coenzyme A dehydrogenase (HADH), (previously known as Short-chain L-3-Hydroxyacyl-CoA dehydrogenase SCHAD), which is an intra-mitochondrial enzyme that catalyses the penultimate step in the ß-oxidation of fatty acids, the NAD^+ dependent dehydrogenation of 3-hydroxyacyl–CoA to the corresponding 3-ketoacyl-CoA. Human *HADH* encodes a 314-amino acid protein with 8 exons and spans approximately 49 kb (Vredendaal *et al.,* 1996). It is composed of a 12-residue mitochondrial import signal peptide and a 302-residue mature HADH protein with a calculated molecular mass of 34.3kD (Vredendaal *et al.,* 1998).

Loss-of-function mutations in the *HADH* gene are associated with CHI (Clayton *et al.,* 2001; Molven *et al.,* 2004; Hussain *et al.,* 2005c). The clinical presentation of all patients reported is heterogeneous, with either mild late onset intermittent HH or severe neonatal hypoglycaemia. All reported cases so far have presented with increased 3-hydroxyglutarate in urine and hydroxybutyrylcarnitine in blood which may be used as diagnostically useful markers for HADH deficiency. In the first patient reported sequencing of the *HADH* genomic DNA from the fibroblasts showed a homozygous mutation (c.773C>T) changing proline to leucine at amino acid 258 (Clayton *et al.,* 2001). Analysis of blood from the parents showed they were heterozygous for this mutation. Western blot studies showed undetectable levels of immunoreactive HADH protein in the patient's fibroblasts. Expression studies showed that the P258L enzyme had no catalytic activity. This patient presented with intermittent hypoglycaemia at 4 months of age.

A novel, homozygous deletion mutation (deletion of the six base pairs CAGGTC at the start of *HADH ex*on 5) was found in the second patient who presented with severe neonatal hypoglycaemia (Molven *et al.,* 2004). The mutation affected RNA splicing and was predicted to lead to a protein lacking 30 amino acids. The observations at the molecular level were confirmed by demonstrating greatly reduced HADH activity in the patients' fibroblasts and enhanced levels of 3-hydroxybutyryl-carnitine in their plasma. Urine metabolite analysis showed that HADH deficiency resulted in specific excretion of 3-hydroxyglutaric acid. Finally the third patient reported to date was found to be homozygous for a splice site mutation (IVS6-2 a>g) in the *HADH* gene with western blotting with an anti-HADH antibody indicating a decrease in the amount of immunoreactive protein in fibroblasts from the patient consistent with the observed decrease in enzyme activity (Hussain *et al.,* 2005c). This patient presented at 4 months of age with HH that responded to treatment with diazoxide.

The molecular mechanism of how loss of function in the *HADH* gene leads to unregulated insulin secretion is still unclear. Several recent studies in rodents have begun to give some insight into how *HADH* regulates insulin secretion and its interaction with other genes involved in ß-cell development and function (Hardy *et al.,* 2007; Martens *et al.,* 2007; Lantz *et al.,* 2004). The normal ß-cell phenotype is characterized by a high expression of *HADH* and a low expression of other ß-oxidation enzymes. Down-regulation of *HADH* causes an elevated secretory activity suggesting that this enzyme protects against inappropriately high insulin levels and hypoglycaemia (Hardy *et al.,* 2007; Martens *et al.,* 2007). Hence HADH seems to be a negative regulator of insulin secretion in ß-cells. Further studies will be required to fully understand the biochemical pathways by which defects in *HADH* lead to dysregulated insulin secretion.

2.2.1.5 Mutations in the promoter region of *SLC16A1* gene: the cause of exercise induced hyperinsulinism (EIHI)

In the glycolytic pathway glucose is metabolised to pyruvate which then enters in the mitochondria. Pyruvate can be converted into lactate or enters into the tricarboxylic acid (TCA) cycle, generating reducing equivalents. This leads to stimulation of the respiratory chain and ATP synthesis. The transport of monocarboxylates such as lactate and pyruvate is mediated by the SLC16A family of proton-linked membrane transport proteins known as monocarboxylate transporters (MCTs). Fourteen MCT-related genes have been identified in mammals and of these seven MCTs have been functionally characterized. Despite their sequence homology, only MCT1–4 have been demonstrated to be proton-dependent transporters of monocarboxylic acids (Halestrap and Price, 1999).

The *SLC16A1* gene encodes for MCT1 that mediates the movement of lactate and pyruvate across cell membranes. The *SLC16A1* gene maps to chromosome 1p13.2-p12, spans approximately 44 kb, and is organized as 5 exons intervened by 4 introns (Cuff and Shirazi-Beechey, 2002; Garcia et al., 1994). The first of these introns is located in the 5' UTR-encoding DNA, spans >26 kb, and thus accounts for approximately 60% of the entire transcription unit (Cuff and Shirazi-Beechey, 2002). Analysis of a 1.5 kb fragment of the *MCT1* 5' flanking region shows an absence of the classical TATA-Box motif. However, the region contains potential binding sites for a variety of transcription factors (Cuff and Shirazi-Beechey, 2002).

Studies in whole rat and mouse islets have shown that pyruvate and lactate cannot mimic the effect of glucose on insulin secretion despite active metabolism (Zhao and Rutter, 1998; Ishihara *et al.*, 1999). This is postulated to be due to low expression of MCT in ß-

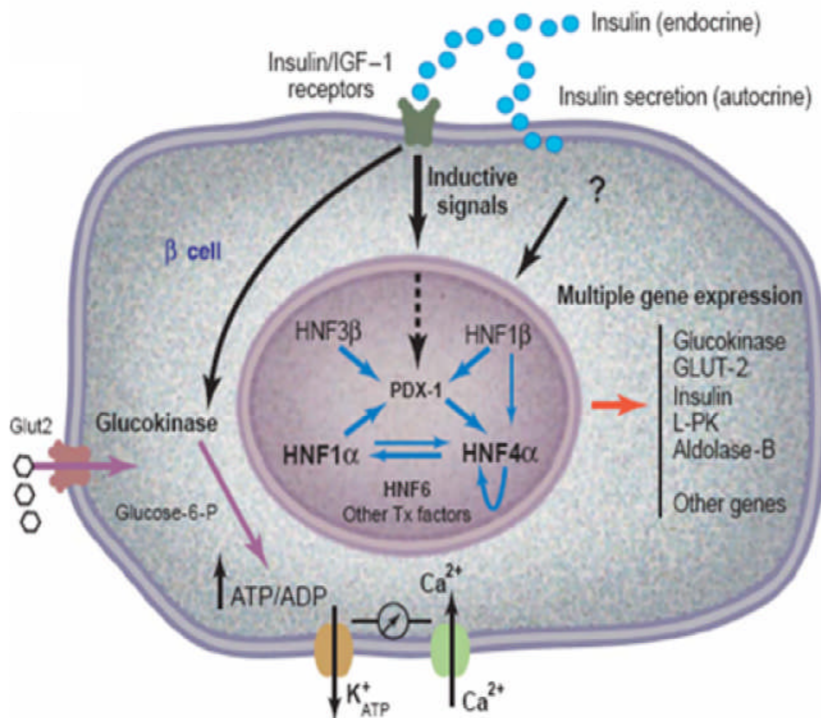
cells. However over expression of LDH and MCT1 leads to pyruvate induced insulin secretion (Ishihara *et al.*, 1999). In exercise induced CHI there is increased expression of MCT1 transporter in ß-cells due to dominant mutations in *SLC16A1* (Otonkoski *et al.*, 2007). In these patients anaerobic physical exercise induces HH that is preceded by an inappropriate increase in the circulating insulin concentration (Meissner *et al.*, 2001; Meissner *et al.*, 2005). Affected patients become hypoglycemic within 30 min after a short period of anaerobic exercise. A pyruvate load test causes a brisk increase in the serum insulin concentration suggesting that pyruvate metabolism or transport is in some way involved in signaling insulin secretion from ß-cells in these patients (Otonkoski *et al.*, 2003). A genome scan performed on two families with 10 affected patients first mapped the gene to chromosome 1 (Otonkoski *et al.*, 2007). Mutations in the promoter region of *SLC16A1* gene were confirmed in all patients. Studies on cultured fibroblasts from affected patients showed abnormally high *SLC16A1* transcript levels, although the MCT1 transport activity was unchanged in fibroblasts (possibly reflecting additional posttranscriptional control of MCT1 levels in extrapancreatic tissues).

The mutations identified in two separate pedigrees were in the binding sites of several transcription factors (nuclear matrix protein 1, albumin negative factor (ANF) and AML-1a, simian- virus-40-protein-1 (Sp1), upstream stimulatory factor (USF), myeloid zinc finger 1 (MZF1) and GATA-1–binding site). They resulted in increased protein binding to the corresponding promoter elements and marked (3- or 10-fold) transcriptional stimulation of *SLC16A1* promoter-reporter constructs (Otonkoski *et al.*, 2007). These studies suggested that activating mutations in the promoter region of *SLC16A1* induce increased expression of MCT1 in the ß-cell (where this gene is not usually transcribed) allowing pyruvate uptake and pyruvate-stimulated insulin release despite ensuing hypoglycaemia (Otonkoski *et al.*, 2007).

2.2.1.6 Mutations in the *HNF4A* gene: a novel cause of CHI

HNF-4α (hepatocyte nuclear factor 4alpha) is a transcription factor of the nuclear hormone receptor superfamily and is expressed in liver, kidney, gut, and pancreatic islets (Sladek *et al.*, 1990). In combination with other hepatocyte nuclear factors, HNF4α has been proposed to form a functional regulatory loop that regulates the development and function of the pancreas and the liver (Boj *et al.*, 2001; Odom *et al.*, 2004). In ß-cells, HNF4α has been shown to be the most widely acting transcription factor and regulates several key genes involved in glucose stimulated insulin secretion (Odom *et al.*, 2004; Wang *et al.*, 2000) (figure 2.5).

Mutations in the *HNF4A* gene (encoding the hepatocyte nuclear factor 4alpha, HNF-4α) are a novel cause of transient and persistent HH, associated with macrosomia (Pearson *et al.*, 2007). Mutations in this gene are known to cause maturity onset diabetes of the young type1 (MODY1), characterized by autosomal dominant inheritance and impaired glucose-stimulated insulin secretion from pancreatic ß-cells (Yamagata *et al.*, 1996).



Cited by Kulkarni *et al.*, *Science.* 2004,**303**;1311-1312

Figure 2.5: Figure published by Kulkarni *et al.* (2004) demonstrating the interaction of HNFs in the pancreatic ß-cells and the regulation of several target genes by the transcription factors. HNF4α is the most widely acting transcription factor, regulating the expression of other transcription factors and that of several key genes involved in glucose stimulated insulin secretion

The *HNF4A* gene is located on human chromosome 20q13.1-13.2. The gene has two promoters P1 and P2 with P2 being upstream to P1. At least nine isoforms (*HNF4A1-9*) are known to be produced by the gene by means of alternate splicing, polyadenylation (addition of poly(A) tail to the RNA molecule) and production of different transcripts by the two promoters (Harries *et al.,* 2008). The P1 derived isoforms are most abundant in the hepatic tissues while the P2 promoter represents the major transcription site in ß-cells

(Harries *et al.,* 2008). Transfection assays with various deletions and mutants of the P2 promoter revealed the presence of functional binding sites for *HNF1A, HNF1B*, and *IPF1* (Thomas *et al*., 2001). The HNF4α protein consists of an N-terminal ligand-independent transactivation domain (amino acids 1-24), a DNA binding domain containing two zinc fingers (amino acids 51-117), and a large hydrophobic portion (amino acids 163-368) composed of the dimerization, ligand binding, cofactor binding, and ligand-dependent transactivation domain (Hadzopoulou-Cladaras *et al*., 1997).

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Heterozygous mutations in the human *HNF4A* gene classically lead to maturity onset diabetes of the young subtype 1 (MODY1). MODY is a type of monogenic diabetes that usually presents in early adulthood and is characterized by autosomal dominant inheritance and impaired glucose-stimulated insulin secretion from pancreatic ß-cells (Yamagata *et al*., 1996). These mutations in the *HNF4A* gene cause multiple defects in glucose stimulated insulin secretion and in expression of *HNF4A* dependent genes (Wang *et al*., 2000; Stoffel and Duncan, 1997). Recently mutations in the *HNF4A* gene were reported to cause macrosomia and transient HH (Pearson *et al*., 2007). In this retrospective study the birth weight of the *HNF4A* mutation carriers compared to non-mutation family members was increased by a median of 790 g. Transient hypoglycaemia

was reported in 8/54 infants with heterozygous *HNF4A* mutations and documented HH in three cases (Pearson *et al.*, 2007).

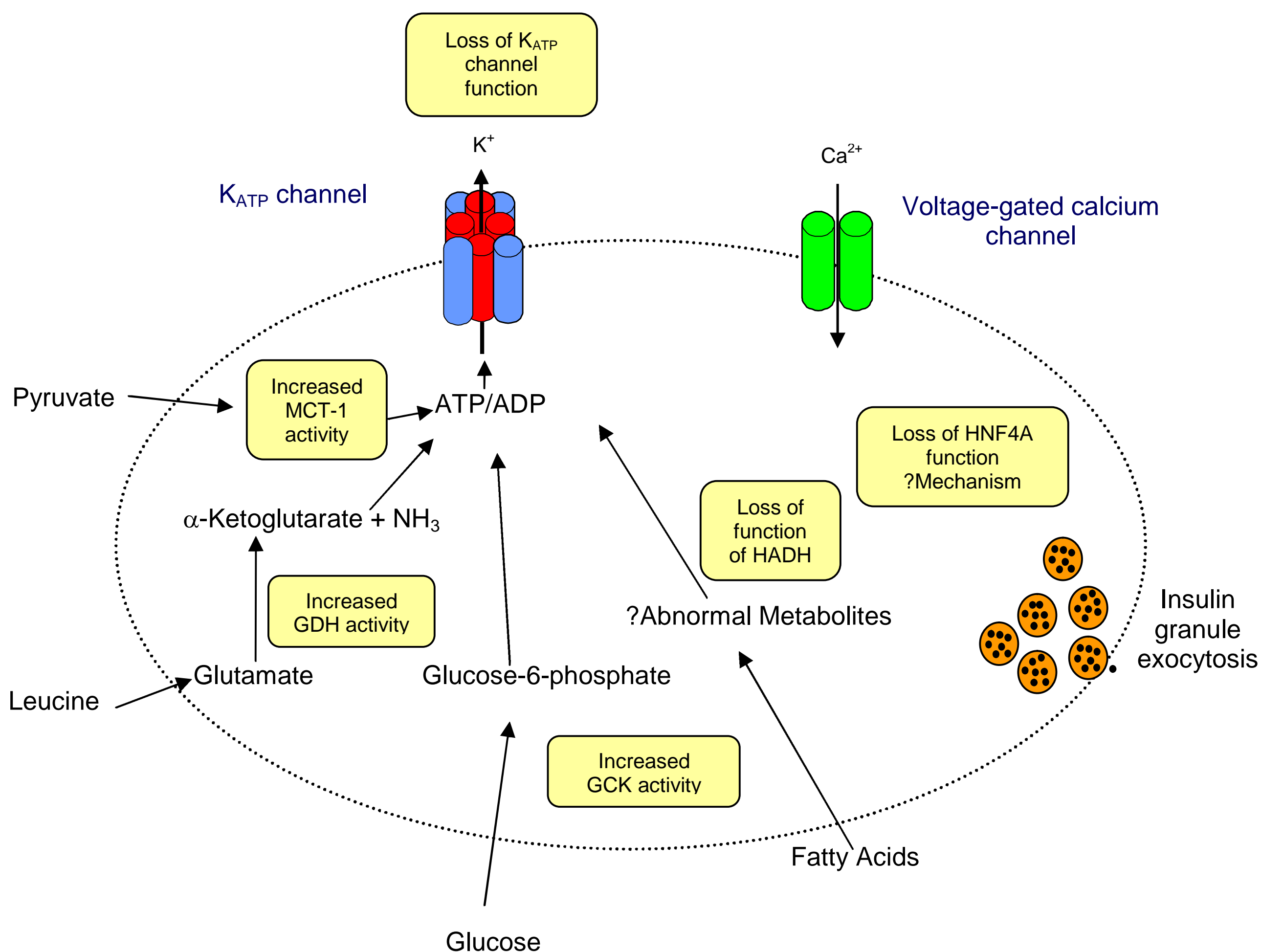
At present it is unclear how heterozygous mutations in *HNF4A* lead to hypoglycaemia. Using the conditional Cre-loxP-based inactivation system to delete *HNF4A* specifically in the pancreatic ß-cell, Gupta et al studied glucose homeostasis in the adult mice (Gupta *et al.*, 2005). These mice were hyperinsulinaemic in fasted and fed state but also paradoxically had impaired glucose tolerance with inadequate insulin secretion after glucose stimulation. Cotransfection assays demonstrated that these mice had a 60% reduction in the expression of the Kir6.2 subunit of the potassium channel. However two further studies (Pearson *et al.*, 2007; Miura *et al.*, 2006) have reported no change in the expression of Kir6.2 in *HNF4A*- deficient mice suggesting that this loss of expression of Kir6.2 in the pancreatic ß-cell may not be the cause of the dysregulated insulin secretion. Hence, it is unclear how heterozygous mutations in the *HNF4A* gene cause HH in the newborn period followed by the opposite phenotype of MODY-1 in young adulthood.

In summary, several molecular mechanisms due to mutations in seven different genes are known to cause CHI. These mechanisms are enlisted in table 2.3 and are schematically represented in a cartoon model of the pancreatic ß-cell in figure 2.6.

Table 2.3: The genes implicated in CHI with the gene loci, proteins affected and patterns of inheritance (AD- autosomal dominant, AR- autosomal recessive) (James *et al.,* 2009).

Gene (locus)	#OMIM	Protein	Mechanism	Inheritance
ABCC8 (11p15.1)	600509	Sulphonylurea receptor1 (SUR1)	Defects in K_{ATP} biogenesis and turnover, trafficking and nucleotide regulation	AR/ AD
KCNJ11 (11p15.1)	600937	Inward rectifying potassium channel (Kir6.2)	Defects in K_{ATP} biogenesis and turnover, trafficking and nucleotide regulation	AR/ AD
GLUD1 (10q23.3)	138130	Glutamate dehydrogenase (GDH)	Loss of inhibition of GDH by GTP and increased basal GDH activity	AD
GCK (7p15-13)	138079	Glucokinase	Increased affinity of GCK for glucose	AD
HADH (4q22-26)	601609	3-hydroxyacyl-CoA dehydrogenase	Unknown	AR
SLC16A1 (1p13.2-p12)	600682	Monocarboxylate transporter 1 (MCT1)	Increased expression of MCT1	AD
HNF4A (20q12-13.1)	600281	Hepatocyte nuclear factor 4 alpha	Unknown	AD

Figure 2.6: Schematic representation of the known genetic causes of CHI (published Kapoor *et al.,* 2009c). The commonest causes of CHI are mutations in the genes (*ABCC1 and KCNJ11*) that encode the proteins of the K_{ATP} channels (SUR1 and KIR6.2), respectively.



2.2.2 Clinical management of CHI

Early diagnosis and immediate meticulous management are the cornerstones for preventing brain injury in patients with CHI. The mainstay of medical therapy is diazoxide- a drug that binds to the intact SUR1 component of the K_{ATP} channels. It acts by keeping the K_{ATP} channels open, thereby preventing depolarisation of the ß-cell membrane and insulin secretion. Diazoxide therapy may sometimes be supplemented by dietary manipulation in patients where a dietary trigger is established.

In patients that are unresponsive to diazoxide it is essential to differentiate focal from diffuse disease as the surgical approaches are radically different. The precise preoperative localisation and limited surgical removal of the focal domain "cures" the patient (Otonkoski *et al.*, 2006). In contrast, patients with diffuse disease may require a near total pancreatectomy which will have lifelong implications (high risk of diabetes mellitus, pancreatic exocrine insufficiency).

Some infants who fail to respond to diazoxide may be managed with long term subcutaneous octreotide injections in combination with frequent feeding. Octreotide is a long-acting analog of the natural hormone, somatostatin. Somatostatin inhibits insulin release via activation of the somatostatin receptors (SSTR) which belong to the G-protein-coupled receptor family. Five distinct SSTRs are known, SSTRs 1-5. Octreotide is known to bind to SSTR2 and SSTR5, both of which mediate inhibition of insulin release from the pancreatic islets (Singh *et al.,* 2007). Octreotide is used in the short and long term management of some patients with CHI. In the short term (with and without glucagon) it is used to stabilise patients pending further investigations. Octreotide has been successfully used in the long term management of some CHI patients in combination with frequent

feeding (Glaser *et al.*, 1989). The principle of this treatment is based on the fact that the hypoglycaemia in some patients gradually gets milder over time. The long term medical management of diffuse disease with frequent subcutaneous injections of octreotide and frequent feeding may impose a huge burden and is extremely stressful on the family. A gastrostomy is often required in these patients for the delivery of bolus and continuous overnight feeds.

2.2.3 Clinical relevance of the molecular genetic classification:

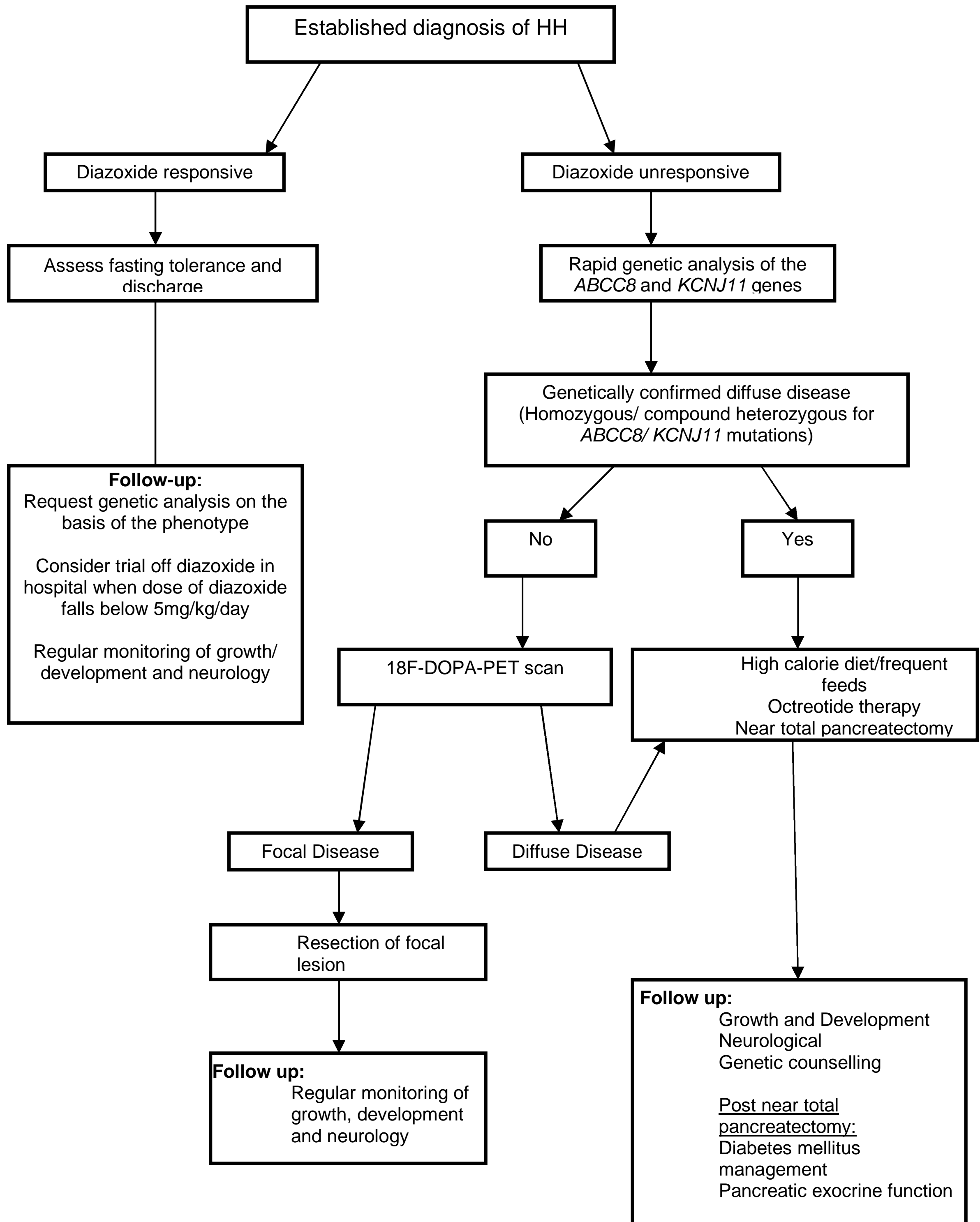
Diazoxide is ineffective in diffuse forms of CHI due to recessively inherited inactivating mutations in *ABCC8* and *KCNJ11* and in patients with focal CHI as it requires a functional channel to act upon. All of the other forms of CHI are responsive to diazoxide, although there are recent reports that CHI due to a mutation in the *GCK* gene can cause diazoxide unresponsive disease (Cuesta- Munoz *et al.,* 2004; Sayed *et al.,* 2009).

The availability of rapid genetic analysis for mutations in *ABCC8* and *KCNJ11* allows early confirmation of the diagnosis and identification of the majority of patients with diffuse disease (homozygous or compound heterozygous mutations in *ABCC8* and *KCNJ11*). This information helps in predicting the likely course of the disease and institution of appropriate treatment. Patients with a paternal mutation in *ABCC8* and *KCNJ11* (or those with no mutations in these genes) potentially have focal disease and thus enables selection of patients who require further imaging studies with 18F-DOPA-PET scan for precise pre-operative localisation of the focal lesion. The clinical approach to this complex disease has hence been transformed by the understanding of the molecular and histological classification and is summarized in figure 2.7.

The confirmation of a *GLUD1* mutation informs clinicians of the protein sensitive nature of hypoglycaemia and allows the manipulation of diet (protein restriction) as a useful (sometimes mandatory) adjunct to diazoxide therapy in controlling recurrent hypoglycaemic episodes.

The identification of a disease causing mutation in a proband has implications for other family members too. In a dominantly inherited mutation, unless the mutation is '*de novo*', the proband's siblings will have a 50% chance of inheriting the mutation. In contrast, siblings of probands with recessively inherited mutations will have a 25% risk of inheriting both the mutations and developing the disease. Furthermore, the identification of a *HNF4A* mutation in a proband would identify infants who are at a risk of developing diabetes (MODY) in adulthood. Unless the mutation has arisen *de novo*, one of the parents (and potentially other family members) will also be heterozygous for the *HNF4A* mutation and at risk of developing diabetes, if not already developed. A molecular genetic diagnosis in the proband may therefore also be of benefit for other relatives.

Figure 2.7: Flow chart outlining the management cascade of CHI (Kapoor *et al.,* 2009c).



2.2.4 Aims of the thesis

As outlined in the introduction, the knowledge of the molecular genetic mechanisms underlying the different subtypes of CHI has transformed the clinical management of the patients and has provided unique insights into pancreatic ß-cell physiology. However, the genetic basis of CHI and phenotypic characterisation of the sub groups is only just beginning to be unravelled. Although CHI due to mutations in the K_{ATP} channel genes has been well studied, novel aspects of the phenotypic variation are still emerging. For example, controversy exists on whether these mutations are a risk for future development of diabetes. This knowledge not only has implications for the patients and family members, but also may help in understanding the pathophysiology of a common condition such as diabetes mellitus.

The less well studied sub groups, where little is known about the molecular mechanisms and the phenotype, include CHI due to mutations in the *HADH* and *HNF4A* genes. Further understanding of the genotype-phenotype correlations due to these known and novel causes of CHI will be very important for the diagnosis and subsequent management of these patients. With the knowledge of the lacunae within the field of CHI, the aims of this thesis include:

4. To investigate genotype/phenotype correlations in a large cohort of patients with CHI by comparing different genetic aetiologies

5. To examine the prevalence and phenotype of patients with HH resulting from *HNF4A* gene mutations

6. To study the phenotype of dominantly inherited K_{ATP} channel mutations causing CHI and functionally characterize the novel dominant mutations identified

Chapter 3- General Methods

This section gives an overview of the methodologies used throughout this thesis. Further details are provided in the methods section for each chapter.

3.1 Ethical Approval

Ethical approval for the study was obtained from the Research Ethics Committee at the Institute of Child Health and Great Ormond Street Children's Hospital NHS Trust (GOSH) in 2006 by Dr Hussain. In 2007, the protocol was amended and a 'Parent Information Sheet' and 'Proforma and Consent' pack was designed (appendix 12.1-12.2) by me. I then submitted a notice of substantial amendment to the Research Ethics Committee. This notice was reviewed at the meeting of the Sub-Committee of the REC held on 24 April 2007 and was given a favourable opinion (appendix 12.3). The study was also exempted from Site-Specific assessment at other NHS sites.

3.2 Recruitment of patients

Patients with congenital hyperinsulinism are referred to GOSH, a national centre for the management of children with CHI. The study was discussed with all the patients referred to the CHI service at GOSH who met the inclusion criteria. The information sheet was also provided to the parents and an informed consent was obtained prior to recruitment.

A thorough assessment of the phenotype of each patient was carried out at the initial admission/ referral. Along with this a blood sample (2-5 ml) for extraction of DNA was also

collected. Blood samples from parents or any other family member were requested depending on the family history.

Information regarding the study was disseminated to the neonatalogists in the UK via the bulletin published by the BAPM (British Association of Perinatal Medicine). National and international referring clinicians were also informed about the study. Hence, patients have also been recruited from other NHS trusts in the UK and worldwide with the help of local and international collaborators.

3.3 Inclusion Criteria

All patients with HH, usually defined as:

a. Hypoglycaemia (blood glucose concentration <3.0 mmol/L) with detectable concentrations of insulin and/or C-peptide with
b. Glucose requirement of > 8mg/kg/min (intravenous glucose infusion rate of >8mg/kg/min and
c. Inappropriately low concentrations of ketones and fatty acids during the episode of hypoglycaemia

3.4 Exclusion criteria

All patients with a secondary cause of HH were excluded. This included patients with the following risk factors:

- Perinatal asphyxia
- Intra-uterine growth restriction (IUGR)
- Rhesus isoimmunisation

– Infant of diabetic mothers with HH <48 hours

3.5 Clinical and biochemical phenotyping

3.5.1 Data collection using a proforma

A proforma (see appendix 12.1) was designed to collect data on patients with CHI that met the inclusion criteria. Apart from identifying details (name, date of birth, hospital number, gender), personal data included ethnic background of the patients according to the Office for National Statistics (ONS) classification (Office for National Statistics, 2003).

Phenotype assessment included a detailed clinical and biochemical profiling for each patient. Clinical assessment included a thorough history with a focus on birth-weight, gestational age, age at presentation, treatment details and family history of hypoglycaemia or diabetes mellitus. Examination details of the patient (presence or absence of syndromic features) were recorded. Biochemical assessment carried out at the time of investigating hypoglycaemia whether at the primary referring hospital or at our center was also recorded. This included serum insulin, c-peptide, ammonia and acylcarnitines.

Response to medical therapy and results of 18F-DOPA-PET scan, if applicable were also documented. Finally, for the patients who required surgery, the type of the surgical procedure and the results of the histological analysis of the pancreas were documented.

In cases where the child had been previously treated in the hospital and discharged, a review of the medical records and telephone interviews were conducted to complete the data collection. Home visits were also conducted to obtain blood samples from the patient and/ or family members for genetic analysis.

Children with CHI who had an established molecular diagnosis were not excluded from the study. The phenotypic data on these children were retrospectively collected from them and their medical case notes.

A database using Microsoft Access was created to allow ease of entry and access to the data. The database has been designed, completed and updated by myself throughout the period of the study.

3.5.2 Further phenotypic data

In some patients, the phenotype was further defined with the help of the following provocation tests, as detailed in individual chapters:

a. Oral glucose tolerance test
b. Oral protein tolerance test
c. Leucine tolerance test

3.5.3 Statistics

In order to correct for the gestational age, birth weight standard deviation scores (birth weight SDS) were calculated for each patient. The mean birth weight SDS and age of presentation of patients with diazoxide responsive CHI due to a K_{ATP} channel mutation, *GLUD1* mutation, *HADH* mutation or an *HNF4A* mutation were compared using one-way analysis of variance (ANOVA) followed by the Fisher's Least Significant Difference (LSD) post-test to test for statistical significance (SPSS, version 16).

The one way ANOVA test is a common statistical test used to test the difference between three or more groups. The test can be applied to test the equality of means between samples that are independent and normally distributed. Prior to applying this test, it was

ensured that the samples within the groups were normally distributed. The one way ANOVA test showed that the mean birth weight SDS and age of presentation of the groups were different. The LSD post-hoc test (which makes individual comparisons) was then applied to understand where the differences were.

3.6 Molecular genetic testing

3.6.1 Sequence analysis of genes known to cause CHI

The DNA samples were analysed for mutations in genes known to cause CHI (*ABCC8, KCNJ11, HNF4A, GCK, GLUD1* and *HADH* genes) at the Diagnostic Molecular Genetics Laboratory at Peninsula Medical School in Exeter under the supervision of the Consultant Clinical Molecular Geneticist, Professor Sian Ellard. DNA was extracted from blood samples by the staff at Peninsula Medical School using their standard protocol (appendix 12.3). The amplification of specific regions of DNA by polymerase chain reaction (PCR) was the principal methodology used, followed by detection of mutation using DNA sequencing.

Polymerase chain reaction (PCR): Initially, the DNA is denatured, separating the double helix, allowing the primers to bind to their complementary DNA sequences. Primers extend in the presence of DNA polymerase and deoxynucleotide triphosphates, allowing the synthesis of a complementary DNA sequence. The process is repeated approximately 35 times resulting in over 2 million copies of the target DNA sequence.

Dideoxy Chain Termination Sequencing: Purified PCR products were sequenced using the ABI Prism Big Dye Terminator Cycle Sequencing ready reaction kit 1.1 (Applied Biosystems, Warrington, UK). The method utilises a single- stranded PCR product which

acts as a template for the synthesis of a complementary strand of DNA. The sequencing reactions contain a DNA polymerase, primer and four dideoxynucleotides which lack a 3' hydroxyl group. Each ddNTP is labelled with a base specific fluorescent dye. The absence of the 3' hydroxyl group prevents phosphodiester bonding resulting in the formation of many hundreds of oligonucleotide fragments which differ in length. The sequencing reactions are then separated by size on a capillary sequencer (ABI 3730 Sequencer, Applied Biosytems, Warrington, UK). The DNA sequence is generated by computer software and analysed using either Staden software (hhtp://staden.sourceforge.net) or Mutation Surveyorv2.61 (Softgenetics, State College, PA, USA).

The single exon of *KCNJ11* gene was amplified in five patient samples by me using three primer pairs (primer sequences published in Flanagan *et al.,* 2006). I performed sequencing in both directions using the BigDye Terminator Cycler Sequencing Kit v1.1 (Applied Biosystems, Warrington, UK) and analysed the five reactions on the ABI 3730 Capillary sequencer (Applied Biosystems). The sequences were then compared by me with the published sequence (NM_000525) using the Staden analysis software. Analyses of these five samples taught me the principles and details of the techniques employed (primer designing, polymerase chain reaction, gel electrophoresis and sequencing) in analysing the samples. Sequencing of the other patient samples and analysis was carried out by the laboratory staff at Peninsula Medical School. Sequencing of genes known to cause CHI is routinely carried out at this large Diagnostic Molecular Genetics Laboratory in Exeter. After recruitment, detailed phenotyping and collection of DNA samples from the patient and family members, I organized genetic testing at this laboratory where sequencing of the genes was carried out by the diagnostic service.

3.6.2 Sequencing strategy (Figure 3.1)

ABCC8 and *KCNJ11* genes were sequenced in all patients with diazoxide unresponsive CHI.

In patients with diazoxide responsive CHI,

i) mutations in *GLUD1* were sought if the patient had hyperammonaemia or leucine sensitivity

ii) *HADH* was sequenced if there was raised plasma 3-hydroxybutyrylcarnitine

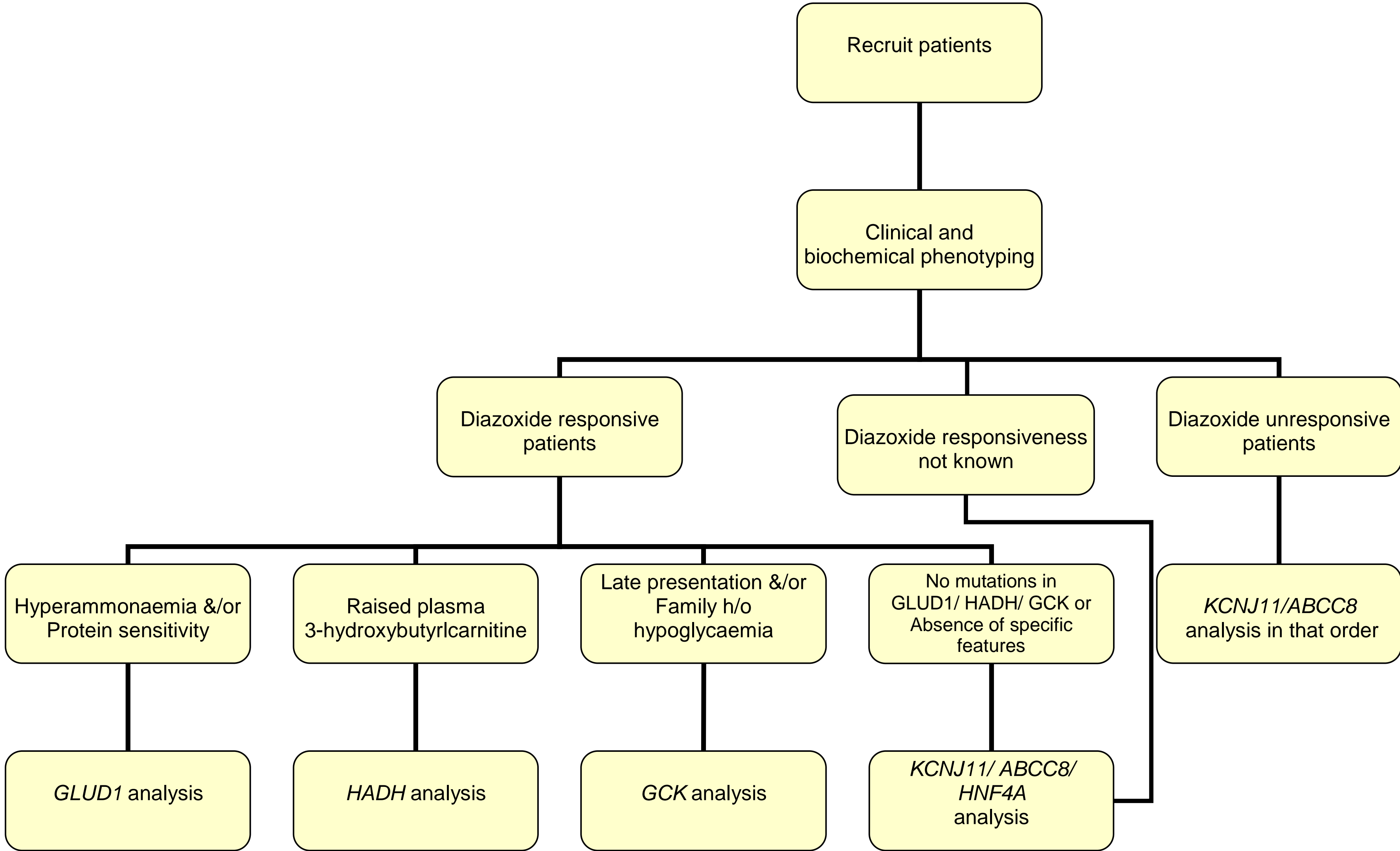
iii) if no mutations were identified and in the absence of above features; *KCNJ11, ABCC8* and *HNF4A* genes were sequenced in that order

iv) Sequencing of *GCK* was considered if there was a positive family history of hypoglycaemia or delayed presentation of hypoglycaemia

If it was not possible to establish whether a patient with CHI was diazoxide responsive or not, *KCNJ11, ABCC8* and *HNF4A* genes were sequenced in that order.

Sequencing of *SLC16A1* was planned in case of a history of exercise induced hypoglycaemia. However, no patients with EIHI were recruited during the study period.

Figure 3.1: Schematic representation of the Experimental Design



3.7 Functional analysis of novel mutations

The functional consequences of certain novel mutations identified in the *ABCC8* or *KCNJ11* genes were undertaken under the supervision of Professor Tinker at the Rayne Institute, University College London. Briefly, mutations were introduced into hamster SUR1 cDNA or mouse Kir6.2 cDNA using the QuickChange site-directed mutagenesis kit. Functional consequences were then examined by reconstituting the K_{ATP} channel in HEK293 cells and evaluating the effect of drugs (diazoxide, glibenclamide) and metabolic poisoning on the channels using Rb^{86} flux assay. Western blotting was carried out to confirm expression of the mutant proteins. The details of these techniques and the statistics used for functional evaluation of the novel mutants are provided in chapter 9.

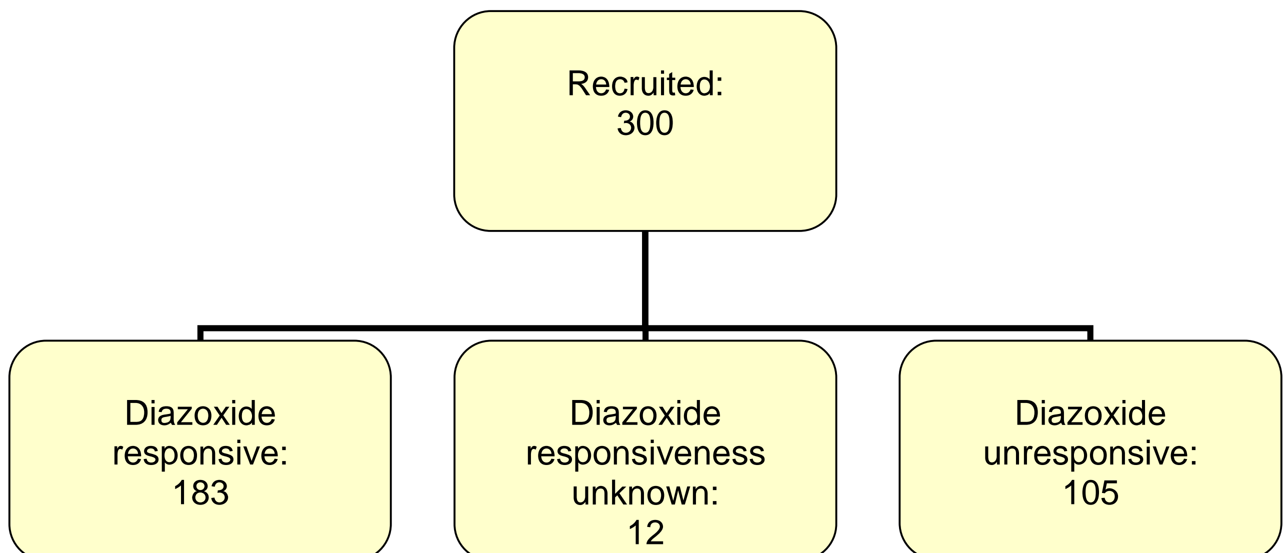
Through established collaboration with experts in functional characterisation of *GLUD1* and *HADH* mutations (Dr Bernadette Chadefaux and Dr Simon Eaton respectively), functional consequences of selected novel *GLUD1* and *HADH* gene mutations were investigated to provide insight into the mutational mechanisms.

Chapter 4- Overview of Results

Patient Recruitment and Prevalence of the Different Genetic Aetiologies

Three hundred patients with CHI were recruited in this study, forming the largest cohort of children with CHI in the world. As outlined in chapter 3, mutational analysis of various genes known to cause CHI was carried out depending on the phenotype of the patient. The patients were broadly classified into diazoxide responsive (n=183) and diazoxide unresponsive (n=105) groups (figure 4.1), diazoxide responsiveness being defined as normoglycaemia maintained during normal feeding volume and frequency with normal fasting tolerance for age of the child. Twelve patients with CHI were not able to be grouped within these two broad categories. These included patients with transient CHI that did not require treatment with diazoxide (n=9) and 1 patient who was not treated with diazoxide following an anaphylactic reaction to the drug.

Figure 4.1: Categorisation of the 300 recruited patients, according to their responsiveness to diazoxide



4.1 Diazoxide responsive CHI

The largest category of patients with CHI in this cohort included patients with diazoxide responsive CHI. Of the 183 patients with diazoxide responsive disease, a genetic diagnosis was possible in 51 patients (Fig 4.2).

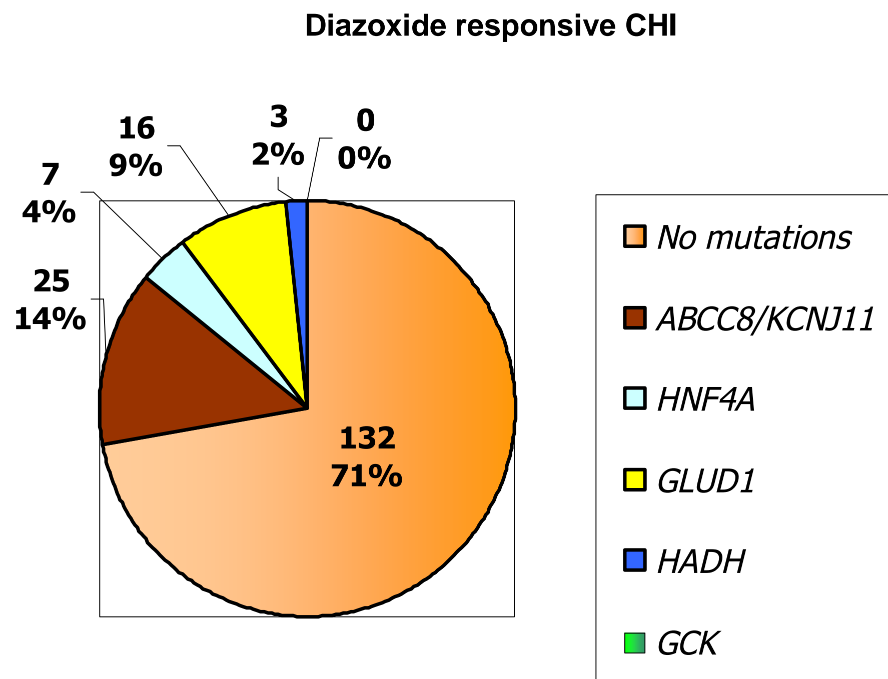


Figure 4.2: Figure demonstrating the genetic aetiologies identified in patients with diazoxide responsive CHI

168 patients had mutational analysis of the K_{ATP} channel genes, of which 25 were identified to have a mutation in the *KCNJ11* or *ABCC8* genes. The phenotypic characteristics of these patients are outlined in chapter 5.

20 patients with diazoxide responsive CHI (19 patients with hyperammonaemia and 1 with leucine sensitivity) had mutational analysis of *GLUD1* gene, of which 16 were positive for a mutation in this gene. The genotype-phenotype correlations of these patients are discussed in chapter 6.

Of the 105 patients with diazoxide responsive CHI who had mutational analysis of the *HNF4A* gene, 7 were identified to have a mutation in this gene. The phenotypic characteristics of these patients are presented in chapter 8.

This cohort also contains 3 patients with a mutation in the *HADH* gene, of which 2 patients were known to have CHI due to recessively inherited mutations in the *HADH* gene and have been described previously (Clayton *et al.,* 2001; Hussain *et al.,* 2005c). The novel phenotypic characteristics of the patients with *HADH* mutations causing CHI are presented in chapter 7.

GCK was analysed in patients with a delayed age of presentation (> 6 months) if sufficient DNA was available after analysis of the *KCNJ11* and *ABCC8* genes. No patients with *GCK* mutation were identified on analysis of the DNA of 17 patients.

4.2 Diazoxide unresponsive CHI

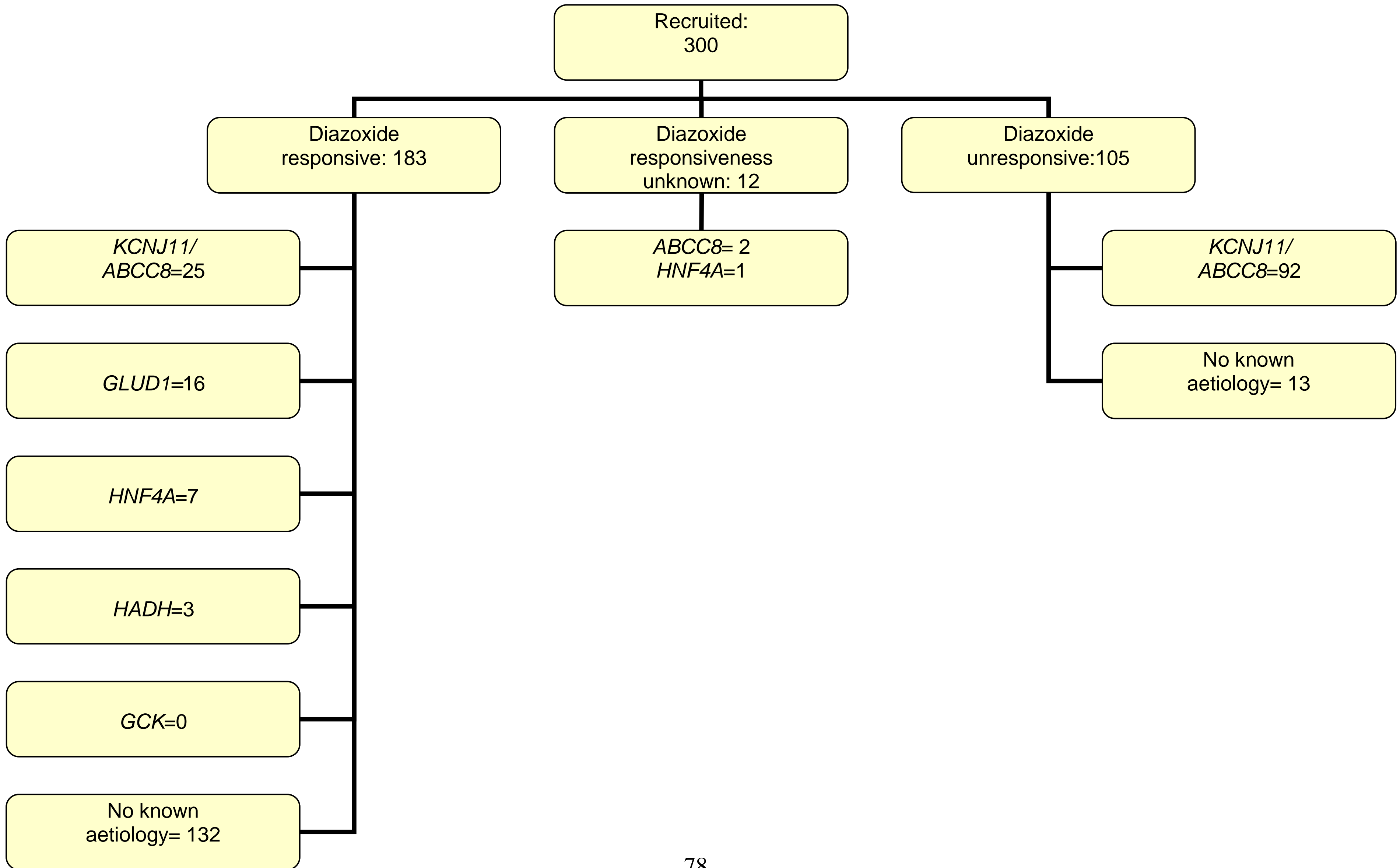
In this cohort, there were 105 patients who did not respond to 1st line treatment with diazoxide and required additional treatment measures. All of these patients had sequencing of the *ABCC8* and *KCNJ11* genes. A significant proportion (92/105; 87.6%) of these patients had a mutation in one of these genes. This included 10 patients with a mutation in the *KCNJ11* gene and 82 patients with a mutation in the *ABCC8* gene. No mutation in these genes were found in 13 patients (12.4%).

4.3 Diazoxide responsiveness not known

There were 12 patients where it was not possible to establish whether they were responsive to diazoxide or not. These included 11 patients who had transient HH (lasting 1-10 days) that was managed with supportive treatment (intravenous dextrose solutions) alone. The remaining patient had a severe reaction to the first dose of diazoxide and hence diazoxide was discontinued. A further attempt to re-start diazoxide was met with a similar reaction where the child developed a generalized rash and difficulty in breathing. Hence, diazoxide was not continued and the child underwent a partial pancreatectomy to manage the HH. All these patients had sequencing of the *ABCC8* and *KCNJ11* genes and a mutation in the *ABCC8* gene was identified in two patients. Six patients had sequencing of the *HNF4A* gene (DNA sample was insufficient for the remaining 4), of which one patient with transient HH had an *HNF4A* mutation.

These results are summarized below in figure 4.3.

Figure 4.3: Figure summarizing the results of mutational analysis of 300 patients with CHI:



4.4 Discussion

In this cohort of patients, with the designed strategy of the order of sequencing and the limitation of low DNA sample in some patients, a genetic diagnosis was possible in 48.6% of patients. The vast majority of patients with an unknown genetic cause were diazoxide responsive (132/154; 85.7%). This may be reflective of the sequencing strategy rather than the actual prevalence of the genetic aetiologies. For example, *GCK* was only sequenced in patients with a delayed age of presentation as patients with GCK-CHI were initially reported to be diagnosed at a much later age (Glaser *et al.,* 1998; Christensen *et al.,* 2002; Cuesta-Munoz AL *et al.,* 2004). A recent study looking at the prevalence of *GCK* mutation in patients with CHI reported five patients with *GCK* mutation who presented in the neonatal period with hypoglycaemia (Christensen *et al.,* 2008). Hence, this low prevalence of *GCK* mutations in our group of patients with diazoxide responsive CHI may be an underestimate and a thorough sequencing of all patients with diazoxide responsive CHI and no mutations in the *ABCC8/ KCNJ11* genes is indicated. Similarly, sequencing of *GLUD1* and *HADH* were limited to patients with specific phenotypic features (hyperammonaemia for *GLUD1* and abnormal plasma acyl carnitine profile for *HADH*). In the chapters to follow, it will be clear that patients may have CHI due to mutations in these two genes in the absence of these biochemical markers that were previously thought to be diagnostic. Hence, sequencing of these two genes in this large cohort of patients with diazoxide responsive CHI may also be indicated. This large cohort of patients with an unknown aetiology forms a unique cohort for further studies investigating the known and novel genetic causes of CHI.

Unlike the cohort with diazoxide responsive CHI, a genetic diagnosis was possible in the vast majority of patients with diazoxide unresponsive CHI that underwent sequencing of

the *ABCC8* and *KCNJ11* genes. A genetic diagnosis in this group of patients helps in the clinical management by determining whether a patient may have focal disease that is potentially curable by a limited pancreatectomy or whether the patient has diffuse disease and is a candidate for near-total pancreatectomy (Kapoor *et al.,* 2009c). Hence, the high possibility of finding a K_{ATP} channel mutation in patients within this cohort confirms the importance of rapid sequencing of the *ABCC8* and *KCNJ11* genes in these patients. Only 13/105 (12.4%) patients with diazoxide unresponsive patients did not have a mutation in these genes. Mutations in the *GCK* gene have recently been described to cause medically unresponsive CHI (Cuesta-Munoz *et al*., 2004; Sayed *et al.,* 2009). Sequence analysis of the *GCK* gene in this cohort of patients is hence indicated, prior to further research investigating novel genetic aetiologies.

The patients identified to have a genetic mutation are varied in their phenotypes depending on their genotypes. In the chapters to follow, I describe the phenotype of patients with K_{ATP} channel mutations (chapter 5), *GLUD1* mutations (chapter 6), *HADH* mutations (chapter 7) and *HNF4A* mutations (chapter 8) and compare the clinical characteristics of the patients according to their genetic aetiology (chapter 8).

Chapter 5

Congenital Hyperinsulinism due to mutations in the *ABCC8/ KCNJ11* genes

5.1 Background

Mutations in the *ABCC8* gene were the first genetic cause identified and are the commonest cause of CHI. So far over 150 mutations in the *ABCC8* gene have been identified that are distributed throughout the gene (Flanagan *et al.,* 2009). Relatively, fewer mutations have been described in the *KCNJ11* gene (Flanagan *et al.,* 2009). The mutations are commonly recessively inherited and cause diazoxide unresponsive disease in the vast majority of patients. However, there have been reports of dominantly inherited mutations in these genes causing diazoxide responsive disease (Huopio *et al.*, 2000; Thornton *et al.*, 2003; Pinney *et al.,* 2008). Compound heterozygous mutations in the *ABCC8* have also been reported to cause diazoxide responsive disease (Dekel *et al.*, 2002). Mutations in these genes are also associated with the pathogenesis of focal CHI, where a focal area of the pancreas is affected which is amenable to curative surgery.

The identification of an *ABCC8* or *KCNJ11* mutation in a patient helps to predict the likely course and influences the management of the patient (Kapoor *et al.,* 2009c). In patients with medically unresponsive disease, rapid genetic testing of these genes identifies those who have diffuse disease (homozygous/ compound heterozygous mutations) where a near-total pancreatectomy may be considered. It also helps in identifying patients who may have a focal lesion (single, paternally inherited mutation) that is potentially curable with resection of the focal lesion.

In this chapter, the phenotype and genotype of the 119 patients with a mutation in the *ABCC8/ KCNJ11* genes is discussed. This section reports several novel mutations identified in these genes and summarises the associated clinical picture in each patient.

5.2 Methodology

Of the 300 patients recruited, 2 patients with known *HADH* mutations and 15 patients with hyperammonaemia that screened positive for a *GLUD1* mutation did not have sequence analysis of the K_{ATP} channel genes. The remaining 283 patients had mutational analysis of the *KCNJ11* and *ABCC8* genes, in that order. *KCNJ11* was sequenced as outlined in section 3.6.1. The coding regions and conserved splice sites of the *ABCC8* gene were amplified using 38 primer pairs (primer sequences published in Proks *et al.*, 2006). PCR products were sequenced using standard methods on an ABI 3730 (Applied Biosystems, Warrington, UK) and compared to the published sequence (NM_000352.2) at Peninsula Medical School, Exeter. When a mutation in the *ABCC8/ KCNJ11* gene was identified, the parents were tested (if available) to ascertain the mode of inheritance. Novel mutations were confirmed by testing 500 control chromosomes, of mixed ethnicites (as are our patients). In addition, these genes are also analysed by Peninsula Medical School in patients with neonatal diabetes (>300 and increasing). These patients also serve as additional controls, although ethnic differences cannot be guaranteed.

All the patients had standard clinical and biochemical phenotyping, as outlined in chapter 3. The patients positive for a mutation in the *ABCC8* gene were categorized into those with homozygous mutations, compound heterozygous mutations and single, heterozygous mutations. The clinical data were tabulated, according to these categories summarizing

the most pertinent clinical features, with particular emphasis on the responsiveness to diazoxide therapy and outcome.

Clinical features of the patients with a *KCNJ11* mutation were tabulated separately.
Data on ethnic backgrounds of the patients was also tabulated to study whether certain mutations were more prevalent in certain ethnic populations. The Office for National Statistics (ONS) classification was used to categorise ethnicity (Office for National Statistics, 2003).

5.3 Results

Of the 119 patients with a mutation in the K_{ATP} channel gene, 106 patients had a mutation in the *ABCC8* gene while 13 patients had a *KCNJ11* mutation.

5.3.1 *KCNJ11* gene mutations

Thirteen different *KCNJ11* mutations were identified; eleven of which are novel. The genotype and phenotype of these patients is summarized in table 5.1. The mean age of presentation of these patients was 4 days (median of 1 day) and the mean birth weight SDS was +1.69 standard deviation score (SDS).

<u>Diazoxide unresponsive disease:</u>
Six patients had diazoxide unresponsive disease associated with homozygous mutations in the *KCNJ11* mutation. Four (table 1- patients 26, 323, 379 and 395) of these patients underwent a near total pancreatectomy while two (67, 74) were treated conservatively with octreotide. Diffuse disease was confirmed histologically in the four patients who underwent

a near- total pancreatectomy and in patient 74 via a pancreatic tail biopsy. The histological diagnosis was not established in patient 67.

Three patients (133, 244 and 365) with diazoxide unresponsive disease and a paternal mutation in *KCNJ11* had focal disease, confirmed on histological examination following a limited/ sub-total pancreatectomy. One of the patients (148) with diazoxide unresponsive disease who was heterozygous for a novel paternal mutation had diffuse disease requiring a near total pancreatectomy.

Diazoxide responsive disease:

Three others who were heterozygous for a mutation in the *KCNJ11* gene had diazoxide responsive disease. Two (22 and 56) of these had transient disease, requiring diazoxide therapy for 4- 6 months. Patient 56 has inherited the mutation from an unaffected mother and the inheritance of the mutation in the second patient could not be determined (mother's DNA was negative and unable to obtain paternal DNA sample). The third patient (285) has inherited the mutation from her affected father (who has a history of neonatal hypoglycaemia), confirming dominant inheritance.

There were no common *KCNJ11* mutations in our cohort and hence an intra-gene genotype-phenotype correlation was not possible.

Table 5.1: Summary of patients with a *KCNJ11* gene mutation: Patients presented early (mean age of presentation was 1 day) and were large at birth with a mean birth weight of +1.69 standard deviation score. The majority (10/13) were diazoxide unresponsive.

ID	Genotype	Type of mutation	Novel	Inheritance	Ethnicity	Age of presentation (days)	Birth weight SDS	Management	Histology/ 18F-DOPA-PET scan
Diazoxide responsive patients with a *KCNJ11* mutation									
56	R347H/N	Missense	Yes	Maternal	Asian Indian	1	-0.00	Diazoxide for 6 months	n/a
22	R54H/N	Missense	Yes	Not determined	Black African	1	-0.27	Diazoxide for 4 months	n/a
285	I284del/N	Deletion	Yes	Paternal	White British	1	+1.48	Continuing Diazoxide at 1.9 yrs	n/a
Diazoxide unresponsive patients with a *KCNJ11* mutation									
26	A161V/ A161V	Missense	Yes	Both parents	Asian Pakistani	2	+1.33	Near total pancreatectomy	Diffuse
395	G40A/ G40A	Missense	No	Both parents	Other Asian	1	+2.46	Near total pancreatectomy	Diffuse
323	G132D/ G132D	Missense	Yes	Both parents	Other Asian	1	+0.07	Near total pancreatectomy	Diffuse
379	E51X/ E51X	Missense	Yes	Both parents	White British	1	+4.73	Near total pancreatectomy	Diffuse
74	H259Q/ H259Q	Missense	Yes	Both parents	Asian Pakistani	1	+1.3	Continuing octreotide at 5.7 yrs	Diffuse- tail biopsy
67	A213T/ A213T	Missense	Yes	Both parents	Asian Pakistani	1	+3.22	Continuing octreotide at 5.5 yrs	n/a
133	E292K/N	Missense	Yes	Paternal	White British	1	+2.36	Near total pancreatectomy	Focal
244	R136fs/ N	Frameshift	No	Paternal	White British	1	+2.2	Near total pancreatectomy	Focal
365	R301H	Missense	Yes	Paternal	White British	50	+0.91	Resection of focal lesion	Focal
148	T294M/N	Missense	Yes	Paternal	White British	1	+2.23	Near total pancreatectomy	Diffuse

5.3.2 *ABCC8* gene mutations

Of the 106 patients with an *ABCC8* gene mutation, 19 patients were compound heterozygous, 38 patients were homozygous and 49 patients were heterozygous for a mutation in the gene. A variety of missense, frameshift, nonsense, deletions and splice site mutations, located throughout the gene, were identified. Sixty-eight novel mutations were identified in this study. These mutations affected residues that were conserved across species and were not found in the control chromosomes. An intragene genotype-phenotype correlation was not possible due to the small numbers of mutations found in common. The most common mutations were the splice site mutation IVS32-9G>A and the missense mutation G111R, found in six patients each. However, even within these groups, the mutations were either homozygous or heterozygous or compound heterozygous and hence a true intra-gene genotype-phenotype was not possible.

5.3.2.1 CHI due to homozygous/ compound heterozygous mutations in *ABCC8* (tables 5.2 and 5.33):

All the patients with recessively inherited CHI due to *ABCC8* mutations were unresponsive to diazoxide therapy (n= 57). Of these 57 patients, 38 patients underwent a near total pancreatectomy and diffuse disease was confirmed histologically. The remaining 19 patients were treated conservatively with octreotide therapy. Diazoxide was used as an adjunct in 7 patients and nifedipine was added to octreotide therapy in 1 patient.

The mean age of presentation of these patients was 20 days (median of 1 day) and the mean birth weight SDS was +1.67 SDS.

Table 5.2: Summary of the patients with compound heterozygous mutations in the *ABCC8* gene: All of the 19 patients were diazoxide unresponsive. Diffuse disease was confirmed in all the patients that underwent a pancreatectomy.

ID	Genotype	Type of mutation and location (Exon/ Intron)	Novel	Ethnicity	Age of Presentation (day)	Birth Weight SDS	Diazoxide responsive	Management	Histology
213	c.1012-2A>G/ c.3870+7G>A	Splicing In Intron 6 and 31	c.3870+7G>A	White Irish	1	+0.79	No	Continuing octreotide	N/A
20	G735E/R1215W	Missense In Exons 16 And 29	G735E	White British	1	+2.38	No	Continuing diazoxide + octreotide	N/A
252	K329X /P1414L	Nonsense In Exons 6 and 35	K329X	Other White	1	+1.4	No	Continaquing octreotide + nifedipine	N/A
346	H125Q/E529K	Missense in Exons 3 and 10	E529K	Other White	250	+1.51	No	Continuing diazoxide + octreotide	N/A
104	R74Q/G111R	Missense In Exons 2 And 3	No	Asian Indian	1	+2.66	No	Continuing diazoxide +octreotide	N/A
184	R74Q/G111R	Missense In Exons 2 And 3	No	Asian Indian	1	-0.58	No	Continuing Octreotide	N/A
23	V222M/E515K	Missense In Exons 5 And 10	Both Novel	White British	60	+1.58	No	Continuing octreotide	N/A
88	R168C/S606T	Missense In Exons 4 And 12	S606T	Asian Indian	1	+4.63	No	Octreotide for 8 weeks	N/A
191	G70E/R1419H	Missense In Exons 2 And 35	No	White British	1	+4.53	No	Near Total Pancreatectomy	Diffuse
142	M429fs/H627fs	Frameshift In Exon 8 And 13	M429fs	White British	1	+1.56	No	Near Total Pancreatectomy	Diffuse

138	[I320N;W739C]/ c.1176+2T>C	Two Missense in Exons 6 And 16 and Splicing In Intron 7	All Three Novel	White Irish	1	+2.05	No	Near Total Pancreatectomy + Octreotide	Diffuse
85	R1421C/T1532 A	Missense In Exons 35 And 38	T1532A	White British	1	+2.89	No	Near Total Pancreatectomy	Diffuse
114	G1343E/c.2116 +3A>G	Missense In Exon 10 And Splicing In Intron 15	No	White British	1	+1.57	No	Near Total Pancreatectomy	Diffuse
200	c.725delA/R143 7L	Framehsift In Exon 5 And Missense In Exon 35	Both novel	White British	1	+1.63	No	Near Total Pancreatectomy	Diffuse
261	R16P/c.2116+3 A>G	Missense In Exon1 And Splicing In Intron 15	R16P	White British	56	+0.79	No	Near Total Pancreatectomy	Diffuse
262	R16P/c.2116+3 A>G	Missense In Exon1 And Splicing In Intron 15	R16P	White British	1	+2.72	No	Near Total Pancreatectomy	Diffuse
175	R248X/c.4311-2A>G	Nonsense In Exon 10 And Splicing In Intron 35	C.4311-2A>G	White British	4	+1.85	No	Near Total Pancreatectomy	Diffuse
155	R526C/S1482R	Missense In Exons 10 And 37	Both Novel	White British	1	+2.85	No	Near Total Pancreatectomy	Diffuse
231	V21D/c.1176+2 T>C	Missense In Exon1, Spilcing In Intron 7	No	White British	1	Not known	No	Near Total Pancreatectomy	Diffuse

Table 5.3: Summary of the patients with homozygous mutations in the *ABCC8* gene: Simlar to the patients with compound heterozygous mutations, all of the 38 patients were diazoxide unresponsive. Diffuse disease was confirmed in all the patients that underwent a pancreatectomy.

ID	Genotype	Type	Exon/ Intron	Novel	Ethnicity	Age of presentation (Days)	Birth Weight SDS	Diazoxide Responsive	Management	Histology
31	c.1877del57/ c.1877del57	Frameshift	Exon 13	Yes	Asian Pakistani	1	+1.01	No	Near Total Pancreatectomy	Diffuse
77	c.945delC/ c.945delC	Frameshift	Exon 6	No	Asian Pakistani	1	+4.1	No	Near Total Pancreatectomy + Octreotide	Diffuse
250	c.1672-5C>G/ c.1672-5C>G	Splicing	Intron 11	Yes	Asian Pakistani	1	+1.85	No	Near Total Pancreatectomy	Diffuse
105	c.3871-1G>A/ c.3871-1G>A	Splicing	Intron 31	Yes	Asian Indian	2	+0.79	No	Near Total Pancreatectomy	Diffuse
102	c.3871-1G>A/ c.3871-1G>A	Splicing	Intron 31	Yes	Other Asian	30	-).86	No	Near Total Pancreatectomy	Not Known
193	E786del/ E786del	Deletion	Exon 19	Yes	Asian Pakistani	3	+0.53	No	Near Total Pancreatectomy	Diffuse
292	G111R/ G111R	Missense	Exon 3	No	Asian Indian	1	+4.71	No	Near Total Pancreatectomy	Diffuse
321	G111R/ G111R	Missense	Exon 3	No	Asian Indian	1	+2.66	No	Near total pancreatectomy	Diffuse
291	G111R/ G111R	Missense	Exon 3	No	Asian Indian	1	+1.92	No	Near total pancreatectomy	Diffuse
243	[c.554delT;Q664I]/ [c.554delT;Q664I]	Frameshift- C.554delt, Missense Q664I	Exons 4 and 14	Yes	Asian Bangladeshi	1	Not known	No	Near Total Pancreatectomy	Diffuse
207	L1471Pfs/ L1471Pfs	Frameshift	Exon 36	Yes	Chinese	2	-1.59	No	Near Total Pancreatectomy	Not Known

108	N32K/ N32K	Missense	Exon1	Yes	Other Asian	1	+2.59	No	Near Total Pancreatectomy	Diffuse
41	R1251X/ R1251X	Nonsense	Exon 30	No	Other Asian	1	+0.59	No	Near Total Pancreatectomy	Diffuse
61	R1494W/ R1494W	Missense	Exon 37	No	Other Asian	11	Not known	No	Near Total Pancreatectomy	Post Pancreatectomy
62	Q1342X/ Q1342X	Nonsense	Exon 33	Yes	Other Asian	1	+1.29	No	Near Total Pancreatectomy	Not Known
63	Q1342X/ Q1342X	Nonsense	Exon 33	Yes	Other Asian	1	-0.92	No	Near Total Pancreatectomy	Not Known
208	Q1488X/ Q1488X	Nonsense	Exon 37	Yes	Chinese	1	+2.15	No	Near Total Pancreatectomy	Diffuse
290	A390E/ A390E	Missense	Exon 7	Yes	Other Asian	1	+4.75	No	Near total Pancreatectomy	Diffuse
303	IVS32-9G>A/ IVS32-9G>A	Splicing	Intron 32	No	Other Asian	120	+1.1	No	Near total pancreatectomy	Diffuse
205	IVS32-9G>A/ IVS32-9G>A	Splicing	Intron 32	No	Asian Bangladeshi	1	-0.92	No	Near Total Pancreatectomy	Diffuse
183	G706S/ G706S	Missense	Exon 15	Yes	Black African	1	+3.24	No	Near total pancreatectomy	Diffuse
388	A736fs/ A736fs	Frameshift	Exon 16	Yes	Other Asian	1	+1.92	No	Near Total Pancreatectomy + Octreotide	Diffuse
377	IVS32-9G>A/ IVS32-9G>A	Splicing	Intron 32	No	Other White	1	+2.44	No	Near total pancreatectomy	Diffuse
362	W1339X/ W1339X	Missense	Exon 33	Yes	Other Asian	1	+2.99	No	Near total pancreatectomy	Diffuse
266	IVS9+5G>A/ IVS9+5G>A	Splicing	Intron 9	Yes	Other Asian	1	Not known	No	Near Total Pancreatectomy	Diffuse
343	IVS9+5G>A/ IVS9+5G>A	Splicing	Intron 9	No	Asian Pakistani	1	+1.01	No	Near total pancreatectomy	Diffuse
64	IVS9+5G>A/ IVS9+5G>A	Splicing	Intron 9	Yes	Asian Pakistani	1	Not known	No	Near Total Pancreatectomy	Diffuse
65	IVS9+5G>A/ IVS9+5G>A	Splicing	Intron 9	Yes	Asian Pakistani	1	+1.75	No	Continuing Diazoxide+ Octreotide at 6 years	N/A

331	L362fs/ L362fs	Frameshift	Exon 7	Yes	Other Asian	1	+3.36	No	Continuing Octreotide at 2yrs	N/A
332	L362fs/ L362fs	Frameshift	Exon 7	Yes	Other Asian	1	+0.30	No	Continuing Octreotide + Diazoxide at 1.8 yrs	N/A
333	N188S/ N188S	Missense	Exon 4	No	Other Asian	1	+0.57	No	Continuing Octreotide + Diazoxide at 1.3 yrs	N/A
326	G1376R/ G1376R	Missense	Exon 34	Yes	Black African	14	+1.12	No	Continuing Octreotide at 1.5 yrs	N/A
327	Homozygous deletion of exons 1-22	Deletion	Exons 1-22	No	Other Asian	3	-0.81	No	Continuing Octreotide at 1.5 yrs	N/A
364	R74Q/ R74Q	Missense	Exon 2	No	Asian Pakistani	540	+0.35	No	Continuing Octreotide at 2.4 yrs	Diffuse
302	R837X/ R837X	Nonsense	Exon 21	No	Other Asian	1	+0.12	No	Continuing Octreotide at 1.9 yrs of age	N/A
234	Q822X/ Q822X	Nonsense	Exon 20	Yes	Other Asian	1	+0.8	No	Octreotide For 10 Weeks	N/A
69	IVS32-9G>A/ IVS32-9G>A	Splicing	Intron 32	No	Other White	2	+4.07	No	Octreotide- Till 1 Year	N/A
152	E490X/ E490X	Nonsense	Exon 10	No	Asian Pakistani	1	+1.52	No	Continuing Octreotide + Diazoxide at 5.4 yrs of age	N/A

5.3.2.2 CHI due to heterozygous mutations in *ABCC8* (table 5.4):

Forty-nine patients were heterozygous for a single mutation in *ABCC8*. This is a heterogeneous group of patients that includes 25 patients with diazoxide unresponsive CHI and 22 patients with diazoxide responsive CHI. A single mutation in the *ABCC8* was also identified in 2 patients where the diazoxide responsiveness is not known as they had transient CHI that resolved without trial of diazoxide therapy. This includes patient 172 who is heterozygous for a novel dominantly inherited mutation (G1479A) that arose *de novo*, and patient 281 who is heterozygous for the R1539Q mutation known to cause mild, dominantly inherited CHI (Pinney *et al.,* 2008). The clinical phenotype of these and other patients with mild, dominantly inherited CHI is detailed in chapter 9.

Diazoxide unresponsive CHI with a single mutation in *ABCC8*:

Of the 25 patients with diazoxide unresponsive CHI and a single mutation in the *ABCC8* gene, 19 patients had a paternal mutation, 3 had a *de novo* mutation, 1 had a maternally inherited mutation and in 2 patients (with histologically confirmed focal disease), the inheritance was not established due to unavailability of the paternal DNA.

Of the 19 patients with a paternal mutation, 12 patients had confirmed focal disease (confirmed histologically or via an 18-F-DOPA PET scan) that was managed by resection of the focal lesion/ limited pancreatectomy. However, six patients had diffuse disease with a mutation inherited from an unaffected father. Two of these (patient 97 and 270) are heterozygous for the same novel mutation (A1263T). One other patient with a paternally inherited mutation was treated conservatively with octreotide and it was not determined whether they had a focal lesion or not.

Four patients with three novel dominant mutations (D1506E (c.4518C>A), G1485E (c.4454G>A) and M1544K (c.4541T>A)) were identified to cause severe diazoxide unresponsive diffuse disease requiring a near total pancreatectomy. The D1506E mutation was identified in two patients from different families. Testing of the parents demonstrated that the mutation had arisen *de novo* in one (patient 57) and from an affected mother in the other patient (patient 295). The mother of patient 295 had diazoxide unresponsive CHI for which she underwent a sub-total pancreatectomy at 5 weeks of age. Post operatively she continued to have HH that was managed on a combination of subcutaneous octreotide injections and frequent high calorie carbohydrate feeds until she outgrew the HH at 11 years of age. Testing of the maternal grandparents confirmed that the mutation had arisen *de novo* in the mother. The G1485E and M1544K mutations were each identified in one patient (patients 9 and 300). Testing of their unaffected parents demonstrated that the mutations had arisen *de novo*.

<u>Diazoxide responsive CHI with a single mutation in *ABCC8*:</u>

There were 22 patients who had diazoxide responsive CHI associated with a single mutation in the *ABCC8* gene. The patients in this category have varying durations of HH, ranging from transient disease of 4 weeks duration (patient 185) to persistent disease continuing diazoxide therapy at 10.7 years of age (pt 194). Similar to the other groups of patients with an *ABCC8* mutation, the mean age of presentation in this group was early at 35.5 days of age (median of 1 day) and the mean birth weight SDS was 1.27 SDS.

This category includes 11 patients (four related and seven unrelated) with seven different dominantly inherited mutations occurring in NBD2 area of the SUR1 protein. Five of these mutations are novel. The clinical phenotype of these patients and the functional characterisation of the mutations are detailed in chapter 9. Apart from the patients with

dominantly inherited mutations, there are 11 patients where a single mutation was inherited from an unaffected parent, seven (A309G, A355T, V601I, A726T, E1443D, A1303V, A1185E) of which are novel.

CHI due to a single mutation in *ABCC8*, where diazoxide responsiveness was not known:

Two patients (patients 281 and 172) with mutations G1479A and R1539Q in *ABCC8* had mild CHI limited to the neonatal period that did not require treatment with diazoxide. The R1539Q mutation is known to be a dominant mutation (Pinney *et al.,* 2008) while the G1479A mutation is a novel dominant mutation. The genotype and phenotype of the patients with mild CHI due to dominantly inherited mutations in the K_{ATP} channel genes are described in detail in chapter 9.

Table 5.4: Summary of the patients heterozygous for a mutation in *ABCC8*: Twenty-three novel mutations were identified. A paternal mutation was associated with both focal and diffuse disease. Dominantly inherited mutations were identified to be associared with both diazoxide responsive and unresponsive disease.

ID	Mutation	Exon/intron	Novel	Ethnicity	Inheritance	Age of Presentation (days)	Birth weight SDS	Management	Histology/ PET
Diazoxide unresponsive CHI due to heterozygous mutations in *ABCC8*									
378	L1171fs	Frameshift in exon 28	Yes	White Irish	Paternal	1	+3.16	Resection of focal lesion	Focal- Giant lesion
236	M1V	Missense in exon1	No	White British	Not determined	1	+2.4	Subtotal pancreatectomy	Focal
203	c.580-1G>C	Splicing in intron 4	Yes	White British	Not determined	2	-1.13	Subtotal pancreatectomy	Focal
356	A1493T	Missense in exon 37	No	White Irish	Paternal	1	+2.15	Resection of the focal lesion	Focal- Giant
260	E128K	Missense in exon3	No	White British	Paternal	14	+1.27	Subtotal pancreatectomy	Focal
304	c.2669_2675delAGCTACA	Frameshift deletion	Yes	White British	Paternal	1	+2.5	Resection of focal lesion	Focal
197	R934X	Nonsense in exon 23	No	White Irish	Paternal	2	+2.6	Resection of focal lesion	Focal
235	S12X	Nonsense in exon 1	Yes	White British	Paternal	Not known	-0.87	Resection of focal lesion	Focal
136	R1494W	Missense exon 37	No	White British	Paternal	1	+0.45	Subtotal pancreatectomy	Focal
230	R74W	Missense in exon 2	No	Other White	Paternal	1	+0.48	Resection of focal lesion	Focal

110	Q1373X	Nonsense in exon 33	Yes	Other Asian	Paternal	70	+1.54	Subtotal pancreatectomy	Focal
86	c.1629-2A>C	Intron 10	Yes	White British	Paternal	1	+1.73	Resection of focal lesion	Focal
182	G111R	Missense in exon 3	No	Asian Indian	Paternal	3	-1.98	Resection of focal lesion	Focal
129	H627fs	Frameshift in exon 13	No	White Irish	Paternal	1	+0.78	Resection of focal lesion	Focal
211	H627fs	Frameshift in exon 13	No	White British	Paternal	90	+0.07	Octreotide for 18 months, dietary mgmt till 7 yrs	Not known
132	G92D	Missense in exon 2	No	Other Asian	Paternal	1	+1.6	Subtotal pancreatectomy	Diffuse
97	A1263T	Missense in exon 31	Yes	White British	Paternal	1	+3.06	Subtotal pancreatectomy	Diffuse
270	A1263T	Missense in exon 32	Yes	White British	Paternal	900	-1.82	Near total pancreatectomy	Diffuse
87	IVS32-9G>A	Splicing intron 32	No	White British	Paternal	1	+0.23	Octreotide	Diffuse (PET scan)
98	A113V	Missense in exon 3	No	White British	Paternal	1	Not known	Near total pancreatectomy x2	Diffuse
279	IVS38-2A>G	Splicing in intron 38	No	Other White	Paternal	84	-1.59	Subtotal pancreatectomy	Diffuse
Diazoxide unresponsive CHI due to a dominant mutation in *ABCC8*									
300	M1514K	Missense in exon 37	Yes	White British	De novo	1	+1.01	Near total pancreatectomy	Diffuse
295	D1506E	Missense in exon 37	Yes	White Irish	Maternal	1	+2.54	Subtotal pancreatectomy	Diffuse
57	D1506E	Missense in exon 37	Yes	White British	De novo	1	+2.61	Near total pancreatectomy x2	Diffuse

9	G1485E	Missense in exon 37	Yes	White	De novo	1	+1.94	Subtotal pancreatectomy	Diffuse
Transient CHI due to heterozygous *ABCC8* mutation (diazoxide responsiveness not known)									
281	R1539Q	Missense in exon 39	No	White Irish	Maternal	1	+0.07	Nil- Transient	n/a
172	G1479A	Missense in exon 37	Yes	White British	De novo	3	+1.02	Nil- transient	n/a
Diazoxide responsive CHI due to heterozygous mutations in *ABCC8*									
15	L1390R	Missense in exon 34	Yes	White British	De novo	1	+3.22	Continuing diazoxide at 11 yrs of age	n/a
146	A1508P	Missense in exon 37	Yes	White British	Paternal	1	+4.09	Continuing diazoxide at 9.2 yrs of age	n/a
194	G1479R	Missense in exon 37	No	White British	Maternal	1	+1.37	Continuing diazoxide at 10.1 yrs of age	n/a
383	G1479R	Missense in exon 37	No	White Irish	Paternal	2	+1.65	Continuing diazoxide at 8.5 yrs of age	n/a
382	G1479R	Missense in exon 37	No	White Irish	Paternal	2	+2.23	Continuing diazoxide at 5.1 yrs of age	n/a
70	Q1459E	Missense exon 36	Yes	White British	Maternal	1	+3.14	Continuing diazoxide at 5.9 yrs of age	n/a
50	R1539Q	Missense in exon 39	No	White Irish	Maternal	1	+2.19	Continuing diazoxide at 3.3 yrs of age	n/a
8	A1537V	Missense in exon 38	Yes	White British	Maternal	1	+4.66	Continuing diazoxide at 3.1 yrs of age	n/a
310	L1431F	Missense in exon 35	Yes	Black African	Paternal	365	-0.98	Continuing diazoxide at 2.5 yrs of age	n/a

313	G1479R	Missense in exon 37	No	White Irish	Maternal	1	+2.82	Continuing diazoxide at 1.4 yrs of age	n/a
306	G1479R	Missense in exon 37	No	White Irish	Paternal	2	+1.93	Continuing diazoxide at 1.5 yrs of age	n/a
271	A355T	Missense in exon 7	Yes	Not known	Maternal	90	+0.88	Continuing diazoxide at 5.9 yrs of age	n/a
2	A309G	Missense in exon 6	Yes	White British	Maternal	1	-0.0	Continuing Diazoxide at 2.1 yrs of age	n/a
128	D1472N	Missense in exon 36	No	Asian Indian	Paternal	3	-1.91	Diazoxide for 1 year	n/a
329	IVS32-9G>A	Splicing in intron 32	No	Not known	Paternal	28	+0.11	Diazoxide stopped due to severe hypertrichosis. Continuing octreotide at 1 yr	n/a
344	V601I	Missense in exon 12	Yes	White British	Paternal	3	+1.19	Diazoxide for 9 months	n/a
156	E1443D	Missense in exon 36	Yes	Mixed-White and Black African	Maternal	14	-1.41	Diazoxide for 9 months	n/a
368	A1185E	Missense in exon 28	Yes	White Irish	Paternal	1	Not known	Diazoxide for 8 months	n/a
10	IVS32-9G>A	Splicing in intron 32	No	Other White	Paternal	1	+3.43	Diazoxide for 5 months	n/a
224	A726T	Missense in exon 16	Yes	Other White	Not determined	1	+0.79	Diazoxide for 3 months	n/a
186	A1303V	Missense in exon 32	Yes	White British	Not determined	1	Not known	Diazoxide for 4 weeks	n/a
185	C418R	Missense in exon 8	No	Other White	Not known	1	+2.27	Diazoxide for 4 weeks	n/a

5.3.3 Ethnicity

The ethnic backgrounds of the 119 patients are summarized below. The five major groups include White British (n= 42), Other Asian (n=21), White Irish (n= 14), Asian Pakistani (n= 12), and Asian Indian (n=10). The missense mutation G111R was found in 6/10 unrelated patients from the Asian Indian background. This mutation was not found in patients in any other ethnic group. Apart from this mutation, no other mutation was strikingly associated with a particular ethnic background.

Ethnic background	**Number of patients**
White British	42
White Irish	14
Other White	9
Asian Indian	10
Asian Pakistani	12
Asian Bangladeshi	2
Other Asian	21
Chinese	2
Black African	4
Mixed White and Black African	1
Not known	2

5.4 Discussion

This is the largest cohort of patients with CHI due to mutations in the *ABCC8* and *KCNJ11* genes encoding the two subunits of the pancreatic ß-cell adenosine triphosphate (ATP)-sensitive potassium (K_{ATP}) channel. The study confirms that recessive mutations in these two genes are the most common cause of CHI (Thomas *et al.,* 1995; Thomas *et al.,* 1996). It also confirms that these recessive mutations cause severe CHI that is unresponsive to medical therapy with diazoxide and requires a near total pancreatectomy in the vast majority of patients. Histologically, the recessive mutations in this study, as reported in the literature, were associated with diffuse CHI. The study also confirms that dominant K_{ATP} channel mutations are a cause of mild, diazoxide responsive CHI (detailed in chapter 9). In addition, this study extends the clinical phenotype associated with recessive K_{ATP} channel mutations to include transient CHI; and that of dominant K_{ATP} channel mutations to severe CHI requiring near total pancreatectomy. These two novel phenotypic features associated with K_{ATP} channel mutations are discussed in detail below.

Recessive mutations in the K_{ATP} channel genes have not been reported to cause transient CHI. In this study, a neonate with transient CHI was identified to be compound heterozygous for the R168C and S606T mutations in the *ABCC8* gene. This patient (pt 88) presented with severe HH at two hours of age and failed to respond to maximum doses of diazoxide. However, he unexpectedly had complete resolution of his HH at eight weeks of age. This is the first case of transient HH due to a compound heterozygous mutation in *ABCC8* (now published Kumaran *et al.,* 2010).

Compound heterozygous mutations in *ABCC8* and *KCNJ11* account for approximately 13% of CHI mutations and are usually associated with medically unresponsive CHI (Suchi *et al.,* 2003). To date, there is one report of a patient with a compound heterozygous

ABCC8 mutation who was responsive to diazoxide (Dekel *et al.,* 2002). Dekel et al described a patient with compound heterozygosity for two common mutations previously associated with CHI in Ashkenazi Jews, the splicing mutation of intron 32(IVS32-9G>A) and the deletion of phenylalanine at codon 1388 (F1388del) of the *ABCC8* gene. The patient had a relatively low glucose utilisation (<10mg/kg/min) and interestingly responded to diazoxide. The authors postulate that the splicing mutation of intron 32 was responsible for the diazoxide responsiveness as patients homozygous for F1388del have been reported to have severe diazoxide resistant disease (Nestorowicz *et al.,* 1996).

Greer et al (2007) have reported a patient who was compound heterozygote for the R168C/R1421C mutations in the *ABCC8* gene and had severe, medically unresponsive CHI. No functional characterisation of the R168C mutation has yet been undertaken. Along with the R168C mutation, our patient has a novel mutation S606T. The S606T mutation results in a substitution of a G>C at the last base of exon 12 and is predicted to cause aberrant splicing. Compound heterozygote mutations in CHI result in complex interactions (Muzumbya *et al.,* 2007) and it is possible that the combination of mutations and their interactions may modify the potential disease pathogenesis and this requires further evaluation using functional analysis.

Muzyamba et al (2007) studied a patient with diffuse disease who had inherited the mutations D1193V and R1436Q in the *ABCC8* gene. Channel complexes containing the D1193V mutant were delivered to the plasma membrane and were functional and those containing R1436Q were also present at the plasma membrane but were nonfunctional. Combining the two mutations (SUR1D1193V/R1436Q) led to intracellular retention of the channel complex. This suggests that mutations may interact to modify K_{ATP} channel function and influence disease severity. Although the precise molecular mechanism/s leading to transient CHI in this patient is/are not known it is possible that the interaction of

the two mutations (R168C/S606T) may lead to transient impairment of K_{ATP} channel function. In vitro studies will be needed to assess the functionality of the mutated protein and the interactions of these two mutations.

This case illustrates that some patients with compound heterozygous mutations may have resolution of CHI even as early as eight weeks. It also highlights the importance of delaying pancreatic surgery (while maintaining normoglycaemia) even in genetically confirmed diffuse disease unresponsive to diazoxide therapy, as the natural course of many of the novel mutations is unknown.

This study also identifies three novel heterozygous *ABCC8* missense mutations (G1485E, D1506E and M1514K) in 4 patients from 3 families causing diazoxide unresponsive disease. The mutations had arisen *de novo* in all four pedigrees. A second mutation was not found in any of the 4 patients by sequence analysis. Histological examination of the pancreatic samples was consistent with diffuse disease. In conclusion, G1485E, D1506E and M1514K mutations are dominantly acting *ABCC8* mutations associated with diffuse disease with a 50% risk of HH in future offspring. This clinical presentation is very different to that described in previous reports of dominant *ABCC8* missense mutations which were associated with a milder phenotype (Huopio *et al.,* 2007, Pinney *et al.,* 2008). These mutations identified are novel, suggesting a genotype/phenotype correlation where different missense mutations may result in a variable severity of CHI. This hypothesis is supported by the identification of the same mutation, D1506E, in two individuals from different families with diazoxide-unresponsive CHI.

Pancreatic ß-cell K_{ATP} channels are regulated by intracellular nucleotides (ATP and ADP). SUR1 has two cytoplasmic nucleotide binding domains (NBD1 and NBD2) that sense changes in intracellular [ATP]/[ADP] and transmit the signal to the pore. NBD1 appears to

be the principal site for ATP binding, whereas NBD2 binds MgADP (Conti *et al.,* 2001). These binding domains cooperate with each other in mediating the nucleotide regulation of the pore function (Ueda *et al.,* 1999). MgADP and diazoxide activate K_{ATP} channels in the presence of inhibitory concentrations of ATP, and both processes require Mg^{2+} (Shyng *et al.,* 1997; Dunne and Peterson 1986; Nichols *et al.,* 1996). Mutations in NBD2 can abolish channel activation by diazoxide or MgADP (Shyng *et al.,* 1997). This loss of metabolic inhibition by MgADP on K_{ATP} channel activity has been reported for patients with CHI due to recessive *ABCC8* mutations and seems to be a common molecular mechanism of CHI (Nichols *et al.,* 1996, Shyng *et al.,* 1998).

Nine of the 11 previously reported dominant *ABCC8* mutations affect residues within the NBD2 region and functional studies have demonstrated that these mutations significantly diminish or completely abolish the channels' response to MgADP and diazoxide (Huopio, *et al.,* 2000; Pinney *et al.,* 2008). Further studies on 10 of the *ABCC8* mutations, under simulated heterozygous conditions, indicated that while the mutant subunits do reduce channel function, the wild type *ABCC8* allele is sufficient to confer partial channel response to changes in the ATP/ADP ratio expected during glucose metabolism and to diazoxide (Pinney *et al.,* 2008).

In this study, thirteen patients (from nine families) were identified to be heterozygous for eight different dominantly inherited mutations occurring in NBD2 area of the SUR1 protein. Six of these *ABCC8* mutations (L1451F, L1390R, Q1459E, A1537V, A1508P and Q1479A) are novel. The phenotype associated with these dominantly inherited mutations (discussed in detail in chapter 9) confirms the reported phenotype associated with dominant mutations in the NBD2 area (Pinney *et al.,* 2008; Huopio *et al.,* 2000), ranging from asymptomatic macrosomia to persistent diazoxide responsive CHI. However, the three mutations (G1485E, D1506E and M1514K) identified with diazoxide unresponsive

CHI are also located in the NBD2 region of SUR1 and it is likely that they also abolish the stimulatory effects of MgADP/ATP. However, the reasons underlying the lack of response to diazoxide in the 4 patients with the G1485E, D1506E and M1514K mutations is not known.

These findings suggest that the clinical presentation of patients with dominant inactivating *ABCC8* missense mutations is variable, ranging from mild medically responsive to severe early onset CHI requiring a near total pancreatectomy. The clinical presentation of patients at the time of diagnosis cannot distinguish between recessive and dominantly acting *ABCC8* mutations. A genetic diagnosis is important since finding a *de novo* dominant mutation in a sporadic case with diazoxide unresponsive CHI confers a lower recurrence risk for future siblings compared to a recessive mutation, but a higher risk of CHI (50%) for the next generation.

In addition, this study demonstrates that a paternal mutation in the *ABCC8/ KCNJ11* in association with diazoxide unresponsive disease is not synonymous with a focal lesion. Of the 23 patients with diazoxide unresponsive disease and an identifiable paternal mutation (4- *KCNJ11* and 19- *ABCC8*), 15 had confirmed focal disease, 7 had diffuse disease (confirmed by an 18F-DOPA-PET scan or on histological analysis) and the histological diagnosis was not confirmed in one. The mechanism of diffuse disease in these patients is not understood. It is possible that the sequencing technique was unable to pick a second *ABCC8* mutation. It is also possible that there is a second hit within the pancreas for e.g. loss of a part of the maternal 11p15 affecting the entire pancreas (leading to a large focal lesion affecting the entire pancreas) or a second pancreatic specific mutation. Genetic analysis of the pancreatic samples is warranted to test this hypothesis.

Thirteen patients with diazoxide responsive CHI were heterozygous for a single mutation in the *ABCC8/ KCNJ11* gene. Four of these mutations are known to act recessively and it is possible that the second mutation has not been identified by the sequencing technique. However, the phenotype of these patients does not fit in with recessively inherited CHI that is generally diazoxide unresponsive (as is also seen in this series). The remaining nine mutations are novel. These have been classified as disease causing mutations as they have not been found in control chromosomes and occur in residues that are conserved across species. These mutations have not been classified as dominant mutations as they are inherited from a parent unaffected by hypoglycaemia or diabetes or the parental origin has not been determined. However, it is possible that they act dominantly (with variable penetrance) as many of the adult mutation carriers in the only series of dominantly acting mutations (Pinney *et al.,* 2008) were asymptomatic. The identification of these mutations in other pedigrees displaying recessive/ dominant patterns of inheritance would help in establishing the inheritance of these mutations.

Finally, this series identified a missense mutation G111R in 6/10 unrelated families of Indian background. Three of these patients were homozygous for the mutation; two were compound heterozygous for the mutation and one patient with focal disease was heterozygous for the mutation. All of these patients had severe, diazoxide unresponsive disease. Two *ABCC8* mutations, splicing mutation IVS32-9G>A in intron 32 and the delF1388 mutation have been identified to be associated with CHI in the Ashkenazi Jewish population (Nestorowicz *et al.,* 1996) and the V187D mutation has been associated with the Finnish population (Otonkoski *et al.,* 1999). No mutation has yet been reported to be associated with CHI in the Indian population. The recognition of the G111R mutation exclusively in 6/10 patients from an Indian ethnic background suggests a founder effect.

5.5 Conclusions

This is the largest study of patients with CHI due to mutations in the K_{ATP} channel genes. This study confirms that the commonest known cause of CHI is mutations in the K_{ATP} channel genes, of which the majority were inherited in a recessive manner. It also confirms that recessively inherited mutations in the K_{ATP} channel genes are associated with diazoxide unresponsive disease while the dominantly inherited mutations in these genes are generally associated with mild, diazoxide responsive disease. In addition, the study identified several novel mutations in the *ABCC8* and *KCNJ11* genes. Of the novel mutations, the highlights of the study were the identification of dominant mutations in *ABCC8* as a cause of diazoxide unresponsive disease and the identification of a compound heterozygous mutation in *ABCC8* in association with severe CHI that was transient in nature. The identification of these mutations would be important in future patients in predicting the clinical course and in estimating the risk of CHI in their future siblings and offspring. Finally, the study identified, for the first time, a known mutation G111R to be a common mutation in the Indian population with CHI.

Chapter 6

Congenital Hyperinsulinism due to mutations in the *GLUD1* Gene

6.1 Background

The hyperinsulinism/hyperammonemia (HI/HA) syndrome is a form of CHI in which affected children have recurrent symptomatic HH together with a persistently elevated plasma ammonia concentration. It has been shown that this disorder is caused by dominant mutations of the *GLUD1* gene that encodes for the mitochondrial enzyme, glutamate dehydrogenase (GDH) (Stanley *et al.*, 1998). GDH is highly expressed in the liver, pancreatic ß-cells, kidney and the brain (Hudson and Daniel, 1993). It catalyzes the oxidative deamination of glutamate to α-ketoglutarate and ammonia. In the pancreatic ß-cells, α-ketoglutarate enters the tricarboxylic acid cycle and leads to an increase in the concentration of cellular ATP. This rise in the cellular ATP causes closure of the ATP sensitive potassium channel (K_{ATP} channel); resulting in cell membrane depolarisation, Ca^{2+} influx via voltage gated calcium channels and insulin exocytosis.

GDH is allosterically regulated by a wide array of inhibitors (e.g. GTP) and activators (e.g. leucine) (Fahein *et al.*, 1980). Activating mutations in the *GLUD1* gene reduce the sensitivity of the enzyme to allosteric inhibition by GTP and ATP (Stanley *et al.*, 2000) or less frequently cause an increase in the basal GDH activity (Yorifugi *et al.*, 1999). The loss of inhibition by GTP leads to increased leucine induced glutamate oxidation to α-ketoglutarate. Hence leucine sensitivity is manifested by postprandial HH (following protein-rich meals) which is a classical feature of this condition (Hsu *et al.*, 2001). The mechanism of persistent hyperammonaemia, a striking and consistent feature of this condition, is not completely understood.

This chapter discusses the phenotype and genotype of twenty patients with HI/HA syndrome. A patient with a normal serum ammonia concentration is described who has severe leucine sensitivity with a novel *GLUD1* mutation. The chapter also highlights the high risk of epilepsy in patients with *GLUD1* mutations.

6.2 Research design and methods

6.2.1 Patients

All the recruited patients with a raised serum ammonia concentration (n=19) had mutational analysis of the *GLUD1* gene. The exons of the *GLUD1* gene which encode the catalytic and allosteric domains of glutamate dehydrogense (exons 6, 7, 10, 11 and 12) were amplified by PCR (for primer sequences see appendix 12.4 A) and sequenced using Big Dye terminator v3.1 (Applied Biosystems, Warrington, UK). Sequencing reactions were analysed on the ABI 3730 (Applied Biosystems, Warrington, UK) and compared to the published sequence (M37154).

In one patient, *GLUD1* analysis was requested in view of leucine sensitivity. This patient (patient 12) presented at one year of age with a tonic–clonic seizure associated with HH (serum insulin concentration of 9.6mU/L with a concomitant blood glucose concentration of 1.7mmol/L). She had a normal serum ammonia concentration (21μmol/L) on presentation and on repeated measurements (30μmol/L and 41μmol/L) during childhood. She responded to diazoxide and demonstrated a normal fasting tolerance on a moderate dose of diazoxide (12-15mg/kg/day). However she continued to experience intermittent hypoglycaemic episodes in the post-prandial period even on a high dose of diazoxide (15-17mg/kg/day). Leucine sensitivity was hence evaluated at 13 years of age by an oral leucine tolerance test.

Oral leucine tolerance test: Following a fast of four hours, an oral dose of 0.15gm/kg of L-Leucine was administered. Blood glucose and plasma insulin concentrations were then measured at -30, 0, 30, 60, 90 and 120 minutes. Diazoxide was not discontinued for the purpose of the test. The test was stopped at 60 minutes following the oral leucine load as she developed symptomatic hypoglycaemia (sweating, disordered consciousness and blurred vision) with a blood glucose concentration of 2.2mmol/L and simultaneous serum insulin concentration of 69.5mU/L (table 6.1). Sequencing of the *GLUD1* gene was requested in view of the severe leucine sensitivity, a feature of HI-HA syndrome.

Table 6.1: Oral Leucine Tolerance Test in patient 12 demonstrating leucine induced hyperinsulinaemic hypoglycaemia:

Time (minutes)	Glucose (mmol/L)	Insulin (mU/L)
-30	4.5	4.1
0	4.0	8.5
+60	2.2	69.5

Hence, a total of 20 patients had mutational analysis of the *GLUD1* gene. Sixteen patients were unrelated and four were related demonstrating vertical transmission of disease consistent with a dominant pattern of inheritance. Along with the standard clinical information (birth weight, age of presentation, treatment details, family history of hypoglycaemia / diabetes) as outlined in chapter 3, information regarding seizure history/ epilepsy was also collated. Birth weight SDS was calculated for each patient. The clinical characteristics are presented as mean. Comparison of the phenotypic characteristics (birth weight, age of presentation) with patients with diazoxide responsive/ transient CHI and a K_{ATP} mutation (n= 27), *HNF4A* mutation (n= 8) and a *HADH* mutation (n=3) was done

using the one-way analysis of variance (ANOVA) followed by LSD post-test to test for statistical significance. The comparative statistics are presented in chapter 8. Birth weights of the three patients with the S445L mutation in *GLUD1* was compared with the birth weights of the rest of the cohort with a *GLUD1* mutation using ANOVA followed by LSD post-test.

6.2.2 Functional analysis of GDH activity (Performed by Dr Chadefaux at Necker Hospital, Paris)

In order to confirm the pathogenecity of a novel mutation, P436L, GDH activity was analysed (in collaboration with colleagues at the Necker Hospital, Paris) in lymphoblast homogenates according to the method of Wrzeszczynski & Colman (Wrzeszczynski & Colman, 1994). Lymphocytes were isolated from peripheral blood of the patient with the novel P436L mutation and transformed with Epstein-Barr virus to establish lymphoblast cultures. Lymphoblasts were harvested and washed with PBS. Cells were resuspended in homogenising buffer (10mM Tris pH 7.4 + 0.1% Triton – X100) then homogenised using a mechanical homogeniser for 3 x 30sec before being centrifuged at 18000g for 10 minutes. The supernatant was retained to be used in the assay. An aliquot of lymphoblast supernatant was added to the following reaction mixture made up in 10mM Tris buffer pH 8.0 to a reaction volume of 980µl (final concentrations: NH_4Cl 35mM, NADH 90µM, rotenone 2.5µM and EDTA 1mM.)

Assays were carried out at 30˚C in 1ml cuvettes .Absorbance was recorded at 340nM to establish a baseline. Another absorbance measurement was taken at 5 minutes. Then 8mM α-ketoglutarate was added and absorbance was recorded after a further 5 minutes. Blank cuvettes contained all the reaction mixture apart from α-ketoglutarate. Change in absorbance was recorded at all time points and the extinction coefficient for NADH

($6.22x10^3 M^{-1}cm^{-1}$) was used to calculate a rate (de Lonlay *et al.,* 2001). Enzyme activity was expressed as nmol/min/mg protein. Protein was measured according to the method of Lowry *et al* (Lowry *et al.*, 1951).

To measure sensitivity of GDH to inhibition by GTP, a dose curve was set up using concentrations of GTP from 50nM-1000nM and absorbance was measured. Absorbances after addition of GTP were expressed as a percentage of basal absorbances and plotted on a dose curve to establish the half-maximal inhibitory concentration (IC50).

6.3 Results

6.3.1 Molecular Genetics

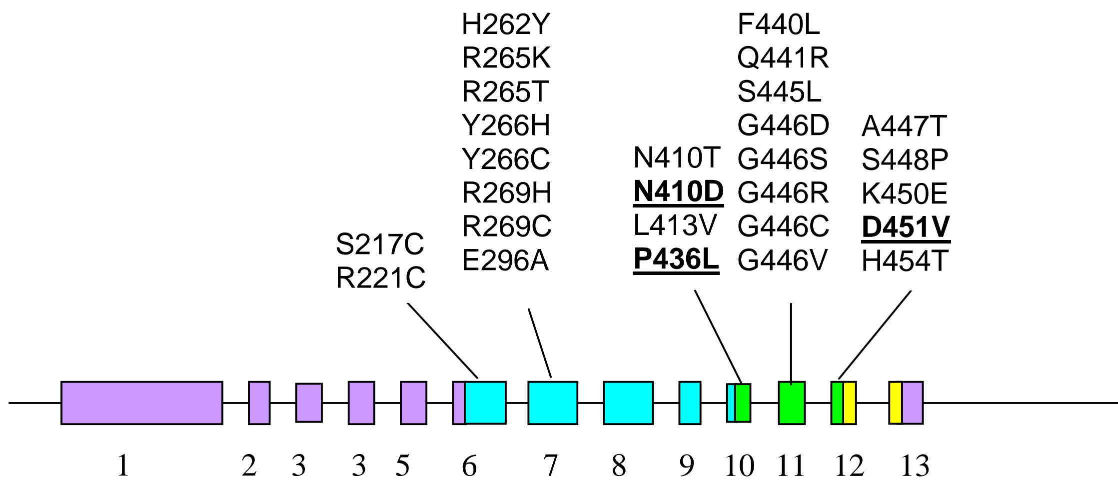
16/20 patients were identified to be heterozygous for a mutation in the *GLUD1* gene. Eight different heterozygous mutations were identified in 13/17 probands (76%) screened. Three mutations; N410D (c.1400A>G), D451V (c.1524A>T) and P436L (c.1479C>T) were novel. These mutations were not present in the probands' unaffected parents and affected residues were conserved across species. Mutations at residue N410 have previously been reported in patients with HI/HA, including 1 patient within this cohort, suggesting that N410 is a functionally important residue. The location of the novel mutations in context of previously described mutations is demonstrated in figure 6.1.

Two mutations, S445L and R269H, were each identified in three unrelated families. All other mutations were identified in a single proband. Testing of unaffected parents demonstrated that the mutation had arisen de novo in 11/13 probands. For one patient, with a S445L mutation, a spontaneous mutation could not be confirmed as DNA was unavailable from the unaffected father. In a second patient, the R269H mutation was inherited by the proband from their affected mother. Testing of further family members

demonstrated that the R269H mutation was also present in the proband's maternal uncle and grandmother, all of whom have HI/HA.

Four patients referred with HI/HA were negative for a *GLUD1* mutation. The mean serum ammonium concentration of this group of patients was 66.7μmol/l (50-87μmol/l). All four patients had diazoxide responsive hyperinsulinism with no clinical symptoms suggestive of protein/leucine sensitivity.

Figure 6.1: Cartoon showing location of mutations along a representation of the *GLUD1* gene, the different colours signify the subunits of the protein (the purple region interacts with NADP, the blue with GTP and ATP, the yellow structure is the pivot helix and the green antenna region interacts with other proteins). The novel mutations identified in this study are underlined and in bold.



6.3.2 Clinical characteristics of patients with *GLUD1* mutation:

The clinical characteristics of the sixteen patients with a *GLUD1* mutation is summarised in table 6.2. The mean birth weight SDS of our cohort with a mutation was -0.11. Macrosomia was not a common feature; with birth weight >90th centile in only three patients. All of these three patients had the S445L mutation. The difference in the birth weights of these three patients and the rest of the cohort with a *GLUD1* mutation was statistically significant (P=0.027).

The age of presentation was delayed (mean of 22.4 weeks), in comparison with the patients with diazoxide responsive CHI due to K_{ATP} channel mutations (mean of 27 days, P<0.005) and those due to *HNF4A* mutations (mean of 1 day, p=0.001), with only three presenting in the neonatal period. Two patients were successfully managed with manipulation of diet alone (low protein diet); all others required treatment with diazoxide. Interestingly, two members of family M due to mutation R269H were successfully treated with dietary management alone whilst two have required diazoxide therapy (figure 6.2), suggesting a degree of clinical heterogeneity within the same family. None of our patients required a pancreatectomy.

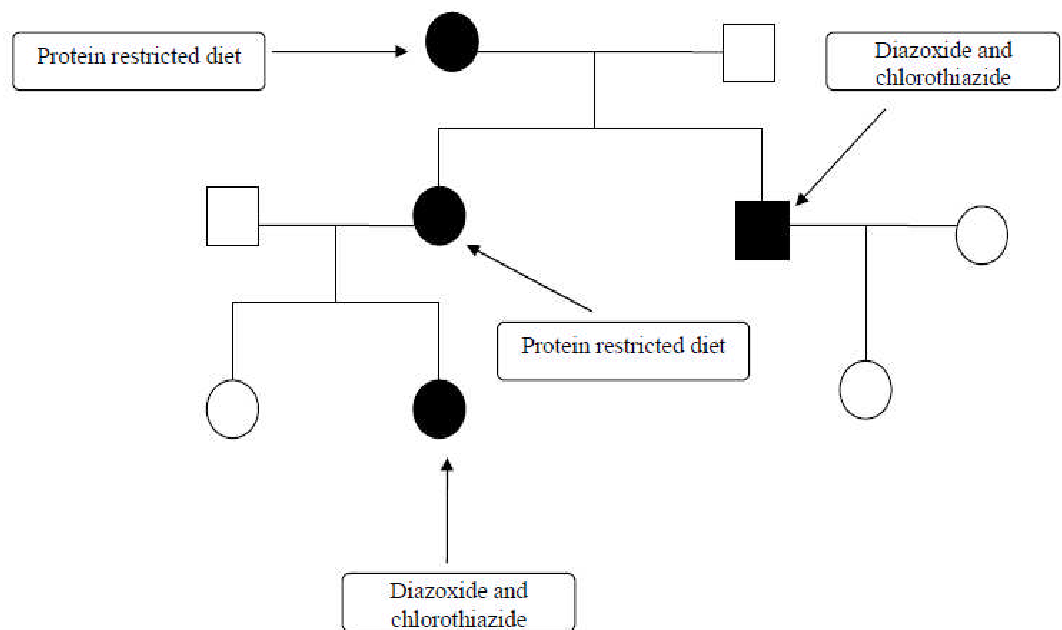
There was a high incidence of generalised seizures in the patients with a *GLUD1* mutation. 15/16 patients presented with seizures and seven (43%) of them have developed epilepsy. All of the patients who developed epilepsy had generalised seizures. 3/7 patients with epilepsy had mutations in exons 6 and 7. The other four patients had mutation in exons 10 and 11, of which three had the mutation S445L in exon 11.

Table 6.2: Clinical characteristics of the patients with a *GLUD1* mutation (Kapoor *et al.*, 2009b): The mean birth weight SDS was -0.11 and the mean age of presentation was 22.4 weeks. None of the patients with a *GLUD1* mutation required a pancreatectomy.

Family	No	Sex	Age Of presentation	Current age	Birth weight SDS	Epilepsy	Ammonia (umol/L) (<50umol/L)	Management	Mutation	Novel
A	1	F	52 weeks	13.8 yrs	-0.58	No	21	Diazoxide +protein restriction	P436L (c.1479C>T)	Yes
B	2	F	1 week	10 yrs	+0.47	Yes	120	Diazoxide	N410D (c.1400A>G)	Yes
C	3	F	0.28 weeks (2 days)	7 yrs	+0.57	Yes	58-95	Diazoxide	R221C (c.833C>T)	No
D	4	F	6 weeks	18 yrs	+1.2	Yes	244	Diazoxide	S445L (c.1506C>T)	No
E	5	F	1 week	1.6 yrs	+1.73	Yes	125-161	Diazoxide	S445L (c.1506C>T)	No
F	6	F	32 weeks	2.5 yrs	+0.29	Yes	128	Diazoxide	R269H (c.978G>A)	No
G	7	F	28 weeks	2.6 yrs	-1.2	No	73-144	Diazoxide	D451V (c.1524A>T)	Yes

H	8	M	24 weeks	1.6 yrs	-1.3	Yes	258-307	Diazoxide	R265K (c.966G>A)	No
I	9	M	8 weeks	4.8 yrs	+1.63	Yes	150-190	Diazoxide	S445L (c.1506C>T)	No
J	10	M	16 weeks	0.9 yrs	-1.34	No	250	Diazoxide	N410T (c.1401A>C)	No
K	11	M	28 weeks	1.4 yrs	-0.41	No	165	Diazoxide	R269H (c.978G>A)	No
L	12	M	72 weeks	5.9 yrs	Not known	No	60-100	Diazoxide	R296H (c.978G>A)	No
M	13	F	24 weeks	2.5 yrs	-2.37	No	96	Diazoxide	R269H (c.978G>A)	No
M	14	F	52 weeks	35 yrs	Not known	No	78	Protein restricted diet	R269H (c.978G>A)	No
M	15	F	Not known	56 yrs	Not known	No	Not known	Protein restricted diet	R269H (c.978G>A)	No
M	16	M	52 weeks	26 yrs	-2.05	No	103	Diazoxide	R269H (c.978G>A)	No

Figure 6.2: Family tree of patients 13-16 with the R269H mutation. Circles represent females and squares represent males. Individuals with HI/HA and the R269H mutation in *GLUD1* are represented by filled symbols. Two members with HI/HA due to this mutation were successfully treated with dietary management alone whilst two have required diazoxide therapy.



6.3.3 GDH activity in lymphoblasts:

The activity and allosteric response of GDH in lymphoblasts from patient 12 (with the P436L mutation in *GLUD1*) is shown in table 6.3. She had normal basal GDH activities. However, the half-maximal inhibitory concentration of GTP expressed in nmol/L (IC50) was 470% higher than that of controls (n=20) i.e. a higher concentration of GTP (625nmol/l) was required to inhibit 50% of the P436LGDH in comparison with the control GDH (mean of 133nmol/l). This is consistent with a loss of GTP inhibition, a mechanism known to cause HI/HA.

Table 6.3: Activity and allosteric responsiveness of GDH in lymphoblasts from patient 12 and normal subjects (n=20) (Kapoor *et al.,* 2009b): The GTP concentration required to inhibit GDH activity in patient lymphoblast was 470% higher than that of controls.

	Patient 12	**Normal subjects**
Basal GDH activity (nmol/min/mg protein) Measured in the absence of effectors	11.2	13.2±3.2 (mean±SD)
IC50 GTP concentration (nmol/L)	625	133±77 (mean±SD) or <275

6.4 Discussion

The phenotype of CHI due to mutations in the *GLUD1* gene is well characterised. It is reported to be milder, not usually associated with macrosomia at birth and tends to escape recognition till later in infancy (Stanley *et al.,* 2000; Hsu *et al.,* 2001; de Lonlay *et al.,* 2002). This was confirmed in this study where the mean age of presentation was 22.4 weeks with only 3/17 patients presenting in the neonatal period. Interestingly, only three patients had a birth weight of >90th centile and all three had the S445L mutation (*P*=0.027). This may indicate that this mutation causes more severe fetal hyperinsulinism than the other mutations identified in our cohort. This mutation, along with the R269H mutation, was also the most prevalent mutation in our cohort. Three patients each were heterozygous for these two mutations (S445L and R269H) accounting for 46% of our patients with CHI due to mutations in the *GLUD1* gene.

Missense mutations causing HI/HA were first described to occur in exons 11 and 12 that form the hinge and antenna regions of GDH and lies close to the binding site for GTP (Stanley *et al.,* 1998 and Stanley *et al.,* 2000). Mutations were then described in exons 6 and 7 that lie in the GTP binding region (Miki *et al.,* 2000; Santer *et al.,* 2001). Finally, mutations in exon 10 were described located in the α-helix of the antenna like structure of the GDH (Fujioka *et al.,* 2001; Yorifuji *et al.,* 1999). Of the 13 probands with a *GLUD1* mutation, 6 had mutations in the GTP binding domain (exons 6 and 7), 2 had mutations in exon 10 while 5 had a mutation in exons 11/ 12.

Epilepsy has been frequently reported in association with HI/HA syndrome (Raizen *et al.,* 2005; Bahi-Buisson *et al.,* 2008a). The increased frequency of epilepsy is thought to be either the result of a) hypoglycaemic brain injury due to recurrent hypoglycaemia or b)

chronic hyperammonaemia or c) decreased concentrations of glutamine and the neurotransmitter GABA (gamma-aminobutyric acid) in the brain due to raised GDH activity (Bahi-Buisson *et al.,* 2008a). This study confirms that a high risk of epilepsy is associated with hyperinsulinism due to mutations in the *GLUD1* gene as 7/16 (43%) of the patients developed epilepsy. The study by Bahi- Buisson et al (Bahi-Buisson *et al.,* 2008b) reported that epilepsy is associated more frequently with mutations in exons 6 and 7 (GTP binding site). This was not confirmed in this study as 4/7 patients with epilepsy had mutations in exons 10 and 11. Hence, no genotype-phenotype association according to the location of the mutations was found in this study. However, all the three patients with the S445L mutation developed epilepsy. Hence, this mutation was associated with an increased frequency of epilepsy in this cohort.

The most consistent feature of the hyperinsulinism due to mutations in *GLUD1* is the persistent hyperammonaemia and hence the term 'Hyperinsulinism/ Hyperammonaemia Syndrome' is used synonymously with CHI due to *GLUD1* mutations (Stanley *et al.,* 2000; de Lonlay *et al.,* 2001; Miki *et al.,* 2000). It is proposed that hyperammonaemia is the result of excessive GDH activity in the hepatocytes leading to a) either an increase in the production of ammonia from glutamate and/ or b) depletion of glutamate with reduction in the production of N-acetylglutamate, an allosteric activator essential for the first step of ammonia detoxification (Stanley *et al.,* 2004). However, the second mechanism is not supported by results of a study by de Lonlay *et al* where they showed that the administration of N-carbamylglutamate (an analog of N-acetylglutamate) does not normalise the concentrations of venous ammonia (de Lonlay *et al.,* 2001) in the patients. Additionally, they also described a patient with the R265T mutation who had normal venous ammonia concentrations during muscle relaxation (curarization) suggesting that hyperammonaemia may have a muscular origin (de Lonlay *et al.,* 2001). In summary, the

exact mechanism causing hyperammonaemia remains unknown, but is an important feature of CHI due to *GLUD1* gene mutations.

In this cohort, patient 12 with the novel P436L mutation has a completely normal serum ammonium concentration (highest being 41µmol/L). So far, there has been one other report of two patients (father and son) with hyperinsulinism due to the R269H mutation in the *GLUD1* gene and normal serum ammonia concentrations (35 and 28µmol/L) (Santer *et al.,* 2001). Six patients (4 from the same family) with HI/HA in this cohort who are heterozygous for the R269H mutation have elevated ammonia concentrations proving that the lack of hyperammonaemia is not mutation specific. A possible explanation for the lack of hyperammonaemia is the occurrence of mosaicism. If the origin of the hyperammonaemia is in the liver, it is possible that patients with lack of hyperammonaemia and a *GLUD1* mutation are mosaic for the mutation with the mutation being absent or at <50% in the hepatic tissues. And hence this absence of hyperammonaemia may not be genotype specific. However, this theory cannot be proved or disproved without a liver/ pancreatic biopsy. Similarly, mosaicism may be the underlying mechanism in the four patients with HI/HA syndrome and no *GLUD1* mutation with the mutation affecting the liver and pancreas, but not blood.

A genotype-phenotype correlation between serum ammonium concentration and sensitivity to GTP inhibition (MacMullen *et al.,* 2001) has been previously reported with higher serum ammonia concentrations being measured in patients with higher GTP IC50. This was not confirmed in this study where enzymatic analysis of the lymphoblastic GDH from patient 12 with the P436L mutation showed a loss of GTP inhibition with the half-maximal inhibitory concentration of GTP being 470% higher than that of controls despite the absence of hyperammonaemia. Loss of GTP inhibition is known to increase glutamate

oxidation in the presence of leucine causing leucine sensitive hyperinsulinism, a classical feature of HI/HA syndrome. This was clinically evident in patient 12 with this novel P436L mutation and was confirmed by the oral leucine tolerance test. The confirmation of leucine sensitivity prompted the sequencing of the *GLUD1* gene and led to the molecular diagnosis. This suggests that analysis of the *GLUD1* gene must be considered in all patients with leucine sensitivity even in the absence of hyperammonaemia.

Leucine sensitivity in HI/HA syndrome is thought to be responsive to treatment with diazoxide. However, anecdotal evidence suggests that some patients with HI/HA syndrome may continue to have protein induced hypoglycaemic episodes even while on diazoxide (Hsu *et al.*, 2001). This study provides evidence to support these reports as patient 12 is severely leucine sensitive despite normal fasting tolerance on a high dose of diazoxide. This emphasises the importance of evaluating leucine tolerance in patients who experience hypoglycaemic episodes on seemingly adequate diazoxide therapy with normal fasting tolerance.

The study also reports two other novel mutations (N410D and D451V) in the *GLUD1* gene. N410 is located in the antenna-like structure connecting to the pivot helix of the GDH structure. A mutation (N410T) previously described at the same residue is known to cause HI/HA associated with a higher basal GDH activity (Yorifuji *et al.,* 1999). D451 is located in the allosteric domain of the *GLUD1* gene; a common site for mutations causing HI/HA syndrome and hence likely to be pathogenic by interfering with the allosteric binding sites. Conservation data suggests that these residues are functionally important as they are both well conserved among species.

6.5 Conclusion

In conclusion, this study of 16 patients with *GLUD1* gene mutations confirms that mutations in this gene cause a mild form of CHI associated with normal birth weight, delayed age at presentation and a high risk of epilepsy. The high risk of epilepsy was not associated with the location of the mutation in this cohort of patients, as described previously (Bahi- Buisson *et al.,* 2008b). However, all three patients with S445L mutation in this cohort were large at birth and developed epilepsy.

Most importantly, this study confirmed that mutations in the *GLUD1* gene are not always associated with hyperammonaemia. This raises the possibility that the current prevalence of *GLUD1* mutations in CHI, based on screening in the presence of hyperammonaemia may be an underestimate.

The study also demonstrates that even in the absence of hyperammonaemia, patients with *GLUD1* gene mutations may be extremely leucine sensitive. Hence leucine tolerance test (performed in an experienced centre due to the associated dangers) may give an important clue to the genetic diagnosis and must be considered on all patients with hyperinsulinism who have post-prandial hypoglycaemia/ intermittent hypoglycaemic episodes. The prevalence of *GLUD1* gene mutations in patients with leucine sensitive CHI and normal serum ammonia concentrations is not known and further studies are required to understand this.

Chapter 7

Congenital Hyperinsulinism Due To Mutations in The *HADH* Gene: Characterisation Of A Novel Mutation And Observation Of Severe Protein Sensitivity

7.1 Background

The *HADH* gene encodes for the enzyme 3-hydroxyacyl-coenzyme A dehydrogenase (HADH), which is an intra-mitochondrial enzyme that catalyses the penultimate reaction in the ß-oxidation of fatty acids; the NAD^+ dependent dehydrogenation of 3-hydroxyacyl–CoA to 3-ketoacyl-CoA. Mutations in the *HADH* are a rare cause of recessively inherited CHI. Four patients have been described with HH due to homozygous mutations in this gene (Clayton *et al.,* 2001; Molven *et al.,* 2004; Hussain *et al.,* 2005c). The clinical presentation of all these reported patients was heterogeneous either with mild late onset hypoglycaemia or severe neonatal hypoglycaemia. The blood acylcarnitine profile in all reported patients revealed raised hydroxybutyrylcarnitine and urine organic acids analysis showed raised 3-hydroxyglutarate. Functional analysis of the HADH enzyme in skin fibroblasts from all these patients showed reduced expression and activity.

7.2 Protein sensitive hyperinsulinaemic hypoglycaemia

Protein sensitivity was first described in 1955 by Cochrane *et al.* (Cochrane *et al.,*1955) who described three infants with hypoglycaemia exacerbated by a high protein diet and demonstrated that these patients developed dramatic hypoglycaemia in response to a leucine load. This hypoglycaemia was associated with an excessive rise in plasma insulin.

Subsequently, leucine sensitivity was described in association with the hyperinsulinism-hyperammonaemia syndrome (HI/HA syndrome) caused by missense mutations of *GLUD1* that reduce the sensitivity of the enzyme glutamate dehydrodgenase (GDH) to allosteric inhibition by the high-energy nucleotide triphosphates GTP and ATP. GDH is allosterically activated by leucine and inhibited by GTP (Fahein *et al.*, 1990). Mutations which cause loss of inhibition by GTP cause leucine to increase the oxidation of glutamate, thereby raising the ratio of ATP/ADP in the pancreatic β-cell. The increased ratio of ATP/ADP then triggers closure of the K_{ATP} channel and triggers the release of insulin.

More recently Fourtner *et al.* (2006) described protein induced hypoglycaemia in a group of patients with K_{ATP} CHI without leucine sensitivity and demonstrated that protein sensitivity is not synonymous with leucine sensitivity. This protein sensitivity can be as severe as that seen in the HI/HA syndrome and unlike the protein sensitivity seen in HI/HA syndrome it does not respond to diazoxide.

This section describes the phenotype of the fifth case of CHI associated with a novel missense mutation in the *HADH* gene. Unlike all other previously reported cases, this patient has normal acylcarnitine and urine organic acid profile. In addition, the novel observation of severe protein sensitivity (specifically due to leucine sensitivity) in patients with mutations in the *HADH* gene is discussed.

7.3 Methodology

7.3.1 Clinical case

The index case, born to consanguineous parents of Bangladeshi origin presented at four months of age with hypoglycaemic seizures. Investigations revealed a biochemical picture

consistent with hypoketotic HH with raised serum insulin concentration of 58mU/L and low concentrations of serum fatty acids during hypoglycaemia (blood glucose concentration of 1.8mmol/L). The hypoglycaemia responded to diazoxide but she continued to have episodes of HH especially when taking a meal rich in protein. Interestingly, results of hypoglycaemia screens performed on two occasions were similar to the two patients described previously (Clayton *et al.,* 2001; Hussain *et al.,* 2005c). During a controlled fast she developed significant hypoglycaemia down to 2.4mmol/L within 5 hours of the fast with undetectable insulin and presence of serum fatty acids and ketones. However, on another occasion, she was admitted with a hypoglycaemic seizure and investigations revealed a blood glucose concentration of 2.1 mmol/L with simultaneous insulin concentration of 3.9mU/L. On this occasion, she again had low but detectable levels of non esterified fatty acids (0.9mmol/L) and ketone bodies (1.84mmol/L). However, no abnormal metabolites were detected on repeated measurements of the acylcarnitine and urinary organic acids. The hypoglycaemia responded to diazoxide but she continued to have episodes of HH especially when taking a meal rich in protein.

Given the clinical suspicion, history of consanguinity and the absence of mutations in *ABCC8* and *KCNJ11*, the *HADH* gene was sequenced and in order to investigate the intermittent nature of the hypoglycaemia, the patient was evaluated for protein sensitivity. Apart from this patient, the two patients reported (Clayton *et al.,* 2001; Hussain *et al.,* 2005c) previously from Great Ormond Street Hospital form the three patients with *HADH* mutations in this cohort.

7.3.2 *HADH* sequencing (Analysed in Peninsula Medical School, Exeter)

The eight exons of the *HADH* gene were amplified from genomic DNA of the index case and the products were sequenced at Peninsula Medical School in Exeter. The primer

sequences are outlined in appendix 12.4B. The sequences were compared to the published sequence (NM_005327.2) using Mutation Surveyor version 2.61 (Softgenetics PA, USA), as described in chapter 3.

7.3.3 Measurement of 3-hydroxyacyl-CoA dehydrogenase activity (Performed by Dr Simon Eaton, ICH, London)

HADH enzyme activity was measured following the method of El-Fakhri & Middleton (1982). Each measurement was performed on 6 fibroblast pellets from the index patient, and on 12 different controls, and data were compared by Student's t-test. Briefly, fibroblasts were thawed and resuspended in 25mM phosphate, 0.2mM EDTA, pH 8. They were then sonicated on ice (3 x 10sec). Triton X-100 was added to give a Triton:protein ratio of 1:1, then samples were incubated on ice for 30 minutes before being centrifuged at 18000g in a bench microfuge for 10 minutes.

Assays were carried out at 30˚C in 1ml cuvettes in a Uvikon spectrophotometer. Fibroblast homogenate was added to assay buffer (0.1M potassium phosphate pH 7.0/ 0.1mg/ml NADH/ 0.3mg/ml BSA (fatty acid free)). Baseline absorbance at 340nM was obtained and then 40μM of substrate was added (2-methyl-acetoacetyl-CoA for short chain, 3-ketooctanoyl-CoA for medium, 3-ketohexadecanoyl-CoA for long chain). Change in absorbance was recorded at all time points and the extinction coefficient for NADH ($6.22x10^3 M^{-1} cm^{-1}$.) was used to calculate a rate. Enzyme activity was expressed as nmol/min/mg protein.

7.3.4 Protein Tolerance Test

An oral protein load was carried out to investigate the possibility of protein induced hypoglycaemia. Following a positive result, two previously described patients with CHI due

to mutations in the *HADH* gene were investigated similarly (Clayton *et al.,* 2001; Hussain *et al.,* 2005c). The results were compared with 3 control subjects with ketotic hypoglycaemia. Patients with ketotic hypoglycaemia were selected as controls as the cause of hypoglycaemia is not related to insulin action in these patients (as evidenced by the presence of elevated ketone bodies during hypoglycaemia). All the patients had previously demonstrated normal fasting tolerance on diazoxide. The diazoxide dose for patients with *HADH* mutations was between 13-15mg/kg/day. Diazoxide was not discontinued for the purpose of the test as stopping diazoxide in patients with hyperinsulinism may itself lead to hypoglycaemia and false positive results. Following the observation of severe protein sensitivity, *GLUD1* was sequenced in all three patients and no mutations were found.

The patients and controls were fasted for four hours prior to the test. Baseline bloods (insulin, glucose) were collected prior to oral administration of the protein mixture. The oral protein load was given in the form of a standard protein powder (Vitapro) dissolved in water. The amount of amino acids present in 100 gram of this mixture is as follows: L-Alanine 3.6g, L-Arginine 1.9g, L-Cystine 1.7g, L-Glutamine 14.8g, L-Glycine 1.4g, L-Histidine 1.2g, L-Isoleucine 4.7g, L-Leucine 7.5g, L-Lysine 6.6g, L-Methionine 1.7g, L-Phenylalanine 2.2g, L-Proline 4.9g, L-Serine 3.2g, L-Threonine 5.9g, L-Tryptophan 1.8g, L-Tyrosine 1.9g, L-Valine 4.0g and L-Asparagine 8.9g. 1.5gm/kg of the mixture was administered and bloods were collected at 30 minute interval post load. The test was stopped at 180mins or earlier if hypoglycaemia developed; hypoglycaemia was defined as blood glucose level of <3.0mmol/L for the purpose of the test.

Serum insulin and blood glucose measurements were performed in the biochemistry department at Great Ormond Street Hospital by the laboratory staff.

Serum insulin was measured using the IMMULITE 2500 immunoassay system (Siemens, USA). IMMULITE 2500 insulin immunoassay is a chemiluminescent immunometric assay. The procedure involves incubation of the patient's sample with a bead coated with anti-insulin antibody and a reagent containing alkaline phosphatise conjugated to anti-insulin antibody for 60 minutes. During incubation, insulin forms a complex with the anti-insulin antibody on the bead and the enzyme conjugated anti-insulin antibody in the reagent. Unbound patient sample and enzyme conjugate are then removed by washes. The chemiluminescent substrate is then added to the reaction which generates a light signal in proportion to the bound enzyme which in turn is proportional to the amount of insulin in the patient's sample.

Blood glucose was measured using the VITROS Chemistry system (Johnson and Johnsons Clinical Diagnostics, UK). First, the sample glucose is converted by glucose oxidase in the presence of oxygen to hydrogen peroxidise and gluconate. Hydrogen peroxidise then reacts with 4-aminophenazone and phenol to yield a coloured complex. The intensity of the complex is measured at 505 nm and is proportional to the glucose concentration in the patient's sample.

7.3.5 Leucine Tolerance Test

To investigate whether the protein sensitivity was due to the leucine component in the protein mixture as seen in patients with HI/HA syndrome, an oral leucine load test was performed on the three patients. Following a fast of four hours, an oral dose of 0.15gm/kg of L-Leucine was administered. Blood glucose and plasma insulin levels were then measured at -30, 0, 30, 60, 90 and 120 minutes.

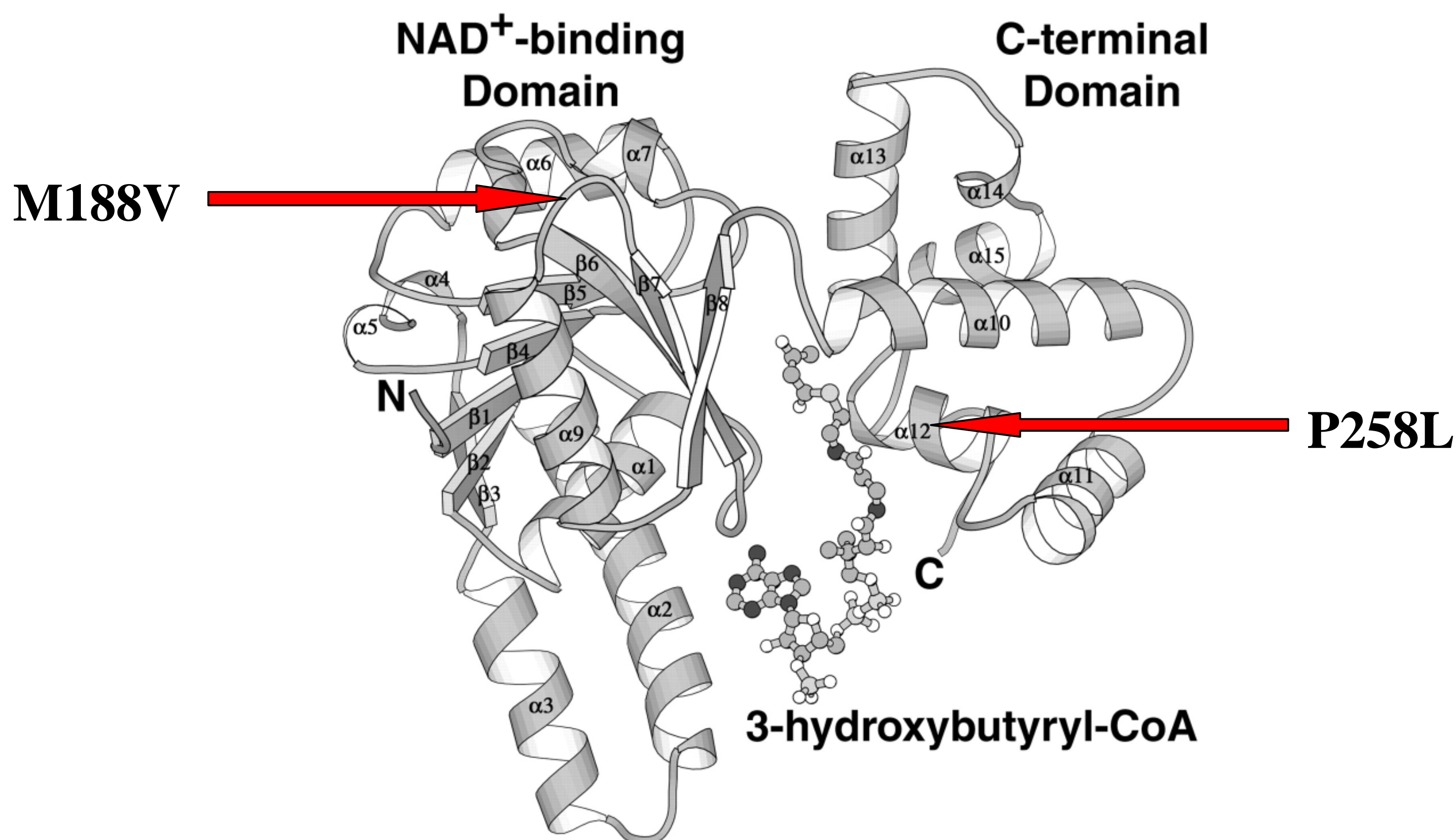
7.4 Results

7.4.1 *HADH* mutation analysis

A novel homozygous missense mutation, M188V (c.562A>G, p.Met188Val) was identified in exon 5 of the index patient's *HADH* gene. Her parents and two unaffected siblings were heterozygous for the M188V mutation. The approximate position of the mutation within the HADH structure is depicted in figure 7.1. Both methionine and valine are nonpolar amino acids, but the methionine residue at position 188 is conserved from human to *Xenopus tropicalis*. 72 control ethnically matched chromosomes were sequenced and the homozygous missense mutation, M188V (c.562A>G, p.Met188Val) was not detected.

The other two patients positive for a *HADH* mutation include a patient with the P258L mutation in exon 7 (Clayton *et al.,* 2001) and a splice site mutation IVS6-2a>g (Hussain *et al.,* 2005c).

Figure 7.1: Diagram depicting the location of the novel mutation M188V within the HADH protein structure. The first 200 amino acids comprise the NAD$^+$ binding domain and the remaining residues comprise the C-terminal domain (Barycki *et al.,* 2000). 3-Hydroxybutyrl-CoA binds to the cleft between the two domains.The location of the P258L mutation is also depicted.



7.4.2 Activity of L-3-hydroxyacyl-CoA dehydrogenase (Performed by Dr Simon Eaton, ICH, London)

Short chain L-3-hydroxyacyl-CoA dehydrogenase activity was significantly decreased compared with controls (index patient mean 26.8 ± SEM 4.8 mU/mg protein vs. controls 48.0 ± 8.1; $p=0.029$), although the residual activity was much higher than in the other reported cases (Clayton *et al.,* 2001; Hussain *et al.,* 2005c). Activities of medium and long chain L-3-hydroxyacyl-CoA dehydrogenase were also mildly decreased compared with controls although this difference was not significant (medium chain index patient 25.8 ± 4.2 vs. controls 40.1 ± 6.1; long chain index patient 28.8 ± 5.0 vs. controls 43.8 ± 6.2).

7.4.3 Protein induced hyperinsulinaemic hypoglycaemia with leucine sensitivity

The blood glucose response to the oral protein load in the patients with HADH deficiency is illustrated in fig 7.2. As shown, all three subjects with HADH deficiency demonstrated a dramatic decline in blood glucose concentration. In contrast, the control subjects had no change in the blood glucose concentration in response to protein. Table 7.1 shows the blood glucose and insulin responses to the oral protein load in subjects and controls. In response to the oral leucine load, all the three patients demonstrated a fall in the blood glucose concentrations with simultaneous increase in the serum insulin concentrations, demonstrating leucine sensitivity (Table 7.2).

Figure 7.2: Blood glucose concentrations in response to oral protein load in patients and controls. All patients with mutations in the *HADH* gene demonstrated marked HH in response to a standard protein load. In contrast, there was no hypoglycaemia in the control patients (Kapoor *et al.,* 2009a).

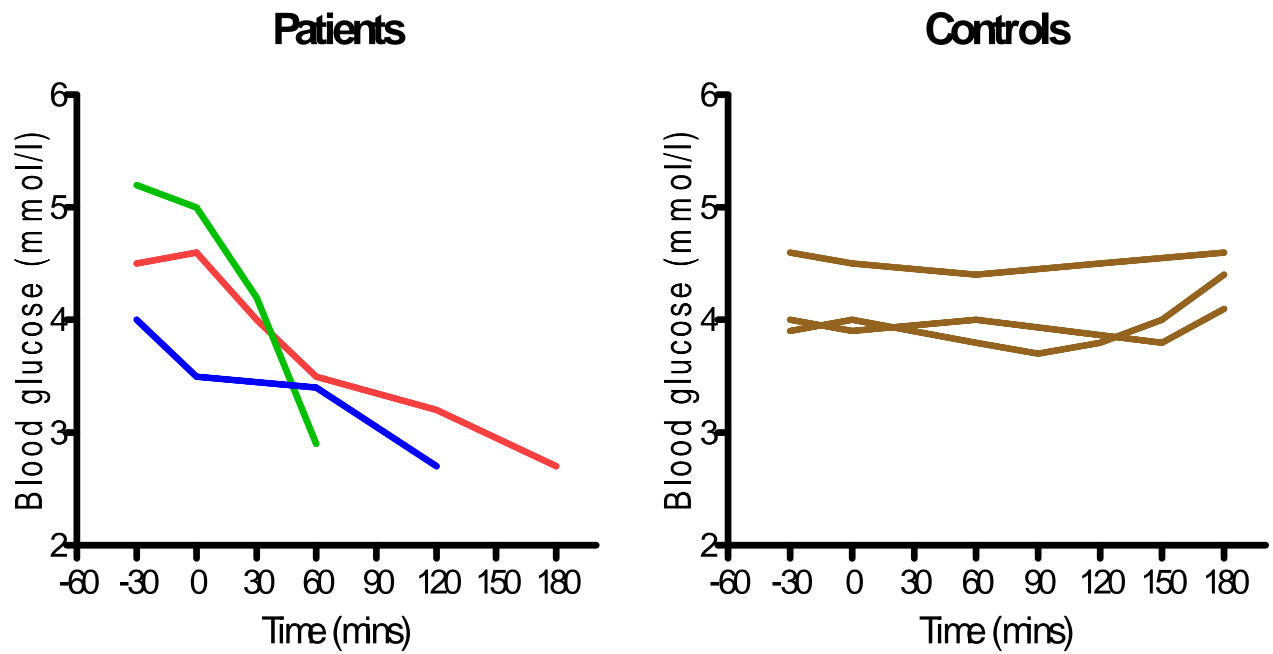


Table 7.1: Comparison of blood glucose and insulin concentrations between subjects (Pt 1, Pt 2 and Pt 3) and controls (C 1, C 2 and C 3) during protein load. All the three subjects with mutations in the *HADH* gene developed hypoglycaemia with a concomitant rise in plasma insulin levels. In contrast, the control subjects had no change in blood glucose levels in response to a protein load (Kapoor *et al.,* 2009a).

Subject	**Age at testing (yrs)**	***HADH* Mutation**	**Baseline BG (mmol/L)**	**Nadir BG (mmol/L)**	**Baseline insulin (mU/L)**	**Peak insulin (mU/L)**	**Time to nadir (mins)**	**Fasting Tolerance (hrs)**
Pt 1	10.3	P258L	4.5	2.7	6.1	67.7	180	16
Pt 2	8.4	IVS6-2a>g	5.2	2.9	<2.0	24	60	14
Pt 3	1.2	M188V	4.0	2.8	4.7	7.8	120	16
C 1	1.1	Nil	4.0	4.1	3.1	6.1	-	10
C 2	8.8	Nil	3.9	4.4	2.6	5.1	-	16
C 3	11	Nil	4.6	4.6	4.1	12.7	-	18

Table 7.2: Oral leucine tolerance test in the three subjects with mutations in the *HADH* gene showing development of HH with a concomitant rise in plasma insulin concentrations.

	Time (minutes)	-30	00	15	30	45
Patient 1	Glucose (mmol/L)	4.4	4.4	4.1	3.4	2.7
	Insulin (mU/L)	9.5	6.6	25.4	34.7	45
Patient 2	Glucose (mmol/L)	4.4	4.4	4.1	1.7	
	Insulin (mU/L)	6.5	3.5	26.5	63.8	
Patient 3	Glucose (mmol/L)	5.1	4.8	4.2	2.7	
	Insulin (mU/L)	8.6	3.4	27.4	31.7	

7.5 Discussion

This data on the study of three patients with CHI due to mutations in *HADH* has highlighted two important phenotypic features of patients with mutations in this gene. Firstly, it suggests for the first time that CHI due to mutations in *HADH* may not be accompanied by abnormalities in the urinary organic acids and plasma acylcarnitine profiles. Secondly, it demonstrates that these patients are highly protein sensitive (and leucine sensitive), a completely novel phenotypic feature of these patients. In the section that follows, the implications of these results on the clinical management of the patients and on the current understanding on the pathophysiology of the condition are discussed.

<u>What is known about the role of HADH in the pancreatic ß-cell?</u>

Mitochondrial HADH is involved in the second dehydrogenation step of the ß-oxidation pathway. It catalyses the conversion of L3-hydroxyacyl CoA to 3-Ketoacyl CoA, and this

reaction also utilises NAD^+ as cofactor producing NADH. It is known that HADH is expressed at high levels in pancreatic ß-cells (Agren *et al.,* 1977). Two recent studies have highlighted the pivotal role of HADH in ß-cell physiology: Firstly, Hardy *et al* (Hardy *et al,* 2007) showed that RNA interference-mediated gene suppression of *HADH* in insulinoma cells and primary rodent islets caused enhanced basal insulin secretion. This demonstrated for the first time that *HADH* is required directly in pancreatic ß-cells for the regulation of basal insulin release. In a second study, Martens *et al* (Martens *et al.,* 2007) compared the expression of *HADH* and other enzymes of the ß-oxidation pathway in rat ß-cells and in the ß-cell line INS1 with that of other cell types. They found that compared with other cell types, pancreatic ß-cells express high levels of *HADH* mRNA and low levels of other ß-oxidation enzymes. They also demonstrated that *HADH* suppression resulted in increased basal and glucose stimulated insulin secretion and proved that this was not caused by increased rates of glucose metabolism or an inhibition of fatty acid oxidation. This study provides evidence for the existence of a crucial *HADH* dependent regulatory pathway that is independent of ß-oxidation and K_{ATP} channel pathways. It also supports the existence of dysregulated ß-cells as the cause of HH in patients with *HADH* mutations. However the exact mechanism of regulation of insulin secretion by *HADH* remains to be elucidated.

<u>What has this data added to the understanding of insulin regulation by *HADH*?</u>

It has been hypothesized that fatty acids increase insulin secretion by affecting the concentrations of long-chain fatty acyl derivatives as a result of the inhibitory effect of citrate and malonyl-CoA on the rate controlling enzyme carnitine palmitoyltransferase-1 (CPT1) (Eaton *et al.,* 2003; Prentki *et al.,* 1992; McGarry 2002). Various potential mechanisms have been put forward to explain HH associated with HADH deficiency (Eaton *et al.,* 2003). The previously reported cases of CHI due to mutations in *HADH* have

presented with increased 3-hydroxyglutarate in urine and hydroxybutyrylcarnitine in blood (regarded as diagnostically useful markers for HADH deficiency). The presence of these circulating abnormal acylcarnitine metabolites in the blood in all previously reported patients led to speculations that accumulation of short chain acyl Co-A esters originating from circulating metabolites or the metabolites themselves may lead to dysregulated insulin secretion either by inhibition of CPT1, or via K_{ATP} channels or via the G-protein coupled receptor GPR40 (reviewed by Eaton *et al.,* 2003). The case described above now demonstrates that hyperinsulinism associated with HADH deficiency in fact may not always manifest itself with the presence of these abnormal metabolites. Hence the accumulation of 3-hydroxybutyrylcarnitine may not be the trigger for insulin secretion in patients with *HADH* deficiency. This supports the study by Martens *et al.* (Martens *et al.,* 2007) that demonstrated that knockdown of HADH in β-cells increases insulin secretion not as a consequence of a perturbation of β-oxidation but possibly by another metabolic mechanism that is currently unknown.

Interestingly, the residual enzyme activity in the patient described is higher than the previously described cases of HADH deficiency with hyperinsulinism. The lack of detectable abnormal acylcarnitines is indeed in keeping with the mild defect in enzyme activity. This suggests that the mechanism of insulin dysregulation in patients with HADH mutations (and/ or the mechanism of insulin regulation by HADH) may be unrelated to the activity of the enzyme. The HADH protein has been previously shown to be associated with other proteins which may be involved in insulin secretion e.g. GDH (Filling *et al.,* 2008) or complex I (Sumegi and Srere, 1984). Indeed, Filling *et al* (Filling *et al.,* 2008) conclude that protein interactions rather than changes in activity are likely to lead to the

phenotype. It is thus possible that mutations in *HADH* resulting in minor changes in enzyme activity can cause major changes in the interactions of HADH with other proteins.

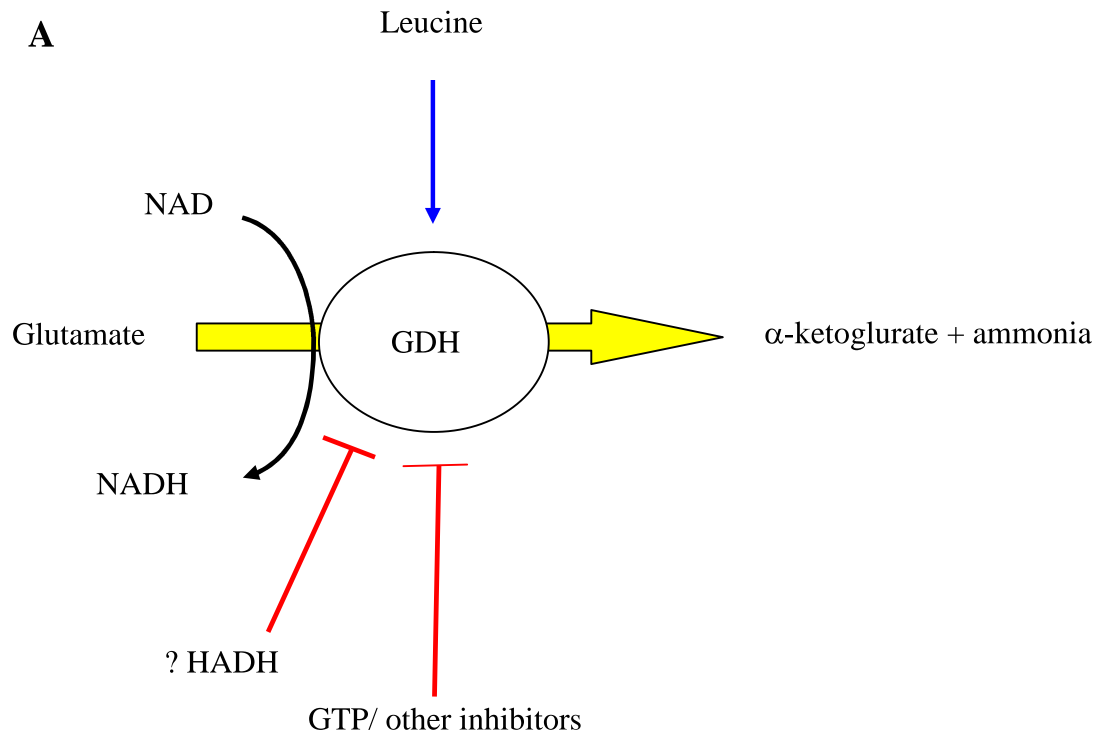
This study also demonstrates for the first time that children with HH due to mutations in *HADH* are severely protein/ leucine sensitive (table 7.1). This novel clinical observation leads to the proposal that defects in *HADH* may reveal an unidentified biochemical pathway by which leucine triggers insulin secretion. Leucine sensitive HH occurs in patients with *GLUD1* mutations whereas protein sensitive (without leucine sensitivity) HH is observed in patients with defects in the K_{ATP} channel genes (Kelly *et al.,* 2001; Fourtner *et al.,* 2006). However protein/ leucine sensitivity due to mutations in the *HADH* gene has not been described before. This clinical observation implies that HADH inhibits leucine induced insulin secretion.

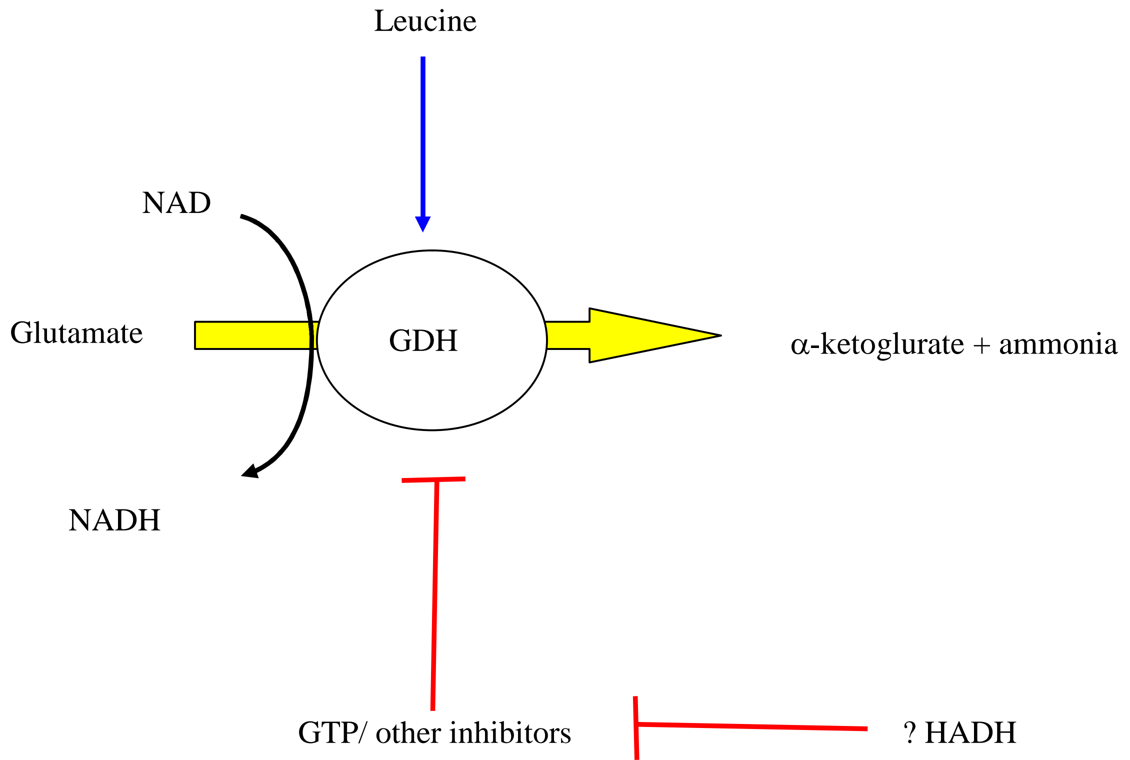
Interestingly, the study by Filling *et al.* (Filling *et al.,* 2008) proposes the existence of an interaction between HADH and glutamate dehydrogenase (GDH) in the pancreatic ß-cell. It could be hypothesized that mutations in *HADH* cause protein sensitive HH via the GDH axis. In this model of HADH and GDH interaction, HADH would act as an inhibitor of GDH, opposing leucine stimulated insulin secretion. This inhibition could be a direct inhibition of GDH (like that exerted by GTP), or indirect via its effect on the interaction of GDH and its inhibitors (such as GTP) or stimulators (such as leucine). Mutations in *HADH* could then cause protein-induced hypoglycaemia due to enhanced sensitivity to leucine-mediated insulin secretion as a consequence of:

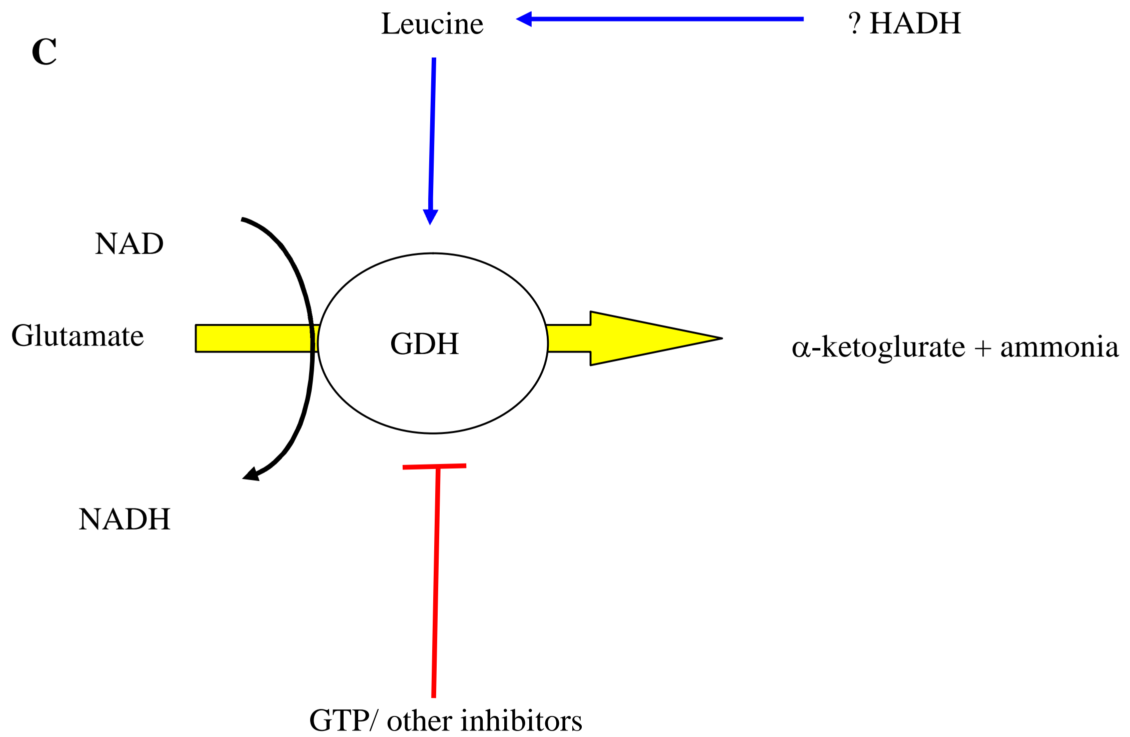
i) impaired inhibitory control of GDH, as in patients with HI/HA syndrome or
ii) impaired inhibition of GDH itself or
iii) excessive stimulation by leucine

All of the above mechanisms (illustrated in figure 7.3) would lead to leucine sensitive HH, as was seen in the patients in this study. Unravelling this biochemical pathway will provide unique novel insights into fatty acid and amino acid induced insulin secretion.

Figure 7.3: Figure demonstrating the three hypothesised mechanisms linking GDH, HADH and amino-acid stimulated insulin secretion. Glutamate is converted to α-ketoglurate + ammonia in the presence of GDH. GDH is activated by leucine and inhibited by GTP. HADH may act by (A) direct inhibition of GDH, (B) inhibition of the inhibitory control of GDH or (C) excessive stimulation of GDH by leucine




B
Leucine
NAD
Glutamate
GDH
α-ketoglurate + ammonia
NADH
GTP/ other inhibitors
? HADH


C
Leucine
? HADH
NAD
Glutamate
GDH
α-ketoglurate + ammonia
NADH
GTP/ other inhibitors

7.6 Clinical Implications of this study

The observation that patients with *HADH* mutations do not necessarily have abnormalities in the urinary organic acid or acylcarnitine profiles indicates that these markers can no longer be used exclusively to screen for *HADH* mutations. It also implies that the current prevalence of *HADH* mutations in CHI may be an underestimate. All the three patients had diazoxide responsive CHI with marked leucine sensitivity, similar to patients with HI/HA syndrome. Furthermore, there is considerable overlap between CHI due to mutations in the *HADH* gene and *GLUD1* gene in terms of other phenotypic features studied, such as age of presentation and birth weight SDS (refer chapter 8). The only distinguishing feature apparent so far is the mode of inheritance, with *HADH* mutations being inherited in a recessive manner as opposed to *GLUD1* mutations that are dominantly inherited. Hence, it may be recommended that in patients with leucine sensitive, diazoxide responsive CHI; *HADH* is sequenced (prior to *GLUD1*), if the patient belongs to a consanguineous family.

The second important clinical implication of this data relates to the observation of protein induced hypoglycaemia in these patients. This novel observation has helped us in understanding the basis of unpredictable hypoglycaemic episodes and in instituting the right management strategy for the patients i.e. protein restriction in the diet. As in patients with HI/HA syndrome, protein restriction is proving an invaluable aid in the clinical management of these patients.

7.7 Conclusions

This in-depth study of the phenotype of three patients with *HADH* gene mutations has provided invaluable knowledge, both to the clinical management of patients with CHI and to the further understanding of how *HADH* regulates insulin secretion. The observation of severe protein/ leucine sensitivity in these patients has demonstrated that *HADH* somehow regulates leucine induced insulin secretion. Further studies are required to understand the link between HADH, ß-oxidation pathway, insulin secretion and protein sensitivity. This may be investigated by studying the effects of *HADH* knockdown on ß-cell gene expression and by identifying proteins that are closely associated with HADH, specifically exploring protein-protein interaction between GDH and HADH. These studies will provide invaluable insights into pancreatic ß-cell biochemistry and physiology.

Chapter 8

Congenital Hyperinsulinism due to Mutations in the *HNF4A* Gene

8.1 Background

Hepatocyte nuclear factor 4 alpha (HNF-4α encoded by the *HNF4A* gene) is a transcription factor of the nuclear hormone receptor superfamily and is expressed in liver, kidney, gut, and pancreatic islets (Sladek *et al.,* 1990). It plays a key role in the regulation of pancreatic insulin secretion. Loss-of-function *HNF4A* mutations have been identified in MODY (maturity-onset diabetes of the young) families, characterized by an autosomal dominant inheritance pattern and impaired glucose-stimulated insulin secretion from pancreatic ß-cells (Yamagata *et al.,* 1996). In addition, variations in the ß-cell specific promoter (P2) elements of the *HNF4A* gene in humans are associated with increased risk of type 2 diabetes (Silander *et al.,* 2004).

HNF-4α plays an important role in regulating insulin secretion as well as interacting with regulatory elements in promoters and enhancers of genes whose products are involved in diverse functions, including cholesterol, fatty acid, amino acid, and glucose metabolism.

Heterozygous mutations in the transcription factor HNF-4α have recently been associated with a mild form of transient HH and considerable risk of macrosomia (Pearson *et al.,* 2007). The finding of transient mild HH is unexpected as heterozygous mutations in the *HNF4A* gene lead to loss of glucose induced insulin secretion with glucose intolerance in patients.

This section discusses the clinical characteristics of the eight patients with CHI that have a mutation in the *HNF4A* gene. This is the largest cohort of patients with CHI due to mutations in this gene. The study of this group of patients extends the observations of Pearson *et al.* (Pearson *et al.,* 2007) reporting that heterozygous *HNF4A* mutations can also cause severe and persistent CHI along with mild, transient CHI described previously.

8.2 Methodology

Of the patients with no mutations in the K_{ATP} channel genes (or features of *GLUD1/ GCK/ HADH* mutations), 111 patients had DNA sequence analysis of the *HNF4A* gene. This included 105 patients with diazoxide responsive CHI and 6 patients with transient CHI, that did not require treatment with diazoxide. *HNF4A* analysis included amplification of the coding exons 1d-10 and the P2 pancreatic promoter using published primer sequences (Thomas *et al.*, 2001 (P2 promoter) and Yamagata *et al.*, 1996 (exons 1d-10)). PCR products were sequenced using standard methods as described in chapter 3, and compared to the published sequence NM_000457.3 (Ellard and Colclough, 2006) using Mutation Surveyor v3.2. The analysis was carried out at Peninsula Medical School, Exeter.

The phenotypic characteristics of the patients found to have a *HNF4A* mutation were collated (as outlined in chapter 3) and compared with the phenotypic characteristics of patients with transient and/ or diazoxide responsive CHI and a K_{ATP} mutation (n= 27), *GLUD1* mutation (n= 13) or a *HADH* mutation (n=3). The phenotypic data are presented as mean and the one-way analysis of variance (ANOVA) followed by LSD post-test was used to test for statistical significance.

8.3 Results

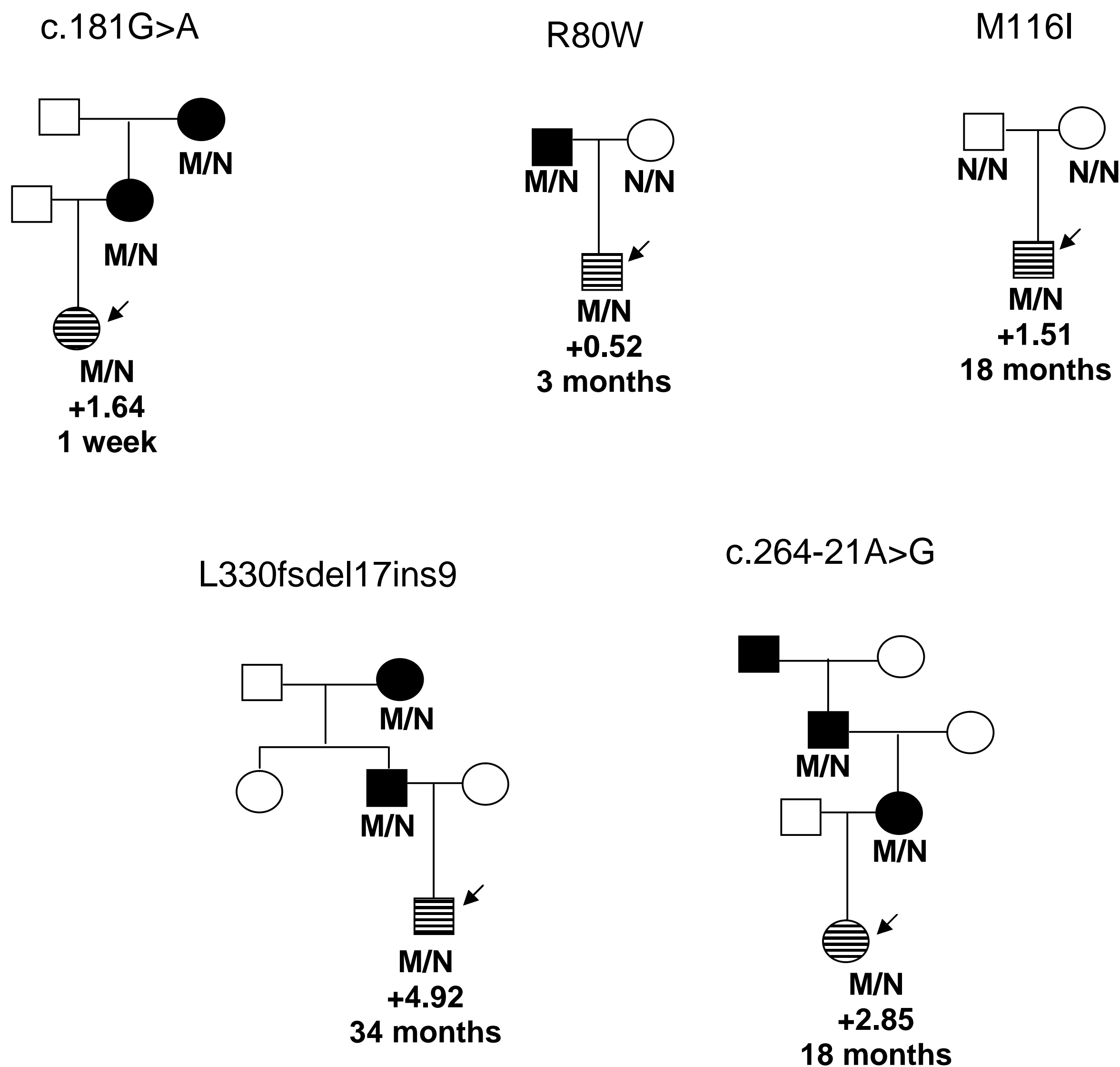
8.3.1 Genetic results

A total of eight different *HNF4A* mutations were identified in the eight probands, all of which are novel. These include a frameshift mutation L330fsdel17ins9 (c.987_1003del17ins9; p.Leu330fs), a mutation affecting the conserved adenine nucleotide of the intron 2 branch site (c.264-21A>G), a nonsense mutation, Y16X (c.48C>G, p.Tyr16X); and missense mutations R80W (c. 238C>T; p.Arg80Trp), M116I (c.348G>A; p.Met116Iso), Y319Valfs (c.953dupA; p.Tyr319ValfsX2) and Q362X (c.1084C>T; p.Gln362X). In addition, a mutation within the P2 promoter of the *HNF4A* gene, c.-181G>A, was identified. This G>A mutation occurs within the HNF1A/HNF1B transcription factor binding site (c.-181G>A) and has been shown to affect the transcription of the *HNF4A* gene (Hansen *et al.*, 2002). Analysis of 7 orthologous sequences demonstrated that these 4 novel missense mutations occurred at residues that are conserved through evolution and these mutations were not present in 300 Caucasian control chromosomes.

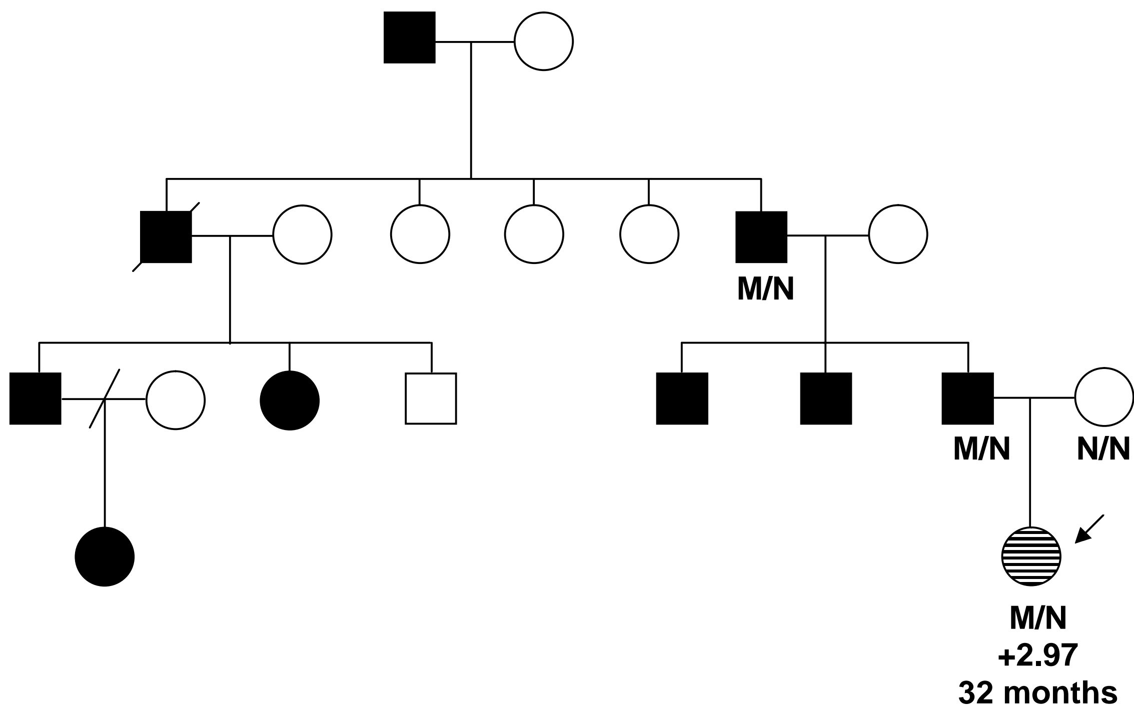
8.3.2 Inheritance of *HNF4A* mutations

In 5/8 families the mutation was inherited from a diabetic parent (for pedigrees see figure 8.1). Two mutations (c.264-21A>G, c.-181G>A) were inherited from an affected mother while three mutations (L330fsdel17ins9, R80W, Y16X) were inherited from an affected father. One patient had inherited the mutation (Y319Valfs) from her unaffected father (current age 39 years) but her paternal aunt who had gestational diabetes at 30 years was also found to carry the Y319Valfs *HNF4A* mutation. Two mutations, M116I and Q362X, were proven by microsatellite analysis to have arisen *de novo*.

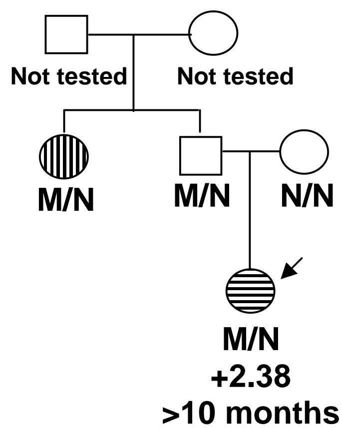
Figure 8.1: Family pedigrees: Partial pedigrees showing inheritance of *HNF4A* mutations in the 8 families. Circles represent females and squares indicate males. Probands are indicated by an arrow. Horizontal hatching denotes patients with HH, vertical hatching represents gestational diabetes and filled symbols show diabetic individuals. The genotype is given below each symbol: M/N denotes a heterozygous *HNF4A* mutation and N/N a normal genotype. For each proband, birth weight SDS and duration of diazoxide treatment are provided, >indicates the minimum duration when treatment is ongoing. The *HNF4A* mutation identified in each family is shown above each pedigree.



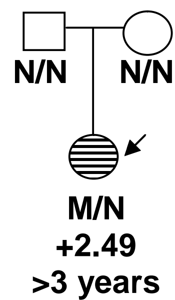
Y16X



Y319Valfs



Q362X



8.3.3 Clinical characteristics of *HNF4A* mutation carriers

The clinical characteristics of the patients with CHI due to a *HNF4A* mutation are summarised in table 8.1. The mean birth weight SDS of the infants was +2.41 SDS (0.52-4.92). All the infants presented with hypoglycaemia on the first day of life. The maximum glucose requirement was 25mg/kg/min. Three infants required an intravenous infusion of glucagon to stabilise blood glucose levels. Seven infants responded to diazoxide therapy (1.5mg/kg/day – 10mg/kg/day). In the eighth infant, hypoglycaemia resolved within the first week of life without diazoxide therapy. Hyperinsulinism resolved within six months in three infants. However, five children developed more persistent hyperinsulinism with one child continuing treatment at 48 months of age (table 8.2). There were no common mutations in our cohort of patients.

The father carrying the L330fsdel17ins9 mutation was macrosomic (birth weight 2.79 kg) at 26 weeks gestation. This raises possibility that *HNF4A* mutations lead to fetal hyperinsulinaemia at an early gestational age, subsequently resulting in macrosomia. Neonatal hypoglycaemia had not been reported in any of the heterozygous relatives.

Table 8.1: Clinical characteristics of patients with CHI due to *HNF4A* mutations: The mean birth weight was +2.41 SDS and all the infants presented at day 1. A history of diabetes in the parents was absent in 3/8 patients. The duration of HH varied between 1 week and 48 months.

Pt	Mutation	Birth Weight SDS	Age of presentation	Diazoxide Responsive	Duration	Family history of diabetes	Inheritance
1	c.181G>A	+1.64	Day 1	Not Treated	1 Week	Mother, Maternal Grandmother	Maternal
2	R80W	+0.52	Day 1	Yes	3 Months	Father, Paternal Aunt, Grandfather And Great Grand Father	Paternal
3	M116I	+1.51	Day 1	Yes	18 Months	Nil	De Novo
4	L330fsdel17 Ins9	+4.92	Day 1	Yes	34 Months	Father, Paternal Aunt And Grandmother	Paternal
5	c.264-21A>G	+2.85	Day 1	Yes	Continuing at 48 months	Mother, Maternal Grandfather	Maternal
6	Y16X	+2.97	Day 1	Yes	32 Months	Father, Paternal Uncles, Grandfather	Paternal
7	Q362X	+2.49	Day 1	Yes	Continuing treatment at 39 months	Nil	De novo
8	Y319Valfs	+2.38	Day 1	Yes	6 months	Paternal aunt- GDM	Paternal

Table 8.2: Clinical characteristics of the patients with persistent HH: The patients had severe HH with requirements of high concentrations of intravenous glucose and subcutaneous glucagon infusions.

	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7
Maximum Glucose Infusion Rate	Not Known	25 Mg/Kg/Min	12.5 Mg/Kg/Min	11 Mg/Kg/Min	Not known
Glucagon Infusion Required	No	Yes	Yes	Yes	No
Diazoxide Responsive (Dose)	Yes, 1.5mg/kg/day	Yes, 10 mg/kg/day	Yes, 10 mg/kg/day	Yes, On 6 mg/kg/day	Yes, on 10mg/kg/d
Family History Of Diabetes	No	Yes	Yes	Yes	No
Attempted Withdrawal Of Diazoxide	Successful At 18 Months	Not Successful At 7 Months	Not Successful At 18 Months	Successful At 32 Months	Continuing treatment at 39 months
Mutation	M116I	L330fsdel17ins9	C.264-21>G	Y16X	Q362X

8.3.4 Clinical characteristics according to genetic aetiology

The clinical characteristics of the probands according to genetic etiology were compared (table 8.3). Patients with a *HNF4A* mutation presented earlier and were born heavier than patients with a *GLUD1* mutation (1 day vs 22.4 weeks, p = 0.001 and +2.41 birth weight SDS vs -0.11 birth weight SDS, p = 0.001 respectively). In comparison to patients with an *HADH* mutation, patients with an *HNF4A* mutation were heavier (birth weight SDS +2.41 (*HNF4A*) vs -1.09 (*HADH*), p= 0.001) and presented earlier (1 day vs 125 days), although the difference in the age of presentation was not statistically significant (p=0.063). No differences in the mean age at presentation were observed between patients with an *HNF4A* or K_{ATP} channel mutations with diazoxide responsive CHI (the range varied from 1 day to 1 year for patients with a K_{ATP} channel mutation). The difference in the birth weights between these two groups were also not significant (+2.41 birth weight SDS vs +1.47 birth weight SDS, p = 0.141).

Within the other groups of patients, the age of presentation and birth weight SDS of patients with a *GLUD1* or *HADH* mutation were similar with no significant differences (mean age of presentation 157 days vs 125 days and mean birth weight SDS -0.11 vs -1.09, p values of 0.6 and 0.3 respectively). The mean birth weight SDS and age of presentation of the patients with a *GLUD1* mutation was significantly different from patients with a K_{ATP} channel mutation (p=0.006 and p<0.005 respectively). Patients with a *HADH* mutation were also significantly smaller at birth than patients with a K_{ATP} channel mutation (p=0.008). The mean age of presentation of patients with a *HADH* mutation was 125 days in comparison with 27.8 days in patients with K_{ATP} channel mutations; however the difference was not statistically significant (p=0.1). These results are displayed in tables 8.4 (birth weight SDS) and 8.5 (age of presentation).

Table 8.3: Summary of the clinical characteristics for the patients with diazoxide responsive CHI according to the genetic aetiology. The data are represented by the mean (interquartile range).

	***HNF4A* mutation positive**	**K_{ATP} channel positive**	***GLUD1* mutation positive**	***HADH* mutation positive**
Number of patients	8	27	13	3
Sex (% males)	37.5%	73%	38.4%	33.3%
Age of presentation	1 (1- 1)	27.8 (1-365)	157.3 (2- 504)	125 (120-135)
Birth weight (grams)	4153 (3360-5900)	3720 (1100- 5600)	3133 (2495- 3990)	3040 (2570-3450)
Gestational age (weeks)	37 (35-40)	37 (31- 41.5)	38.6 (35-40)	40 (40-40)
Birth weight SDS	2.41 (0.52- +4.92)	1.47 (-1.96- +4.66)	-0.11 (-2.37- +1.73)	-1.09 (0.01- -2.45)

Table 8.4: Comparison of the birth weight SDS between different groups of patients using the One Way Anova Post Hoc LSD test: Patients with an *HNF4A* or a K_{ATP} channel mutation were significantly large at birth than patients with a *GLUD1/ HADH* mutation.

Pt group (A)	Pt group (B)	Mean Difference (A-B)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
HNF4A	***GLUD1***	2.51917*	.68334	.001	1.1381	3.9002
	HADH	3.49667*	1.01355	.001	1.4482	5.5451
	K_{ATP}	.93524	.62201	.141	-.3219	2.1924
GLUD1	***HNF4A***	-2.51917*	.68334	.001	-3.9002	-1.1381
	HADH	.97750	.96638	.318	-.9756	2.9306
	K_{ATP}	-1.58393*	.54177	.006	-2.6789	-.4890
HADH	***HNF4A***	-3.49667*	1.01355	.001	-5.5451	-1.4482
	GLUD1	-.97750	.96638	.318	-2.9306	.9756
	K_{ATP}	-2.56143*	.92404	.008	-4.4290	-.6939
K_{ATP}	***HNF4A***	-.93524	.62201	.141	-2.1924	.3219
	GLUD1	1.58393*	.54177	.006	.4890	2.6789
	HADH	2.56143*	.92404	.008	.6939	4.4290

*. The mean difference is significant at the 0.05 level.

Table 8.5: Comparison of the age of presentation between different groups of patients using the One Way Anova Post Hoc LSD test: Patients with an *HNF4A* or K_{ATP} channel mutation presented significantly earlier than patients with a *GLUD1* mutation. There was no significant difference in the age of presentation between other groups.

Pt group (A)	Pt group (B)	Mean Difference (A-B)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
HNF4A	***GLUD1***	-156.38462*	43.25264	.001	-243.4999	-69.2693
	HADH	-124.00000	65.16439	.063	-255.2478	7.2478
	K_{ATP}	-26.80000	39.09864	.497	-105.5487	51.9487
GLUD1	***HNF4A***	156.38462*	43.25264	.001	69.2693	243.4999
	HADH	32.38462	61.65202	.602	-91.7889	156.5582
	K_{ATP}	129.58462*	32.91317	<.005	63.2941	195.8751
HADH	***HNF4A***	124.00000	65.16439	.063	-7.2478	255.2478
	GLUD1	-32.38462	61.65202	.602	-156.5582	91.7889
	K_{ATP}	97.20000	58.81228	.105	-21.2540	215.6540
K_{ATP}	***HNF4A***	26.80000	39.09864	.497	-51.9487	105.5487
	GLUD1	-129.58462*	32.91317	<.005	-195.8751	-63.2941
	HADH	-97.20000	58.81228	.105	-215.6540	21.2540

*. The mean difference is significant at the 0.05 level.

8.4 Discussion

CHI is clinically a very heterogeneous condition in terms of severity of disease, persistence of disease and responsiveness to medical therapy. From this study, it was evident that the heterogeneity was a feature of CHI within the group of patients with *HNF4A* mutations as well. So far, it is known that mutations in *HNF4A* gene cause mild, transient HH. However, at least 3 of the 8 patients with an *HNF4A* mutation had severe HH requiring large amounts of intravenous glucose and subcutaneous glucagon infusions to maintain normoglycaemia. With regards to persistence of disease, three patients had persistent requirement of diazoxide therapy till 18 months, 32 months and 34 months and two are continuing treatment at 39 months and 48 months (tables 8.1 and 8.2). These results illustrate that heterozygous *HNF4A* mutations can cause severe and persistent hyperinsulinism in addition to the mild, transient hyperinsulinism that has been reported previously (Pearson *et al.,* 2007).

The previous study examined families of patients with diabetes with *HNF4A* mutations and concluded that CHI due to *HNF4A* mutations caused hypoglycaemia associated with macrosomia and a family history of diabetes. This study of eight families confirmed that CHI due to *HNF4A* mutations was associated with increased birth weight (mean birth weight +2.41 SDS) which is likely to result from increased insulin secretion in utero. However, three of the eight probands (37.5%) did not have a parent with diabetes and in two cases a *de novo* mutation was confirmed. In the third case, the mutation was inherited from an asymptomatic father and there was history of gestational diabetes in the paternal aunt who also carried the same mutation (family 8). Therefore this study illustrates that the absence of a history of diabetes in the parents should not preclude sequencing of the *HNF4A* gene in patients presenting with diazoxide responsive HH.

Neonatal hypoglycaemia has been reported in only a minority of patients (11%) with *HNF4A* mutations who were ascertained by their family history of MODY (Pearson *et al.,* 2007; Fajans and Bell, 2007) and none of the other heterozygous relatives in this study were known to have had neonatal hypoglycaemia. The reasons for the incomplete penetrance of symptomatic hypoglycaemia are not known, but it appears to be a general feature rather than mutation specific. The hyperinsulinaemic *HNF4A* phenotype hence, ranges from increased birth weight, to neonatal hypoglycaemia managed by intravenous glucose only (Pearson *et al.,* 2007; Fajans and Bell, 2007) for 1-9 days, or neonatal hypoglycaemia requiring diazoxide therapy for between 3 months and 3.3 years. It is therefore likely that other environmental and genetic factors are influencing the severity of the hyperinsulinaemic phenotype associated with *HNF4A* mutations.

It was striking that all the eight patients with *HNF4A* mutations presented on the first day of life with hypoglycaemia. Also, the mean birth weight SDS of patients with *HNF4A* mutations was the highest when compared to the patient groups of diazoxide responsive CHI due to other aetiologies, with 5 out of 8 patients having a birth weight SDS of >2. However, patients with a K_{ATP} channel mutation were also large at birth (birth weight SDS ranged between -1.96 to +4.66) with an early age of presentation (ranging from 1 day to 365 days). Due to this overlap in the age of presentation and birth weight between patients with an *HNF4A* mutations and K_{ATP} channel mutations, it is not possible to distinguish between the two groups on the basis of these features. Although a family history of diabetes is more likely to suggest an *HNF4A* mutation it is also not a distinguishing feature. Hence, given the higher prevalence of K_{ATP} channel mutations and the high rate of diabetes phenocopies in the population, it would be recommended to sequence *KCNJ11* and *ABCC8* first in all patients with diazoxide responsive CHI, followed by *HNF4A* in case of a family history of diabetes.

In contrast, patients with a *GLUD1/ HADH* mutation were diagnosed later (mean of 157 and 125 days respectively) and were of normal birth weight (mean birth wt SDS of -0.11 and -1.09 respectively). It was not possible to distinguish patients with a *GLUD1* mutation from those with a *HADH* mutation based on these two features. Patients with *GLUD1* mutation or *HADH* mutation share further similarities in terms of being leucine sensitive (chapter 7). In the vast majority of patients, a serum ammonia concentration would help in distinguishing between the two aetiologies (raised in the majority of patients reported with *GLUD1* mutation). The other distinguishing feature would be a history of consanguinity as *HADH* mutations reported so far have been recessively inherited and described to occur in consanguineous families while *GLUD1* mutations are dominantly inherited.

How do mutations in the *HNF4A* gene cause CHI?

HNF-4α has a key role in regulating the multiple transcriptional factor networks in the islet and in combination with other hepatocyte nuclear factors (such as HNF-1α) has been proposed to form a functional regulatory loop in the adult ß-cell (Boj *et al.,* 2001; Odom *et al.,* 2004). It interacts with regulatory elements in promoters and enhancers of genes whose products are involved in diverse functions such as cholesterol, fatty acid, amino acid, and glucose metabolism, as well as liver development and differentiation (Mietus-Snyder *et al.,* 1992, Hall *et al.,* 1995). HNF-4α seems to be indispensable for development as targeted disruption of this gene results in defective gastrulation of mouse embryos due to abnormal development of the visceral endoderm (Chen *et al.,* 1994). Since loss of HNF-4α function leads to multiple defects (defective mitochondrial metabolism, impaired mitochondrial tricarboxylic acid cycle enzyme activity and partial uncoupling of the mitochondrial respiratory chain) (Wang *et al.,* 2000) in glucose stimulated insulin secretion it is unclear how heterozygous *HNF4A* mutations can also cause HH in the newborn period. Using the conditional Cre-loxP-based inactivation system and deleting the *HNF4A*

gene in ß-cells, Gupta *et al.* (Gupta *et al.,* 2005) were able to show that fasted and fed mice were hyperinsulinaemic but paradoxically also displayed impaired glucose tolerance. These mice showed a 60% reduction in expression of the potassium channel subunit Kir6.2 with cotransfection assays demonstrating that the *Kir6.2* gene is a transcriptional target of HNF-4α. However two further studies have reported no change in the expression of Kir6.2 in *Hnf4a*- deficient mice (Pearson *et al.,* 2007; Miura *et al.,* 2006). This suggests that the reduction in expression of the potassium channel subunit Kir6.2 may not be the only mechanism responsible for the HH in *Hnf4a*- deficient mice.

HNF-4α also been shown to have an interaction with the nuclear receptor peroxisome proliferator-activated receptor alpha (PPARα) with low levels of PPARα reported in HNF-4α deficient ß-cells (Gupta *et al.,* 2005). PPARα acts as a general sensor of overall tissue lipid supply and activates genes encoding enzymes of the ß-oxidation pathway of fatty acids. A decrease in ß-oxidation of fatty acids results in the accumulation of lipids (such as malonyl-CoA) in the cytoplasm. Increased malonyl-CoA inhibits the enzyme carnitine-palmitoyltransferase 1 (CPT-1) thereby increasing cytosolic long-chain acyl-CoA (LC-CoA) levels, which signal directly or indirectly via fatty acid esterification and/or protein acylation processes to augment insulin release (Prentki *et al.,* 2002).

Given the postulated role of PPARα in the regulation of ß-cell lipid metabolism, it is possible that the lower level of PPARα in the HNF-4α mutants partially contributes to the elevated basal insulin levels (Sugden and Holness, 2004). In support of this hypothesis PPARα null mice develop fasting HH suggesting that PPARα is important for regulated insulin secretion during fasting (Gremlich *et al.,* 2005). HNF-4α binds to the promoters of 11% of islet genes and it is quite likely that HNF-4α deficiency probably exhibits its phenotype via abnormal expression of one or more of these target islet genes (Odom *et*

al., 2004). Hence, further studies are required to study the effect of HNF-4α deficiency on these genes.

Apart from understanding the mechanism of HH and diabetes due to *HNF4A* mutations, it would be interesting to study the glucose tolerance of these patients in the interim period after resolution of HH and development of overt diabetes. A long term prospective study assessing glucose physiology in these patients may provide valuable clues in the further understanding of the function of *HNF4A* and pancreatic insulin secretion.

8.5 Conclusions

Very little is known about CHI due to mutations in the *HNF4A* gene. Our previous knowledge about the phenotype of the babies with a *HNF4A* mutation was limited to one study that retrospectively studied the birth weights and history of hypoglycaemia in children of patients with *HNF4A* MODY. These data have shown that CHI due to heterozygous mutations in the *HNF4A* gene is not limited to mild, transient hypoglycaemia. In fact, CHI due to this genetic aetiology can vary in severity and duration and must be thought of even in cases with severe, persistent HH.

The study confirms that *HNF4A* mutations are associated with large for gestational age babies, but this is not a distinguishing feature due to overlap of this feature with K_{ATP} channel mutations. The data also points out that a history of MODY in the family is also not an essential (though important) feature of CHI due to mutations in the *HNF4A* gene.

A genetic diagnosis is important for these patients as it predicts the likelihood of later sulfonylurea-sensitive diabetes and a high risk of having macrosomic babies. Hence, on

the basis of this study, it would be recommended that neonates with diazoxide responsive CHI without a K_{ATP} channel mutation should be screened for mutations in the *HNF4A* gene, especially in the presence of macrosomia and/ or a family history of young onset diabetes.

Chapter 9

Diabetes Mellitus and Congenital Hyperinsulinism due to Dominant *ABCC8/KCNJ11* Mutations

9.1 Introduction

Dominantly inherited mutations in the *ABCC8/ KCNJ11* genes are known to cause mild, diazoxide responsive CHI. The dominantly inherited mutations have been reported to impair the ability of MgADP to stimulate channel activity with normal expression of the K_{ATP} channels at the surface membrane (Pinney *et al.,* 2008; Abdulhadi-Atwan *et al.,* 2008). Two reports suggest that CHI due to dominantly inherited mutations in the *ABCC8* gene may progress to diabetes mellitus in later life (Huopio *et al.,* 2000, Abdulhadi-Atwan *et al.,* 2008). However, this was not supported by a case series by Pinney *et al.* where only 4 out of 29 adults developed diabetes. Therefore, it is not yet clear whether the dominantly acting mutations in these genes predispose to diabetes mellitus in adulthood or not.

In our cohort, there are 14 patients (from 10 families) with nine dominantly inherited K_{ATP} mutations and diazoxide responsive CHI. This chapter focuses on the phenotype of these 10 families, with the aim to characterise the phenotype of the dominantly inherited *ABCC8/KCNJ11* mutations causing CHI and to study the prevalence of diabetes mellitus in the adult mutation carriers. Of the nine mutations, six mutations are novel, not previously described to be associated with CHI. In order to confirm the pathogenicity of these novel mutations, functional studies were carried out at the Rayne Institute, UCL under the supervision of Professor Andrew Tinker. This chapter gives details of these studies where functional consequences of the mutations were examined by reconstituting the K_{ATP}

channel in HEK293 cells and evaluating the effect of drugs (diazoxide, glibenclamide) and metabolic poisoning on the channels using ^{86}Rb flux assay.

9.2 Methodology

9.2.1 Patients

The phenotype of the fourteen children reported to be heterozygous for a dominantly inherited mutation in the K_{ATP} channel genes were studied in detail. This includes thirteen patients with eight different heterozygous mutations in the *ABCC8* gene and one patient heterozygous for a mutation in the *KCNJ11* gene. Six of the eight *ABCC8* mutations were novel (L1390R; c.4169T>G, L1431F; c.4291C>T, Q1459E; c.4375C>G, G1479A; c.4436G>C, A1508P; c.4522G>C, A1537V; c.4610C>T) and two have been reported previously in patients with dominantly inherited CHI (G1479R; c.4435G>A and R1539Q c.4616G>A) (Pinney *et al.,* 2008). Each mutation was identified in a single family with the exception of G1479R which was identified in two unrelated families. All mutations affected residues that are highly conserved across several species and occurred within the nucleotide-binding domain 2 (NBD2) of SUR1, a previously reported hotspot for dominantly acting mutations. The remaining patient was heterozygous for an inframe deletion (I284del; c.850_852delATC) in the *KCNJ11* gene. The isoleucine at residue 284 in Kir6.2 is conserved across species.

Family member testing was undertaken when DNA was available (for the pedigrees see figure 9.1). For two probands there was no family history of hypoglycaemia or diabetes (family I, II-1 and family J, II-1). Testing of the unaffected parents demonstrated that the L1390R and G1479A *ABCC8* mutations had arisen *de novo* in the proband. In 7/8 families the proband had inherited the mutation from a parent who was affected with

hypoglycaemia or diabetes. In the remaining case (family C, III-2) although the mutation had been inherited from the unaffected father, G1479R was shown to co-segregate with HH in the family having been identified in the proband's two affected siblings and maternal cousin.

To understand the phenotype of the 14 patients with hyperinsulinism, data was collated on:

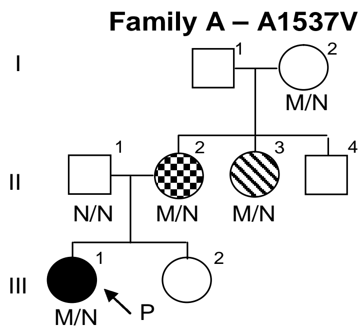
1) birth weight,
2) age of presentation,
3) diazoxide-responsiveness and
4) duration of hyperinsulinism

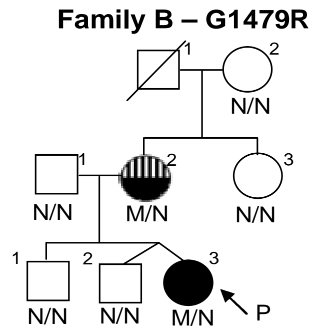
For the adult mutation carriers (n=16), the phenotypic features studied, included:

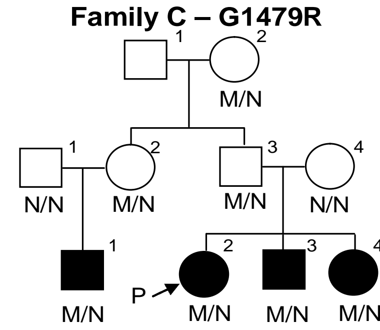
1) birth weights (where available),
2) presence/ absence of hypoglycaemia (past and current),
3) duration of hypoglycaemia (where applicable),
4) presence/ absence of diabetes mellitus
5) type of diabetes mellitus (gestational or other)
6) age of presentation of diabetes mellitus (where applicable)
7) and treatment details, if diagnosed with non-gestational diabetes mellitus

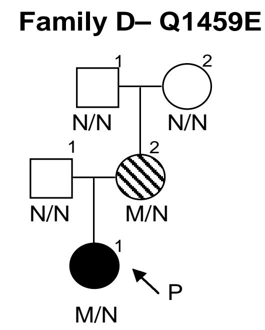
Birth weight SDS of the patients and the adult mutation carriers was calculated. The clinical characteristics (birth weight SDS and age of presentation) are presented as mean.

Figure 9.1: Partial pedigrees showing inheritance of the dominant mutations in the 10 families. Circles represent females and squares indicate males. Probands are indicated by an arrow. Vertical hatching denotes diabetic individuals and diagonal hatching represents gestational diabetes. Filled symbols denote children with HH while symbols with square hatching represent adults with past/ current hypoglycaemia. Individuals who progressed from hypoglycaemia to diabetes are indicated by symbols that are half filled and half hatched horizontally. The genotype is given below each symbol. M/N denotes a heterozygous *ABCC8/ KCNJ11* mutation and N/N a normal genotype.


Family A – A1537V
I
II
III
1
2
M/N
1
2
3
4
N/N
M/N
M/N
1
2
M/N
P


Family B – G1479R
1
2
N/N
1
2
3
N/N
M/N
N/N
1
2
3
N/N
N/N
M/N
P


Family C – G1479R
1
2
M/N
1
2
3
4
N/N
M/N
M/N
N/N
1
2
3
4
M/N
P
M/N
M/N
M/N


Family D– Q1459E
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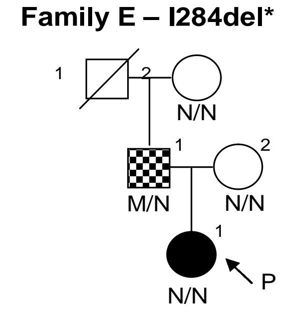
2
N/N
N/N
1
2
N/N
M/N
1
M/N
P


Family E – I284del*
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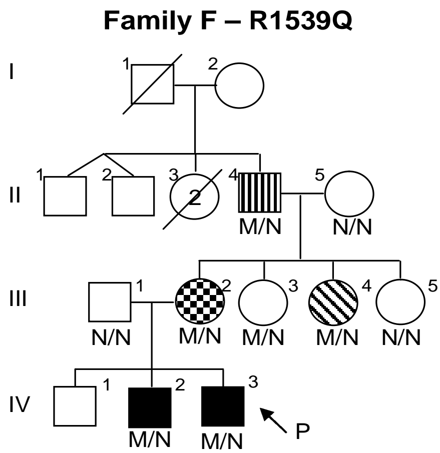
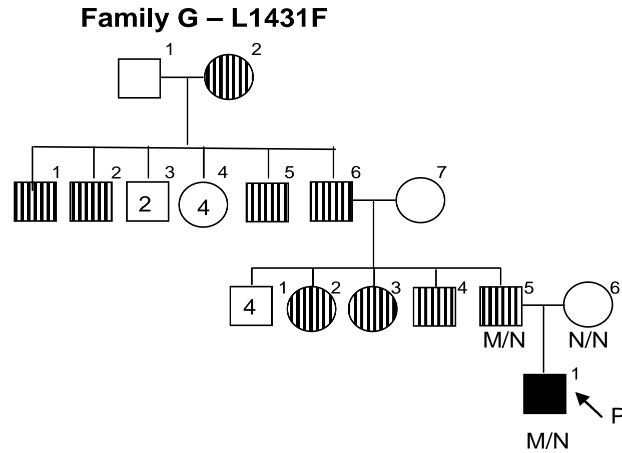
2
N/N
1
2
M/N
N/N
1
N/N
P


Family F – R1539Q
I
II
III
IV
1
2
1
2
3
2
4
5
M/N
N/N
1
2
3
4
5
N/N
M/N
M/N
M/N
N/N
1
2
3
M/N
M/N
P


Family G – L1431F
1
2
1
2
3
2
4
4
5
6
7
4
1
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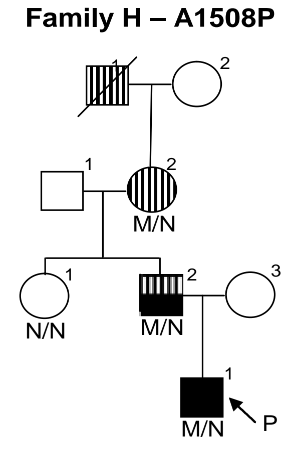
3
4
5
6
M/N
N/N
1
M/N
P


Family H – A1508P
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2
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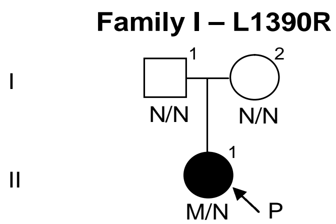
2
M/N
1
2
3
N/N
M/N
1
M/N
P


Family I – L1390R
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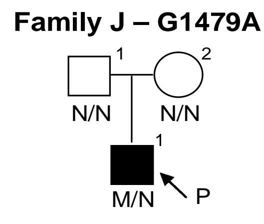
II
1
2
N/N
N/N
1
M/N
P


Family J – G1479A
1
2
N/N
N/N
1
M/N
P

9.2.2 Functional analysis of novel mutations

Mutations were introduced into SUR1 or Kir6.2 using the Stratagene QuikChange site-directed mutagenesis kit (*Stratagene, La Jolla, CA*). Mutated plasmid DNA was then transformed into bacteria, purified and sequenced to ensure that the mutation had been incorporated into the cDNA. Upon sequencing, the plasmid DNA was amplified in a large scale culture providing large yields of mutated plasmid DNA. The functional consequences of the mutations were then examined by expressing the mutant SUR1+WT-Kir6.2/ mutant Kir6.2+ WT-SUR1 (by cell transfection) in HEK293 cell line and the effect on the K_{ATP} channels was evaluated using $^{86}Rb^{+}$ efflux assay. The details of each molecular biology technique are described below.

9.2.2.1 Site directed mutagenesis

Mutations were introduced into hamster SUR1 cloned into the vector pcDNA3 (appendix 12.3, *Invitrogen, Paisley, UK*) (Giblin *et al.,* 1999) and mouse Kir6.2 cloned into pcDNA3.1+/Zeo (appendix 12.4, *Invitrogen, Paisley, UK*) (Giblin *et al.,* 1999) with site-directed mutagenesis using the QuikChange site-directed mutagenesis kit. Hamster SUR1 and mouse Kir6.2 proteins are very similar to the human counterparts and have been extensively used to study the functional consequences of *ABCC8/ KCNJ11* mutations (Shyng *et al.,* 1998; Taschenberger *et al.,* 2002; Pinney *et al.,* 2008). Moreover, we have previous expertise in manipulating these clones (Muzumbya *et al.,* 2007). The human SUR1 sequence (NM_000352.2) was aligned to the hamster SUR1 sequence (L40623.1) and the human Kir6.2 sequence (NM_000525) was aligned to the mouse Kir6.2 sequence (NM_010602). It is worth clarifying that the mutations L1390R, L1431F, Q1459E, A1508P, A1537V in human SUR1 are homologous to the L1390R, L1431F, Q1459E, A1508P,

A1537V in hamster SUR1 and the mutation I284del in human Kir6.2 is homologous to the I284del mutation in mouse Kir6.2.

Site directed mutagenesis utilizes two oligonucleotide primers, complementary to the opposite strands of the cDNA, both containing the desired mutation. The primers are extended during temperature cycling by *PfuUltra* HF DNA polymerase which results in a mutated plasmid containing staggered nicks. The product is then treated with *Dpn* I, an endonuclease that specifically digests the methylated and hemimethylated DNA. Since DNA isolated from G dam (+) *E. coli* strain is dam methylated, *Dpn* I digestion selectively digests parental DNA. The nicked vector DNA containing the desired mutations is then transformed into XL1- Blue supercompetent cells (*Stratagene, La Jolla, CA*) in which the nicks are repaired and the DNA amplified.

9.2.2.2 Primer design

Individual oligonucleotide primers containing the mutation and complementary to the opposite strands of the cDNA, were designed according to the desired mutation. Each primer was between 25-45 bases and designed such that the mutation was in the middle of the primer with 10-15 bases of sequence on both sides. The melting temperature (Tm) of each primer was >/= 78°C, using the formula

Tm= 81.5 + 0.41 (%GC)- 675/N- %mismatch

where, N is the primer length in bases and values for %GC and % mismatch are whole numbers. For calculating Tm for primers introducing deletions, the formula was modified to: Tm= 81.5 +0.41(%GC) -675/N, where N does not include the bases that are being deleted.

The mimimum GC content of each primer was 40% and the primers terminated in one or more C or G bases.

The following primers were designed to create point mutations in the hamster SUR1 cDNA:

1. L1390R (Tm- 85.48)

SENSE: GGGAAGTCCTCCTTCTCCCGGGCCTTTTTCCGAATGGTG

ANTISENSE: CACCATTCGGAAAAAGGCCCGGGAGAAGGAGGACTTCCC

2. A1537V (Tm- 86.7)

SENSE: CGCACGGTGGTCACCATCGTGCATCGTGTGCACACCATC

ANTISENSE: GATGGTGTGCACACGATGCACGATGGTGACCACCGTGCG

3. Q1459E (Tm- 86.7)

SENSE: GAGGCCCTGGAGATCGCCGAGCTGAAGCTGGTAGTGAAG

ANTISENSE: CTTCACTACCAGCTTCAGCTCGGCGATCTCCAGGGCCTC

4. L1431F (Tm= 85.48)

SENSE: CTACAGGACCCCGTCTTCTTCAGCGGCACGATCAG

ANTISENSE: ATCGTGCCGCTGAAGAAGACGGGGTCCTGTAGGATG

5. A1508P (Tm=81.32)

SENSE: ATCTTCATCATGGATGAACCAACCGCCTCCATCGACATG

ANTISENSE: CATGTCGATGGAGGCGGTTGGTTCATCCATGATGAAGAT

A1508P mutation primers (Tm 85.2) were redesigned as mutagenesis reaction with the above primer pair was unsuccessful (2 attempts):

SENSE: CAGCATCTTCATCATGGATGAACCAACCGCCTCCATCGACATGG

ANTISENSE: CCATGTCGATGGAGGCGGTTGGTTCATCCATGATGAAGATGCTG

The following primers were designed to create the I284del mutation in the mouse Kir6.2 cDNA:

6. I284del (Tm=87.7)

SENSE: CACCAGGACCTCGAGATCGTCATCCTGGAAGGCGTG

ANTISENSE: CACGCCTTCCAGGATGACGATCTCGAGGTCCTGGTG

I284del primers were also re-designed (Tm=88.3) following two unsuccessful attempts at mutagenesis with the above primers:

SENSE: CACCACCAGGACCTGGAGATCGTCATCTTGGAAGGCGTG

ANTISENSE: CACGCCTTCCAAGATGACGATCTCCAGGTCCTGGTGGTG

The mutagenesis reaction was prepared in a thin-walled PCR tube as below:

5µl 10x reaction buffer

50ng of dsDNA template

125ng of primer #1

125ng of primer #2

1µl dNTP mix

ddH2O to a final volume of 50µl

Finally, 1µl of PfuUltra HF DNA polymerase was added and the reaction was cycled as follows:

Step 1: Initial denaturation	95°C for 30 seconds
Step 2: Denaturation	95°C for 30 seconds
Step 3: Annealing of primers to template	55°C for 60 seconds
Step 4: Elongation of mutant primers	68°C for 2 minutes per kilobase (kb) of plasmid length

Step 2 to step 4 was repeated for 16 cycles and the reaction was cooled to 4°C.

Following temperature cycling, 1µl of Dpn I restriction enzyme was added to each reaction and thoroughly mixed by pipetting the reaction several times. The reaction mix was then spun in a microcentrifuge for 1 minute and incubated at 37°C for 1 hour to digest the parental DNA.

9.2.2.3 Glycogen precipitation of DNA

Glycogen is insoluble in ethanol and forms a precipitate that traps nucleic acids. After *Dpn* I treatment, 0.5µl of glycogen (20mg/ml) and 120µl of cold 100% ethanol was added to the reaction. The reaction was mixed and stored in -20°C for 2-3 hours. The mix was then centrifuged for 15 minutes at 14,000xg at 4°C to pellet the precipitated nucleic acids. The supernatant was discarded and the pellet was re-dissolved in 5µl of molecular biology grade water after air drying at room temperature for 10 minutes.

9.2.2.4 Transformation of XL1- Blue Supercompetent Cells

Transformation is the process by which foreign DNA is introduced into bacteria. 50µl of XL1- Blue supercompetent cells gently thawed on ice, were incubated with 2.5µl of DNA after glycogen precipitation. This reaction was incubated on ice for 30 minutes and then heat shocked at 42°C for 45 seconds to introduce the DNA into the bacterium. Immediately after heat shocking, the cells were placed on ice for 2 minutes, followed by incubation at 37°C for 1 hour after adding 500µl of Lucia-Bertani (LB) broth. LB broth was prepared by adding 1 LB tablet (*Sigma- Aldrich, USA)* per 50mls of ddH_2O. The cells were then pelleted and re-suspended in 100µl of warm LB before plating. LB plates containing carbenicillin at a final concentration of 80µg/ml were used. Discrete colonies were observed after the plates were incubated for 12-16 hours at 37°C.

9.2.2.5 Purification of plasmid DNA

Following bacterial transformation, a single colony was picked and added to 5mls of LB broth containing 5µl of carbenicillin (100 mg/ml). The culture was incubated overnight with orbital shaking (~225rpm) at 37°C. The following morning, the cells were harvested by centrifugation at 6,000xg for 15 minutes at 4°C. The supernatant was discarded and the plasmid DNA was purified using the QIAGEN plasmid purification Mini-prep kit (*QIAGEN, Crawley, Sussex*) according to the manufacturer's guidelines.

The QIAGEN purification kits use an anion-exchange resin to bind to plasmid DNA. It removes the impurities, dyes, proteins and RNA by washing with a medium-salt buffer and then eluting plasmid DNA with a high salt buffer. Plasmid DNA is then concentrated and desalted by isopropanol precipitation. Various QIAGEN purification kits are available that yield different quantities of DNA. Generally, 10-20µg DNA is obtained using the Mini-prep kit; 100µg of DNA is obtained using the Midi-prep kit and up to 500µg may be obtained using the Maxi-prep kit.

9.2.2.6 Sequence analysis

After purification of the plasmid DNA, the DNA was sequenced (*Geneservice, UCL, London)* to confirm that mutagenesis was successful.

I designed the following primers to sequence the regions:

For mutations L1390R, L1431F, Q1459E (*ABCC8*):

AGCGTGCGCTATGACAGC

For mutation A1537V (*ABCC8*):

CGGGCCTTCGTGAGGAAG

For mutation A1508P (*ABCC8*):

ACACTGTGGGAGGCCCTG

And for mutation I284del (*KCNJ11*):

GAGGGCGAAGTTGTGCCTCTC

9.2.2.7 Re-transformation and DNA purification

Following confirmation of the DNA sequences, the mutated plasmid DNA was re-transformed and purified using the QIAGEN Maxi-prep kit as large quantities of DNA were required for subsequent experiments.

100µl of thawed Top10 competent *E.coli* cells were incubated with 1µl of purified plasmid DNA on ice for 30 minutes and then heat shocked at 42^0C for 90 seconds. The reaction was then chilled on ice for 2 minutes and 800µl of warm LB media was added.
As described above, the reaction was then centrifuged at 14,000xg for 1 minute and the cell pellet was re-suspended in 100µl of warm LB broth before plating on LB-carbenicillin agar plates. Discrete colonies from transformed bacteria were observed after the plates were incubated for 12-16 hours at 37^0C. Following re-transformation, a single colony was picked and cultured as before. Mutated plasmid DNA was then purified using the QIAGEN Maxi-prep kit according to the manufacturer's guidelines.

9.2.2.8 Cell culture

Studies were performed using the HEK293 cell line, an immortalised human cell line derived from primary embryonic kidney cells (obtained from Dr LY Jan, UCSF).

Growth and maintenance of the cell line:

The cells were grown in a T75 ($75cm^2$) (*VWR, Merck House, UK*) tissue culture flask in humidified atmosphere of 95% oxygen and 5% CO_2 in Minimum Essential Medium (MEM) containing Earle's salt and L-Glutamine (*Invitrogen*, Paisley, *UK*). The medium was also

supplemented with 10% Foetal Bovine Serum (FBS, *Invitrogen*, Paisley, *UK*) and 1% penicillin- streptomycin (from a stock of 1mg/ml streptomycin and 10,000 units/ml penicillin). The cells were subcultured when 90% confluent (generally once a week).

Preparation of cells for Rb flux experiments: Splitting cells into 35mm dishes-

To prevent contamination, the cells and media were handled in a vertical laminar air flow hood, using sterile techniques. After removing the media from the T75 flask, the cells were washed twice with 10mls of Ca^{2+}, Mg^{2+} free Dulbeccos' phosphate buffered saline (PBS, *Invitrogen, Paisley, UK*). The cells were then treated with 2mls of 0.25% trypsin EDTA mix in PBS buffer for 2-4 minutes to detach the cells. To stop trypsinisation, 8mls of MEM was added and the cells were then transferred in the media to a 50ml tube (*Strastedt, UK)*. The cells were then pelleted by centrifugation for 2-3 min at 340g. After discarding the supernatant, the cell pellet was re-suspended in 31mls of MEM. 1ml each of the cells was added to each well of a 6 well plate (with wells of 35mm diameter, *Triple Red, UK*), resulting in 30 wells. To each well, a further 1ml of the medium was added so that the volume was 2mls/ well. The remaining 1ml of cells was used to seed a new T75 flask, to which 9mls of MEM was added.

9.2.2.9 Transfection

Transfection is the process by which foreign DNA is introduced into a cell line. Once in the cytoplasm, DNA uses the host cell's transcriptional and translational machinery to express proteins. For these studies, FUGENEHD (*Roche, UK*) was used which is a nonliposomal transfection reagent that uses a polycationic head group to attach to the negatively charged DNA. The complex is then taken up by the cell via endocytosis.

Since the K_{ATP} channel is an octameric compound comprising of SUR1 and Kir6.2 components, mutant SUR1 was co-transfected with WT Kir6.2 and a small amount of the enhanced variant of the green fluorescent protein (eGFP) cloned into peGFP-n1 (appendix 12.5, *Clontech laboratories).* Similarly, while studying the effect of a mutant Kir6.2 protein, it was co-transfected with WT SUR1 and eGFP. Control cells were transfected with WT SUR1, WT Kir6.2 and eGFP. The co-transfection of eGFP enabled me to assess the transfection efficiency using epifluorescence.

Transfection was carried out 24 hours after sub-culturing the HEK293 cells in the 6 well plates. Individual transfection mixtures were prepared containing 2µg of SUR1, 500ng of Kir6.2, 50ng of eGFP, 94µl of serum free MEM and 6µl of FUGENE HD in 1.5mls centrifuge tubes. The mixtures were vortexed thoroughly and left at room temperature for 30 minutes to allow complex formation. The individual mixtures were then added directly to individual wells and incubated overnight.

9.2.2.10 Loading of ^{86}Rb and ^{86}Rb Flux assay

If transfection was successful (>80 % efficiency, as evidenced by >80% cells expressing eGFP), the cells were loaded with ^{86}Rb 24 hours after the transfection of cells. The existing medium was removed from each well and replaced by MEM containing 0.037 MBq/ml of ^{86}Rb. The cells were then left for 24 hours in optimum conditions (5% CO_2, 37°C) in a Perspex box. The lid of the box was left slightly open to allow for gas exchange.

^{86}Rb Flux assay

^{86}Rb is an ideal element to study the conductance of K^+ via the K^+ channels as it is similar in size and charge and is handled by the channels in a manner similar to K^+. The ^{86}Rb efflux assay is based on the principle that when cells are loaded with rubidium, its

distribution between intracellular and extracellular spaces is an indicator of channel activity. Hence, the function of the channel can be studied by measurement of Cherenkov radiation following exposure of cells to channel activators, inhibitors or genetic manipulations (Muzyamba *et al.,* 2007).

The ^{86}Rb flux assay experiment was carried out in a designated area for handling radioactive substances. It was necessary to handle ^{86}Rb in a specific room behind a lead shield to minimise exposure to ß-radiation. Whole body dosimeters were worn to monitor the exposure to ^{86}Rb. A Geiger-Mueller detector was used during the experiment to monitor spillage. Solid waste was collected in sealed, shielded containers and liquid waste was disposed in the designated sinks. Detailed records were maintained throughout each experiment regarding the amount of ^{86}Rb in the vial (by calculating decay), amount used for each experiment and the amount of solid/ liquid waste disposed.

Procedure: First, the loading media was removed from each well and the cells were washed twice with 2mls of HEPES Buffered Saline (HBS). The constituents of HBS included 10mM HEPES, 10mM anhydrous Glucose, 130mM Nacl, 7mMKCl, 2mM $CaCl_2$ ($2H_2O$) and 1mM $MgCl_2$, ($6H_2O$) adjusted to a pH of 7.4 (using NaOH) and autoclaved prior to use. The cells were then incubated with 2ml of HBS medium with or without channel stimulants and/ or inhibitors. The following conditions were used:

- control cells,
- cells treated with 100µM diazoxide,
- cells treated with 100µM diazoxide plus 10µM glibenclamide,
- cells treated with 2.5mM NaCN and 20mM 2-deoxy-D-glucose (metabolic inhibition, MI) and

- cells treated with 2.5mM NaCN, 20mM 2-deoxy-D–glucose and 10μM glibenclamide

The concentrations of the drugs stated have been shown to be effective on this cell line (Muzyamba *et al.,* 2007). The supernatant was aspirated after 5 minutes into 6mls polyethylene scintillation vials (*Packard Biosciences*) for counting. The cells were then lysed with 2% Triton/HBS solution and the cell lysates were aspirated into separate scintillation vials. Cherenkov radiation in the supernatant and cell lysates was measured in a liquid scintillation counter (*TriCarb, Packard 2000CA*). The percentage efflux was calculated using:

$$\frac{{}^{86}\text{Rb content of the medium}}{({}^{86}\text{Rb content of medium} + {}^{86}\text{Rb content of cells})}$$

Three wells of cells were individually transfected for exposure of each drug per experiment, which was repeated three times (n=9).

Statistical analysis:

The data were analysed in Microsoft Office Excel 2003 and Graph Pad Prism 4 (*Graph Pad Software, San Diego, CA, USA*, www.graphpad.com). All data are presented as mean +/- SEM. Significant differences were tested for by employing the one-way ANOVA and Bonferroni post-test. Significant differences are reported as: * indicated *P*</= 0.05, ** indicates *P*</= 0.01 and *** indicates *P*</=0.001.

9.2.2.11 SDS-PAGE and Western Blotting

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and Western blotting were used to detect the level of mutant protein expression.

Principle: Gel electrophoresis separates proteins by molecular weight. The proteins are then transferred onto a stable membrane that is probed with an antibody specific to the protein of interest. A secondary antibody (conjugated to horseradish peroxidase (HRP)) is then used to detect the bound (primary antibody) which itself can be detected using enhanced chemiluminescence (ECL). The ECL technique is based on the principle that the light emitting chemical 'luminol' is oxidised by HRP conjugated to the secondary antibody. ECL is achieved by activating oxidation of luminol by HRP in the presence of chemical enhacers (such as phenol) which enhances the emission of light by 100 fold, making it easier to detect light. The light from the reaction is captured on a photographic film creating an image of the antibodies bound to the blot.

Method:

Sample preparation:

HEK293 cells were first transfected with 2µg of WT SUR1 (control) or SUR1 mutants (A1537V, Q1459E, L1431F, L1390R), 500ng of WT Kir6.2 and 50ng of eGFP in 6-well dishes (35mm diameter). Untransfected HEK293 cells were used as control. After 24 hours, the cells were harvested in Phosphate Buffered Saline or PBS (PBS tablets, *Sigma-Aldrich, USA*) and resuspended in 50µl of PBS + 50µl of 2x SDS loading buffer (0.0625M Tris-HCl (pH 6.8), 2%SDS, 10%glycerol, 0.001% bromophenol blue. 0.1M dithiothreitol (DTT)). The samples were then sonicated for 15 seconds and the protein denatured by heating to 95°C for 5 minutes.

SDS-PAGE:

Each sample was then resolved on an 8% polyacrylamide gel. The resolving gel was first prepared (6.66 mls acrylamide 29:1 (30%), 5mls 1.5M Tris pH 8.8, 200µl 10% ammonium persulphate, 200µl 10% SDS, 10µl TEMED, 7.9mls ddH_2O) and allowed to set. Then a 4% stacking gel (1.7mls acrylamide 29:1 (30%), 1.25mls 1.5M Tris pH 8.8, 100µl 10% ammonium persulphate, 100µl 10% SDS, 10µl TEMED, 6.8mls ddH_2O) was prepared and poured on top of the resolving gel. Combs were inserted and the gel was allowed to set. The protein samples were then loaded in the wells and separated by electrophoresis (120V for 90 minutes). On application of the voltage, the proteins travel towards the anode at different speeds based on their size. To help determine the molecular weight of the seperated protein, a ladder was also loaded onto each gel which is a commercially available mixture of proteins with defined molecular weights (Biorad plus protein standards).

Western Blotting:

The proteins were then transferred by blotting to nitrocellulose membrane in transfer buffer (25mM Tris, 192mM glycine, pH 8.3). The gels were sandwiched with a nitrocellulose membrane between filter paper and sponges. Transfer was performed at 100V for 60 minutes at 4^oC.

Antibody detection:

Remaining nonspecific binding sites on the membrane were blocked using 5% skimmed milk blocking buffer (5% skimmed milk powder in PBS + 0.1% Tween 20 (PBST)) for 1 hour at room temperature. Membranes were then probed with a sheep anti-SUR1 NBD2 polyclonal antibody raised to the peptide sequence ETLLSQKDSVFASFVRADK(C) (developed at *The University of Dundee*) (1:1000 dilution in PBST+ 5% non fat milk) or

mouse anti-GFP monoclonal antibody (*Roche, UK)* (1:1000 dilution in PBST+ 5% non fat milk) overnight at 4°C. The membranes were then washed 1 x 15 minutes in PBST and 2 x 5 minutes. The primary antibody was then incubated with an HRP-conjugated secondary antibody for 1 hour at room temperature. An anti-sheep IgG raised in donkey (*Sigma-Aldrich, USA)* was used to probe the primary anti-SUR1 antibody at 1:5000 dilution and an anti-mouse IgG raised in goat (*Jackson Immunoresearch, USA*) was used to probe the primary anti-GFP antibody at 1:10,000 dilution. The membrane was finally washed with PBST 1x 15 minutes and 4 x 5 mins, prior to detection.

ECL Detection:

Protein-antibody complexes were detected using an ECL plus western blotting detection system as per the manufacturer's instructions (Amersham Biosciences UK Ltd). Molecular masses were determined by comparison to the Bio-Rad Precision Plus protein standards.

9.3 Results

9.3.1 Phenotype of the patients with CHI

The clinical characteristics of the 14 children are summarised in table 9.1. The mean age of presentation with hypoglycaemia was 27.3 days, with all except one presenting within the first week of life. The mean birth weight SDS of the children was +2.06. 12/14 patients responded to diazoxide and have persistent HH, while two others had resolution of hypoglycaemia within the neonatal period and hence were not treated with diazoxide. Two siblings who were heterozygous for the same mutation (R1539Q) in the *ABCC8* gene had varying duration of HH, with one persisting at 3.2 years whilst resolving within a week after birth in the other.

Table 9.1: Clinical characteristics of the 14 children with HH: The mean birth weight was +2.06 SDS. The patients presented early at a mean age of 27.3 days. The duration of HH was variable between few days to 11 years.

Patient	Family	Mutation/ gene	Birth wt SDS	Age of presentation	Diazoxide responsive	Length of treatment with diazoxide
1	A	A1537V/ *ABCC8*	+4.66	1 day	Yes	Continuing at 4 years
2	B	G1479R/ *ABCC8*	+1.37 (twin II)	1 day	Yes	Continuing at 11 years
3	C	G1479R/ *ABCC8*	+2.82	1 day	Yes	Continuing at 1.4 years
4	C	G1479R/ *ABCC8*	+1.65	2 days	Yes	Continuing at 8.5 years
5	C	G1479R/ *ABCC8*	+2.23	2 days	Yes	Continuing at 5.1 years
6	C	G1479R/ *ABCC8*	+1.93	2 days	Yes	Continuing at 1.5 years
7	D	Q1459E/ *ABCC8*	+3.14	1 day	Yes	Continuing at 5.8 years
8	E	I284del/ *KCNJ11*	+1.48	1 day	Yes	Continuing at 1.8 years
9	F	R1539Q/ *ABCC8*	+2.19	1 day	Yes	Continuing at 3.2 years
10	F	R1539Q/ *ABCC8*	+0.07	1 day	n/a	n/a
11	G	L1431F/ *ABCC8*	-0.98	1 year	Yes	Continuing at 2.5 years
12	H	A1508P/ *ABCC8*	+4.09	1 day	Yes	Continuing at 9.2 years
13	I	L1390R/ *ABCC8*	+3.22	1 day	Yes	Continuing at 11 years
14	J	G1479A/ *ABCC8*	+1.02	3 days	n/a	n/a

n/a: not applicable, as had transient HH that did not require treatment with diazoxide

9.3.2 Phenotype of the adult mutation carriers

Birth weight SDS data were available for 10 adult mutation carriers. The mean birth weight SDS of the adult mutation carriers was +0.88 (range -2.1 to +3.05) and five adults were macrosomic. Figure 9.1 shows the pedigrees of the 10 families with a dominant K_{ATP} channel mutation. As shown in figure 9.1, of the sixteen mutation carriers identified, two adults have persisting hypoglycaemia at 28 and 39 years of age (family A, II-2 and family F, III-2) whilst one adult has a history of neonatal hypoglycaemia (family E, II-1). Two other adults have a past history of hypoglycaemia progressing to overt diabetes mellitus at 30 and 38 years of age (individuals II-2 and III-2 in families B and H respectively). Of the remaining eleven adult mutation carriers, three were diagnosed with diabetes mellitus at 20, 47 and 50 years of age (family G, III-5; family F, II-4 and family H, II-2 respectively) whilst three other adult mutation carriers have developed gestational diabetes (II-3, II-2 and III-4 in families A, D and F respectively). The remaining five adults (family A I-2, Family C I-2, II-2, II-3 and family F III-3) have remained asymptomatic at ages 57, 73, 32, 37 and 35 years respectively.

9.3.3 Functional analysis of mutant channels

Expression and functional effects of six novel mutations were studied using SDS-PAGE and western blot and ^{86}Rb flux assays. Of the remaining three mutations, *ABCC8* R1539Q and G1479R mutations have been functionally characterised previously (Pinney *et al.,* 2008) and hence these mutations and another mutation (G1479A) occurring at the same residue were excluded from the functional studies. 85% of transfections carried out were successful. ^{86}Rb flux assay showed that the channels expressing the mutant proteins (SUR1- A1508P, L1431F, L1390R, Q1459E and A1537V or Kir 6.2- I284del) were non-functional (figure 9.2) as evidenced by the lack of ^{86}Rb efflux on exposure to the channel agonist diazoxide or metabolic inhibition. Expression of the mutant SUR1 proteins L1431F,

L1390R, Q1459E and A1537V did not differ from the WT SUR1 protein on western blot analysis (figure 9.3).

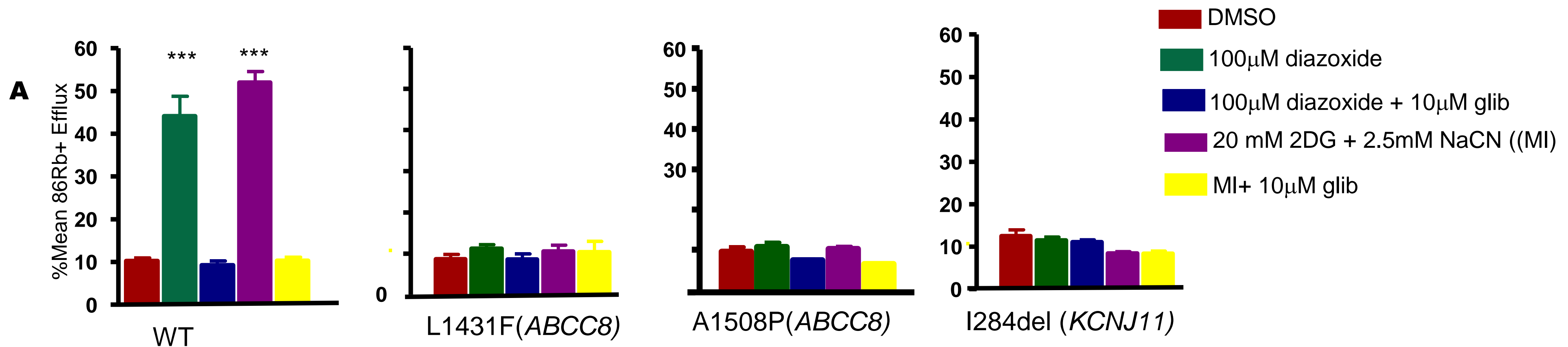


Figure 9.2: The effects of SUR1/ Kir6.2 mutants on K_{ATP} channel function studied with ^{86}Rb efflux. The ^{86}Rb efflux was assayed in control cells (red; no drugs), cells treated with 100μM diazoxide (green), cells treated with 100μM diazoxide plus 10μM glibenclamide (blue), cells treated with 2·5mM NaCN and 20mM 2-deoxy-D-glucose (MI) (purple) and cells treated with MI and 10μM glibenclamide (yellow). Efflux was calculated as %efflux of initial ^{86}Rb content. Data are shown as +/- SEM, whereby n=9. Significant differences are reported as: * indicates $P \leq 0.05$, ** indicates $P \leq 0.01$ and *** indicates $P \leq 0.001$.

A: Channel agonist diazoxide and MI activated the wild-type (WT) channels, evidenced by an increase in the ^{86}Rb efflux from baseline. In contrast, there was no significant increase in the ^{86}Rb efflux when the mutant channels (expressing the *ABCC8* mutations L1431F, A1508P or the *KCNJ11* mutation I284del) were stimulated, suggesting a loss of function of the K_{ATP} channels.

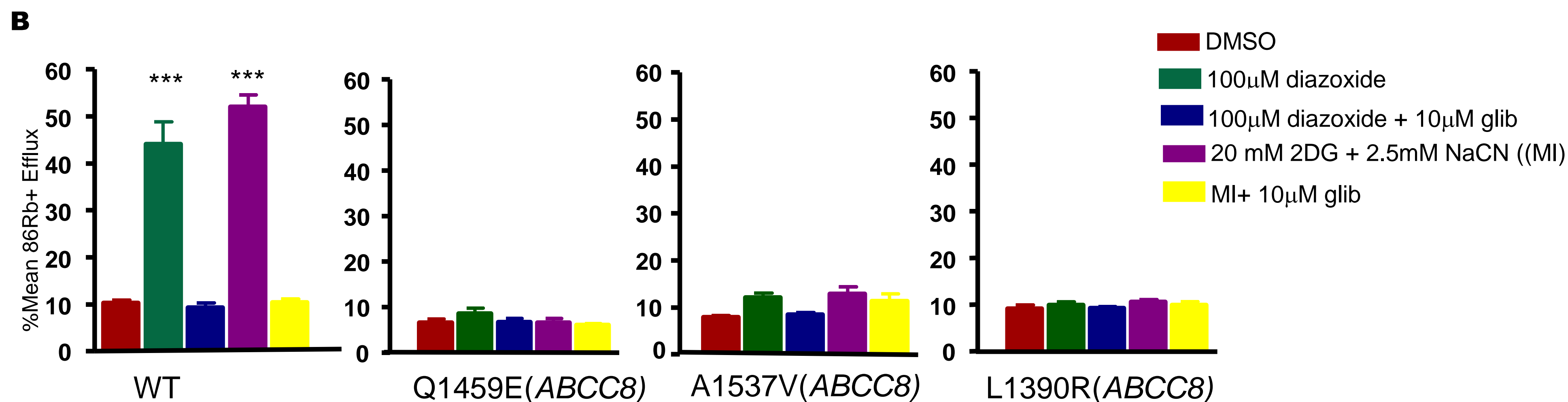


Figure 9.2B: The effect of the *ABCC8* mutations Q1459E, A1537V and L1390R on the function of the K_{ATP} channels as shown by ^{86}Rb efflux. As opposed to the wild-type (WT) channels, there was no significant increase in the ^{86}Rb efflux when the mutant channels were exposed to channel agonist diazoxide or metabolic inhibition, suggesting a loss of function of the K_{ATP} channels. Data are shown as +/- SEM, whereby n=9.

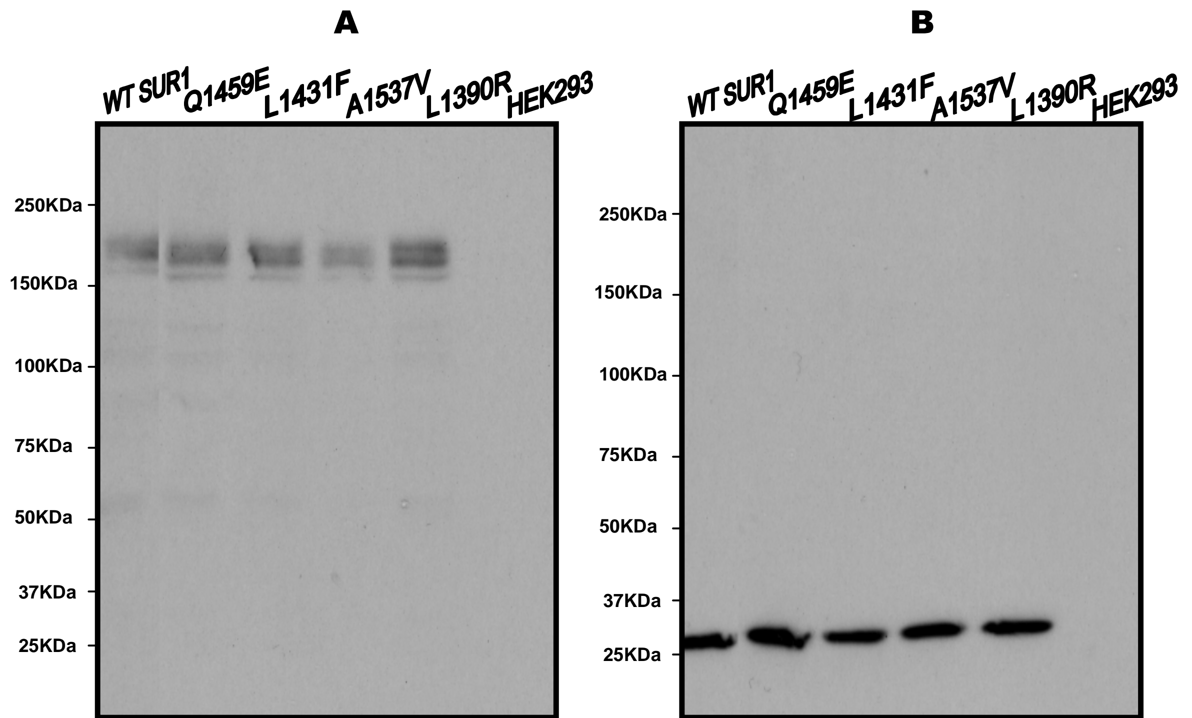


Figure 9.3: Western blot analysis showing the expression of mutant and WT SUR1. Lanes containing WT SUR1 and mutants are indicated and are co-transfected with Kir6.2-GFP. Untransfected HEK293 cells were used as control. The blotting shows that the mutant proteins (A) were expressed at similar levels to WT SUR1. GFP protein bands (B) (~27KDa) were also visualised confirming successful transfections.

9.4 Discussion

This case series confirms the mild, diazoxide responsive HH reported with dominantly inherited mutations in the *ABCC8* and *KCNJ11* genes (Pinney *et al.,* 2008; Huopio *et al.,* 2000; Abdulhadi-Atwan *et al.,* 2008). 12/14 children that were heterozygous for a mutation in these genes and developed HH responded to diazoxide; whilst the remaining two had transient hypoglycaemia limited to the neonatal period. This includes a pair of siblings (family F- figure 9.1) heterozygous for the previously described R1539Q mutation in the *ABCC8* gene who had hypoglycaemia for varying durations. The elder sibling required brief treatment with intravenous glucose after birth while the younger sibling continues to require diazoxide therapy at 3 years of age, demonstrating that the phenotype of HH can be varied within the same family.

Of the 16 adult mutation carriers, two were recognized to have hypoglycaemic episodes after the diagnosis of the children with HH. Three others have a previous history of hypoglycaemia of which one (family E) had transient hypoglycaemia requiring intravenous glucose in the neonatal period while the other two (families B and H) had more persistent hypoglycaemia that remitted in young adulthood. The remaining adult mutation carriers (11/16) have no current/ past history of hypoglycaemia. This is consistent with the study by Pinney *et al.* (Pinney *et al.,* 2008) where 14/29 adults were asymptomatic. The incomplete penetrance of symptomatic hypoglycaemia was a general feature rather than mutation specific. This variability in the phenotype may be the effect of environmental factors or modifying genes. It may also be related to the number of mutant channels expressed on the pancreatic ß-cell membrane in different individuals. The K_{ATP} channels are hetero-octameric complexes and the combination of mutated and wild type SUR1 and Kir6.2 proteins on the pancreatic ß-cells may differ in different individuals. Nevertheless, in-utero

hyperinsulinism was evident by the increased birth weights of 5/10 mutation carriers. Hence the hyperinsulinism phenotype of these dominant mutations varies from asymptomatic macrosomia to transient hypoglycaemia that requires brief supportive treatment to persistent HH requiring diazoxide for prolonged periods. The heterogeneity in the clinical picture due to these mutations is comparable to the heterogeneity observed in HH due to recessive mutations in the K_{ATP} channel genes (Nestorowicz *et al.,* 1998).

It has been debated whether dominant mutations in the K_{ATP} channel genes causing HH predispose to the development of diabetes mellitus in adulthood (Pinney *et al.,* 2008; Abdulhadi-Atwan *et al.,* 2008; Huopio *et al.,* 2000; Huopio *et al.,* 2003, Magge *et al.,* 2004). Huopio *et al*. first reported a dominant inactivating *ABCC8* mutation, E1506K that caused HH progressing to hypoinsulinemic diabetes during middle age (Huopio *et al.,* 2000; Huopio *et al.,* 2003). In their study of 11 adult individuals heterozygous for this particular mutation; four had overt diabetes, five had impaired glucose tolerance, one had impaired fasting glucose, and only one individual had normal glucose tolerance (Huopio *et al.,* 2003). Assessment of glucose tolerance of the adult mutation carriers showed severe blunting of the first phase of glucose stimulated insulin secretion and reduced maximal glucose stimulatory insulin capacity, suggesting that the primary defect lay in the insulin secretory capacity. The individuals who developed diabetes showed a 15% reduction in insulin sensitivity in comparison with those without diabetes with three of the four diabetic individuals being overweight. The authors hypothesized that the primary insulin secreting defect may be related to ß-cell apoptosis due to raised intracellular calcium concentrations and that the accompanying insulin insensitivity may be the reason for conversion from impaired glucose tolerance to diabetes. In support, insulin deficiency (attributed to apoptosis) is seen in transgenic mice with congenital hyperinsulinism over expressing a dominant negative form of Kir6.2 in pancreatic ß-cells (Miki *et al.,* 1998). A recent report

(Vieira *et al.,* 2009) also confirms the association of this mutation (E1506K) with CHI in childhood and diabetes mellitus in adulthood. In addition, Abdulhadi-Atwan *et al.* (Abdulhadi-Atwan *et al.,* 2008) have also described a patient with HH who was heterozygous for a *de novo* mutation in the *ABCC8* gene *(*R370S*)* and presented with diabetes at 10.5y of age. This child was significantly obese (BMI of 30.2kg/m^2), supporting the hypothesis that insulin insensitivity in conjunction with a dominant mutation in the K_{ATP} channel genes can predispose to the development of diabetes mellitus.

However, a recent study that looked at the prevalence of diabetes mellitus in a large group of families with dominant K_{ATP} hyperinsulinism mutations (Pinney *et al.,* 2008) did not report an increased prevalence of diabetes mellitus in the adult mutation carriers. Only 13.8% of the carrier adults in this study were diagnosed with overt diabetes mellitus and 3 of 13 women were diagnosed with gestational diabetes during pregnancy. This prevalence of diabetes in the adult mutation carriers was comparable with the estimated prevalence of type 2 diabetes in adults in the United States, which ranges from 9.6% to 21% (Signorello *et al.,* 2007).

In contrast to the study by Pinney *et al.* (Pinney *et al.,* 2008), our study found an increased prevalence of diabetes in the adult mutation carriers. Two of the adult mutation carriers (family B, II-2 and family H, III-2) have a clear progression of hypoglycaemia in early life to diabetes in adulthood, with one of them being treated with insulin while the other is being treated with metformin. Three others were diagnosed with diabetes at ages 20, 47 and 50 years. Of these three adults, individual II-5 in family G is being treated with insulin while the other two are on oral hypoglycaemic agents. Interestingly, there is a strong family history of young onset diabetes spanning three generations typical of MODY in family G. Co-segregation of diabetes in this family would have confirmed the association of this

mutation with early onset diabetes mellitus. However, unfortunately it has not been possible to recruit the family members into the study. Apart from these five adult mutation carriers who have developed overt diabetes mellitus, three other mutation carriers have developed gestational diabetes. Excluding the two adults who currently have symptoms of hypoglycaemia, the prevalence of diabetes (gestational or other) is high at 57% (8/14) in the adult mutation carriers of our cohort. This may even be an underestimate as some of these adults who do not have diabetes mellitus may go on to develop it later (two adults in this cohort are < 30 years of age).

Studies looking at the functional consequences of dominant mutations in *ABCC8/KCNJ11* so far have shown that these mutations cause decreased K_{ATP} channel responses to MgADP/ diazoxide or impaired channel opening as opposed to the trafficking/ biogenesis effects of recessive *ABCC8/KCNJ11* mutations (Pinney*et al.,* 2008; Huopio *et al.,* 2000). Western blotting carried out on the four mutant SUR1 proteins demonstrated that the mutant SUR1 proteins were expressed in the cell (Fig. 9.3). Using homozygous mutants expressed in HEK293 cells I studied the effects of SUR1/ Kir6.2 mutants on K_{ATP} channel function with ^{86}Rb efflux. Neither the K_{ATP} channel agonist diazoxide nor metabolic inhibition stimulated the mutant channels confirming that these mutations are disease causing and not rare polymorphisms. However *in-vivo* it is likely that the pancreatic ß-cell contains a combination of channel hetero-octamers (a mixture of mutated and wild type SUR1 and Kir6.2 channels with ratios of 0 to 4 SUR1 or Kir6.2 mutant subunits) thus explaining the *in-vivo* responsiveness to diazoxide. Further studies are required to understand the molecular mechanism of disease due to these mutations. Along with ^{86}Rb efflux studies under heterozygous conditions (to mimic patient conditions), it would be beneficial to study the effect of metabolic regulators (viz. MgADP, ATP) and drugs by

inside out patch clamp studies (Pinney *et al.,* 2008, Muzyamba *et al.,* 2007; Cartier *et al.,* 2001).

9.5 Conclusions

This study demonstrates that dominant mutations in *ABCC8/ KCNJ11* cause a varying phenotype ranging from asymptomatic macrosomia to medically responsive HH in childhood. In the adult mutation carriers the prevalence of diabetes mellitus (gestational or other) was significantly high at 57% (8/14). Hence, dominant mutations in *ABCC8/ KCNJ11* may be an important cause of MODY X i.e. MODY where no genetic aetiology has been found. This has important implications for follow up of the children diagnosed with dominant *ABCC8/KCNJ11* mutations and the management of the adult carriers.

This study also opens up further avenues for translational research. Further studies are required to establish the molecular mechanism of HH and diabetes due to these novel mutations. It is also important to study why certain individuals heterozygous for loss of function mutations in these genes develop diabetes mellitus and whether these individuals have additional predisposing factors that lead to the development of diabetes. Clinical studies looking at the glucose physiology of the adult mutation carriers would help in classifying whether the diabetes is due to insulinopenia (due to ß-cell exhaustion) or insulin insensitivity or a combination of both, as hypothesized by Huopio *et al.* (Huopio *et al.*, 2000). This cohort of adult mutation carriers hence forms a unique group of individuals which could give further insights into the development of a common and important health issue –diabetes mellitus. Finally, this study may have implications for the diagnosis of patients with MODY-X. Screening a cohort of patients will help determine whether

mutational analysis of the *ABCC8/KCNJ11* genes should be incorporated into the molecular diagnostic work-up of patients with MODY.

Chapter 10

Summary and Future Work

This is the largest study of patients with Congenital Hyperinsulinism that have been genotyped and phenotyped. The phenotype of the patients recruited in this study was studied in detail with regards to age of presentation, birth weight, biochemical features, family history of hypoglycaemia/ diabetes, responsiveness to treatment with diazoxide, persistence of disease and histopathological outcome (in cases where surgery was performed). Sequence analysis of genes known to cause CHI was conducted following the strategy detailed in chapter 3. The clinical features were then correlated to the results of the genetic analysis. A genetic diagnosis was possible in 48.6% of study subjects providing further evidence to the literature that the genetic cause of CHI is not known in approximately 50% of patients (Hussain, 2008a).

In this cohort, the commonest identifiable cause of CHI was a mutation in *ABCC8/ KCNJ11* genes that encode the components of the K_{ATP} channel in the pancreatic ß-cells. Mutations in these genes accounted for 119/146 (81%) patients with an identifiable cause and 87.6% of all patients with diazoxide unresponsive CHI. Recessively inherited mutations in these genes were confirmed to cause diazoxide unresponsive CHI (chapter 5). So far, dominantly inherited mutations have been described in association with diazoxide responsive disease. Apart from identifying novel dominant mutations that lead to diazoxide responsive CHI, the study also identified novel dominant mutations that led to diazoxide unresponsive disease (Flanagan *et al.,* 2010). This novel finding has implications for the prognosis of children identified to be heterozygous for a mutation in the K_{ATP} channel genes and for genetic counselling of the patient and family. Interestingly, the

dominant mutations causing diazoxide responsive CHI were associated with a high risk of adult onset diabetes in our study (Chapter 9) with important implications for the follow up of the children with these mutations and also for pathogenesis of a common condition such as diabetes. Our findings are in contrast with a previous study by Pinney *et al.,* (Pinney *et al.,* 2008) where the prevalence of diabetes mellitus in a large cohort of families with dominantly inherited CHI was not increased. Interestingly, not all of the adult mutation carriers in our study developed diabetes raising the question as to why only certain individuals with these mutations develop diabetes. The study demonstrates that the association of diabetes was not mutation specific. However, the effect of body mass index (BMI) or any other additional factors was not studied. Future studies looking at the type of diabetes (insulinopenia or insulin resistance or both) and effect of BMI on this unique cohort of patients will help understand the pathogenesis of diabetes. These studies could include an oral glucose tolerance test to assess the glucose tolerance, an intravenous tolerance test and hyperglycaemic clamp to study the insulin secretory capacity and a hyperglycaemic euglycaemic clamp study to assess the insulin sensitivity. Functional work in this study was limited to proving the pathogenecity of the six novel dominant mutations identified that cause diazoxide responsive CHI. Previous studies have shown that the dominant mutations cause impairment of the responses of the K_{ATP} channel to MgADP and diazoxide. Further patch-clamp studies (Muzumbya *et al.,* 2007) are required to understand the functional consequences of these novel dominant *ABCC8/ KCNJ11* mutations that cause diazoxide responsive and unresponsive CHI. In the long term, understanding molecular mechanisms of disease would inform development of novel therapeutic strategies; especially required in the management of the severe, medically unresponsive CHI.

Chapter 5 also discusses the case study of a patient with compound heterozygous mutations in the *ABCC8* gene and transient CHI (Kumaran *et al.*, 2010). This novel association of transient, diazoxide unresponsive disease with recessively inherited mutations in the K_{ATP} channel mutations has important clinical implications. It highlights the importance of delaying pancreatic surgery (while maintaining normoglycaemia) even in genetically confirmed diffuse disease unresponsive to diazoxide therapy, as the natural course of many of the novel mutations is unknown.

Chapter 6 discusses the phenotype of 16 patients with CHI due to *GLUD1* gene mutations (published Kapoor *et al.,* 2009b). The study confirms that mutations in this gene cause diazoxide responsive CHI associated with normal birth weight, delayed age at presentation and a high risk of epilepsy. Although the small number of patients limit the study of the correlations between genotype and phenotype, interestingly all the three patients with S445L mutation in this cohort were large at birth and developed epilepsy, suggesting that this mutation may be associated with a severe phenotype. The highlight of the study was the identification of a mutation in the *GLUD1* gene in a patient with a normal serum ammonia concentration as mutations in this gene are known to cause the Hyperinsulinism-Hyperammonaemia (HI/HA) syndrome. Like other patients with HI/HA syndrome, this patient was extremely leucine sensitive suggesting that G*LUD1* mutational analysis may be indicated in patients with leucine sensitivity; even in the absence of hyperammonaemia. The study raises the possibility that the current prevalence of *GLUD1* mutations based on screening patients with hyperammonaemia might be an underestimate. Further studies are required to study the prevalence of *GLUD1* mutations in patients with leucine sensitive CHI and normal serum ammonia concentrations.

In chapter 7, a case of CHI due to a novel missense mutation in the *HADH* gene, a rare cause of CHI, is discussed. Unlike all other previously reported cases of CHI due to this genetic aetiology, this patient has normal acylcarnitine and urine organic acid profile, previously thought to be diagnostic biochemical markers of CHI due to *HADH* mutations. In addition, the novel observation of severe protein sensitivity (due to leucine sensitivity) in patients with mutations in the *HADH* gene is described (published Kapoor *et al.,* 2009a). These novel clinical findings have implications for the diagnosis and management of children with CHI. Additionally, the occurrence of severe protein/ leucine sensitivity in these patients has provided a novel link between *HADH* and leucine induced insulin secretion. In view of the reported interaction between HADH and GDH proteins (Filling *et al.,* 2008), it is possible that HADH causes leucine induced insulin secretion via the GDH axis. Studies looking at direct protein-protein interactions involving HADH would help confirm this hypothesis. This could be achieved by performing protein pull-down experiments using anti-HADH or anti-GDH antibodies in ß-cells, followed by mass spectrometric analysis of the pull-down proteins. It would also be interesting to study the activity of GDH enzyme in patients with *HADH* mutations and confirm if there is a loss of inhibitory effect of GTP on GDH (as in patients with HI/HA syndrome). This could be determined by measuring the basal GDH activity and the concentration of GTP required to inhibit GDH by 50% (IC50) in patient lymphoblast homogenates. These studies would help in understanding the link between HADH, GDH and regulation of insulin secretion and will provide invaluable insights into pancreatic ß-cell biochemistry and physiology.

Chapter 8 discusses the clinical characteristics of the eight patients with CHI that have a mutation in the *HNF4A* gene. The study of this group of patients extends the observations of Pearson *et al.* (Pearson *et al.,* 2007) reporting that heterozygous *HNF4A* mutations can also cause severe and persistent CHI along with mild, transient CHI described previously

(published Kapoor *et al.,* 2008). The only other previous study on *HNF4A* mutation causing CHI examined families of patients with diabetes due to *HNF4A* mutations and concluded that CHI due to *HNF4A* mutations caused hypoglycaemia and was associated with a family history of diabetes. In this study, three of the eight probands did not have a diabetic parent, illustrating that the absence of a history of diabetes in the parents should not preclude sequencing of the *HNF4A* gene in patients presenting with diazoxide responsive CHI.

In chapter 8, the clinical characteristics of the four groups of patients with diazoxide responsive CHI (with a K_{ATP} channel, *GLUD1, HNF4A* and *HADH* mutation) were also compared. There was overlap in the birth weight and age of presentation between the K_{ATP} channel and the *HNF4A* groups, with patients in these two groups presenting earlier and being heavier at birth than the patients with a mutation in the *GLUD1* and *HADH* gene. Similarly, these two clinical characteristics were not statistically different between the *HADH* and *GLUD1* group. This extends the phenotypic similarities shared by the patients with a mutation in the *HADH* or *GLUD1* genes, in addition to the leucine sensitivity described previously. Additionally, the association of diabetes in the adult carriers of a dominant K_{ATP} channel mutation (Chapter 9) suggests that a family history of diabetes is not a feature exclusive to patients with *HNF4A* mutations.

The mechanism of CHI due to *HNF4A* mutations is not known. It is likely that HNF-4α deficiency probably exhibits its phenotype via abnormal expression of one or more of its many target islet genes. Further studies are required to study the effect of HNF-4α deficiency on the genes expressed in the ß-cells of the pancreatic islets, to help understand the molecular mechanism underlying the opposite phenotypes of CHI and diabetes caused by mutations in this gene.

Finally, the genetic mechanisms causing CHI are yet to be elucidated in the remaining 152 (51.4%) patients from this cohort. This cohort forms a unique group of patients for future research. The majority of the patients in this group with an unidentified genetic aetiology are diazoxide responsive (132/ 152). Within this large group of patients with diazoxide responsive CHI, there are unique and rare groups of patients with unusual types of CHI where the phenotypic features are not limited to hyperinsulinaemic hypoglycaemia. These include:

a) Renal cysts and CHI (n=6): These patients have clinical and imaging features of autosomal recessive polycystic kidney disease along with diazoxide responsive CHI.
b) Hypopituitarism and CHI (n=5). These patients have hypopituitarism, ranging from isolated growth hormone deficiency to multiple pituitary hormone deficiencies.
c) Cerebellar dysfunction and CHI (n=4). Four patients with cerebellar signs (structurally normal brain on MRI scan) and CHI have been identified, indicating a possible genetic link between the cerebellum and pancreas. Two candidate genes (*NEUROD1* and *Kv2.1* gene) expressed in the cerebellum and the pancreas, have been sequenced in these patients so far (*Kv2.1* gene sequencing was performed by me).

The occurrence of groups of patients with additional phenotypic features suggests that a genetic aetiology may link these rare phenotypes. Future studies including candidate gene studies, homozygosity mapping studies (in consanguineous families) and the use of next generation sequencing techniques may lead to discovery of novel genetic aetiologies and may give novel insights into the development of organs such as the cerebellum, pituitary, kidney and pancreas.

Chapter 11

References

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Chapter 12

Appendices

12.1 Proforma and consent forms:

PLEASE RETURN THIS FORM WITH EDTA BLOOD OR DNA WHEN GENETIC TESTING FOR HYPOGLYCAEMIA IS REQUESTED

Defining phenotype-genotype correlations in patients with hypoglycemia

Consultant Paediatric Endocrinologist: Dr Khalid Hussain (02079052128 or K.Hussain@ich.ucl.ac.uk)
Consultant Molecular Geneticist: Prof S. Ellard (01392-402910 or Sian.Ellard@rdeft.nhs.uk)

Please contact Dr Ritika Kapoor, Clinical Research Fellow at the Institute of Child Health (07814149531, 0207 2429789/ Extn 2274, r.kapoor@ich.ucl.ac.uk) as soon as diagnosis is established.
Please fill in as fully as possible and circle Yes/ No where appropriate.

Patient details
Surname...
First name(s)...
Date of birth..
Gender..
Hospital No..
Address...
Tel No...
Ethnic origin ....

Requestor details
Clinician name...........................
Telephone ....
Email Address..............................
Address for report......................
....

Type of Test: NHS ☐ Research ☐
If NHS referral, has consent for research been obtained? Yes/No

Clinical information
Age at presentation (weeks): ....
Duration of hyperinsulinism:................................
Insulin level: .... (mU/L) C-peptide: .... (pmols/l) Glucose level: .... (mmol/L)
Details of Treatment
    Current Treatment: ....
    Responsive to current treatment? : Yes/No
    Any other medications tried? Please give details (name of drug, duration tried):
    ....
    Pancreatectomy performed? : Yes/No
    If yes, focal/diffuse disease: ....
Neonatal History
Birth weight (g): ....Gestation (weeks): ....

IUGR: Yes/No
Perinatal Asphyxia: Yes/No
Any syndromic features (e.g. Beckwith Weidemann Syndrome). Please give details:
………..

Hyperammonaemia? : ………………………………………………………(give level and normal range)
Any other medical problems? : ………………………………………………………………………………

Family history
Are parents related? If yes – how? : ……………………………………………………
Any family history of hypoglycaemia? : ……………………………………………………
Any family history of diabetes? Please give details of affected family members (age of onset, treatment and duration of diabetes):
………
……………………………………………………………………………………
(*NB. A pedigree showing clinical details of affected family members would be very helpfu*l).

Testing required (If no boxes are ticked, testing will be performed according to the clinical information provided)

SUR1 sequencing ☐ *KCNJ11* (gene encoding Kir6.2) sequencing ☐ *GCK* (glucokinase) sequencing ☐
GLUD1 sequencing ☐ LOH analysis for 11p15 (paraffin-embedded pancreatic tissue required + blood DNA) ☐

DNA sample taken from patient: Yes/No

Consent form

Consent by parent or guardian for genetic testing to determine the cause of hypoglycemia in a child less than 16 years old

Date:…………………………………………………………...

Child's full name:……………………………………………..

Date of birth:…………………………………………………..

Parent or guardian's full name:……………………………………..

I have given permission for a blood sample to be taken from my child to allow genetic testing to be performed. I understand that this testing will be only for the purpose of determining the cause of hypoglycaemia in my child or a member of my family. The sample will not be used for any other purpose. The testing will be performed in the molecular genetic laboratory, Peninsula Medical School, Exeter UK.

The details of the testing have been explained by …………………………………….

Signed …………………………………………………

Consent for genetic testing to determine the cause of hypoglycemia (patient)

Date:…………………………………………………………...

Name of the person:……………………………………………..

Date of birth:…………………………………………………..

I have given a blood sample to allow genetic testing to be performed on my blood. I understand that this testing will be only for the purpose of determining the cause of hypoglycaemia that affects myself or a member of my family. The sample will not be used for any other purpose. The testing will be performed in the molecular genetic laboratory, Peninsula Medical School, Exeter UK.

The details of the testing have been explained by …………………………………….

Signed …………………………………………………

If samples from other family members have been sent previously please give details:

....

Consent for genetic testing to determine the cause of hypoglycemia (family member, if applicable)

I have given a blood sample to allow genetic testing to be performed on my blood. I understand that this testing will be only for the purpose of determining the cause of hypoglycaemia that affects a member of my family. The sample will not be used for any other purpose. The testing will be performed in the molecular genetic laboratory, Peninsula Medical School, Exeter UK.

The details of the testing have been explained by ……………………………………..

1) Name of the person:………………………………………

Date of birth:…………………………………………………

Relationship with the patient:………………………………..

Signed …………………………Date……………………….

2) Name of the person:………………………………………

Date of birth:…………………………………………………

Relationship with the patient:……………………………….

Signed …………………………Date……………………….

3) Name of the person:………………………………………

Date of birth:…………………………………………………

Relationship with the patient:……………………………….

Signed …………………………Date……………………….

4) Name of the person:………………………………………

Date of birth:…………………………………………………

Relationship with the patient:……………………………….

Signed …………………………Date……………………….

Samples taken
The samples should be labeled with name and date of birth and can either be:
1. Our preferred option is Blood 5 mls: taken in tubes containing EDTA and couriered fresh (not frozen). This should be sent in a package with at least two layers of wrapping and include absorbent material to absorb any blood that leaks out of the tube.
2. Or made into DNA and 2-10 micrograms (to allow repeats) sent at room temperature. Again please make sure the tube is very securely sealed.

Where should samples be sent?

Please discuss with Dr Ritika Kapoor (r.kapoor@ich.ucl.ac.uk/ 07814149531/ 02072429789, Ex 2274) before sending samples.
The samples should be sent to:
Dr. Ritika Kapoor
NIHR Clinical Research Associate
Institute of Child Health
1st Floor, Clinical & Molecular Genetics Unit
30, Guilford Street
London
WC1N 1EH
Email: r.kapoor@ich.ucl.ac.uk

Other questions
Please do contact me if there are any other questions. We would be very keen to help in any way we can.

12.2 Parent/Guardian Information Sheet:

Project Title: Defining genotype-phenotype correlations in patients with hypoglycemia.

1. The aim of the study

The aim of this project is to investigate the genetic causes of hypoglycaemia (low blood glucose) in newborns, infants and children.

Previous research has already identified a number of abnormalities in certain genes that can cause hypoglycaemia, but we still don't know the cause in the majority of children. Recently, we found a new genetic cause of hypoglycaemia due to excessive insulin secretion (hyperinsulinism). The aim of this study is to characterize this novel type of hyperinsulinism along with trying to unravel further genetic causes.

2. Why is the study being done?

A normal blood glucose level is important for brain function. Hypoglycemia (low blood glucose levels) can cause significant brain injury. We currently have very little understanding as to the genetic mechanisms that cause hypoglycaemia in childhood. This study will allow us to understand why hypoglycaemia develops in some children and the genetic basis of this. This will have important implications for the future treatment of our children.

3. How is the study being done?

If you agree to participate in this study, your child will have an additional blood sample taken when they are having their routine investigations. We will use this blood sample to extract DNA (genetic material). The DNA will then be used to analyze certain genes. The extra blood sample that we take will do no harm to your child, as the amount of blood that we will take is extremely small (5 mls). As a part of the study, we might request blood samples from you or other related family members; especially if there is a family member affected with diabetes mellitus/ hypoglycemia. This blood sample will be used to extract DNA that will be used only for the purpose of establishing a genetic cause for hypoglycaemia.

4. What are the risks and discomfort?

No risk to the child can be foreseen. Your child will have a cannula inserted for routine medical treatment and we will then take the blood sample from the cannula. There is discomfort from the insertion of the cannula but we would normally numb the skin anyway with a local anaesthetic cream.

5. Who will have access to the case/research records?

Only the researchers and a representative of the Research Ethics Committee will have access to the data collected during this study.

6. What are the arrangements for compensation?

An independent Research Ethics Committee who believes that it is of minimal risk to your child has approved this research. However, research can carry unforeseen risks and we are required to tell you that *if your child is harmed and this is due to someone's negligence, you may have grounds for legal action for compensation in respect of any harm arising out of participation in the study.*

7. What are the potential benefits?

This study will not bring any immediate benefits to your child. However, if we find the genetic mechanism that is causing the hypoglycaemia in your child we will explain this to you and it will give us a better understanding of why some children do develop this condition.

8. Do I have to take part in this study?

No, you do not have to take part in the study if you do not wish to. If you decide, now or at a later stage, that you do not wish to participate in this research project, that is entirely your right and will not in any way prejudice any present or future treatment.

9. Who do I speak to if problems arise?

If you have any complaints about the way in which this research project has been, or is being conducted, please, in the first instance, discuss them with the researcher (Dr. Ritika Kapoor: 07814149531; 02072429789/ Extn 2274; r.kapoor@ich.ucl.ac.uk).

12.3: Letter of approval from the Research Ethics Committee

Institute of Child Health/Great Ormond Street Hospital Research Ethics Committee

The Institute of Child Health
30 Guilford Street
London
WC1N 1EH

Tel: 020 7905 2620
Fax: 020 7905 2201

27 April 2007

Dr Khalid Hussain
Consultant Paediatric Endocrinologist
Unit of BEM, 30 Guilford Street
London
WC1N 1EH

Dear Dr Hussain

Study title:	**The genetics of childhood hypoglycaemia**
REC reference:	**06/Q0508/58**
Amendment number:	**1**
Amendment date:	**22 March 2007**

The above amendment was reviewed at the meeting of the Sub-Committee of the REC held on 24 April 2007.

Ethical opinion

The Subcommittee asked for extra clarifications which were dealt with by Dr Kapoor in writing. The further information was considered by Chairman's action.

When they are available, the Subcommittee asks for copies of the ethical approval from the countries outside the UK.

The Chairman on behalf of the Subcommittee present gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
~~Protocol~~	~~new - The genetics of childhood hypoglycaemia~~	
~~Participant Information Sheet~~	~~new Parent/ Guardian~~	
~~"Pro-forma" entitled Defining phenotype-genotype correlations in patients with hypoglycemia~~	~~new~~	
Notice of Substantial Amendment (non-CTIMPs)		22 March 2007
Covering Letter		22 March 2007
~~Participant Consent Form: Consent Form~~	~~new~~	
~~Protocol~~	~~old protocol~~	
~~Participant Information Sheet~~	~~old information sheet~~	
~~Participant Consent Form~~	~~old consent form~~	
Protocol	Version II	March 2007
Parent/ Guardian Information Sheet	Version II	March 2007
Participant Consent Form, “Pro forma” entitled Defining phenotype-genotype correlations in patients with hypoglycaemia	Version II	March 2007
Email from Dr Kapoor with further clarifications		26 April and 27 April 2007

Membership of the Committee

The members of the Committee who were present at the meeting are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

06/Q0508/58:	**Please quote this number on all correspondence**

Yours sincerely

Taki Austin
Research Ethics Co-ordinator

E-mail: t.austin@ich.ucl.ac.uk

Enclosures List of names and professions of members who were present at the meeting and those who submitted written comments

Copy to: Great Ormond Street Childrens Hospital NHS Trust/ Institute of Child Health R&D Department

Institute of Child Health/Great Ormond Street Hospital Research Ethics Committee

Attendance at Sub-Committee of the REC meeting on 24 April 2007

Name	*Profession*	*Capacity*
Dr V Larcher	Consultant Paediatrician	
Mr R Selby	Lay member	

Also in attendance:

Name	*Position (or reason for attending)*
Taki Austin	Research Ethics Co-ordinator

12.4 DNA Extraction -Promega Wizard Genomic DNA purification Kit

The following DNA extraction protocol is used by staff at Peninsula Medical School, Exeter for volumes of blood greater than 1ml

Safety precautions

Standard laboratory precautions apply and powder free gloves should be worn at all times. Please refer to COSHH forms/safety data sheets for other chemicals utilised in this protocol and risk assessments for procedures employed. The extraction should be carried out in a Class II cabinet.

Materials

Product	Supplier
Promega Wizard Genomic DNA Purification kit	Promega Cat. No. A1620
Ethanol (99.7-100%)	BDH Cat. No. 10107
Isopropanol	Sigma Aldrich Cat. No. 190764

Notes:

Ensure all reagents are mixed before using.

Methods

1. Place blood samples to be extracted into a rack in the order that they are on the extraction workbatch. Check details on blood tube against workbatch.
2. Label 50ml centrifuge tubes with the patient name as per workbatch.
3. Transfer 1-7.5ml blood to the centrifuge tube. Add Cell Lysis Solution to make a total volume of 30ml. Invert tube twice to mix.
4. Incubate at room temperature for 10 minutes, inverting 2-3 times during this period.
5. Centrifuge at 3,000rpm in Sigma 4-15 centrifuge for 10 minutes.
6. Tip off supernatant carefully (into 2% bleach solution) without disturbing the visible white pellet.
7. Add 5ml Cell Lysis Solution, vortex to mix and repeat steps 7-8. Proceed to step 10.
8. Vortex the tube vigorously until the white blood cells are resuspended.
9. Add 5ml Nuclei Lysis Solution to the cells. Using a pasteur, pipette the solution 5-6 times to lyse the white blood cells. The solution should become very viscous.
10. Add 1.65ml Protein Precipitation Solution to the nuclear lysate and vortex vigorously for 10 seconds.
11. Centrifuge at 3,000rpm in Sigma 4-15 centrifuge for 10 minutes. A dark brown protein pellet should be visible.
12. Label a 15ml centrifuge tube with the patient name as per workbatch. Add 5ml isopropanol.
13. Transfer the supernatant to the 15ml centrifuge tube containing the isopropanol. Gently mix the solution by inversion until the white thread-like strands of DNA form a visible mass.
14. Centrifuge at 3,000rpm in Centaur 2 for 2 minutes.

15. Carefully decant the supernatant into a beaker. Add 1ml 70% ethanol to the DNA.
16. Centrifuge at 3,000rpm in Centaur 2 for 2 minutes.
17. Carefully decant the ethanol into a beaker. Air-dry the DNA pellet for 10-15 minutes.
18. Add 500µl DNA Rehydration Solution (0.1mM EDTA see Appendix 12.3.1) to the tube (100µl if there is little visible DNA) and rehydrate the DNA by placing samples in the waterbath for a maximum of 1 hour at 65°C then leave samples overnight to rehydrate DNA. For samples extracted at the end of the day put in the waterbath the following morning. For samples run on Friday afternoon leave for the weekend at room temperature.
19. Generate labels for each patient according to the workbatch. The label should have the patient name, lab number and location (as per DNA data sheet, column N).
20. Nanodrop the DNA sample (refer to Nanodrop SOP – MG/SOP/SPE017) and record concentration and ratio on DNA data sheet (columns B and C) and workbatch.
21. If DNA concentration is <300ng/ul for any test (excluding FVL/PROTH, B27, Haematology tests, HCHRO) then a repeat extraction should be carried out on a spare blood sample if possible. For all other tests, if the concentration is <50ng/ul notify a scientist who will decide whether this is sufficient for the test requested.
22. A second person should now be present to witness the transfer. Read out the details on the blood tube, 50ml tube and 15ml tube and the witness should check the details against the workbatch. Pick up the 15ml tube.
23. Obtain a 2D barcode tube and scan barcode number into column L of DNA data sheet.
24. Transfer entire DNA sample from 15ml tube to 2D barcode tube.
25. Obtain label and check details on label by reading out to witness. If correct stick label on 2D barcode tube.
26. DNA can now be put in appropriate location in -20°C freezer.

Any blood remaining from extraction should be stored (maximum 1 tube per patient). Samples for Clinical Genetics patients are stored for one year and then destroyed. Non-Clinical Genetics patients' spare bloods should be destroyed after 3 months. Any surplus blood tubes should be placed in the autoclave bin.

Appendix 12.3.1:

DNA Rehydration buffer (0.1mM EDTA)

5ml Kit Rehydration Buffer

1ml 1M Tris-HCl (Sigma T2788)

44ml Baxter water

12.5: Primer sequences for amplification of *GLUD1* gene and *HADH* genes:

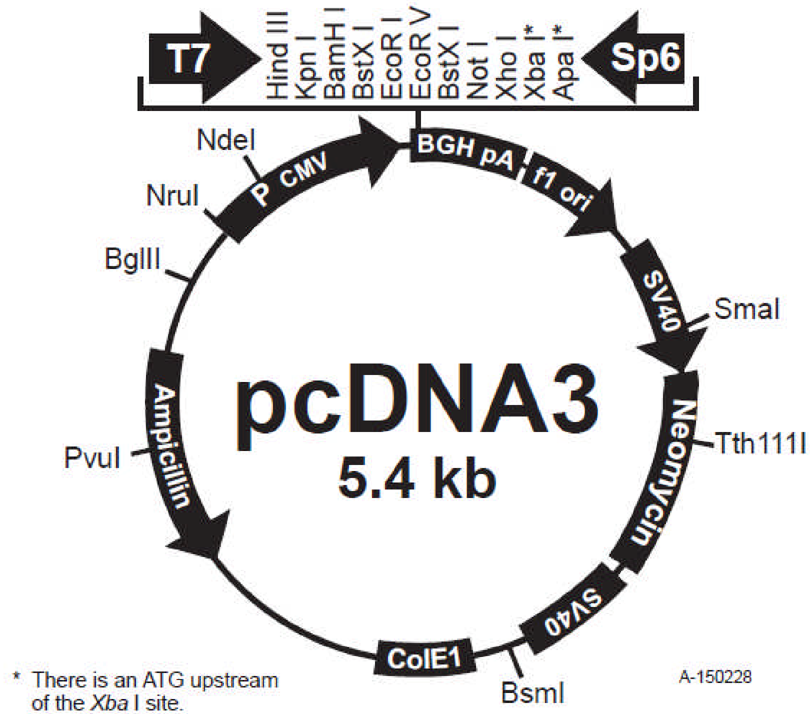
A) Primer sequences for amplification of *GLUD1* genes which encodes the catalytic and allosteric domains of glutamate dehydrogenase:

Exon	Forward	Reverse
6	TTAAATGAGAATGTGCTTTGACTT	TGAATTTGGTGATAGTTTGGTTG
7	GCCCGCTCATCACATTCTT	CTCTGGGTGCGTTGGATTT
10	GTGGGATGGGAAGGAGTGT	TTGTGCATTTTTGGTCTAAGTTTC
11	TCTGTTAGCAATATCTTGATCTCTTG	CAAAGACTATGCCGCAGATG
12	GTTCTGTGTGGTGTCCCTGTT	GGCTGAGATAGCATGGTTGAG

B) Primer sequences for amplification of the eight exons of *HADH* gene:

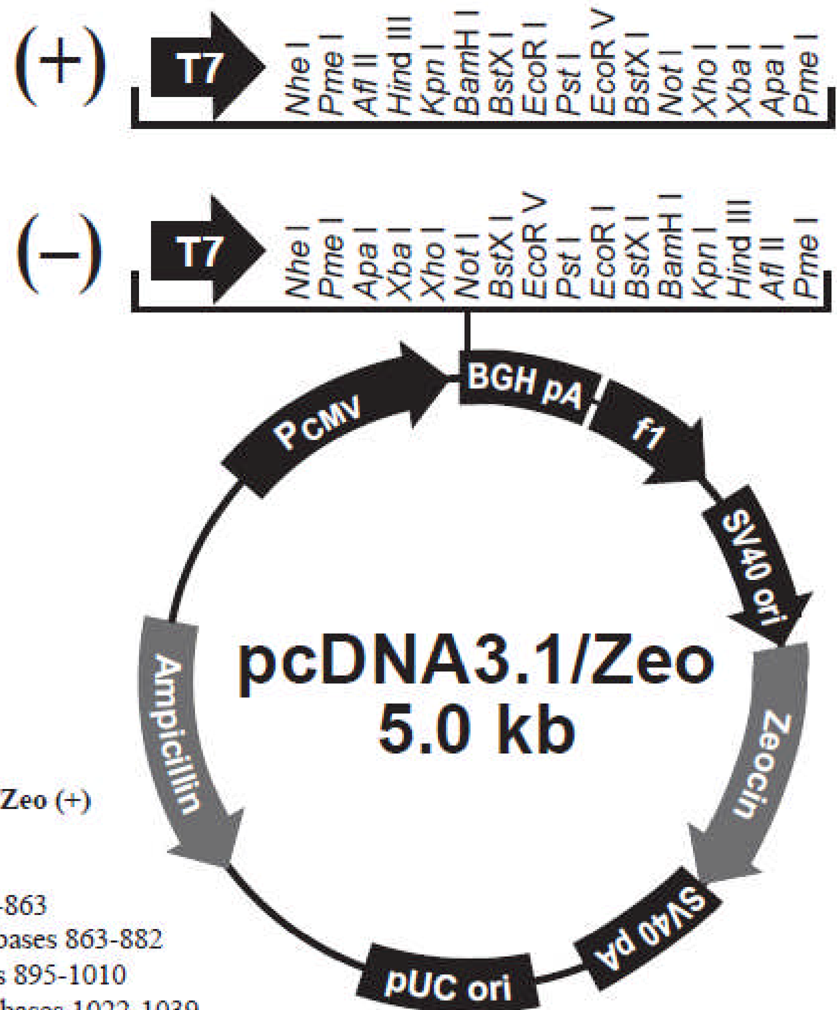
Exon	Forward	Reverse
1	CGTGTATACCCGCTCAACG	GTGAAAACTCCCTGGTGTCG
2	TCGGTATTTTGAATGGAATGG	AACCTCAAATCCCACCCAAC
3	GAACAGATAGGGTAGGCCAAT	GCATGGAACAAAACTGCAC
4	CCCAATGGGTCAGGACAAC	TATGAGGCTCAGGGACAACC
5	TTGTTTCTGGCTTGAGATTCC	AACTAGAACAGGAGGCACGG
6	TGTACAGCTTGATAAATGGGG	TCACAAAGCTAGAAAACAGACACTG
7	CCAAGCCAGAAAGTCTCAGTC	GTGAGGCAGGATTTTGGAAG
8	CACCAGCACAGCCTTCCT	TGCTGTAACTGGTGTAATAATCACTTC

12.6: Map of the vector pcDNA3, a commercially available vector (*Invitrogen, Paisley, UK*).



CMV promoter: bases 209-863
T7 promoter: bases 864-882
Polylinker: bases 889-994
Sp6 promoter: bases 999-1016
BGH poly A: bases 1018-1249
SV40 promoter: bases 1790-2115
SV40 origin of replication: bases 1984-2069
Neomycin ORF: bases 2151-2945
SV40 poly A: bases 3000-3372
ColE1 origin: bases 3632-4305
Ampicillin ORF: bases 4450-5310

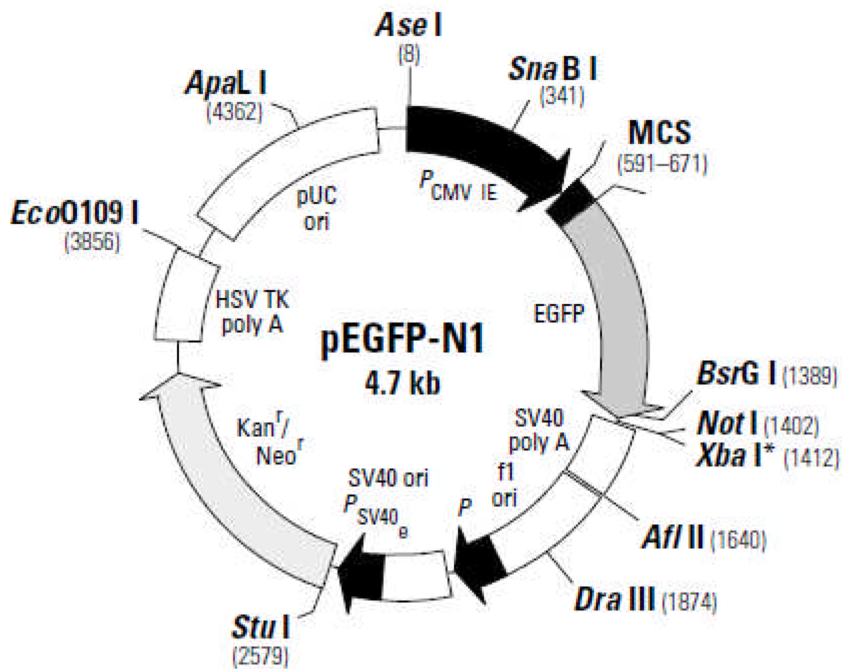
12.7: Map showing the main features of the vector pcDNA3.1/Zeo (*Invitrogen, Paisley, UK*).

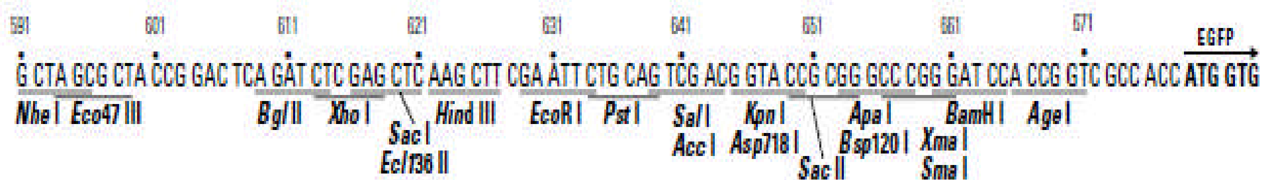


Comments for pcDNA3.1/Zeo (+)
5015 nucleotides

CMV promoter: bases 209-863
T7 promoter priming site: bases 863-882
Multiple cloning site: bases 895-1010
BGH reverse priming site: bases 1022-1039
BGH polyadenylation signal: bases 1021-1235
f1 origin: bases 1298-1711
SV40 promoter and origin: bases 1776-2101
EM7 promoter: bases 2117-2183
Zeocin™ resistance gene: bases 2184-2558
SV40 polyadenylation: bases 2688-2817
pUC origin: bases 3201-3874 (C)
bla promoter: bases 4880-4978 (C)
Ampicillin (*bla*) resistance gene: bases 4019-4879 (C)

12.8: Map of the pEGFP-N1 vector (*CLONTECH Laboratories*), showing the multiple cloning site (MCS) and the various restriction sites.





12.9 Presentations at National and International Conferences

2009

1. Diabetes Mellitus and Hyperinsulinaemic Hypoglycaemia (HH) due to Dominant *ABCC8/KCNJ11* Mutations- Oral Presentation at the British Society of Paediatric Endocrinology and Diabetes (BSPED) meeting, Reading, November

2. Diabetes Mellitus and Hyperinsulinaemic Hypoglycaemia due to Dominant *ABCC8/KCNJ11* Mutations- Poster Presentation at the UCL Partners Symposium, London, September

3. 3-Hydroxyacyl-Coenzyme A Dehydrogenase (HADH) Deficiency and Hyperinsulinaemic Hypoglycaemia: Characterization of a Novel Mutation and Severe Protein Sensitivity. Oral Presentation at the Congenital Hyperinsulinism and Pancreatic Tumors in Childhood International Congress, Greifswald, Germany, September

4. Clinical and Molecular Characterisation of 300 Patients with Congenital Hyperinsulinism- Oral Presentation at the Lawson Wilkins Pediatric Endocrine Society- European Society of Paediatric Endocrinology (LWPES-ESPE) Conference, New York, September

5. Prevalence of *HNF4A* gene Mutations in Patients with diazoxide Responsive Hyperinsulinism- Poster Presentation at the LWPES-ESPE Conference, New York, September

6. Hyperinsulinism-Hyperammonaemia (HI/HA) Syndrome: Novel Mutations in the *GLUD1* gene and Genotype-Phenotype correlations- Poster Presentation at the LWPES-ESPE Conference, New York, September

2008

1. Hyperinsulinism-Hyperammonaemia (HI/HA) syndrome: Novel mutations in the *GLUD1* gene and genotype phenotype correlations- Poster presentation at the BSPED Conference, Swansea, November

2. Genotype-Phenotype Correlations in Patients with Hyperinsulinaemic Hypoglycaemia due to Mutations in the *HNF4A* Gene- Oral presentation at the ESPE Conference, Turkey, September

3. Persistent Hyperinsulinaemic Hypoglycaemia and Maturity Onset Diabetes of the Young (MODY) due to Heterozygous *HNF4A* Mutations- Poster Presentation at the International Update in Paediatric Endocrinology, Mumbai, India, February

2007

1. Severe Hyperinsulinaemic Hypoglycemia and Maturity Onset Diabetes Of the Young -due to mutations in the HNF4A gene. BSPED Conference, Cambridge, UK – September

12.10 Honours and Awards

- **Clinical Research Associate Award** for the research project entitled 'Congenital Hyperinsulinism: Understanding the Genotype-Phenotype Correlations, Molecular Mechanisms and Relationships with Diabetes Mellitus'. Funding awarded by the NIHR- Biomedical Research Centre at the Institute of Child Health, London for 2 years commencing September 2009.

- **President's Best Poster Prize** awarded for 'Prevalence of *HNF4A* gene Mutations in Patients with diazoxide Responsive Hyperinsulinism' presented at the **LWPES/ESPE** Conference, New York, September 09

- **ESPE Travel Grant** awarded for the abstract 'Genotype-phenotype Correlations in Patients with Hyperinsulinaemic Hypoglycaemia due to Mutations in the *HNF4A* Gene', presented at the ESPE (European Society of Paediatric Endocrinology) Conference, Turkey- September 08.

12.11 Publications in peer reviewed journals

1. Flanagan SE, **Kapoor RR**, Banerjee I, Hall C, Smith V, Hussain K, Ellard S (2010). Dominant ABCC8 mutations in patients with medically unresponsive hyperinsulinemic hypoglycaemia. *Clinical Genetics* (in press).

2. Kumaran A, **Kapoor RR**, Flanagan SE, Ellard S, Hussain K. Congenital hyperinsulinism due to a compound heterozygous *ABCC8* mutation with spontaneous resolution at eight weeks. *Horm Res Paediatr*. 2010;73(4):287-92

3. Flanagan S, **Kapoor RR,** Mali G, Cody D, Murphy N, Schwahn B, Siahanidou T, Banerjee I, Akcay T, Rubio-Cabezas O, Shield J, Hussain K, Ellard S. Diazoxide-responsive hyperinsulinemic hypoglycemia caused by *HNF4A* gene mutations. *Eur J Endocrinol.* 2010 Feb 17.

4. **Kapoor RR ,** Flanagan SE, Fulton P, Chakrapani A, Chadefaux B, Banerjee I, Julian Shield, Ellard S, Hussain K. Hyperinsulinism-Hyperammonaemia (HI/HA) syndrome: Novel mutations in the GLUD1 gene and genotype phenotype correlations- *European Journal of Endocrinology* 2009 Nov;161(5):731-5.

5. **Kapoor RR ,** Flanagan SE, James C, Eaton S, Ellard S, Hussain K. 3-Hydroxyacyl-Coenzyme A Dehydrogenase (HADH) Deficiency and Hyperinsulinaemic Hypoglycaemia: Characterization of a Novel Mutation and Severe Protein Sensitivity. *JCEM* 2009 Jul;94(7):2221-5.

6. **Kapoor RR**, Colclough K, Flanagan S, Wales J, Conn J, Ellard S, Hussain K. Persistent Hyperinsulinaemic Hypoglycaemia and Maturity Onset Diabetes of the Young (MODY1) due to Heterozygous Mutations in the HNF4A gene. *Diabetes.* 2008 Jun;57(6):1659-63