Neuroimaging, cognitive and metabolic profiles in children with hypoglycaemia

A thesis submitted by

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I, Anitha Kumaran, confirm that the work presented in this thesis is my own. Any informatiom and contribution derived from other sources and persons have been indicated in the thesis.

I dedicate this thesis to my mother for her unconditional love and support that has encouraged and inspired me – *thank you Amma.*

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Abstract

Background: Glucose is a major fuel for brain function, and persistent and recurrent hypoglycaemia of any aetiology can cause brain injury. Children with hyperinsulinaemic hypoglycaemia (HH) are at a high risk of brain injury, as the inappropriate secretion of insulin during hypoglycaemia inhibits lipolysis thereby reducing the availability of ketone bodies (KB) that are an important source of alternate fuel for the brain during hypoglycaemia.

In contrast children with ketotic hypoglycaemia (KH) are believed to be neurologically protected, due to the presence of abundant ketone bodies during hypoglycaemia. However, in both these groups of children, a comprehensive assessment of the neurocognitive profile with correlation to neuroimaging is not available.

Aims: The aims of this thesis are

1. To understand the magnitude and rate of ketone body response during a diagnostic fast between children with KH and a 'control' group (children with suspected or previous history of hypoglycaemia that were subsequently normoglycaemic on the diagnostic fast).
2. To investigate and compare the neurocognitive profiles of children with HH and KH (used as a control group), and to correlate the profile of deficits in the HH group to the underlying structural abnormalities.

The studies on children with KH (biochemical, neurocognitive and neuroimaging) were utilised to understand the neuroprotective role of KB during hypoglycaemia.

Methods: 30 children with KH and 74 children from the 'control' group underwent a diagnostic fast with measurements of plasma glucose, lactate, catecholamines and serum insulin, cortisol, growth hormone, non-esterified fatty acids (NEFA) and 3-

βhydroxybutyrate (ketone body) concentrations at the beginning, middle and end of fast.

The neurocognitive profile of 21 children with HH was compared to a group of 14 children with KH, using a combination of standardised tests to investigate IQ, memory, attention, academic attainment, movement, emotion and behaviour. The structural integrity of the brain was evaluated using conventional neuroradiological assessments, hippocampal volumetry and diffusion tensor imaging (DTI).

Results: Fasting studies have shown that children with KH demonstrate a significant increase (87% per hour) in the rate and magnitude of 3-βhydroxybutyrate concentrations relative to 'controls'. Cortisol concentrations are a significant predictor of KB concentrations at the end of the fast. The performance of KH children as a group was within the normal range for the neurocognitive measures. However four children in the KH group scored in the borderline (77-79) and low average range (81-84) for full scale IQ, and memory scores in one KH child was lower than predicted by 19 points. Neuroimaging revealed small hippocampi in one child, focal white matter lesions in two children and diffuse white matter lesions in two children with KH.

Children with HH underperformed significantly (relative to KH and standard population means) in the tests for intelligence (especially perceptual reasoning), memory, and sustained attention and manual dexterity. Memory impairments in children with HH did not correlate with hippocampal pathology.

However, analysis of DTI studies has revealed the presence of white matter microstructural deficits that correlate with IQ and perceptual reasoning indices in these (HH) children. The genu and splenium of the corpus callosum were highlighted as specific white matter regions vulnerable to injury in the HH group.

Conclusions: Children with hypoglycaemia are at risk of white matter injury. Children with HH manifest widespread cognitive deficits that are partly explained by the white matter microstructural deficits noted in the DTI studies. The increased rate and magnitude of KB response in KH group during fasting supports increased metabolic utilisation and a glucose sparing effect. However some chidren with KH in this study exhibit white matter injury and a wide variation in the neurocognitive scores is also noted, indicating the presence of neurocognitive impairment in certain children with KH.

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Abbreviations

ACOD - Acyl CoA Oxidase

ACS - Acyl CoA Synthase

ADHD - Attention Deficit Hyperactivity Disorder

ADP - Adenosine Diphosphate

AMP - Adenosine Mono Phosphate

ATP - Adenosine Triphosphate

BGT - Basal Ganglia Thalamus

BI - Brain Injury

BMI – Body Mass Index

BMI SDS - Body Mass Index Standard Deviation Scores

CA - Cerebellar Atrophy

CMS - Childrens Memory Scale

CoA - Coenzyme A

CSF - Cerebrospinal Fluid

CT - Computerized Tomography

DG-Deoxyglycose

DTI - Diffusion Tensor Imaging

DWI - Diffusion weighted imaging EAA -
Excitotoxic Amino Acid Injury FA - Fractional
Anisotropy

FLAIR - Fluid Attenuated Inversion Recovery
Sequence

FLASH - Fast Low Angle Shot

FSIQ - Full Scale IQ

G3PDH - Glycerol-3-Phosphate-Dehydrogenase

GABA - Gamma-Amino-Butyric-Acid

GH - Growth Hormone

GLUT - Glucose Transporters

GOD - Glucose Oxidase

GOSH - Great Ormong Street Hospital NHS Foundation Trust

GM – Grey Matter

HBI - Hypoglycaemic Brain Injury

HH - Hyperinsulinaemic Hypoglycaemia

HIE - Hypoxic Ischaemic Encephalopathy

HV-Hippocampal volume

IQ - Intelligence Quotient

KB - Ketone Bodies

KH - Ketotic Hypoglycaemia

LGA - Large for Gestational Age

MD - Mean Diffusivity

MEHA - 3-Methyl-N-Ethyl-N-Hydroxymethyl Alanine

MR - Magnetic Resonance

MRI - Magnetic resonance imaging

MTL - Medial temporal lobe

NAD - Nicotinamide Adenine Dinucleotide

NADPH - Nicotinamide Adenine Dinucleotide
Dehydrogenase

NEFA - Non-Esterfied Fatty Acids

NL - Norleucine

NMDA - N-Methyl-D-Aspartate-A

NPA - Nitrophenylalanine

OP - Occipito Parietal

PEDS-QL - Paediatric Quality of Life

PEPCK - Phosphoenolpyruvate
carboxykinase

PET - Positron Emission Tomography

PITC - Phenylisothiocyanate

PLIC - Posterior Limb of Internal Capsule

POD - Peroxidase

Ppi - Pyrophosphate

PR - Perceptual Reasoning

PR - Perceptual Reasoning

PS - Processing Speed

PVM - Periventricular White matter

PWM - Periventricular White Matter

RF - Radiofrequency

SCQ - Social Communication Questionnaire

SD - Standard Deviation

SDQ - Strength and difficulties questionnaire

SDS - Standard Deviation Score

SEM - Standard Error of Mean

SGA - Small for Gestational Age

SSA - Sulpho Salicylic Acid

TBSS - Tract Based Spatial Statistics

TEA-Ch - Tests of Everyday Attention

TLE - Temporal Lobe Epilepsy

VBM - Voxel Based Morphometry

VCI - Verbal Compehension Index

WM - White Matter

WM - Working Memory

WIAT – Wechsler Individual Acheivement Test

WISC – Wechsler Intelligence scale for children

1 Maintenance of glucose homeostasis

1.1 Introduction

A constant supply of glucose is critical for the organs that are obligate glucose users, such as the brain (Amiel et al., 1995). Blood glucose concentrations are maintained in a steady state despite wide variations in diet, physical activity, and periods of starvation (Rizza et al., 1980; Wahren et al., 1978; Consoli et al., 1987). This is achieved by a balance between glucose production and utilisation, mediated by a complex interaction between insulin and a counterregulatory neuro-hormonal system comprising of glucagon, catecholamines, cortisol and growth hormone (GH).

Insulin decreases glucose production and increases glucose utilisation, whereas glucagon, catecholamines, cortisol and GH increase glucose production and decrease glucose utilisation (Bolli et al., 1999). Any factor that disrupts this balance will lead to hypoglycaemia (Figure 1).
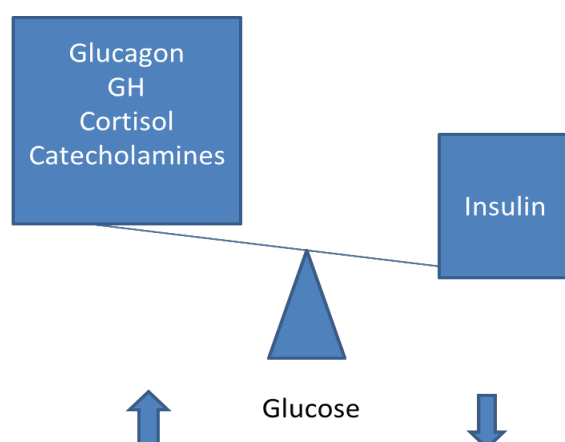
Figure 1 The effects of insulin, GH, cortisol, glucagon and catecholamines on maintenance of glucose homeostasis. Insulin lowers blood glucose concentrations while the actions of glucagon, GH, cortisol and catecholamines result in an elevation of blood glucose concentrations.

1.2 Glucose production

In the fed state, glucose is mainly derived from dietary carbohydrates.

In the fasted state blood glucose concentrations are maintained by mobilizing glycogen stores from the liver (glycogenolysis), or production of glucose from non-carbohydrate sources known as gluconeogenesis (liver and kidney) (Cersosimo et al., 2000)

Glycogenolysis is a process that involves breakdown of glycogen to produce glucose. Liver, kidneys and muscle store glycogen. During fasting glycogen is converted to glucose-1-phosphate and glucose-6-phosphate by glycogen phosphorylase and phosphoglucomutase respectively.

Glucose-6-phosphatase then converts glucose-6-phosphate to free glucose. Due to the high glycogen content and presence of glucose-6-phosphatase, liver is the main organ that contributes to glycogenolysis, in comparison to muscle and the kidney that have only small quantities of stored glycogen. In addition muscle lacks glucose-6-phosphatase required to release glucose into circulation.

When sufficient energy (ATP) is available, excess glucose is converted to glycogen by glycogen synthase and stored (glycogenesis). This balance between glycogenesis and glycogenolysis is regulated by insulin (favours glycogenesis) and glucagon (favours glycogenolysis). Insulin activates glycogen synthase (Larner et al., 1988) and inactivates the enzyme glycogen phosphorylase that causes glycogenolysis (Cohen et al., 1985)
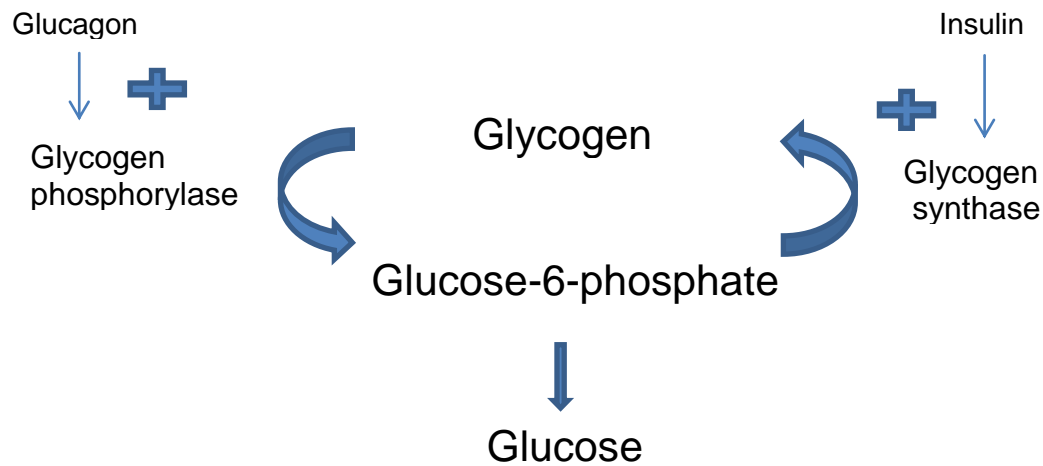
Figure 2 Glycogenesis and glycogenolysis. Insulin favours glycogenesis and glucagon favours glycogenolysis

Gluconeogenesis is the production of glucose from non-carbohydrate sources. The main substrates for gluconeogenesis are pyruvate, lactate, glycerol and glucogenic amino acids alanine and glutamine.

In gluconeogenesis the crucial steps are the conversion of pyruvate to oxaloacetate and to phosphoenol pyruvate and the conversion of fructose-1, 6-biphosphate to fructose-6-biphosphate (rate limiting step in gluconeogenesis). The ultimate step is conversion of glucose-6-phosphate to glucose.

In healthy adults gluconeogenesis contributes to 50% of glucose production after an overnight fast and nearly all glucose production after 42 hours (Landau et al., 1996). In comparison to adults, children have limited glycogen stores that sustain blood glucose concentrations for only 12 -16 hours, beyond that period gluconeogenesis becomes important (Chaussain et al., 1977; Jahoor et al., 1990).

Based on glucose production following glucagon injection after a 24 hour fast in children, Chaussain et al were able to show a wide variability in liver glycogen reserves and its mobilization during starvation (Chaussain et al., 1974). Following a 30 hour fast gluconeogenic substrates (alanine) and glucose concentrations were low in children in comparison to adults (Haymond et al., 1982).

The brain is the principal consumer of glucose (Amiel et al., 1995) and as children have greater brain to bodyweight ratio, they have higher glucose production rates in comparison to adults (Bier et al., 1977; Huidekoper et al., 2008). Accordingly endogenous glucose production has also been shown to decrease with age (Bier et al., 1977).

Renal contribution to glucose homeostasis

Traditionally the liver was assumed to be the key site of gluconeogenesis, with an insignificant contribution from the kidneys (Aber et al., 1966; Bjorkman et al., 1980; Castellino et al., 1990). It is now known that, following an overnight fast the kidney contributes to 20-25% of the glucose released into the circulation (Consoli et al., 1987; Moller et al., 2001; Ekberg et al., 1999).

The renal medulla is the site of glucose utilisation as it contains glycolytic enzymes, whereas glucose production is a function of the renal cortex that contains gluoneogeogenic enzymes (Guder & Ross et al., 1984). Renal gluconeogenesis is inhibited by insulin and stimulated by catecholamines, as epinephrine infusion rather than glucagon infusion during hypoglycaemia was associated with increased and sustained glucose release (Stumvoll et al., 1998; Stumvoll et al., 1995).

Stumvoll et al have demonstrated that in healthy adult humans, the renal contribution to systemic glucose release increases twofold in response to epinephrine infusion (Stumvoll et al., 1995).

In the postprandial state, hepatorenal glucose reciprocity exists whereby a reduction in the hepatic glucose production is compensated by a twofold increase in renal gluconeogenesis (Meyer et al., 2002). This hepatorenal glucose reciprocity is thought to enable replenishment of hepatic glycogen stores and also maintain glucose homeostasis in certain pathological and physiological conditions such as prolonged fasting, liver transplantation and acidosis (Gerich 2002).

In addition kidneys also contribute to glucose homeostasis by reabsorbing glucose from the proximal convoluted tubules through the sodium glucose transporters (SGLT's) (Wright et al., 2001).

1.3 Glucose Utilisation

Glucose is utilised by all tissues. Insulin contributes to glucose utilisation by promoting peripheral uptake of glucose, promoting glycogenesis and inhibiting lipolysis and ketogenesis. Increased blood glucose concentrations in the postprandial state are sensed by the β-cell, resulting in increased insulin secretion. Binding of insulin to its receptor in the peripheral tissues causes up regulation of glucose transporters (synthesis and mobilisation to cell surface) (Thorens et al., 1996) and enables tissue uptake of glucose.

Following a meal, the rate of gastric emptying also influences the circulating glucose concentrations. Mixed meals (carbohydrate combined with protein and fat), decrease gastric emptying and stimulate the production of incretins (hormones produced by the gut), thereby lowering postprandial glucose concentrations (Rayner et al., 2001) (Horowitz et al., 1993).

Glucose is transported into the cells by a process of carrier mediated facilitated diffusion. The carriers are a group of proteins known as glucose transporters (GLUT). Fourteen glucose transporters have been discovered in humans (Thorens et al., 2010), but the most well described are the glucose transporters GLUT 1-5 that are crucial for glucose utilisation and each of them have a specific tissue distribution and function.

GLUT1 and GLUT3 are distributed in all the tissues including the nervous system and glucose transport is insulin independent (Thorens et al., 1996). As they have a high affinity for glucose they transport glucose readily and are crucial in ensuring a constant glucose supply to brain.

GLUT 2 is an insulin independent transporter located in the liver, small intestine, kidney and pancreatic β-cells (Bell et al., 1991). They have a high capacity but low affinity for glucose and function as glucose sensors (Matschinsky et al., 1996). GLUT 4 is an insulin dependent glucose transporter present in the muscle, heart and adipose tissue (James et al., 1989), while GLUT 5 is a fructose transporter in the jejunal brush border (Thorens et al., 1996)

Another family of sodium dependent glucose transporters (SGLT) enable energy dependent glucose uptake. They are predominantly located in the luminal surface of the small intestine (SGLT-1) and the proximal renal tubule of the kidney (SGLT2) (Wright et al., 2001).

Following uptake glucose can undergo glycolysis to generate ATP and/or be stored as glycogen or fat. Glucose uptake by tissues also depends on the availability of plasma glucose, glycaemic state (fed or fasted), presence of alternate substrates, tissue requirements and sensitivity for insulin (Kelley et al., 1988).

Figure 3 Glucose production and utilisation. Adapted from Zijlmans et al., 2009 (Edited)

Role of gut hormones in glucose homeostasis

A multimodal mechanism of glucose regulation has emerged following the discovery of several hormones released by the gut and elucidation of their impact on blood glucose concentrations.

Incretin hormones: Glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP) are the principal incretin hormones produced by the enteric mucosa cells (L and K cells) following a meal. They stimulate insulin secretion and are responsible for the increase in plasma insulin response to an oral glucose load in comparison to an intravenous glucose load (incretin effect) (Perley et al., 1967). GLP-1 also delays gastric emptying, inhibits glucagon secretion, promotes satiety and aids weight loss (Baggio et al., 2007).

Ghrelin is secreted by the cells in the fundus of the human stomach and acts via growth hormone secretagogue receptor (GHSR) (Kojima et al., 1999). Ghrelin concentrations have been shown to increase prior to a meal and reduce following a meal (Ariyasu et al., 2001; Cummings et al., 2001). It is a potent stimulator of GH secretion (Takaya et al., 2000), increases secretion of ACTH, cortisol, epinephrine (Takaya et al., 2000; Nagaya et al., 2001), and increases appetite via action on the hypothalamus (Kamegai et al., 2001). Human studies have also shown a direct effect of ghrelin in inducing glycogenolysis following intravenous infusion of ghrelin (Broglio et al., 2001).

Amylin is a neuroendocrine hormone, co-secreted with insulin by the pancreatic β-cell. It enhances the actions of insulin by suppressing post-prandial glucagon secretion (Gedulin et al, 1997). Amylin also slows gastric emptying (Samsom et al., 2000) and has been shown in animal models to reduce food intake and body weight (Bhavsar et al., 1998; Rushing et al., 2001; Rushing et al., 2000).

2   Metabolic adaptation to birth, fasting and ketone body production

2.1 Introduction

Newborns are vulnerable to hypoglycaemia due to the massive metabolic adaptation required to transition from a metabolically dependent fetus to an independent neonate. Maturity of the enzymes involved in glucose homeostasis, appropriate hormonal changes, adequate storage reserves, presence of alternate fuels are all important for a smooth transition to postnatal life (Ward et al., 2005).

Similarly children have a limited capacity to fast in comparison to adults and any maladaptation to fasting renders them susceptible to hypoglycaemia in times of metabolic stress such as illness or prolonged fasting (Zijlmans et al., 2009).

2.2 Fetal glucose metabolism

In utero the fetus is exposed to a continuous glucose supply via a carrier mediated facilitated diffusion across the placenta (Hay et al., 2006). The fetal glucose concentrations are a direct reflection of maternal glucose concentrations (Mitanchez et al., 2007). Under physiological conditions the fetus produces only minimal amounts of glucose and fetal glucose homeostasis is characterised by high plasma insulin concentrations (which stimulates anabolism and glycogen deposition) and low glucagon concentrations.

However, during prolonged reduced glucose supply, animal studies have shown the ability of the fetus to maintain glucose homeostasis by initiating glycogenolysis and gluconeogenesis at the expense of growth (Van Veen et al., 1987; Carver et al., 1997). The fetal brain adapts to chronic hypoglycaemia by modulating glucose transporters (increasing GLUT1 and decreasing GLUT3), to promote glucose uptake by the brain (Das et al., 1999).

2.3 Metabolic adaptation to birth

Umbilical venous glucose concentration at birth (4.6 mmol/l range 3.7-5.5 mmol/l) is nearly 70% of maternal glucose concentration (Creery et al., 1953). The postnatal period is characterised by a fall in the blood glucose concentrations (1.3 -1.5 mmol/l) during the first 2 hours of life, that stabilises to 3-4 mmol/l by 3-72 hours of life (Tanzer et al., 1997; Hawdon et al., 1992; Srinivasan et al., 1986). The fall in blood glucose concentrations are well tolerated without any symptoms or long term dysfunction probably due to the presence of alternative fuels for the brain such as ketone bodies and lactate (Hawdon et al., 1992) and a compensatory increase in cerebral blood flow (Anwar & Vannucci et al., 1988).

Following birth there is a prominent surge in catecholamine and glucagon concentrations (Girard et al., 1990). The catecholamine surge is secondary to increased fetal catecholamine secretion rather than maternal placental transfer (Padbury et al., 1982).

The surge in glucagon concentrations occur within minutes to hours of birth (Sperling et al., 1984). The rise in glucagon is coupled with the upregulation of glucagon receptors, fall in insulin concentration and downregulation of the insulin receptors. The insulin response to glucose also remains blunted for several days. The resultant decreased insulin: glucagon ratio decreases activity of glycogen synthase and stimulates glycogen phosphorylase thus inhibiting glycogen synthesis and promoting glycogenolysis (Sperling et al., 1984; Ktorza et al., 1985).

Glycogenolysis is the initial primary pathway of glucose production which is exhausted within a few hours; later contribution by gluconeogenesis is derived from lactate, pyruvate and alanine. The capacity for gluconeogeneis is not fully developed at birth. The rate limiting enzyme in gluconeogenesis, phosphoenolpyruvate carboxykinase (PEPCK) is activated by the reduced insulin: glucagon ratio and the infants are capable of gluconeogenesis by 4-6 hours of age, although actual maturation of the enzyme activity to adult levels can take up to 2 weeks (Girard et al., 1990; Girard et al., 1986). Lipolysis and fatty acid oxidation are induced by the elevated concentrations of catecholamines, thyroid stimulating hormone (Marcus et al., 1988) and cortisol and decreased insulin concentrations.

Alternate fuels such as ketone bodies and lactate are extremely valuable in the newborn period as they spare glucose for cerebral utilisation. Although hepatic ketogenesis is limited at birth, there is a significant increase during the first 24 hours following birth. High ketone body concentrations comparable to adult concentrations after several days of fasting are seen from 12 hours of life and continue to be high during the first 3 days of life (Hawdon et al., 1992; Bougneres et al., 1986). Enteral feeding also promotes ketogenesis by providing substrates other than glucose to the liver and the carnitine content of milk facilitates hepatic uptake and metabolism of fatty acids (Hawdon et al., 1992).

Glucose transporters (GLUT) also play a key role in postnatal adaptation. During the immediate postnatal period there is a predominance of GLUT1 in all tissues. As GLUT1 is a high affinity glucose transporter it increases tissue availability of glucose (Mena et al., 2001; Nualart et al., 1999). GLUT 1 gradually decreases after birth, and is replaced by specific isoforms in each tissue (Postic et al., 1994).

2.4 Metabolic adaptation to fasting

In the fasted state glucose homeostasis is maintained by a combination of glycogenolysis, gluconeogenesis and lipolysis (Figure 3). The interplay between insulin and counterregulatory hormones cortisol, glucagon, epinephrine and GH is crucial for these metabolic pathways in the fed and fasted state.
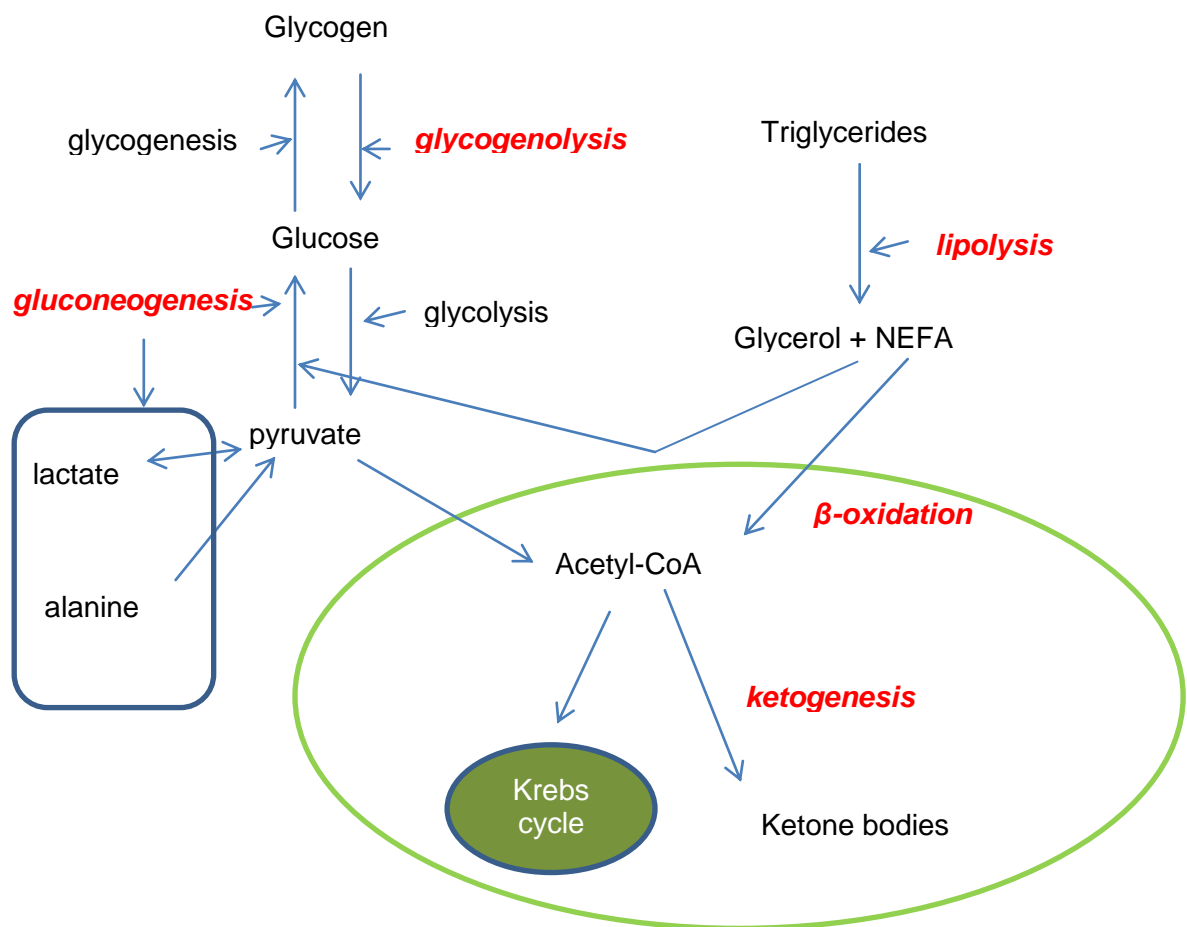
Figure 3 Integration of carbohydrate, lipid and protein metabolism during fasting. The pathways active during fasting are highlighted in red. During the initial period of fasting, glycogenolysis maintains blood glucose concentrations, as fasting progresses gluconeogenesis and lipolysis play a greater role. Non- esterified fatty acids (NEFA) and ketone bodies generated following lipolysis act as alternate fuels for liver, skeletal and cardiac muscle and the brain (ketones only)

Glucagon facilitates glycogenolysis while insulin suppresses endogenous glucose production, enhances peripheral uptake of glucose, promotes glycogenesis and inhibits lipolysis and proteolysis. During the initial period of fasting, the fall in insulin concentrations and increase in glucagon concentrations promote glycogenolysis Catecholamines reduce peripheral glucose uptake, induce glycogenolysis, gluconeogenesis and lipolysis, while growth hormone and cortisol play a permissive role and regulate peripheral tissue sensitivity to insulin and glucagon

The gluconeogenic amino acids are converted to pyruvate and enter the citric acid cycle. Hormone sensitive lipase, activated by glucagon, cortisol and epinephrine is an important step in mobilisation of fatty acids (Murray et al., 1996). Lipolysis also provides glycerol that can be utilised in gluconeogenesis (Jensen et al., 1987). Glycerol is transported to the liver and converted to glucose via glycerol-3- phosphate (Herrera et al., 2000). In the liver, β-oxidation of fatty acids results in production of ketone bodies that are released in to the circulation for peripheral uptake of sensitive tissues.

2.5 Ketone body production

Glucose is the major fuel for brain function but it can adapt to using ketone bodies, namely acetoacetate and 3-βhydroxybutyrate during starvation (Owen et al., 1967; Patel et al., 1975). Thus the presence of ketone bodies during hypoglycaemia has a cerebroprotective effect. Ketone bodies are always present in the blood but increase as a normal physiological response during exercise, starvation, in the neonatal period, pregnancy and high fat diet (Nordli, Jr. et al., 2001; Bonnefont et al., 1990; Huttenlocher et al., 1976).

Ketogenesis begins with adipocyte lipolysis (breakdown of triglycerides into free fatty acids and glycerol) by hormone sensitive lipase (HSL). HSL induced lipolysis comes into play during fasting, stress and exercise. Under basal conditions non hormone dependent lipase ensures lipolysis. The free fatty acids travel to liver bound to albumin, to undergo β-oxidation to acetyl-CoA followed by synthesis of ketone bodies (Laffel et al., 1995).
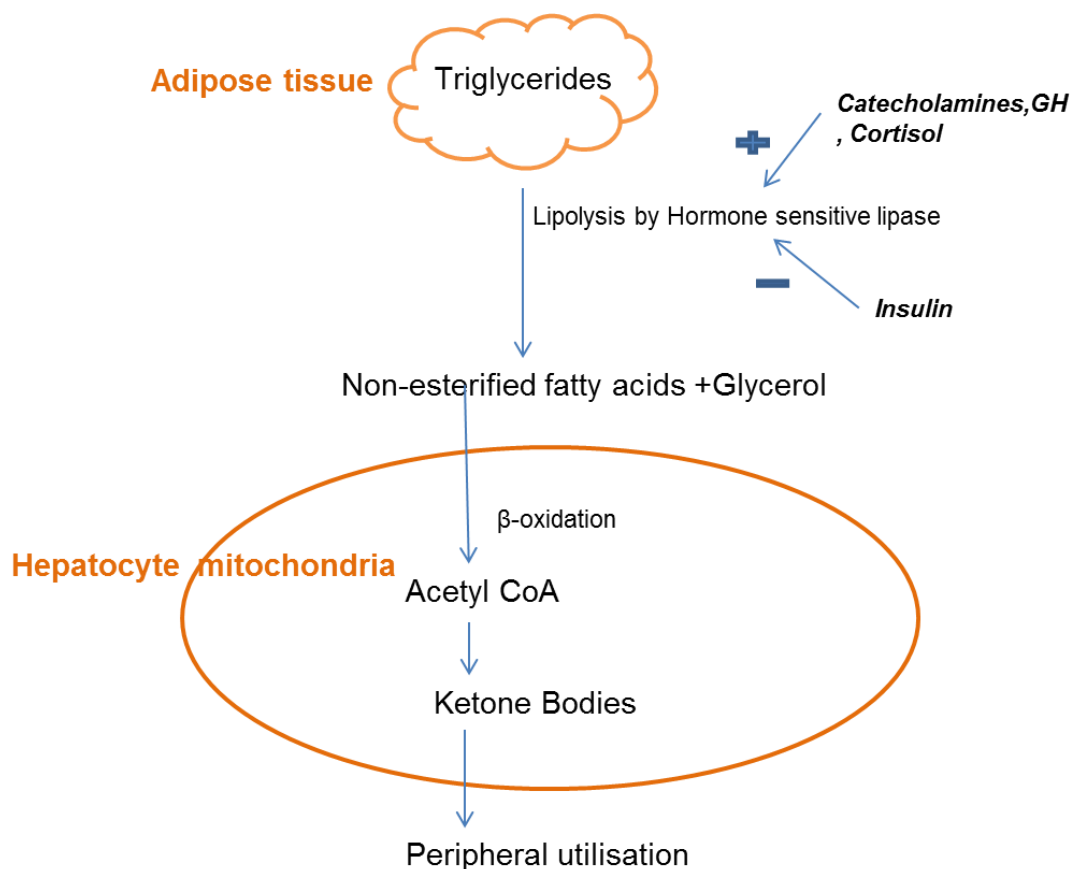
Figure 4 Ketone body production. Adipocyte lipolysis by hormone sensitive lipase (HSL), releases non-esterified fatty acids (NEFA). β-oxidation of fatty acids in hepatic mitochondria leads to generation of ketone bodies. Insulin inhibits HSL, while catecholamines, growth hormone (GH) and cortisol activate HSL

Thus ketone bodies are derived from β-oxidation of fatty acids or from ketogenic amino acids such as leucine, lysine, and tryptophan in the liver (Mitchell et al., 1995). The key substrates for ketogenesis are medium and long chain fatty acids and to a minor extent ketogenic amino acids. β-oxidation of fatty acids in hepatic mitochondria result in production of 2 carbon unit acetyl-CoA. This is then converted into acetoacetate by covalent linkage of the two acetyl groups facilitated by the enzymes thiolase, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase and HMG-CoA lyase. Acetoacetate can be further reduced to 3- βhydroxybutyrate by βhydroxybutyrate dehydrogenase (Figure 6).

Acetoacetate and 3-βhydroxybutyrate are the principal ketone bodies. Acetone is another ketone body that is produced in small quantities by spontaneous slow decarboxylation of acetoacetate. While acetoacetate and βhydroxybutyrate enter peripheral circulation to be utilised by sensitive tissues as energy source (Fukao et al., 2004), acetone is exhaled.

Acetyl-CoA + Acetyl-CoA

↓ **β-ketothiolase**

Acetoacetyl-CoA

↓ **HMG-CoA**

HMG-CoA

↓ **HMG-CoA lyase**

Acetoacetate

βhydroxybutyrate dehydrogenase

Acetone   β-hydroxybutyrate
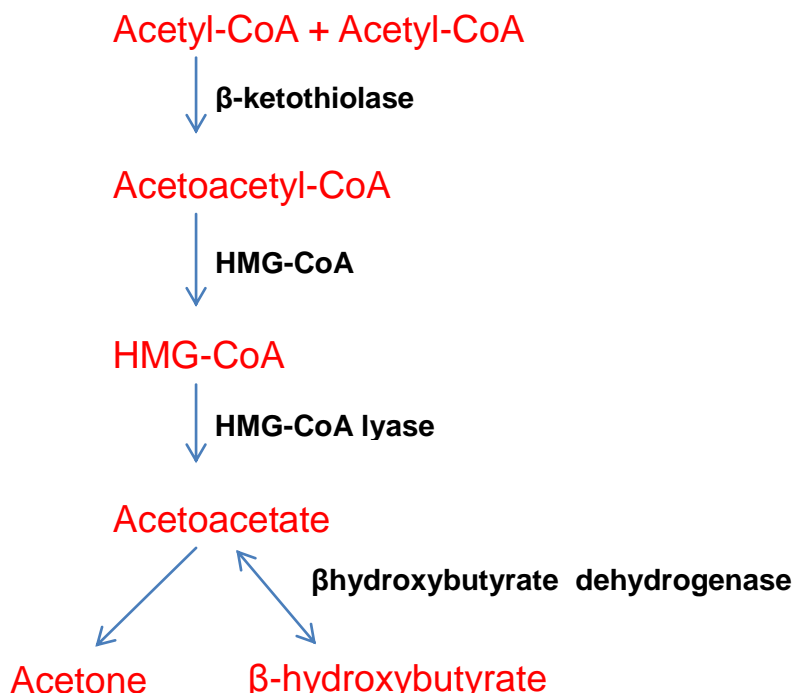
Figure 5 Ketogenesis. Acetyl-CoA is converted into acetoacetate by covalent linkage of the two acetyl groups facilitated by the enzymes thiolase, 3-hydroxy-3-methylglutaryl- CoA (HMG-CoA) synthase and HMG-CoA lyase. Acetoacetate can be further reduced to β-hydroxybutyrate by β-hydroxybutyrate dehydrogenase or decarboxylated to acetone.

Regulation of ketogenesis

The key steps in the the regulation of ketogenesis (Laffel et al., 1999) are:

1. Provision of substrate (FFA) for ketogenesis by lipolysis of triglycerides.

Slavin et al (Slavin et al., 1990) using rat adipocytes were able to show that epinephrine, dexamethasone, GH and glucagon increase HSL activity and promote ketogenesis. They do so by phosphorylating HSL via cyclic AMP dependent protein kinase pathway (Yeaman et al., 1990). Insulin supresses HSL activity by HSL dephosphorylation (Shakur et al., 2001).

In adult humans only catecholamines have been shown to cause lipolysis, mediated via β adrenoreceptors (Bjorntorp et al., 1977). In-vitro studies investigating the effects of various hormones on human adipocyte cells, has shown thyroid stimulating hormone to be the main mediator of lipolysis in the neonatal period. A gradual increase in the contribution of catecholamines to lipolysis was seen with increasing age in older children (Marcus et al., 1988).

2. The next crucial step is the concentration of malonyl CoA within hepatocytes. Low concentrations of malonyl CoA promotes FFA entry into hepatic mitochondria, by inducing carnitine palmitoyl 1 transferase enzyme (CPT I). CPT1 is an enzyme that is necessary for transport of long-chain fatty acids into mitochondria for oxidation.

Malonyl CoA is formed from acetyl CoA by the action of acetyl CoA carboxylase (ACc). Insulin activates (dephosphorylates) ACc, thus inhibiting FFA entry into mitochondria and glucagon deactivates ACc and promotes FFA entry into mitochondria.

Harano et al (Harano et al., 1982) were able to show that mitochondria isolated from adult rat hepatocytes, showed an increase in ketogenesis (50-55%), and an increase in Carnitine palmitoyl I (CPT I) activity by 25-50% under the influence of glucagon, while insulin suppressed CPT I activity by 34%

.3. The final (rate limiting) step in ketogenesis is the action of HMG CoA synthase (mHS) that converts Acetoacetyl CoA to HMG CoA. Starvation, low insulin and ketogenic diet promote activity of HMG CoA synthase.

Studies using tracer techniques to investigate adult human KB production and utilisation in vivo, has confirmed that insulin, epinephrine and thyroid hormone are the main mediators of ketone body homeostasis. These hormones not only affect ketogenesis by their effect on lipolysis but also influence intrahepatic ketogenesis (Keller et al., 1989)

Adipose tissue   **Blood**   **Liver**

Lipolysis by Hormone sensitive lipase

Non-esterified fatty acids

Low malonyl CoA induces CPT-1

Acyl CoA

β-oxidation

Acetyl CoA

Ketogenesis by HMG CoA synthase

Ketone bodies
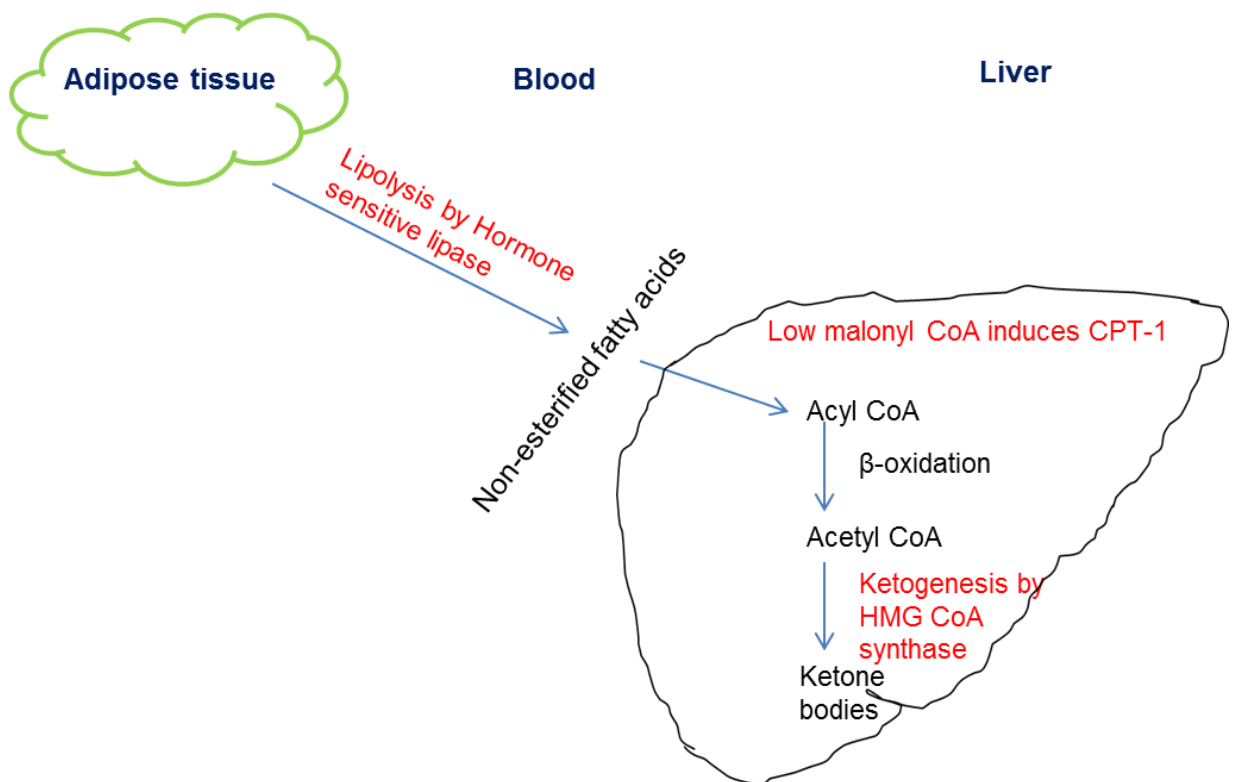
Figure 6 Regulation of ketogenesis. Ketogenesis is regulated at 3 levels (highlighted in red). Lipolysis by hormone sensitive lipase ensures availability of NEFA to the liver. The concentration of malonyl CoA in the hepatocytes influences transport of fatty acids into the hepatic mitochondria. The rate limiting enzyme in ketogenesis HMG CoA synthase is the final step of regulation.

3 Hypoglycaemia

3.1 Definition of Hypoglycaemia

There have been several approaches (based on clinical symptoms, epidemiological studies, threshold of counterregulatory response, neurophysiological impairment and neurodevelopmental outcome) to the definition of significant hypoglycaemia, but none satisfactory (Cornblath et al., 2000).

The variability in perception of hypoglycaemia was highlighted by a survey of 178 paediatricians and 36 textbooks, where definition of hypoglycaemia varied from <1 to < 4 mmol/l (Koh et al.,1988). There are several reasons contributing to the difficulty in defining significant hypoglycaemia which include the significance of symptomatic and asymptomatic hypoglycaemia, level of glycaemia associated with neurological impairment and the difficulty in quantifying duration of hypoglycaemia (Aynseley-green et al., 1996).

Although the definition of hypoglycaemia remains controversial, defining significant hypoglycaemia in relation to brain function has been felt to be the most appropriate approach (Aynsley-Green et al., 1991). In accordance with that, Koh et al (Koh et al., 1988) studied the effect of hypoglycaemia on neural function in 17 children (age 1 day - 16 years). Abnormal brainstem evoked potentials (prolonged latencies and abnormal waveforms) were observed in 10/17 subjects when blood glucose concentration was below 2.6 mmol/l. In one child with blood glucose concentration of 1.9 mmol/l evoked potential was normal, that led to speculation about the protective effect of alternate cerebral fuels such as lactate and ketone bodies.

In the absence of a clear definition of significant hypoglycaemia, operational thresholds for intervention have been recommended (Cornblath et al., 2000) that merely provide guidance. In symptomatic infants intervention is recommended if blood glucose is < 2.5mmol/l, in asymptomatic infants intervention is suggested at a blood glucose concentration < 2.0 mmol/l, and intravenous glucose infusions at glucose concentration between 1.1-1.4 mmol/l.

However, management at these suggested operational thresholds does not necessarily imply normal neurological outcome as the blood glucose concentration associated with brain injury is not known. A recent consensus workshop concluded that the evidence was still lacking to specify a range of glucose concentration that would define significant hypoglycaemia (Hay et al., 2009)

3.2 Causes of Hypoglycaemia

Hypoglycaemia is a biochemical diagnosis and can occur in a wide variety of conditions such as prematurity, intrauterine growth retardation, asphyxia, hypothermia, sepsis, infant of diabetic mother, large for gestational age, polycythaemia, erythroblastosis foetalis, endocrine disorders (hyperinsulinism, hypopituitarism, cortisol deficiency, growth hormone deficiency), inborn errors of metabolism (defects in gluconeogenesis, defects in carnitine metabolism, defects in fatty acid oxidation), insulin producing tumours and insulin therapy for diabetes and ketotic hypoglycaemia in older children.

In reference to the patient group recruited, only two causes of hypoglycaemia are discussed in detail below, namely hyperinsulinaemic hypoglycaemia (HH) and ketotic hypoglycaemia (KH). Children with HH have hypoglycaemia with complete lack of alternative cerebral fuels, whereas children with KH have hypoglycaemia with marked increase in alternative substrates such as ketone bodies (ketotic hypoglycaemia). This unique patient cohort is therefore ideal for undertaking a study that will allow the assessment of the neuroprotective effect of ketone bodies in hypoglycaemic brain injury.

3.2.1 Hyperinsulinaemic Hypoglycaemia (HH)

HH is a major cause of persistent and recurrent hypoglycaemia in the newborn period and in infancy due to dysregulated secretion of insulin (Aynseley-Green et al., 2000).

Biochemically (Table 1) HH is characterised by inappropriately elevated insulin and C-peptide concentrations with low serum fatty acids and ketone bodies during the episode of hypoglycaemia. Importantly even a 'normal' (i.e detectable) insulin concentration in the presence of hypoglycaemia is inappropriate (Aynsley-Green et al., 2000). The presence of excess insulin results in an increased glucose requirement of more than 8 mg/kg/min (normal 4-6 mg/kg/min) to maintain normoglycaemia.

Table 1 Diagnostic criterion for hyperinsulinemic hypoglycaemia

Glucose infusion rate >8mg/kg/min

Blood glucose <3mmol/l

Detectable insulin and/or C-peptide

Undetectable or low ketone bodies

Undetectable or low fatty acids

Clinically HH may be transient or persistent. There are several causes of HH, it may be congenital (CHI - described in the next section), and it may coexist in high risk groups (like small for gestational age, hypoxic ischemic encephalopathy and maternal diabetes mellitus) or may occur associated with syndromes such as Beckwith - Wiedman, Kabuki, Costello, Usher and Trisomy 13. HH has also been reported in metabolic conditions such as congenital disorders of glycosylation type 1a/b/d and Tyrosinemia type 1 and also in association with dumping syndrome.

HH is a heterogeneous condition in terms of the age of onset, clinical presentation, duration, severity, molecular biology, histology and response to medical treatment (Hussain et al., 2007).

The Incidence is 1 case per 40-50,000 in sporadic forms and as high as 1 per 2500 in familial forms (Glaser B et al. 2000). Early recognition and management is crucial to prevent brain injury (Menni et al., 2001; Meissner et al., 2003).

In addition the glucagon and cortisol counter regulatory response has been shown to be blunted in these children, further exacerbating the hypoglycaemia. Due to the high risk of brain injury, it has been recommended that blood glucose concentrations less than 3.5 mmol/l should be considered as hypoglycaemia (Hussain et al., 2007).

Mechanism of insulin secretion

Glucose is the key stimulus for insulin secretion. Within the pancreatic β-cell, glucose is converted to glucose-6-phosphate by the enzyme glucokinase that acts as a glucose sensor and couples the metabolism of glucose to insulin secretion (Figure 7). The ensuing glycolysis increases intracellular ATP/ADP ratio, that triggers closure of the adenosine triphosphate - sensitive potassium channels (K_{ATP}) spanning the β- cell membrane. This results in membrane depolarization and influx of Ca^{2+} via the voltage gated Ca^{2+} channels. This influx of Ca^{2+} results in insulin exocytosis. There are other K_{ATP} independent mechanisms of glucose stimulated insulin secretion (Gembal et al., 1992).
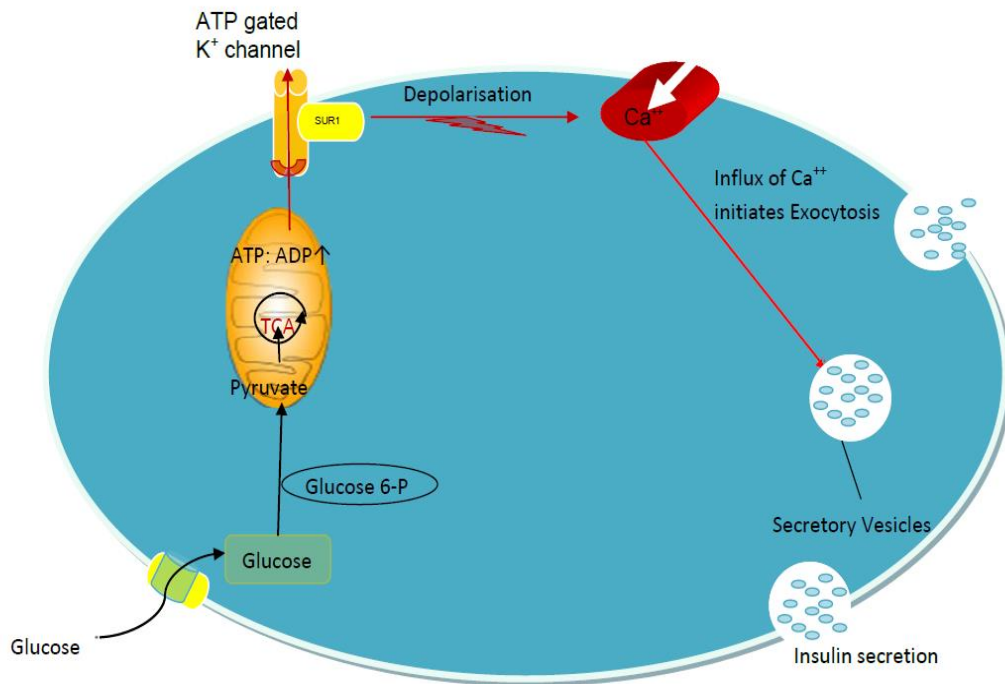
Figure 7 Illustration of mechanism of insulin secretion in β-cell of the pancreas. The β-cell adenosine triphosphate sensitive potassium channels (K_{ATP}) channel consists of 2 essential protein subunits; Kir6.2, which is the pore-forming unit and belongs to the inwardly rectifying potassium channel family, and sulfonylurea receptor 1 (SUR1), which belongs to the ATP-binding cassette (ABC) transporter family. The channel is an octameric complex of four Kir6.2 and four SUR1 subunits. Glycolysis increases intracellular ATP/ADP ratio, which triggers closure of the K_{ATP} channels. This results in membrane depolarization and influx of Ca^{2+} via the voltage gated Ca^{2+} channels triggering insulin exocytosis. Figure reproduced with permission from Mohamed et al., 2012.

Molecular mechanisms of HH

Mutations in seven different genes (*ABCC8, KCNJ11, GLUD1, GCK, HADH, SLC16A1* and *HNF4A*) have been implicated in the etiology of congenital hyperinsulinism (CHI)

ABCC8 and *KCNJ11* encode for the ATP-sensitive potassium (K_{ATP}) channel subunits SUR1 and Kir6.2 respectively in the pancreatic β-cells. Inactivating mutations (dominant and recessive) in these two genes are responsible for severe forms of CHI.

Hyperinsulinism hyperammonaemia HI/HA is the second commonest cause of CHI. These children present with postprandial HH secondary to a protein meal (Stanley et al., 1988). Activating mutations in *GLUD1* gene that encodes intramitochondrial glutamate dehydrogenase (GDH) is the underlying molecular mechanism. GDH causes oxidative deamination of glutamate to α-ketoglutarate and ammonia (Li et al., 2004). The α-ketoglutarate via the citric acid cycle generates ATP that triggers closure of K_{ATP} channels, causing inappropriate insulin secretion.

Activating mutations of the glucokinase (GCK) gene that encodes glucokinase enzyme can also cause CHI. Glucokinase via glycolysis generates ATP that triggers closure of K_{ATP} channels, causing insulin secretion (Glaser et al., 1998).

Loss of function mutations in Hydroxyacl-CoA dehydrogenase (HADH) gene is associated with CHI (Clayton et al., 2001). The exact molecular mechanism of HADH CHI is not known. Mutations in HNF4A gene (encoding transcription factor hepatocyte nuclear 4 α, HNF-4α) is responsible for CHI associated with macrosomia (Pearson et al., 2007). HNF-4α is known to regulate several genes that influence glucose stimulated insulin secretion (Odom et al., 2004)

Dominant mutations in the promoter region of SLC16A1 gene are associated with exercise-induced HH (Meissner et al., 2005). SLC16A gene encodes a monocaboxylate transporter (MCT 1) that enables movement of pyruvate into the mitochondria to enter the tricarboxylic acid cycle and generate energy. Dominant mutations result in overexpression of MCT 1 in β cells (Otonkoski et al., 2003).

Histopathological forms of HH

Histologically they may present as diffuse (increased nuclear size in all β cells) or focal (focal adenomatous hyperplasia confined to a single region) forms depending on the extent of pancreatic involvement. Management is medical (if responsive to diazoxide or octreotide) or surgical (subtotal pancreatectomy) in medically unresponsive children. The aim of the treatment is to prevent hypoglycaemia, while optimizing definitive management (either diet and medications or surgery).

With the advent of 18F-DOPA-PET/CT scans to differentiate focal and diffuse lesions and rapid genetic sequencing and increased knowledge regarding genotype phenotype correlations, clinical management of children with HH has been revolutionized leading to improved care in these complex patients.

3.2.2 Ketotic hypoglycaemia (KH)

KH is the most common form of hypoglycaemia beyond infancy (Pershad et al., 1998). The pathophysiology remains unknown (Pagliara et al., 1972; Haymond et al., 1974) and children tend to manifest between 6 months to 7 years of age.

These children are otherwise healthy but present with recurrent episodes of hypoglycaemia and ketonuria during an intercurrent illness or fasting. These children are believed to represent one end of the normal spectrum of age related adaptation to fasting (Stanley et al., 2006)

Diagnosis is confirmed when hypoglycaemia occurs in association with ketonemia/ ketonuria but without any evidence of metabolic or endocrine abnormalities, either during a spontaneous episode of hypoglycaemia or during a fasting tolerance test. Plasma ketone bodies in these children are significantly higher during hypoglycaemia when compared to controls (Bodamer et al., 2006).

Prompt administration of oral or intravenous glucose reverses the hypoglycaemia. Seizures have been reported, but long term neurological impairment is rare (Colle et al., 1964; Kogut et al., 1969), although follow up neurocognitive studies are lacking. Ketotic hypoglycaemia improves with age and disappears before adolescence (Stanley et al., 2006). Management involves parental reassurance, avoidance of fasting, snack of complex carbohydrates before bedtime for slow release of sugar and glucose supplementation during illness.

An assessment of glucose kinetics in fasting children with KH has shown low alanine concentrations (precursor for gluconeogenesis) and reduced endogenous glucose production indicating an imbalance between glucose production and uptake as the underlying mechanism of hypoglycaemia (Huidekoper et al., 2008).

With the advent of better diagnostic modalities, children previously labelled as ketotic hypoglycaemia have been subsequently diagnosed with hepatic glycogen synthase deficiency, acetoacetyl CoA thiolase deficiency and congenital disorders of glycosylation (Stanley et al., 2006). Hence it is important to be vigilant of any atypical features that may suggest an alternate diagnosis and impact on neurodevelopmental outcome.

3.3 Counterregulation of hypoglycaemia

The brain depends predominantly on glucose for its metabolic needs and mechanisms are in place to detect hypoglycaemia and initiate counterregulatory measures to restore normoglycaemia.

Glucose counterregulation results from an interaction of sensory input with complex neural networks resulting in behavioural and neuroendocrine and autonomic responses, the mechanisms of which are yet to be completely elucidated (Watts & Donovan et al., 2010)

The primary glucose sensors are hepatic portal/mesenteric vein (peripheral) and glucose sensitive neurons within parts of the hindbrain and hypothalamus (venteromedial hypothalamus) (McCrimmon et al., 2012). The glucose sensitive neurons integrate the sensory input with other neural processes and trigger the counterregulatory response.

Counterregulation involves an immediate reactive response (decreased plasma insulin and increased plasma glucagon and catecholamines) as well as a late adaptive response (increased cortisol and GH) that ensures adaptation of metabolic processes over longer period of time (Watts & Donovan et al., 2010).

Recent studies have highlighted the importance of central K_{ATP} channels on glucose regulation. K_{ATP} channels in the β- cell play a crucial role in insulin secretion by coupling metabolic signals to electrical changes in resting membrane potential. K_{ATP} channels have been demonstrated in different regions of the animal brain especially in the hypothalamus (Ashford et al., 1988). Evans et al demonstrated impaired counterregulatory response to hypoglycaemia on closure of these channels (Evans et al., 2004). In children with congenital hyperinsulinism this is important as they can have mutations of K_{ATP} channels.

The reduction of Insulin secretion is the first step in the cascade of the counterregulatory response to hypoglycaemia, that occurs within the physiological range of glucose concentrations (4.6+/- 0.2mmol/l) (Schwartz et al., 1987; Mitrakou et al., 1991). Insulin suppression allows secretion of the other counterregulatory hormones at glucose concentrations of 3.8mmol/l prior to onset of adrenergic symptoms of hypoglycaemia at 3mmol/l. If hypoglycaemia continues neuroglycopenia (cognitive dysfunction) occurs by 2.7mmol/l, followed by convulsions and coma at 1.5mmol/l.

Unlike glucagon and epinephrine, the effects of growth hormone and cortisol manifest only several hours after hypoglycaemia is corrected (Defeo et al., 1989). Growth Hormone and cortisol also promote lipolysis during hypoglycaemia (Defeo et al., 1989), thereby increasing the availability of gluconeogenic precursors and contributing to ketone body synthesis , that act as an alternative fuel for the brain during hypoglycaemia.
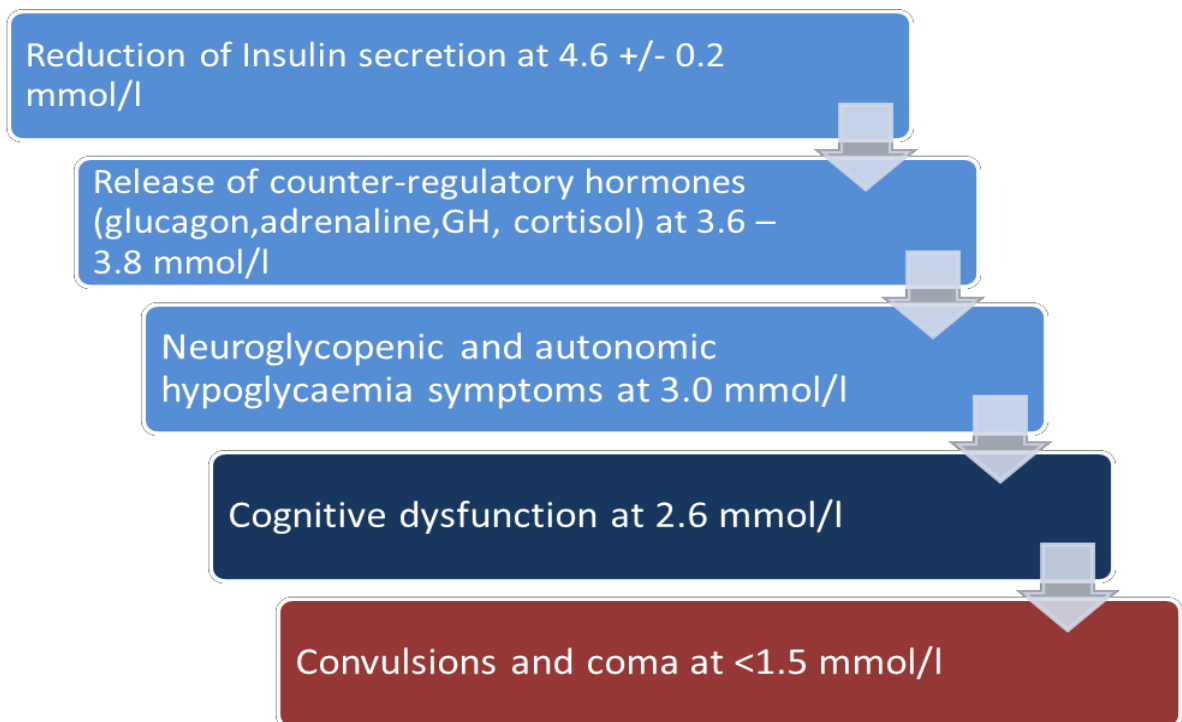
Figure 8 Cascade of the counterregulatory response to hypoglycaemia. This figure shows the hierarchy of events when arterialised venous blood glucose concentrations decrease in adults.

4 Brain Energy Metabolism

The brain develops rapidly during the first few years of life, attaining 90% of adult size by 5 years of age, however further modifications of gray and white matter and development of neural circuits for information processing continue till onset of adulthood (Tau & Peterson., 2010). Any problems in brain metabolism during this critical period of rapid growth can result in brain injury.

4.1 Cerebral blood flow and metabolism

The brain constitutes only 2% of body weight but consumes 20% of oxygen inspired and 15% of cardiac output and 25% of total body glucose utilisation (Sokoloff et al., 1989). This reflects the complex processes of the brain that need to be sustained such as neuronal action potentials, protein synthesis, axoplasmic flow, neurotransmitter release, and maintenance of ionic homeostasis.

Glucose is utilised for purposes beyond oxidation for brain metabolism, the glucose carbon is incorporated in lipids, proteins and glycogen and is a precursor for excitatory neurotransmitters like glutamate and aspartate and inhibitory neurotransmitters like gamma-amino butyric acid (GABA) (Sokoloff et al., 1989)

Sokoloff et al (Sokoloff et al., 1977) using 2- (14C) - deoxyglucose (2DG) enabled regional measurement of cerebral glucose utilisation by the brain in humans while previously only measurement of global brain glucose utilisation was possible. Incorporating this method, Chugani et al (Chugani et al., 1987, 1986, 1994) used PET (positron emission tomography) with 18 fluoro-2-deoxyglucose in humans and described cerebral glucose utilisation from birth to adulthood.

These studies suggest that until 5 weeks of age, the sensorimotor cortex, thalamus, hippocampus, and brainstem show high utilisation of glucose. This was followed by marked increase in glucose utilisation in parietal, temporal and occipital cortices, basal ganglia and cerebellum by 3 months of age. By 8 months of age increased utilisation was seen in frontal cortex and association regions. This pattern of utilisation remained relatively stable with little change till 18 months of age. This evolution in the pattern of cerebral glucose utilisation seem to be linked to the pattern of functional and behavioural maturation of the brain (Chugani et al., 1989)

The overall cerebral utilisation rate of glucose is low at birth, increases rapidly until 4 years of age (twice the adult concentrations), followed by a plateau until 11 years of age and a gradual decline thereafter (Chugani et al., 1994). Studies in newborn dogs have shown increased cerebral blood flow (with regional variations) during hypoglycaemia, although regional utilisation of glucose remained unaltered except in occipital white matter (Mujsce et al., 1989).

4.2 Glucose uptake and utilisation

Glucose is taken up by the brain by an insulin independent carrier mediated facilitated diffusion, via glucose transporters (GLUT) (Lund et al., 1979; Partridge et al., 1983). These homologous carrier molecules transport substrates down a concentration gradient. GLUT1 and GLUT3 have been established as the principal glucose transporters in the brain (Thorens et al., 1998).

GLUT 1 is localised mainly within the brain endothelial cells and GLUT 3 in the neurons (Maher et al., 1994; Vannucci et al., 1997). The pattern of distribution and expression of these glucose transporters seems to be closely related to cerebral glucose utilisation.

In the rat and rabbit brain, GLUT1 and 3 expressions are low at birth in accordance with low rate of cerebral glucose utilisation, and then rapidly increase in the 2nd and 3rd postnatal week reflecting the increased cerebral glucose uptake crucial for the rapid synaptogeneis occurring during this period (Vannucci et al., 2000).

The crucial role of glucose transporters is demonstrated by GLUT1 deficiency (De V et al., 1991), a condition that is characterized by low cerebrospinal glucose concentrations despite normal blood glucose concentrations and commonly associated with significant neurodevelopmental impairment, ataxia and seizures.

On reaching the blood brain barrier glucose is transported into the brain interstitium via GLUT1. In the physiological state, glucose from the brain interstitium is taken up by the astrocytes and converted to lactate producing 2 mol of ATP. Lactate is then transported into the neurons and converted to pyruvate. Pyruvate enters the citric acid cycle followed by oxidative phosphorylation resulting in 34 mol of ATP which is crucial to sustain synaptic activity (Magistretti et al., 1999). Thus astrocytes play a key role in linking synaptic activity to brain energy metabolism.

4.3 Alternative fuels for cerebral metabolism

Ketone bodies primarily acetoacetate and 3-βhydroxybutyrate are produced in the liver from beta oxidation of fatty acids. In certain metabolic conditions such as the fasted state (Owen et al., 1967; Haymond et al., 1982) and in breast fed neonates (Hawdon et al., 1992) there is an increased concentration of ketone bodies that can be utilised by the brain as an alternative fuel. This spares the breakdown of glycogen and skeletal muscle proteins to provide gluconeogenic substrates.

Cerebral utilisation of ketone bodies is linearly dependent on the circulating concentration of ketone bodies (Kraus et al., 1974), the abundance of monocarboxylic acid transporters (MCT1) (Froberg et al., 2001; Gjedde et al., 1975) that determine the permeability of blood brain barrier to ketone bodies and also the maturity of relevant ketolytic enzymes (Middleton et al., 1973).

In suckling rats ketone bodies can provide more than 30% of cerebral energy requirement (Cremer et al., 1982). In hypoglycaemia secondary to hyperinsulinism, lipolysis and gluconeogenesis are inhibited, preventing the availability of ketone bodies, rendering the brain more susceptible to hypoglycaemic brain damage (Hussain et al., 2007).

Plecko et al studied oral supplementation of 3-βhydroxybutyrate (βohb) to two 6 month old infants with hyperinsulinaemic hypoglycaemia, and were able to demonstrate an increase in blood βohb concentration that was equivalent to concentrations seen following 16-24 hour fast and a comparable increase in CSF βohb and more importantly intracerebral uptake of βohb was confirmed by magnetic resonance spectroscopy. This provides an exciting avenue of possible additional neuroprotective therapy in these patients (Plecko et al., 2002).

Ketone body turnover is much higher in fasting children than adults (Bougneres et al., 1986). However they are in very low concentrations in the human newborns prior to commencement of enteral feeding when the neonate is at a high risk of hypoglycaemia. KB concentrations in healthy term human infants progressively increase from 12 hours – 4 days of life and decline thereafter (Hawdon et al., 1992).

During this period (1st day of life), possible alternative fuels in the form of lactate is present in high concentrations (Stanley et al., 1979; Hawdon et al., 1992) and in newborn dogs lactate has been shown to significantly contribute to cerebral energy metabolism during hypoglycaemia (Vannucci et al., 1981).

Rodent studies have shown that enzymes that are crucial for ketogenesis such as carnitine palmitoyltransferase I and β-hydroxy-β-methylglutaryl–coenzyme A synthase, are not transcribed till 12 hours of age. Early introduction of enteral milk feeds are also important as they provide long chain fatty acids that initiate transcription of the genes encoding the beta oxidative enzymes (Pegorier et al., 1998).

Neuroprotective effects of ketone bodies have been demonstrated in certain conditions such as epilepsy, hypoxia, neurodegenerative disorders, and traumatic brain injury although knowledge regarding mechanisms of neuroprotection is still evolving (White & Venkatesh et al., 2011). In rodent models of glutamate excitotoxicity (one possible mechanism of hypoglycaemic brain injury), acetoacetate infusion reduced neuronal damage and increased cellular ATP.

Some of the other neuroprotective mechanisms postulated include protection against glutamate induced apoptosis/necrosis by decreasing free radical formation, increased glutathione peroxidase activity (Ziegler et al., 2003), conversion of excitatory neurotransmitter glutamate to inhibitory GABA (gamma amino butyric acid) (Gasior et al., 2006) and increased cerebral blood flow (Hasselbalch et al., 1996). In children ketogenic diets have been used in the treatment of epilepsy and although the exact mechanism is unknown a modulation of excitatory and inhibitory neurotransmitter balance has been thought to confer the neuroprotection (Hartman et al., 2007).

Although the ketone bodies (KB) are available and the immature brain has the capacity to utilise them, their actual contribution to cerebral metabolism during hypoglycaemia in human newborns and older children is not known. In addition, glucose via glycolysis and its associated pathways is responsible for neurotransmitter homeostasis, defence against oxidative injury and maintaining redox equilibrium, that KB's are unable to compensate for.

Children with idiopathic ketotic hypoglycaemia are anecdotally believed to be neurologically normal suggesting a neuroprotective role of ketone bodies, but no supporting neuroimaging or neurodevelopmental data are available and existence of subtle learning difficulties cannot be excluded in the absence of reliable evidence.

5 Hypoglycaemia and the Brain

There is insufficient understanding regarding the severity and duration of hypoglycaemia associated with brain injury.

5.1 Pattern of Brain Injury reported in Hypoglycaemia

Neuropathological studies following human neonatal hypoglycaemia have revealed neuronal necrosis in cerebral cortex (occipital more that frontal with temporal being least affected), hippocampus and basal ganglia (Anderson et al., 1967).

A distinct pattern of cortical grey matter and subcortical white matter injury (bilateral and unilateral) in the occipital and parietal regions on neuroimaging is traditionally believed to be the outcome of neonatal hypoglycaemic brain injury (Yager et al., 2002).

Alkalay et al (Alkalay et al., 2005) summarized the neuroimaging findings in hypoglycaemia from 9 case reports involving 23 patients with a differing underlying etiology of hypoglycaemia. The median and range at age of clinical presentation was 30 hours and 1-72 hours respectively. Most cases, 17 of 23 (74%) showed persistent abnormal neuroimaging studies and significant neurological sequelae.

Changes were confined predominantly to the occipital (82%) and parietal lobes (29%). Periventricular deep white matter changes, cerebral cortical atrophy and ventricular dilation were described. Involvement of the globus pallidus in a patient with severe cortical injury and another with frontal white matter injury were also noted. A minority of the cases, 6/23 (26%), showed only transient brain oedema.

Seizures as presenting symptom 12/17 (70%), motor and/or psycho-developmental delay 11/17 (65%), visual impairment 7/17 (41%) and microcephaly 6/17 (35%) were all noted as neurological outcomes.

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Recently more diverse changes have been described as a consequence of hypoglycaemia. Burns et al (Burns et al., 2008) performed early (<6 weeks) MRI scans in 35 term infants with symptomatic hypoglycaemia and assessed their neurodevelopment at 18 months. Posterior pattern of brain injury was seen in 29%. 94% showed white matter (WM) injury of variable extent (global, periventricular, posterior and anterior parasagittal) and 51% showed cortical injury (cortical abnormality either as cortical highlighting or loss of grey and white differentiation in areas of WM injury). They also noted haemorrhage and infarction in WM, not previously reported in hypoglycaemia.

Involvement of basal ganglia and thalamus (BGT) was seen in 40% (4 patients had involvement of the posterior limb of the internal capsule (PLIC). Isolated cases with cerebellum and brainstem involvement were also present. It is possible that unrecognized comorbid conditions contributed to this varied presentation. At 18 months cognitive impairment was more common than motor impairment. 9 patients showed motor abnormalities (three with cerebral palsy), 15 showed mild to moderate cognitive impairment, 12 developed seizures, 9 developed microcephaly and 8 developed visual impairment.

Thus the pattern of brain injury following neonatal hypoglycaemia seems widespread, although a greater frequency of posterior cortical and subcortical WM injury is seen.

The reason for occipital lobe involvement in hypoglycaemia is not clear. Postulated mechanisms include reduced regional cerebral glucose uptake during hypoglycaemia (Mujsce et al.,1989), a defect in expression of glucose transport proteins affecting glucose utilisation and increased synaptogenesis in the visual cortex during the neonatal period increasing susceptibility to hypoglycaemia (Alkalay et al., 2005; Filan et al., 2006).

Neuroimaging studies in children following neonatal hypoglycaemia has been commonly performed in the acute period with short duration of follow up. Given the resilience of the immature brain to injury and its plasticity, neuroimaging studies performed beyond the acute phase with correlation to neurocognitive outcome, may be of greater prognostic value to patients.

5.2 Mechanism of Injury in hypoglycaemia

The mechanism of cellular injury in hypoglycaemia is complex and poorly understood and not merely a result of energy failure. Animal studies have been crucial in providing an insight into the possible mechanisms involved in hypoglycaemic brain injury. Depletion of high energy phosphates, accumulation of excitotoxic amino acids, membrane breakdown, release of fatty acids, intracellular calcium influx and cell death have been noted in hypoglycemic bran injury (McGowan et al., 1999; Behar et al., 1985; Auer et al., 1986).

An important mechanism implicated in brain damage is excitotoxic amino acid (EAA) injury. During hypoglycemia there is excess extracellular accumulation of excitatory amino acids such as glutamate and aspartate, due to release from the neurotransmitter pool and reduced reuptake by astrocytes and neurons (Butcher et al., 1987, Lipton et al., 1994).

Although the exact mechanism by which EAA's lead to brain injury is not known, they seem to mediate damage by excessive stimulation of N-methyl D aspartate A (NMDA) and non NMDA receptors that triggers a cascade of reactions (Morris et al., 1998). These include increased sodium and calcium influx, activation of proteases, lipases and superoxide generation leading to acute and chronic neuronal injury (Clarke et al., 1989). NMDA receptor antagonists have been shown to decrease hypoglycemia mediated cell injury (Wieloch et al., 1985) furnishing some hope as a possible neuroprotective therapy.

Glutamate receptors increase with the development of human brain (Lee et al., 1992) and are crucial for long term potentiation, a cellular mechanism that underlies learning and memory. McGowan et al (McGowan et al., 2002) demonstrated modification of glutamate binding sites of these receptors in newborn piglets. This might alter the function of how these receptors manipulate synaptic connections and result in irreversible change in brain structure and function.

In developing rat brain, hypoglycaemia can cause elevation of adenosine levels that activate A1 adenosine receptor and alter intracellular calcium concentrations resulting in neuronal damage (Turner et al., 2004).

Yan et al (Yan et al., 2006) have shown that hypoglycaemia in newborn rats can affect oligodendrocyte precursor cell differentiation, maturation, migration and viability in vitro. Oligodendrocytes are cells that contribute to myelin formation in the central nervous system and form a pathophysiological basis for white matter injury following hypoglycaemia.

Reactive oxygen species (superoxide and hydrogen peroxide) have been implicated in the pathogenesis of brain injury in hypoxic ischemic encephalopathy and neurodegenerative diseases (Traystman et al., 1996; Fachinetti et al., 1998; Lu et al., 2000). McGowan et al (McGowan et al., 2006; Ballesteros et al., 2003) demonstrated a similar increase in free radical generation by mitochondria during insulin induced hypoglycemia in newborn piglets.

These changes occurred within 2 hours of insulin induced hypoglycemia, implicating this as a possible early mechanism that initiates hypoglycemic brain injury. They postulate that the increase in mitochondrial reactive oxygen species is secondary to the imbalance between the normal oxidation potential in hypoglycemia and the decreased availability of reducing equivalents due to reduced glycolysis.

Reactive oxygen species mediate injury by damage to mitochondrial membrane and fragmentation of DNA compromising the protein complexes of electron transport chain that leads to impairment of ATP synthesis. Intracellular calcium accumulation and apoptosis can also be triggered causing further damage.

5.3 Neurodevelopment in Hypoglycaemia

There have been variable, even conflicting reports on the impact of hypoglycaemia on neurodevelopment. This has primarily resulted from the population group studied (ex preterm, infants with transient hypoglycaemia), underlying aetiology of hypoglycaemia (children with hyperinsulinism lack alternate fuels for the brain and are more vulnerable), difficulty in quantifying duration of hypoglycaemia, controversy regarding definition of hypoglycaemia, adequacy of follow up periods and the use of questionnaires or neurological examination to assess development as opposed to standardised tests.

Brand et al (Brand et al., 2005) looked at 60 large for gestational age (LGA) children (non hyperinsulinaemic and non-diabetic parents) with transient hypoglycaemia defined as plasma glucose < 2.2mmol at 1 hour and < 2.5mmol beyond 1 hour on the first postnatal day and compared them to 15 normoglycaemic LGA children. No significant difference was found in the Denver developmental scale after a follow up period of 4 years although reasoning IQ score was lower in the hypoglycaemic group. 64% of eligible patients were lost to follow up in this study.

Boluyt (Boluyt et al., 2006) et al conducted a systematic review to assess neurodevelopmental outcomes after hypoglycaemia in the first week of life. They identified 18 studies, and classified 2 as high quality and 16 as low quality. They concluded based on quality, clinical and methodological heterogeneity of studies, that the effect of hypoglycaemia on neurodevelopmental outcome cannot be reliably estimated.

In a large study by Lucas et al (Lucas et al., 1988), 31 of 661 preterm infants with hypoglycaemia > 5 days, were found to have reduced mental and motor scores on Bayley developmental scales at 18 month follow up. Neurodevelopmental impairment was 3.5 times greater in children with blood glucose < 2.6mmol/l for > 5 days. This study adjusted for other risk factors (eg: days of ventilation) associated with neurodevelopmental impairment.

Cerebral palsy, epilepsy, microcephaly, visual impairment, motor deficits, apraxia, autism, learning difficulties, attention deficit hyperactivity disorder (ADHD) have all been reported following neonatal hypoglycaemia (Yalnizoglu et al., 2007; Kinnala et al., 1999; Creery, 1966; Haworth & McRae, 1967; Pildes et al., 1974; Murakami et al., 1999; Burns, 2008; Udani, 2009)

Caraballo et al (Caraballo et al., 2004) described 15 patients with neonatal hypoglycaemia. Thirteen children had posterior cerebral injury on CT or MRI, 14 developed occipital lobe epilepsy (focal > generalised), 12 suffered mild to moderate mental retardation and 2 had global severe developmental delay. Microcephaly (6 patients) and visual disturbances (8 patients) were also described.

Neurodevelopmental outcome reported at 2 years of age by Burns et al in children with a predominant pattern of global white matter injury on neuroimaging was cognitive impairment more than motor impairment.

In addition, hypoglycaemia in the presence of co-morbid conditions such as sepsis, hypoxia and seizures that increase the cerebral glucose demand, has an additive effect on the resultant brain injury (Basu et al., 2009)

5.3.1 Neurodevelopment in HH

In comparison to other forms of hypoglycaemia children with HH are at an increased risk of brain injury due to lack of alternate fuels (ketone bodies). Even transient forms of HH have been associated with significant neurological damage (Vercellino et al., 2011). Neonatal seizures and occipital brain injury have been reported following transient hyperinsulinaemic hypoglycaemia (IUGR and maternal diabetes mellitus) (Filan et al., 2006).

Al-Rabeeah (Al-Rabeeah et al., 1995) reported brain injury in 12/28 (42%) patients with HH, where late referrals were more likely to sustain brain injury. This was a highly consanguineous pedigree (70%) and brain injury was reported based on retrospective data review. It's not clear if outcome was based on neuroimaging or neurodevelopmental examination. Cresto et al (Cresto et al., 1998) reported neurological abnormalities in 10/26 (38%) patients with HH. Motor difficulties, seizures, neuroimaging changes of diffuse cortical atrophy and low IQ were reported.

Menni et al (Menni et al., 2001) looked at the outcome in 90 children with congenital hyperinsulinism. They collected data retrospectively and classified outcomes as mild (IQ> 80 or 1 minor disability), moderate (IQ 60-80, or 2 or more disability) and severe (IQ <60 or major disability or mental retardation). 26% of patients had psychomotor retardation - 7 with severe psychomotor retardation, 12 with intermediate psychomotor disability and 16 with epilepsy. Visual impairment was reported in 3 patients (1 blind, 2 with strabismus) and deafness in 2 patients.

They concluded that the main risk factors for adverse neurodevelopmental outcome were neonatal onset and surgically treated patients and reported no differences in developmental outcome between different disease types (focal and diffuse). It was a large study but the data was collected retrospectively and it is unclear if standardised measures were used to assess neurodevelopment.

Yet another large study (Meissner et al., 2003) looked at 114 children with persistent hyperinsulinaemic hypoglycaemia and found a high rate of psychomotor retardation (44%) and epilepsy (25%), with infancy onset disease as the main risk factor.

Ludwig et al (Ludwig et al., 2011) assessed the neurological outcome of 20 children with HH (6 subtotal pancreactectomy), age range 7 months – 39 years in comparison to 4 controls (9 months – 13 years). Although limited by the sample size and wide age range they reported a trend to motor and speech delay in 0-3 year old children and, better performance of non-verbal tasks in 2.5 -7 year old in the patient group. 33% (4/12) of patients also had behavioural problems (poor attention, increased aggression).

Children with HI/HA syndrome, a type of hyperinsulinaemic hypoglycaemia, have increased frequency of generalised seizures (absence type), developmental delay and behaviour problems (Bahi-Buisson et al., 2008). This has been attributed to a combination of hypoglycaemic brain damage, hyperammonaemia and abnormal regulation of GDH in the brain. A genotype- phenotype correlation has been suggested with increased incidence of epilepsy noted in children with mutations in exon 6 and 7 of GLUD1 gene (Kapoor et al., 2009; Bahi-Buisson et al., 2008). In a cohort of 20 children with HI/HA, epilepsy was reported in 43% (Kapoor et al., 2009)

Adverse neurodevelopmental outcome following hyperinsulinism has been attributed to – neonatal presentation, poor responsiveness to medical treatment, requirement of surgery, late diagnosis and possible influence of underlying molecular disorder contributing to hyperinsulinism.

HH is associated with a high risk of neurodevelopmental impairment, yet few studies have examined neurodevelopment using standardized neuropsychological measures or in correlation with neuroimaging. Although KH is believed to be a benign condition, existence of subtle neurocognitive abnormalities has not been investigated and neuroimaging studies are not available.

Thus a greater understanding of the impact of hypoglycaemia on neurodevelopment, in HH and KH children with correlation of neurodevelopment to imaging changes are required to provide a clear picture of the outcome and to enable recommendations for clinical practice.

6 Background and general aims of thesis

6.1 Background to fasting studies

Hypoglycaemia is a common problem encountered in clinical paediatric practice. Blood glucose concentrations are measured in virtually every child admitted to the hospital. As glucose is a major fuel for brain function, anything that leads to persistent and recurrent hypoglycaemia can cause brain damage, epilepsy, mental retardation and even sudden death (Siesjo et al., 1988).

However, despite the commonality of hypoglycaemia, there is little consensus as to what constitutes a "safe" blood glucose concentration for normal brain function (Cornblath et al., 2000).

There are many causes of hypoglycaemia in the childhood period ranging from the very severe hyperinsulinaemic hypoglycaemia (HH) to the mild fasting ketotic hypoglycaemia (KH).

Children with HH exhibit hypoglycaemia with complete lack of alternative fuels such as ketone bodies, as the inappropriate insulin secretion inhibits lipolysis and ketogenesis. In contrast children with KH have hypoglycaemia with marked increase in alternative substrates. This unique patient cohort is therefore ideal for undertaking a study that will allow the assessment of the neuroprotective effect of ketone bodies in hypoglycaemic brain injury.

The initial aims of the study were
(a) To examine the relationship between low blood glucose concentrations and brain electrical activity
(b) To determine if changes in intermediary metabolites (ketone bodies) and counterregulatory hormones, modulate the effect of low blood glucose on brain electrical activity
(c) To investigate whether the generation of intermediary metabolites have a protective effect on brain electrical activity when blood glucose levels are low

(d) To investigate the relationship between structural brain abnormality and neuropsychological outcomes in children with KH and HH.

Accordingly, those children undergoing fasting studies for suspected ketotic or hyperinsulinaemic hypoglycaemia was recruited to undergo simultaneous EEG (electroencephalography) recording of the brain. Electrical potentials associated with specific sensory, perceptual or motor events known as event related potentials (ERP) were also recorded. The ERP waveforms are embedded within the EEG recording and time locked averaging of the EEG signal were performed to study the response to event. The ERP waveform is a series of negative and positive waves that are classified as N1, P1, N2, P2 and so on, based on polarity (N=negative, P= positive) and their order of appearance. These waves reflect internal cognitive processing of the stimulus.

In children who became hypoglycaemic during the fasting study, the aim was to assess the amplitude and latency of these waveforms before, during and after hypoglycaemia. As bloods were simultaneously collected at regular intervals for counterregulatory hormones and ketone bodies, any modulating effects of these on brain electrical activity during hypoglycaemia could be assessed. Children who were normoglycaemic at end of fast were utilised as control group.

13 children (age range 1 month - 14 years) were recruited and underwent simultaneous fasting study and EEG/ERP recording. However this study had to be abandoned due to unresolvable methodological issues. As these experiments were conducted on the wards, interference from neighbouring electrical equipment (such as infusion pumps, monitors, mobile phones) led to poor EEG data quality.

I performed the application, recording, processing and interpretation of data under supervision of Dr. Stewart Boyd (consultant neurophysiologist, GOSH). The time required to apply 26 scalp electrodes were nearly 2 hours per patient. These electrodes had to stay in place for 4 hours which was cumbersome for younger patients. Quite often electrodes had to be replaced due to artifacts from sweating or spontaneous displacement.

Thus patient compliance was a critical issue and particularly challenging in children less than 1 year of age. Artifacts due to movements, sweating and chewing further decreased the quality of data. Following artifact rejection only small numbers of events were available to average, and appropriate waveforms were not discernible to study the effects of hypoglycaemia.

Although the EEG/ERP experiment was discontinued, the recruitment of fasting children continued to enable the study of rate and regulation of ketone body response during fasting in children. Thus the fasting studies included in this thesis comprise of children who demonstrated ketotic hypoglycaemia at the end of the fast or remained normoglycaemic during the fasting period.

6.2 Background to neuroimaging and cognitive studies

A pilot study of 17 children with hyperinsulinaemic hypoglycaemia in Great Ormond Street Hospital (GOSH) was conducted prior to this study. Specific cognitive domains such as IQ, memory and academic achievement were assessed with a battery of age appropriate tests. 11 males and 6 females were assessed with a mean age of 8.5 years (range 6-13 years).

Results demonstrated verbal IQ in the average range (mean 91.70 (range 73-114)), non verbal IQ in the average range (perceptual reasoning mean 93.88 (range 69-131)) and general memory score in the low average range (mean 81.62 (range 66-103)).

Both the IQ and the memory scores showed a huge variability, however the presence of lower mean memory score relative to mean IQ score (10 point difference) was suggestive of selective memory impairment. The hippocampus is a key structure in the memory system and neuropathological studies of prolonged human neonatal hypoglycaemia have shown the hippocampus to be susceptible to hypoglycaemic brain injury (Auer et al., 1989). Hence further exploration of selective memory impairment by quantitative assessment of hippocampal volumes with correlation to memory indices was conducted.

Based on recent evidence of predominant and widespread pattern of white matter injury rather than posterior (occipitoparietal lobe) cortical subcortical injury following neonatal hypoglycaemia (Burns et al., 2008) and the pilot study in children with HH children suggestive of cognitive impairment, it was proposed that HH group in addition would manifest white matter damage and related neurocognitive deficits This was explored further by quantitative neuroimaging assessment of white matter (diffusion tensor imaging) with correlation to neurocognitive indices.

Children with HH have profound energy deficiency (recurrent hypoglycaemia and lack of KB) that would render them more susceptible to brain injury and subsequent development of neucognitive deficits in comparison to KH group. In the absence of a normal healthy control group, children with KH were used as the control group in the neuroimaging and cognitive studies.

6.3 Aims of the Thesis

The aims of this thesis are

1. To characterise the KB response and its regulation during fasting in children

2. To assess and compare the structural brain abnormality and neurocognitive profile in children with hyperinsulinaemic hypoglycaemia (HH) and ketotic hypoglycaemia (KH).

6.4 Ethical approval

Ethical approval for the study was obtained from the East London and the City Research Ethics committee. Consent forms, parent and children information leaflets were designed (appendix 1, 2, 3, 4) that were then approved by the ethical committee.

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Metabolic profiles in children with hypoglycaemia

7 Biochemical changes during fasting studies

This chapter outlines the biochemical changes during the fasting test in children with KH and a control group (children with suspected hypoglycaemia or previous history of hypoglycaemia, who were subsequently normoglycaemic on a fast) with an aim to investigate ketone body response between the two groups and factors regulating the response. As children with KH are believed to represent one end of the spectrum of normal healthy with limited fast tolerance, the KB concentrations and regulating factors were also analysed by grouping together the data from KH and control group.

7.1 Introduction

During fasting glycogenolysis, gluconeogenesis, fatty acid oxidation and ketone body formation are activated. Any defect along this pathway would lead to hypoglycaemia during the fast.

In children, glucose homeostasis is maintained predominantly by the mobilisation of glycogen reserves in the initial 4- 8 hours of a fast; gluconeogenesis is initiated within 4-6 hours and plays a greater role in homeostasis by 10-12 hours of age and lipolysis and ketogenesis begins with the suppression of endogenous insulin production and gradually increases as glycogen reserves are exhausted (Haymond et al., 1982; Pagliara et al., 1972). However the timing of these responses may vary with age, adequacy of glycogen reserves and gluconeogenic substrates and concomitant glucose concentrations (Ward et al., 1982).

Children with ketotic hypoglycaemia are biochemically characterised by the presence of ketone bodies during hypoglycaemia. Despite several attempts the pathophysiological basis of KH remains elusive (Huidekoper et al., 2008; Bodamer et al., 2006) and ketosis is considered a consequence rather than a cause of the hypoglycaemia.

A greater KB response to fasting is seen in children in comparison to adults (Haymond et al., 1982). In particular, KB turnover rates during 8-10 hours of fasting in neonates and infants (12.8-26 micromol/kg/min) are comparable to adult rates after several days of fasting. Children with KH demonstrate greater KB concentrations in comparison to controls during 15-24 hour fasts (Chaussain et al., 1977; Saudubray et al., 1971; Bonnefont et al., 1990). The current utility of fasting studies apart from aiding diagnosis of underlying metabolic and hormonal disorders are to determine the safe fasting time in these children.

The duration of current fasting studies are tailored to the age of the child (Morris et al., 1996). While information regarding KB response in children are available after prolonged periods of fast (15-24 hours), it is not known if there is a variation in the pattern and regulation of KB response during physiological periods of fasting between KH and healthy children and the relationship of counterregulatory hormones to KB response in children are not well reported. This knowledge would be crucial in interpreting the results of clinical fasting studies and increase understanding of the biochemical mechanisms underlying KH.

3-βhydroxybutyrate was the circulating KB measured during this study and is used interchangeably with the word 'ketone body (KB)' throughout this thesis.

7.2 Specific Aims

To determine the variation in the magnitude and rate of ketone body response to physiological periods of fasting in KH group and the 'control' group.

To investigate the factors influencing KB response during fasting in children.

7.3 Methods

7.3.1 Patient recruitment

All patients recruited into the study were referred to Great Ormond Street Hospital for investigation of hypoglycaemia during the period between March 2009 - March 2012. Great Ormond Street Hospital (GOSH) is a tertiary referral centre for the diagnosis and management of children with all forms of hypoglycaemia.The children requiring fasting studies were admitted to Kingfisher ward, a designated programmed investigation unit for performing controlled fasting investigations.

Patient group
Patients with the diagnosis of idiopathic ketotic hypoglycaemia (KH) defined as raised plasma NEFA and 3-βhydroxybutyrate at the time of hypoglycaemia during the fasting study and exclusion of other metabolic and endocrine causes of hypoglycaemia.

Control group
Children investigated for suspected or previous history of hypoglycaemia and found to be normoglycaemic on the fast test.

Exclusion criteria
Children with known endocrine (GH or cortisol deficiency) or metabolic causes of hypoglycaemia.

7.3.2 The diagnostic fast

The children admitted for the fasting studies had an intravenous cannula inserted 24 hours before fasting. They underwent an initial 24 hour period of blood glucose profiling (2 hourly measurements) on a normal food and activity schedule to see if they had any spontaneous hypoglycaemia and to ensure normoglycaemia prior to fasting. The children were then fasted.

The length of the fast followed the protocol (Table 2) routinely used for fasting children in our programmed investigation unit by the endocrine and the metabolic team. The duration of the fast was determined by the age of child, with an aim to increase diagnostic yield and establish a maximum safe interval between meals while minimising the risks and discomfort associated with fasting tests (Morris et al., 1996). During the fast all oral and intravenous intake was withheld except sips of water.

Table 2 Duration of the fast according to the age of the child

Age	Length of fast in hours
0-6 months	6
6-8 months	8
8-12 months	12
1-2 years	16
2-8 years	18
>8 years	20

During the fast blood glucose was measured hourly (bedside monitoring and in addition sample was sent to the laboratory) initially and half hourly if hypoglycaemia was anticipated. The fast was terminated

If the blood glucose concentration was <3.0 mmol/l with or without symptoms of hypoglycaemia (nausea, vomiting, drowsiness, headaches, confusion, tremors, sweating) or

At the end of the predetermined period of fast in the absence of hypoglycaemia

A threshold of 3 mmol/l was chosen, as the normal fasting blood glucose concentrations in children range from 3.5 - 5.5 mmol/l and the counterregulatory hormonal responses are initiated by the time blood glucose concentrations reach 3 mmol/l. This threshold is also in agreement with published reports of clinical fasting investigation from other hospitals (Van Veen et al., 2011).

Hypoglycaemia if present was corrected with intravenous fluids (1-2 ml of 10% dextrose bolus) or oral feeds if child was tolerating oral intake. All children were discharged only after normal food intake was established following the fast and normoglycaemia was maintained.

Most of the hormones and intermediate metabolites were assessed as part of their initial clinical investigation plan. Bloods were taken for the measurement of the following (Table 3)

Glucose, lactate, NEFA (non-esterified fatty acids), 3-βhydroxybutyrate, GH, cortisol, insulin (beginning, middle and end of fast), Ammonia, plasma amino acids (at the beginning and end of fast) and plasma catecholamines and serum glycerol (beginning, middle and end of fast)

All samples were transported, for analysis to the biochemistry laboratory at Great Ormond Street Hospital.

Acyl carnitine was measured on Guthrie card samples and urine sample was collected at end of fast for estimation of organic acids. Data from these 2 tests were not included as part of my study, although I checked them to detect any abnormalities.

Table 3 Hormone and intermediary metabolites measured at the beginning and end of the fast or at the time of hypoglycaemia

Hormone/Metabolite	Volume of blood (mls)	sample collection
Glucose	0.2	fluoride
Lactate	0.2	fluoride
NEFA	0.5	lithium heparin
3-βhydroxybutyrate	0.5	lithium heparin
Insulin	0.5	clotted
GH	0.5	clotted
Cortisol	0.5	clotted
Plasma amino acids	0.5	lithium heparin
Ammonia	0.5	Special 'NH3-free' heparin
Catecholamines	1	lithium heparin
Glycerol	0.5	lithium heparin

The blood sample for GH, cortisol, NEFA, 3-βhydroxybutyrate, and amino acids, were centrifuged, separated and stored at -20^{0}C until further analysis. The samples for glucose, lactate, and insulin, ammonia were centrifuged, plasma/serum separated and analysed immediately. The sample for catecholamines was centrifuged, plasma separated and stored at -20^{0}C and transferred to the biochemistry laboratory at Royal Brompton Hospital for analysis. The sample for glycerol was precipitated with perchloric acid in the laboratory or by bedside, centrifuged, plasma separated, frozen to -20^{0}C and analysed within 2 weeks.

7.3.3 Measurement of hormones and intermediary metabolites

All hormones (except plasma catecholamines) and metabolites were measured in the Camelia Botnar biochemistry laboratory of Great Ormond Street Hospital and plasma catecholamines were measured at the biochemistry laboratory at Royal Brompton hospital. I learned the principles of measurement by spending time at the lab and observing the laboratory staff performing the procedures.

Measurement of serum insulin

Serum insulin was measured by immunometric assay using the automated Immulite 2500 analyser. The Immulite 2500 analyser performes automated chemiluminescent immunoassays. The assay utilises a solid-phase bead coated with monoclonal murine anti-insulin antibody, a liquid phase alkaline phosphatase conjugated to polyclonal sheep anti-insulin antibody and alkaline phosphatase conjugated to monoclonal murine anti-insulin antibody and a chemiluminescent substrate.

The patient sample and the enzyme conjugate are incubated with the coated bead for 60 minutes. The insulin in the sample forms an antibody sandwich complex, and the unbound patient sample and the enzyme conjugate are removed by centrifugal washes. The chemiluminescent substrate is then added and the light emission detected is proportional to the bound enzyme and antigen in the patient sample.

The reportable range is 2- 300 mU/l with an analytical sensitivity of 2mU/l. This test for insulin has no cross-reactivity for c-peptide or glucagon and 8% cross reactivity for pro-insulin. The within run coefficient of variation was 3.7 -5.5%.

Measurement of serum GH

Serum GH was measured by a two-site chemiluminescent immunometric assay using the automated Immulite 2500 analyser. The patient sample is incubated with the solid phase (bead) coated with murine monoclonal anti-hGH antibody and reagent alkaline phosphatase conjugated to a rabbit anti-hGH polyclonal antibody. The GH in sample forms an antibody-sandwich complex. Unbound patient sample and enzyme conjugate are removed by a centrifugal wash. The chemiluminescent substrate is then added and the light emission detected is proportional to the bound enzyme antigen in the patient sample.

Measurement of serum cortisol

Serum cortisol was measured by a solid phase competitive chemiluminescent immunoassay, using the automated Immulite 2500 analyser. The assay utilises a solid-phase bead coated with polyclonal rabbit anti-cortisol antibody, a liquid phase alkaline phosphatase conjugated to cortisol and a chemiluminescent substrate.

The patient sample and enzyme conjugate are incubated with the coated bead. The unlabelled cortisol in sample competes with the labelled cortisol for antibody binding sites. The chemiluminescent substrate is then added and the light emission detected is proportional to the bound enzyme and inversely proportional to cortisol in the patient sample.

The measuring range is 28-1380 nmol/L with a detection limit of 5.5 nmol/l. The within run coefficient of variation was 5.1 – 7.4 %. The antibody is highly specific for cortisol, however there is a 62% cross reactivity with prednisolone.

Measurement of 3-βhydroxybutyrate

Plasma 3-βhydroxybutyrate was measured on the automated iLab 650 analyser. The method involves oxidation of 3-βhydroxybutyrate to acetoacetate by the enzyme 3-βhydroxybutyrate dehydrogenase. Alongside this oxidation reaction, cofactor NAD+ is reduced to NADH. There is a corresponding change in absorbance that is correlated directly to βhydroxybutyrate.

$$3 \ \beta\text{-hydroxybutyrate} + NAD^+ \longrightarrow Acetoacetate + H^+ + NADH^+$$
$$(\text{3-}\beta\text{hydroxybutyrate dehydrogenase})$$
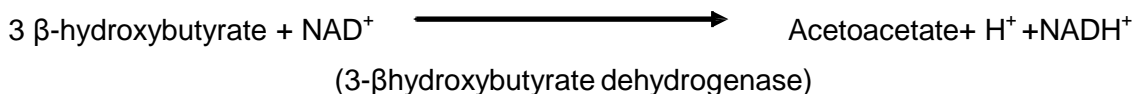
The measuring range is: 0.05 – 3.2mmol/L

Measurement of Non Esterified Fatty Acids (NEFA)

Plasma NEFA was measured on the automated iLab 650 analyser. In this enzymatic assay NEFA reacts with acyl CoA synthase (ACS) in the presence of adenosine triphosphate (ATP), to form acyl CoA, adenosine monophosphate (AMP) and pyrophosphate (PPi). Acl CoA is then oxidised by acyl CoA oxidase (ACOD) to hydrogen peroxide (H_2O_2). The hydrogen peroxide, in the presence of peroxidase (POD), yields a blue purple pigment by quantitative oxidation condensation with 3-methyl-N-ethyl-N (b-hydroxyethyl)-Aniline (MEHA) and 4-amino-antipyrine. The concentration of NEFA in the sample is calculated from the optical density measured at 546 nm.

NEFA + ATP +CoA $\xrightarrow{\text{(ACS)}}$ Acyl-CoA + AMP + PPi

Acyl-CoA + O_2 $\xrightarrow{\text{(ACOD)}}$ 2, 3-trans-Enoyl-CoA + H_2O_2

H_2O_2 + 4-amino-antipyrine + MEHA $\longrightarrow$ Blue purple pigment + H_2O
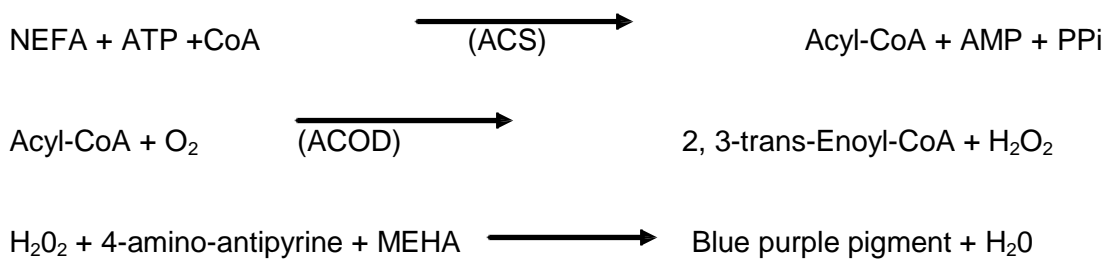
The measuring range is: 0.01 – 4.0mmol/L

Measurement of plasma lactate

Plasma lactate was measured on the Kodak Vitrous 5600 analyser. 10 microlitres of the patient sample is deposited on multi-layered Vitros slide and evenly distributed. Lactate in the sample is oxidised by lactate oxidase in the reagent layer, to pyruvate and hydrogen peroxide. The hydrogen peroxide generated oxidises the 4-aminoantipyrine, 1, 7-dihydroxynapthalene dye system in a horseradish-peroxidase-catalysed reaction and results in a dye complex. The slide is incubated and the intensity of the dye complex is measured spectrophotometrically at 540 nm.

$$L - (+) - \text{lactic acid} + O_2 \xrightarrow{\text{lactate oxidase}} \text{Pyruvate} + H_2O_2$$

$$2\,H_2O_2 + 4\,\text{aminoantipyrine} + 1,7\,\text{dihydroxynapthalene} \xrightarrow{\text{peroxidase}} \text{red dye}$$
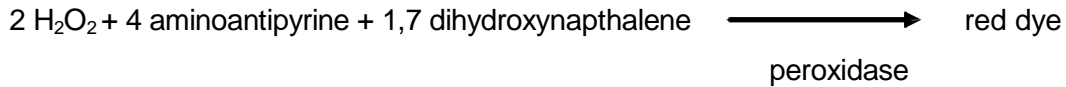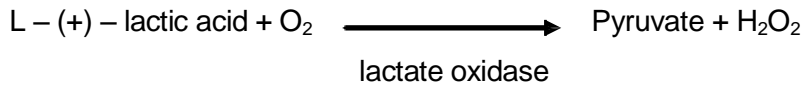
The reference range is between 0.7 – 2 mmol/L. The measuring range is 0.5 – 12.00 mmol/L.

Measurement of plasma ammonia

Plasma ammonia was measured on the Kodak Vitrous 5600 analyser. 10 microlitres of patient sample is spread evenly on the Vitros slide. The Vitros slide contains the reactive ingredient bromophenol blue. The reaction of ammonia with a specific indicator, bromophenol blue, produces a coloured (blue) dye. The absorbance of the dye produced is measured by reflectance spectrophotometry at 600 nm.

$$\text{Ammonia} + \text{bromophenol blue (ammonia indicator)} \longrightarrow \text{blue dye}$$
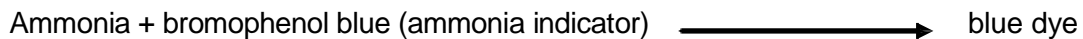
The measuring range is between 1.0 – 500 µmol/L. The sensitivity of the ammonia assay is 1.0 µmol/L.

Measurement of plasma amino acids

Plasma amino acids were measured by high performance liquid chromatography (HPLC). The analysis comprises of four different components. The first step is the removal of proteins by precipitation with 5-sulphosalicylic acid (5SSA) containing a specified quantity of internal standard, norleucine (NL) and 4-nitrophenylalanine (4NPA). The sample is then centrifuged, and a fraction of the supernatant containing amino acids is treated with phenylisothiocyanate (PITC), in the presence of methanol and triethylamine, resulting in a PITC derivative.

In the 3rd step, the PITC amino acid derivatives are separated by reversed phase partition liquid chromatography on a heated silica column, commencing with an acetate buffer pH 6.5 mobile phase, followed by an increasing gradient of organic solvent (acetonitrile, methanol). The eluting PITC amino acid derivatives are detected by their UV absorbance at a wavelength of 254nm.

Electronically integrated detector peak areas are quantitated by using standard amino acid factors calculated from amino acid calibration standards. Before the amino acid concentrations are calculated, each chromatogram is inspected visually on the visual display monitor and the baseline manually adjusted where necessary.

The detection limit ranges from1 µmol/l to 1500 µmol/l. The within batch coefficient of variation for the various amino acids range from 0.8-7.2%.

Measurement of plasma glycerol

Plasma glycerol is measured by an enzymatic method on the Roche Cobas Bio Analyser.The enzymatic reactions involved in the assay are as follows:

Glycerol + ATP $\longrightarrow$ glycerol-3-phosphate + ADP

Glycerokinase

Glycerol-3-phosphate + NAD $\longrightarrow$ dihydroacetone phosphate + NADH + H$^+$

G3PDH
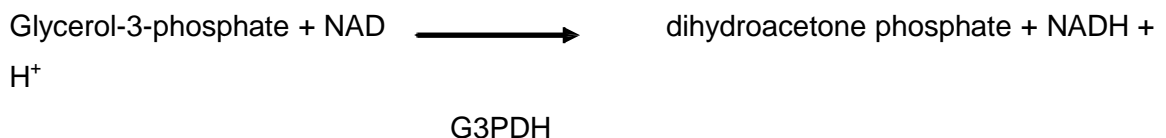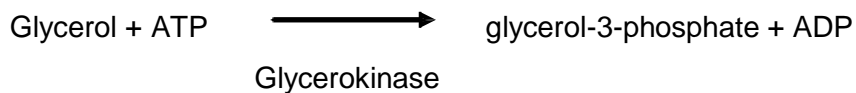
The associated increase in fluorescence emission of light at 450 nm, during the reaction caused by NADH when excited by light at 340 nm, can be directly correlated with the glycerol concentration. No reference ranges are available. The measuring range is between 0 – 700 µmol/L. The coefficient of variation in between batch samples is between 6-8%. This assay requires addition of a fixed amount of blood to perchloric acid precipitation tubes prior to analysis.

Measurement of plasma catecholamines

Free catecholamines are detected in the plasma using high performance (pressure) liquid chromatography (HPLC) with electrochemical detection (HPLC-ECD), using an isocratic system. Extraction of catecholamines is achieved using alumina adsorption. Patient sample is injected by the autoinjector into the flow of mobile phase and onto the column (stationery phase). The sample passes on to the analytical column where separation of its components occurs due to a combination of the mobile phase and the column characteristics.

The column eluate then passes through the detector where an electrochemical reaction occurs at the surface of a working electrode. This produces a current that is amplified and transmitted to the integrator. (The separated molecules require different electrode potential before they can be oxidised or reduced). The integrator monitors the ECD output and allows both qualitative and quantitative evaluation of the separation.

The reference ranges are based on resting adult fasted samples (Plasma Adrenaline: 0.10 – 0.80 nmol/L, Plasma Noradrenaline: 0.15 – 3.50 nmol/L)

Measurement of plasma glucose

Plasma glucose is measured on the Kodak Vitrous 5600 analyser. 10 microlitre of patient sample is spread evenly on a multi-layered (the reagent layer containing glucose oxidase, peroxidase, dye precursors) Vitros slide. This results in an even penetration of solute molecules into the underlying reagent layer. Glucose in the patient sample is converted by glucose oxidase (GOD) to gluconate and hydrogen peroxide in the presence of oxygen. Hydrogen peroxide produced reacts with 4-aminophenazone and phenol in the presence of peroxidase (POD) to yield a coloured complex. The intensity of the complex is measured at 505 nm and is proportional to glucose concentration.

Glucose + O_2 + H_2O $\xrightarrow{\text{GOD}}$ gluconate + H_2O_2

$2H_2O_2$ + 4-aminophenazone + phenol $\xrightarrow{\text{POD}}$ coloured complex + $4H_2O$
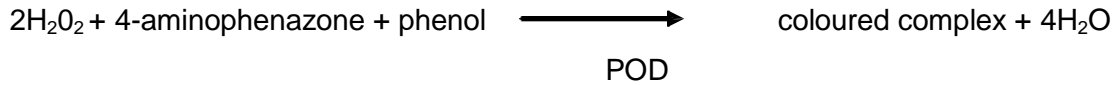
The measuring range is 1.11 – 34.69 mmol/L.

7.3.4 Statistical analysis

As distribution of hormones and metabolites during fasting tests are skewed, non-parametric tests were used. All statistical analysis was performed on IBM SPSS version 20.

Samples at the beginning of the fast are denoted by suffix t = 0 or t0 and at the end of the fast by suffix t = e or te

Within group analysis: Wilcoxon signed-rank test was used to detect significant differences between serum concentrations at beginning (t0) and end of fast (te), within each group. Median, 10th and 90th percentile concentrations at the beginning and end of the fast were generated.

Between group analysis: Mann Whitney-U was used to detect significant differences between 2 groups.

Non parametric correlations (Spearman) were used to investigate the relationship between KB response and counterregulatory hormones.

To estimate if rate of change of KB varied between 2 groups, the relationship between change in KB concentrations and fast duration was assessed using linear regression and subgroup (KH or control group) was entered into the regression model as a dummy variable to compare the difference in the slope of regression coefficient.

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7.4 Results

Patient characteristics

Biochemical data were available for 30 children with ketotic hypoglycaemia and 74 children from the control group. There was no significant difference in the baseline characteristics between the two groups (Table 4).

The duration of fast was predetermined according to age, but shortened if hypoglycaemia occurred (Table 5).

Table 4 Comparison of the baseline characteristics between the control and the KH group

Characteristics	Ketotic hypoglycaemia	Control group	*p* value
Number	30	74	
Gender (Male/Female)	18M/ 12F	43M/ 31F	0.859
Birthweight in grams (mean (SD))	3054.4 (836.2)	3136.03 (1016.9)	0.604
Gestational age in weeks (median, range)	3.1 (0.36 -7.17)	1.2 (0.08 – 16.00)	0.375
Age at fast in years (median, range)	-0.36 (-2.29 to 3.75)	-0.17 (-2.10 to 2.32)	0.133
Height SDS (median, range)	-0.59 (-2.42 to 2.00)	-0.37 (-1.96 to 1.50)	0.799
Weight SDS (median, range)	-0.36 (-2.38 to 1.54)	-0.36 (-2.40 to 3.24)	0.277

SD= Standard deviation, F= female, M= male, SDS= Standard deviation scores

Table 5 Duration of fast and distribution of patients

Fast duration in hours	Ketotic hypoglycaemia	Control group
4	2	0
6	2	3
8	3	17
10	0	1
11	1	0
12	7	17
14	1	2
15	1	0
16	3	11
17	0	1
18	8	16
20	2	5
24	0	1
Total	30	74
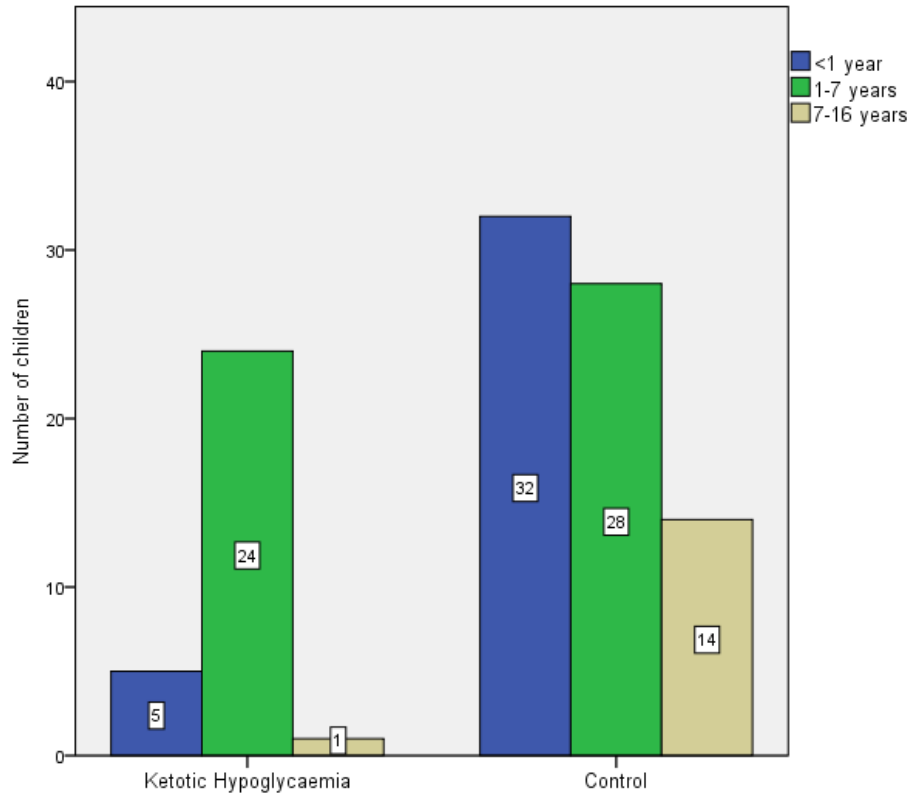
Figure 9 Distribution of the age at fast in the control and the KH group

In the KH group 5 children were < 12 months of age, 24 children between 1-7 years of age and only 1 child > 7years of age. In the control group 32 children were <1 year of age, 28 children between 1-7 years of age and 14 children > 7 years of age. This is in accordance with the diagnosis of KH that presents after 6 months of life and resolves by 7-10 years of life (Figure 9).

Plasma glucose, lactate and serum insulin, cortisol and growth hormone concentrations during the fast

There was a significant decrease (Table 6) in plasma glucose and serum insulin concentrations at the end of the fast, in both KH and the control group, and significant decrease in plasma lactate concentrations in control group. Serum cortisol and GH concentrations showed significant rise at end of the fast in both the groups.

Table 6 Concentrations of plasma glucose, lactate and serum GH, cortisol and insulin at the beginning (t=0) and at the end of the fast (t=e). Median values are presented, with 10th and 90th percentile values in parenthesis.

Hormones and metabolites	KH group t=0	KH group t=e	p value	Control group t=0	Control group t=e	p value
Plasma glucose mmol/l	4.7 (3.6-6.7)	2.6 (2.1-3.0)	<0.001	5.0 (4.2-6.1)	3.9 (3.3-4.5)	<0.001
Plasma lactate mmol/l	1.2 (0.8-1.9)	1.1 (0.8 -5.2)	0.913	1.4 (1-2.2)	1.0 (0.7-2.0)	<0.001
Serum insulin mU/L	9.5 (2-20.5)	2.0 (2.0)	<0.001	14 (2-42)	2 (2-4.5)	<0.001
Serum cortisol nmol/l	113.5 (41-403)	484 (207.6-892.7)	<0.001	85 (38-315)	281 (136-586)	<0.001
Serum growth hormone mcg/L	1.0 (0.1-3.0)	2.9 (0.28 – 11.4)	0.013	0.5 (0.1-3.6)	1.9 (0.2-5.2)	<0.001

Plasma amino acid concentrations during the fast

Apart from leucine, there was a significant decrease in plasma amino acid concentrations (glycine, valine, alanine, glutamine and lysine) in both KH and control group (Table 7)

Plasma alanine concentrations at the beginning (p = 0.009) and end (p = 0.001) of fast are significantly lower in the KH group relative to control group.

Table 7 Plasma amino acid concentrations during the fast. Median values are presented, with 10th and 90th percentile values in parenthesis.

Plasma amino acids in µmol/l	KH t=0	KH t= e	*p* value	Control t=0	Control t=e	*p* value
Glycine	215 (170-334)	158 (102.8-268.2)	<0.001	216.5 (157.8 - 347.3)	197 (149.5 - 264.5)	<0.001
Leucine	129 (89-205)	117 (89.4-166.4)	0.258	127.5 (100-185.5)	101 (76-121)	<0.001
Isoleucine	78 (35-121)	62 (42.8 - 86.6)	0.007	78 (52.3 - 108)	54 (39.5 - 63)	<0.001
Valine	225 (142 - 328)	203 (150.2 - 276)	0.013	236.5 (175.8 - 361.9)	176.5 (143.5 - 229.5)	<0.001
Alanine	331 (242-438)	157 (130.2 - 236.4)	<0.001	402 (282.1 - 566.8)	223 (140 - 298)	<0.001
Glutamine	577(439 - 780)	419 (334.4 - 632.8)	<0.001	585.5 (469.1 - 738.7)	514 (406 -663.5)	<0.001
Lysine	171 (115 - 277)	104 (69.6 -133.4)	<0.001	188.5 (133-268.5)	130 (87-184)	<0.001

Plasma glycerol concentrations in the KH group.

In children with ketotic hypoglycaemia, paired samples (beginning t=0 and end of fast t=e) were available only for 5 patients and end of fast concentration only for 1 patient. There was a significant (p=0.043) increase in glycerol concentrations at the end of the fast. The median concentration of glycerol at baseline was 60 µmol/L (range 30-94 µmol/L), and median concentration at end of the fast was 151 µmol/L (range 120-179 µmol/L).

Plasma glycerol concentrations in the control group

In the control group paired glycerol concentrations were available for 15 patients, 2 patients had only baseline values and 4 only end of the fast values. 9 patients increased their glycerol concentrations during the fast, while 5 patients showed a decrease during the fast. No significant differences were found between the glycerol concentrations at the beginning (glycerol t = 0) and at the end of the fast (glycerol t = e) (p=0.460).

The median glycerol concentration at the beginning of the fast was 50µmol/L (range 11-443 µmol/L) and the median glycerol concentration at the end of the fast was 104 µmol/L (range 51-280 µmol/L). There was no significant difference in glycerol concentrations at the beginning (p = 0.762) or the end of the fast (p = 0.080) between KH and control group.

Table 8 Within and between group comparisons of glycerol concentrations during the fast

Patient type	Glycerol µmol/L t=0 (median, range)	Glycerol µmol/L t=e (median, range)	*p* value (within groups)
Ketotic hypoglycaemia	60 (30-94)	151 (120-179)	.043
n	5	6	
Controls	50 (11-443)	104 (51-280)	.460
n	15	15	
p value (between groups)	0.762	0.080	

Plasma catecholamine concentrations during the fast (Table 9 and Figure 10)

Catecholamine concentrations were available for 21 children in the KH group and 43 - 47 children in the control group (t=0, n=43 and t=e, n=47).

Within group analysis: There was a significant increase in noradrenaline concentrations during the fast in both the KH (p = 0.009) and the control group (p = 0.005). A significant increase in plasma adrenaline concentrations was observed only in the control group (p = 0.003). The baseline plasma adrenaline concentrations were elevated the KH group.

Between group analysis: There was a significant increase (p =0.006) in the baseline plasma adrenaline concentration in the group with ketotic hypoglycaemia. No significant differences between groups were seen in plasma noradrenaline concentrations at the beginning and the end of the fast and plasma adrenaline concentrations at the end of the fast.

Table 9 Catecholamine concentrations during the fast in KH and control group. Median values are presented, with 10th and 90th percentile values in parenthesis.

	KH group	Control group	Between group p value
Noradrenaline nmol/l t = 0	1.56 (0.67 - 2.66)	1.58 (0.93 - 2.55)	0.791
Noradrenaline nmol/l t = e	2.00 (1.16 - 4.33)	1.80 (1.15 - 4.41)	0.947
n	21	43 (t=0), 47 (t=e)	
Within group *p* value	0.009	0.005	
Adrenaline nmol/l t = 0	0.59 (0.30 - 0.76)	0.40 (0.11 - 0.72)	0.006
Adrenaline nmol/l t = e	0.56 (0.13 - 3.1)	0.57 (0.22-1.62)	0.775
n	21	43 (t=0), 47 (t=e)	
Within group *p* value	0.185	0.003	
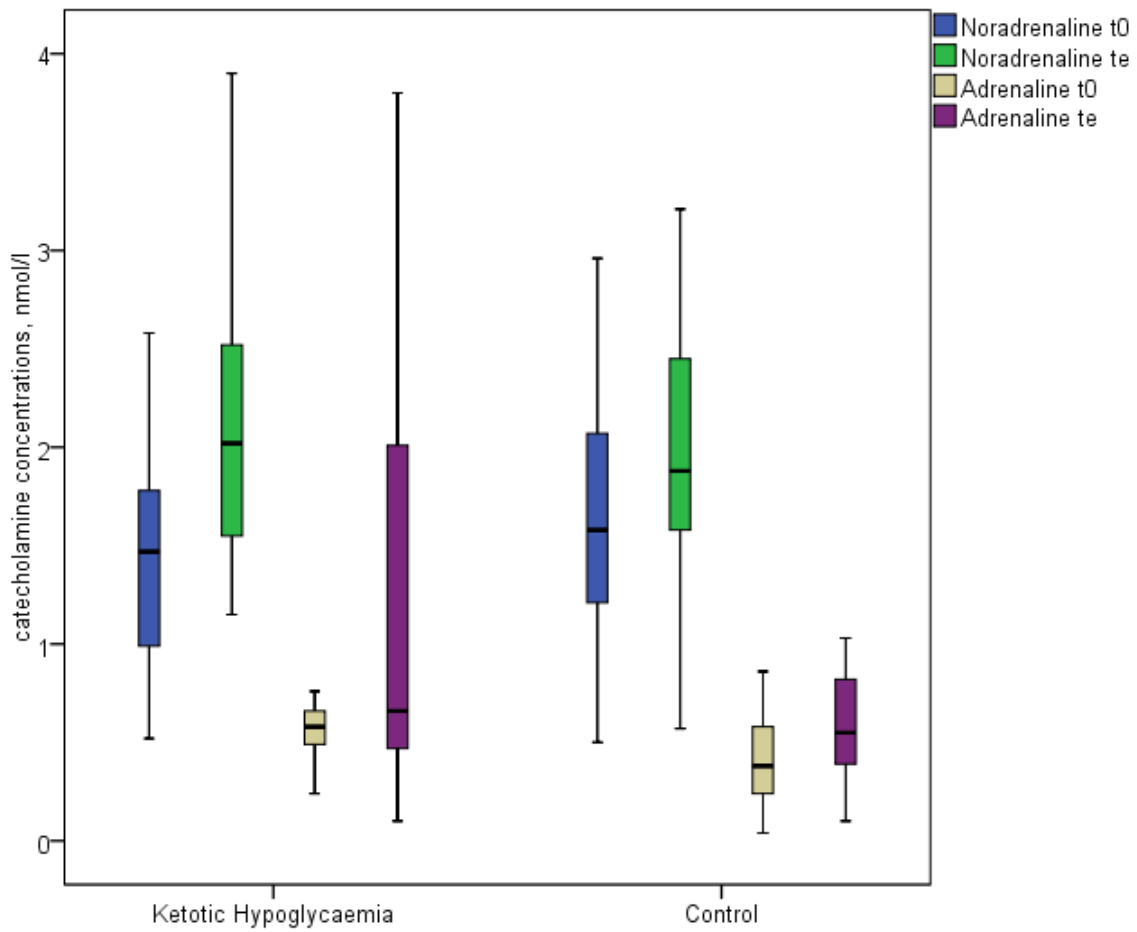
Figure 10 Comparison of noradrenaline and adrenaline concentrations during the fast between KH and control group. Noradrenaline concentrations show a significant increase following the fast in both, KH and control group. Adrenaline concentration rise significantly following the fast only in control group. In KH group, baseline concentrations are significantly elevated compared to control group.

Free fatty acid (FFA) response during the fast (Table 10)

Within group: There was a significant rise in FFA concentrations at the end of the fast, in both KH and control group.

The FFA concentrations at the end of the fast in the KH group, was 0.91 mmol/l (0.70-1.43) in children <1 year (n=5) and 1.75 mmol/l (1.0-3.0) in children between 1-7 years of age (n=24). There was only one patient in the KH group between 7-16 years and hence not included in the analysis.

In the KH group, the end of fast FFA concentrations were significantly (p = 0.015) greater in children 1-7 years of age compared to children <1 year of age.

The FFA concentrations at the end of the fast in the control group, was 0.74 mmol/l (0.41 - 1.16) in children <1 year (n=32), 1.1 (0.67 - 1.88) mmol/l in children between 1-7 yrs of age (n=28) and 0.82 mmol/l (0.44 -1.5) between 7-16 yrs of age (n=14).

In the control group the end of fast FFA concentrations were significantly (p < 0.001) greater in 1-7 year old children compared to children <1 year of age and children between 7-16 years of age (p < 0.008).

Between group: The baseline and end of fast FFA concentrations were compared between the KH and the control group in children <1 year and between 1-7 years of age. In children between 1-7 yrs of age, the baseline (p = 0.045) and end of fast FFA (p = 0.001) concentrations in KH children was significantly greater (p = 0.001) than control group.

Table 10 Free fatty acid (FFA) response during the fast in KH and control group across the 3 age categories

FFA mmol/l	KH t=0	KH t= e	*p* value	Control t=0	Control t=e	*p* value
<1 yr	0.57 (0.52 - 0.67)	0.91 (0.70-1.43)	0.043	0.36 (0.16 - 1.45)	0.74 (0.41 -1.16)	0.033
n	5	5		32	32	
1-7 yr	0.48 (0.19 - 0.98)	1.75 (1.0-3.0)	<0.001	0.34 (0.13 - 0.69)	1.1 (0.67 - 1.88)	<0.001
n	24	24		28	28	
7-16 yrs	0.34	1.59	na	0.28 (0.16 - 0.53)	0.82 (0.44 -1.5)	0.001
n	1	1		14	14	

na = not analysed

Ketone body changes during the fast and relationship to age (Table 11 and Figure 11)

Ketone body response was considered in 3 age categories.

The age categories chosen were based on previous studies that based these classifications on time to hypoglycaemia (time to event analysis) (Van Veen et al., 2011) or on observations indicating later onset of lipid mobilisation in children > 7 years of age (Bonnefont et al., 1990) and more specifically the clustering reflects the duration of fast tolerance expected in clinical practice based on our fasting protocol (Children <1 year of age were fasted <12 hrs, while children between 1-7 years of age were fasted between 12-16 hrs and children between 7-16 years of age were fasted between 18-20 hrs).

Within group: There was a significant rise in KB concentrations at the end of the fast, in both KH and the control group.

The KB concentrations at the end of the fast in the KH group, was 0.66 mmol/l (0.17 - 0.96) in children <1 year (n=5), 1.86 mmol/l (0.37 - 2.97) in children between 1-7 years of age (n=24). There was only one patient in KH group between 7-16 years and was not included in the analysis. In the KH group, the end of fast KB concentrations were significantly (p = 0.011) greater in children 1-7 years of age compared to children <1 year of age.

The KB concentrations at the end of the fast in the control group, was 0.29 mmol/l (0.05 - 0.97) in children <1 year (n=32), 0.79 mmol/l (0.19 -2.1) in children between 1-7 years of age (n=28) and 0.20 mmol/l (0.05 -0.8) between 7-16 years of age.

In the control group the end of fast KB concentrations were significantly (p < 0.001) greater in 1-7 year olds compared to children <1 year old and children between 7-16 years of age (p < 0.001).

Between group: The baseline and end of fast KB concentrations were compared between KH and the control group in children <1 year and between 1-7 years. The end of fast KB concentrations in the KH children was significantly greater (p = 0.001) than the control group.
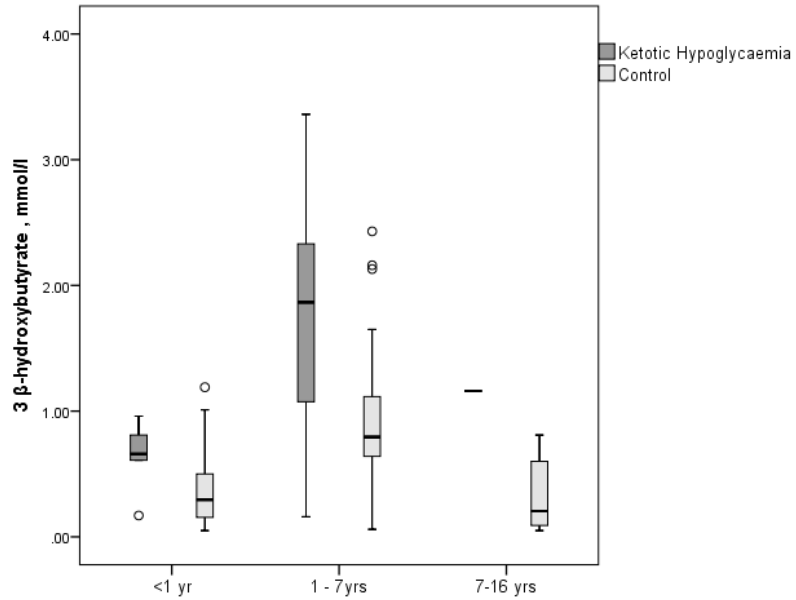
Figure 11 3-βhydroxybutyrate concentrations in KH and control group. Children between 1-7 years of age showed a greater KB response compared children < 1year and >7 years of age, in KH and control group.

Table 11 Ketone body (βohb) changes during the fast in KH and control group across the 3 categories

	KH t=0	KH t= e	*p* value	Control t=0	Control t=e	*p* value
βohb mmol/l <1 yr	0.15 (0.05 - 0.55)	0.66 (0.17 - 0.96)	0.043	0.05 (0.05 - 0.27)	0.29 (0.05 - 0.97)	<0.001
n	5	5		32	32	
βohb mmol/l 1-7 yr	0.06 (0.05 - 0.40)	1.86 (0.37 - 2.97)	<0.001	0.05 (0.05 -0.14)	0.79 (0.19 - 2.1)	<0.001
n	24	24		28	28	
βohb mmol/l 7-16 yrs	0.05	1.16	na	0.05 (0.05 -0.08)	0.20 (0.05 - 0.8)	0.002
n	1	1		14	14	

Bohb = 3-βhydroxybutyrate, na = not available

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Comparison of the rate of change of KB concentrations between KH and control group

The percentage of change in 3-βhydroxybutyrate concentrations at the end of the fast was computed using the following formula = (βohb te- βohb t0/ βohb t0) *100, where t=0 denotes beginning of fast and t =e denotes end of fast concentrations.

Slope of the regression line, examining the effect of fast duration (predictor variable) on percentage change in 3-βhydroxybutyrate concentrations during fast (dependent variable) was compared between the KH and the control group (entered into the model as a dummy variable), yielded the regression equation $Y = -392.1 + 172.6$ (fast duration) -87 d_1, where d_1 denotes control group as dummy variable (Figure 12Figure 12). Thus, for each hour of fast, the KH group showed an 87% increase in 3- βhydroxybutyrate concentrations in comparison to the control group. This difference in the rate of change was statistically significant (p <0.001).
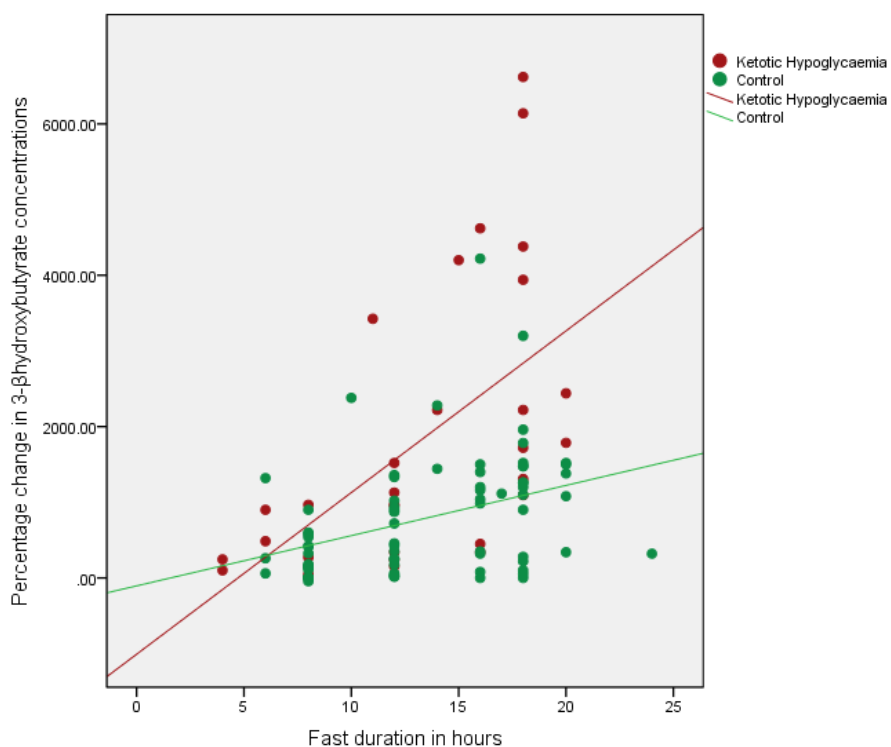
Figure 12 Comparison of the rate of change of KB concentrations between KH and the control group. The percentage increase of KB concentrations at the end of the fast was plotted against duration of the fast. KH children (red line) demonstrated a greater (87%) rise per hour of fast compared to the control group (green).

Relationship between KB response and counterregulatory hormones

a. Comparison between KH and control group

A significant positive correlation was seen between cortisol and 3-βhydroxybutyrate concentrations at the end of the fast, in the KH (r = 0.441, p = 0.015) and control groups (r = 0.297, p = 0.015) (Figure 13). Cortisol concentrations at the end of the fast in KH group was significantly greater than the control group (median 484 nmol/l vs 281 nmol/l, *p* value <0.001)
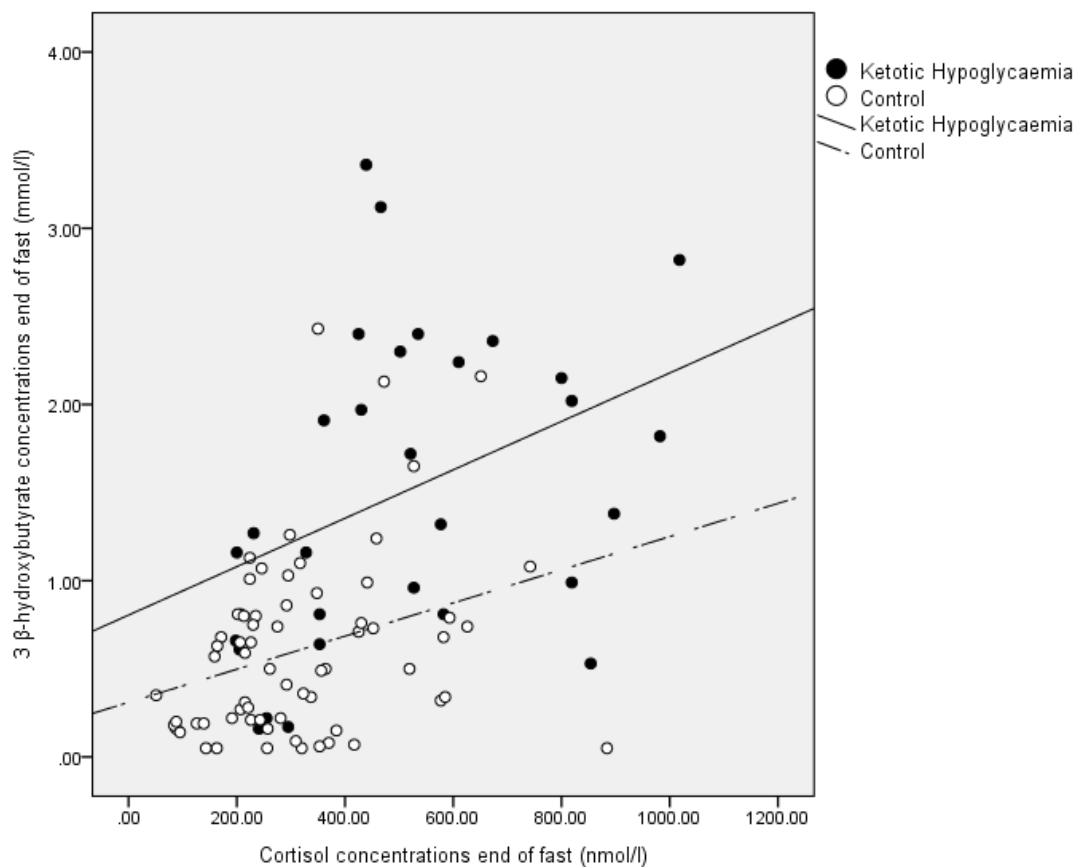
Figure 13 Relationship between cortisol and 3-βhydroxybutyrate concentrations in KH and control group. Positive correlation was seen (p = 0.015) in both groups.

In the control group there was a significant positive correlation (r = 0.435, p = 0.002) between 3-βhydroxybutyrate and plasma adrenaline concentrations at the end of the fast (Figure 14). KH group did not demonstrate any significant correlation (r = 0.271, p = 0.235) between 3-βhydroxybutyrate and plasma adrenaline concentrations at the end of the fast (Figure 15).
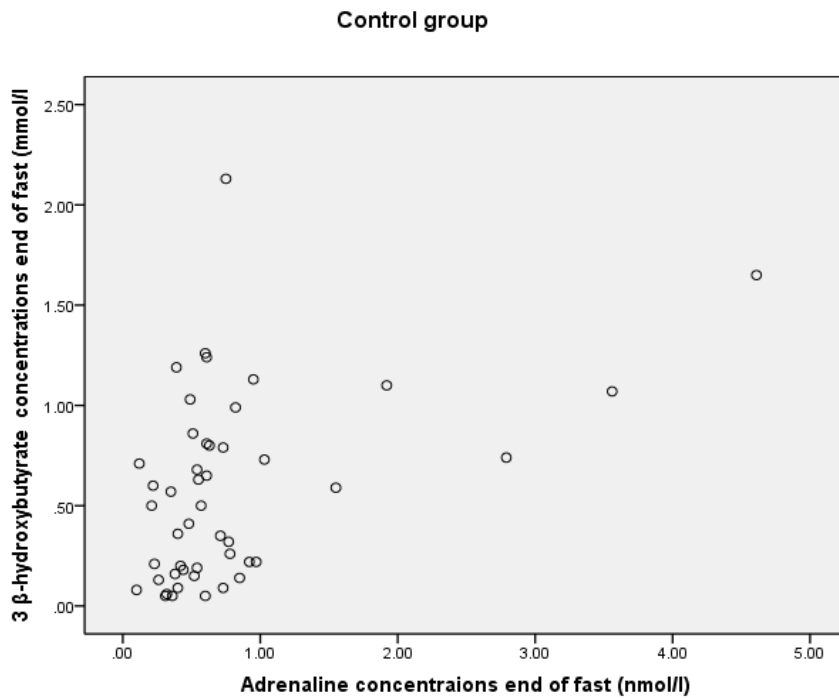
Control group

Figure 14 Relationship between 3-βhydroxybutyrate and adrenaline concentrations at the end of the fast in the control group
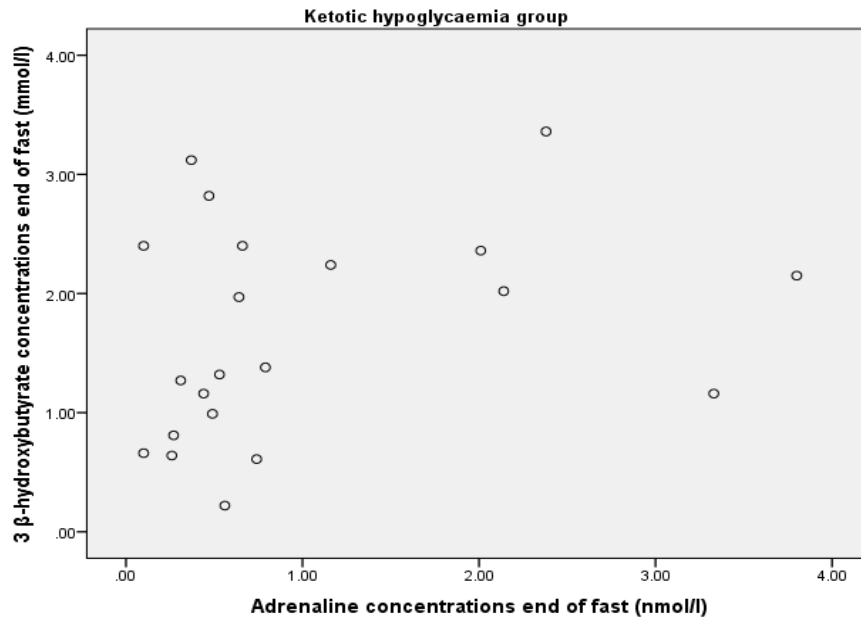
Figure 15 Relationship between 3-βhydroxybutyrate and adrenaline concentrations at the end of the fast in the KH group

Table 12 Comparison of cortisol concentrations at the beginning (t=0) and end of the fast (t=e) between KH and control group

Serum Cortisol	KH group median (range)	Control group median (range)	*p* value
t=0 nmol/l	113.5 (36 -546)	85 (28 - 538)	0.236
t=e nmol/l	484 (198 -1018)	281 (51 -884)	<0.001

b. KH and control group as a continuum.

In this section, the data from the KH and the control group was combined and analysed. As children with KH are considered to be at one end of the spectrum of normal children with limited fast tolerance and classification into the KH group or the control group was performed post data collection, based on glucose concentrations at the end of the fasting test, it was rationalised that a clear understanding of the factors influencing KB concentrations at the end of the fast would be obtained if the data from the two groups was considered as a continuum.

Multiple regression analysis was performed with KB concentrations at the end of the fast as the dependent variable and age at fast, fast duration, BMI SDS and cortisol, glucose, adrenaline and growth hormone concentrations at the end of the fast as the independent or predictor variables. A significant model emerged (F 3, 56 = 16.74, p < 0.0001. Adjusted R square = .445). To correct for multiple testing, bonferroni correction was performed. This was performed using the formula, adjusted p value = α/n (where α is the commonly accepted significance level 0.05 and n is the number of predictors in the model). Thus a *p* value of <0.007 (0.05/7) was considered significant.

Cortisol and glucose concentrations at the end of the fast emerged as the significant predictors of KB concentrations in this model.

Table 13 Results of multiple regression analysis examining the relationship of 7 predictor variables on KB concentrations at the end of the fast

Predictor variables	Beta	*p* value
Cortisol	0.355	0.002
Glucose	-0.392	<0.001
Adrenaline	0.205	0.042
Growth hormone	0.048	0.629
Age at fast	0.013	0.897
Fast duration	0.076	0.463
BMI SDS	-0.193	0.063

7.5 Discussion

Children with KH are believed to exist at one end of the normal gaussian distribution of healthy children with limited fast tolerance. The greater KB response in children with KH compared to the controls has been attributed to an earlier onset of lipolysis and ketogenesis during fasting, rather than a specific variation in KB response (Stanley et al., 2006).

Studies comparing children with KH and controls have reported KB concentrations after similar prolonged durations (15-24 hours) of fasting regardless of age (Bodamer et al., 2006, Haymond et al., 1982).

In this study KH children were compared with the control children at equivalent stages of fasting determined by their age. The results revealed that children with KH have an increased rate of KB response (an 87%/hour increase) in comparison to the control group. This is a novel finding and indicates that the greater magnitude of KB response in KH children is secondary to a quantitative difference in the rate of KB response, rather than merely an earlier onset of ketogenesis.

The mechanisms underlying the variation in the rate of response could be related to the cortisol concentrations during the fast. Cortisol concentrations were found to be a significant predictor of KB response at the end of the fast in both KH and control group. However KH children have significantly greater cortisol concentrations at the end of the fast compared to control population, suggesting that the increase in the rate of KB response in KH children is likely to be mediated by changes in cortisol concentrations.

Glucose concentrations were lower in the KH group and were also shown to be a significant predictor of KB response at the end of the fast. However this is a well known response and our data is in accordance with the literature (Saudubray et al., 1981).

KB body concentrations were positively correlated to NEFA concentrations at the end of the fast in both KH and control children; however NEFA concentrations were not positively correlated to cortisol concentrations in KH children. This may indicate that accelerated lipolysis as well as increased ketogenesis contributes to the variation in KB response in the KH group.

Another consideration is that a combination of increased ketogenesis and reduced KB utilisation may lead to the increased KB concentration in the KH group. Once ketone bodies are formed they are transported to extrahepatic tissues such as the brain, kidney, heart and skeletal muscle where uptake is facilitated via monocarboxylate transporters (MCT1, 2 and 4) (Halestrap et al., 2004) for utilisation while 10-20% are excreted by the kidneys (Owen et al., 1969).

In physiological ketotic states such as fasting, ketogenesis is proportional to ketone body utlisation and excretion (Balasse et al., 1989). This homeostasis is accomplished by the ability of KB to inhibit their own production through a negative feedback mechanism that is mediated by the antilipolytic and insulinotropic actions of ketone bodies. In pathological states KB production in excess to utilisation is invariably associated with ketoacidosis (such as diabetes mellitus and metabolic conditions secondary to ketolytic enzyme deficiencies). Ketoacidosis is not a feature of KH. Thus it may be safe to speculate that ketogenesis may play a greater part in the KB response to fast in KH although reduced capacity to utilise KB cannot be completely excluded.

Regulation of the KB response in children

In adult humans only adrenaline has been shown to effect lipolysis, mediated via β-adrenoreceptors (Bjorntorp et al., 1971). In-vitro tracer studies in children and adults, investigating the effects of various hormones on human adipocyte cells, has shown thyroid stimulating hormone an important mediator of lipolysis in the neonatal period. A gradual increase in the contribution of adrenaline to lipolysis was seen with increasing age in older children and adults (Marcus et al., 1988, Keller et al., 1989).

Adrenaline is believed to stimulate hormone-sensitive lipase mediated lipolysis, thereby increasing substrate (FFA) availability for ketogenesis. In this study cortisol concentration were found to be a significant predictor of KB response at the end of the fast when KH and control group were analysed as a continuum. When the two groups were compared, no significant relationship was seen between NEFA and cortisol concentrations at end of the fast in control or KH group and adrenaline was shown to be positively correlated to KB and NEFA concentrations at end of fast, only in the control group.

This seems to suggest that in children undergoing a fast, adrenaline and cortisol concentrations play a role in regulating ketogenesis. While the role of adrenaline may be mediated by lipolysis, the role of cortisol may well be a direct effect on hepatic ketogenesis. This is a novel finding as cortisol in vivo is not believed to play a significant role in lipolysis and ketogenesis as cortisol induced glucose increase is associated with an increase in insulin and subsequent inhibition of lipolysis. Thus during fasting in children, with the background of suppressed insulin concentrations, cortisol may be an important regulator of ketogenesis.

Plasma catecholamine concentrations during the fast and mechanism of ketotic hypoglycaemia

Fasting was associated with a significant increase in noradrenaline concentrations in the control group and KH group and a significant increase in adrenaline concentrations in the control group. In children, fasting and hypoglycaemia are known to be associated with an increase in adrenaline concentrations (Jackson et al., 2004; Candito et al., 1993).

In children with KH, the baseline adrenaline concentrations were elevated compared to the control group and a further significant increase during the fast was not observed.
The cause for the elevated adrenaline concentrations at baseline in the KH group is not clear as the demographic variables and the baseline concentrations of the various intermediary metabolites and counterregulatory hormones was not significantly different between groups. Increased physical or psychological stress can result in elevated adrenaline concentrations. Although these children were sampled at rest, contribution of psychological stress may be greater in children anticipating hypoglycaemia during the

fasting test. Stress or anxiety scores or dietary levels of sodium or caffeine that are known to augment plasma catecholamine concentrations were not recorded in this study.

As adrenaline is an important mediator of lipolysis, increased adrenaline concentrations would be expected to result in increased lipolysis and increased NEFA and KB concentrations. Although KH group were characterised by brisk lipolysis and ketogenesis, adrenaline concentrations did not show a significant relationship with NEFA or KB concentrations at the end of the fast. It is likely that the rapid onset hypoglycaemia with resultant insulin suppression plays a greater role in promoting lipolysis with an additional contribution of cortisol to ketogenesis.

A positive correlation between cortisol concentrations and KB concentrations at the end of the fast in the KH group noted in this study may lend some support to this hypothesis. No such relationship was observed between GH and KB concentrations. In addition glucagon is known to stimulate ketogenesis however this was not measured in this study.

The observation of elevated adrenaline concentrations at baseline in the KH group, differ from the literature reports. Sizonenko et al (Sizonenko et al., 1973), infused 2-deoxy-D-glucose (2-DG) to simulate hypoglycaemia and trigger counterreulatory response in 5 children with KH. In contrast to controls, infusion of 2-DG failed to elicit an increase in glucose concentrations. As hyperglycaemic response to 2-DG is especially dependent on intact adrenaline counterregulatory mechanism they concluded that children with KH have defective adrenaline production.

Koffler et al (Koffler et al., 1971) also reported defective urinary excretion of adrenaline in 5 children with KH following insulin induced hypoglycaemia or provocative ketogenic diet. However these 2 studies did not assess response to spontaneous hypoglycaemia or changes in plasma catecholamine concentrations.

Huidekoper et al (Huidekoper et al., 2008) studied the rate of endogenous glucose production, glycogenolysis and gluconeogenesis following an overnight fast and at the end of fasting tolerance test in 12 children diagnosed with KH, of who 5 became hypoglycaemic at the end of fast. Compared to normoglycaemic children, hypoglycaemic children had lower glucose production rates and lower rates of glycogenolysis without a compensatory increase in gluconeogenesis. As alanine concentrations were significantly lower at end of fast in the hypoglycaemic group, they concluded that reduced gluconeogenesis was secondary to reduced gluconeogenic precursors.

Pagliara et al (Pagliara et al., 1972) have also reported reduced alanine concentrations in KH children that decrease further during fasting and alanine infusion during hypoglycaemia in these subjects restored normoglycaemia and caused resolution of symptoms. Significantly reduced plasma alanine concentration at the beginning and at the end of the fast in the KH group was also a feature of the fasting study in this thesis.

Plasma glycerol changes during the fast

During fasting, glycerol and fatty acids are released by lipolysis. Glycerol is then taken up by the liver and kidney and converted to glycerol 3-phosphate by glycerol kinase. Following further conversion to dihydroxyacetonephosphate and glyceraldehyde 3-phosphate, it can enter gluconeogenesis.

In this study, there was a consistent increase in the serum glycerol concentrations in all the KH children during the fast. The increased response in KH group cannot be explained by lipolysis alone as, there was no evident correlation between glycerol and free fatty acid and ketone bodies. Thus the elevated glycerol concentrations could reflect a combination of increased lipolysis with decreased utilisation (gluconeogenesis) or additional contribution from other sources such as glycolysis (Jensen et al., 2001).

There was difficulty in obtaining appropriate samples for glycerol measurement. The pre-analytical sample requires perchloric acid precipitation with a fixed amount of blood, to remove proteins in the patient sample. Addition of inappropriate quantities of blood can result in an imbalance of perchlorate and hydrogen ions, and can interfere with the analytical process, leading to falsely elevated glycerol concentrations.

Due to the impact of the pre-analytical conditions on the estimated glycerol concentrations and the small sample size, the increased concentration in the KH group has to be interpreted with caution. Replication of this observation in a larger sample in a standardised manner would be required to confirm this finding.

Limitations of the fasting study

One of the limitations of this study are the control group, that are not a true healthy normal population as they represent children who were referred with suspected hypoglycaemia or had previous history of hypoglycaemia that has resolved. However ethical considerations limit the recruitment of a completely healthy population.

The fasting tests were terminated on the basis of bedside glucose monitoring. However there was no discordance with the laboratory blood glucose concentrations. Another aspect was the unequal fasting times between different age groups. While the duration of fasting may have affected the concentrations of the various intermediary metabolites and counterregulatory hormones, the results were of clinical relevance due to its association with physiological fasting times and spontaneous hypoglycaemia.

Previous studies have suggested that ketone body concentrations during fasting increases with decreasing age (Saudubray et al., 1981, Van Veen et al., 2011). In these studies children were fasted for similar time periods (15-24 hours) regardless of age and only one study looked at children less than 1 year of age (Van Veen et al., 2011). In our study children between 1-7 years of age had a greater response than children between 7-16 years of age. However children <1 year of age had an intermediate response.

This is consistent with the report by bonnefont et al (Bonnefont et al., 1990) who studied normal children in similar age categories however following a longer duration of fast (24 hours).

In contrast to previous studies, children younger than 1 year of age in this study did not demonstrate a greater KB response in comparison to older children. This could be attributed to the shorter duration of fast in this group. The length of the fasts in this study was predetermined by our diagnostic protocol (Morris et al., 1996) that was aimed at diagnosing hypoglycaemia and ascertaining fasting tolerance while minimising harmful complications. In clinical practice children <1 year of age will rarely be subjected to fasting tolerance tests longer than 12 hours.

Assessment of glucagon concentrations were not performed in this study that may have provided additional information regarding the biochemical changes in KH children.

7.6 Conclusion

This study has generated novel information regarding the biochemical changes during KH and the relationship of counterregulatory hormones to KB response.

Results of this study suggest that children with ketotic hypoglycaemia have a greater magnitude and rate of fasting ketone body response in comparison to control group. In both KH and control group, children between 1-7years of age, generated the greatest KB response in comparison to children <1 year and >7years of age, although the duration of fast was unequal between groups. Cortisol concentrations are a significant predictor of KB concentrations at the end of the fast. This suggests that cortisol regulates KB response during fast in children with a possible direct role of cortisol on hepatic ketogenesis.

Neuroimaging and cognitive profiles in children with hypoglycaemia

Patient characterisation

A brief outline of the participants in the neurocognitive and imaging studies is presented below.

Inclusion criteria: HH and KH children (as defined as per criteria given in 3.2.1 and 3.2.2). Children between 5 – 16 years of age were recruited and the scans were performed without sedation or anaesthesia

Exclusion criteria: Children with other additional metabolic and endocrine causes of hypoglycaemia, children with associated abnormalities that can increase risk of brain injury, such as diabetes, parental history of learning difficulties, and family history of epilepsy, hypoxia, cardiac problems, and syndromic forms of hyperinsulinism were excluded.

Patient recruitment: All children with HH (n=24) were recruited from the endocrine clinics. Thirteen children had previously participated in a pilot study investigating neurocognitive function in GOSH. Results showed verbal IQ to be average (mean 91.70 (range 73-114, SD 10.51)), perceptual reasoning was average (mean 93.88 (range 69-131, SD 15.61)), working memory was low average (mean 88.70 (range 68-110, SD 12.18), processing speed was low average (mean 86.95 (range 68-106, SD 11.19), general memory score was low average (mean 81.62 (range 66-103, SD 12.28)) and attention measures low average to average. As general memory scores were lower than the IQ scores, the possibility of injury to hippocampus was contemplated, that led to the investigaion of hippocampal volumes in the neuroimaging investigations.

All children with KH were identified from metabolic and endocrine outpatient lists and invited to participate if >5 years of age.

24 children with HH and 15 children with KH were recruited.

3 children with HH were excluded from study (2 children had additional diagnosis of diabetes, 1 child's parents refused consent as they did not agree with the results of the neurocognitive tests) 1 child with KH was excluded from study as there was a family history (maternal) of learning difficulties that would complicate the interpretation of neuroimaging and neurocognitive data.

Therefore data from 21 children with HH and 14 children with KH were available for analysis

The clinical details of the 21 children with HH are depicted in Table 16 and Table 17. The clinical details of the 14 children with KH are shown in Table 18.

Additional clinical characterisation of the HH group

Nine children in HH group presented within 48 hrs of life, 7 presented beyond the neonatal period (0.16 -0.59 years) and 4 presented between 1-4 years of life. In 6 children parents report a delay in diagnosis (0.15 -2.4 years).

While most of the HH patients (n=15) were managed medically, 6 required surgical intervention (near total or subtotal pancreatectomy). Based on PET scan or histology reports post-surgery, 5 children were found to have focal lesions, 3 diffuse and 1 atypical multifocal. Hyperinsulinism resolved in 8 patients (4 on medical treatment, 4 following surgery).

Eleven children have a genetic diagnosis underlying their hyperinsulinism – 5 have mutations in *ABCC8*, 2 in *KCNJ11*, 2 in *HADH* and 2 in *GLUD1).* 5 children developed epilepsy (1 with *HADH* mutation and 2 with *GLUD1* mutation). 1 child had infantile spasms treated with vigabatrine and lamotrigine; seizures have now evolved into focal epilepsy with secondary generalisation that is well controlled on levetiracetam.

1 child with *HADH* mutation has absence seizures and generalised tonic clonic seizures treated with levetiracetam, 1 child with *GLUD1* mutation has absence seizures responsive to lamotrigine and levetiracetam. Another child with *GLUD1* mutation also has generalised epilepsy treated with valproate. 1 child developed generalised seizures (treated with sodium valproate) after resolution of hyperinsulinism, following an episode of meningitis.

Neurological examination and school support in HH and KH: Neurological findings were normal in all in the KH group and all but one (patient 4, Table 16 had broad based gait that has improved over time) in the HH group. At the time of assessment all the children (HH and KH) were in mainstream schools although 3 children in HH group (patient 2, 5 and 8 in Table 16) and 2 children in KH group (patient 2 and 7 in Table 18) require support with learning. However, as a result of the assessment findings, some children then became eligible for additional funding as evidence for subtle difficulties emerged.

8 Conventional Neuroimaging

8.1 Introduction

This chapter outlines the gross structural changes in the brain of HH and KH children, using conventional neuroradiological assessments (qualitative). Suceeding chapters will discuss the application of objective quantitative MR methods to analyse the hippocampus and the white matter. The quantitative approaches enable detection of subtle abnormalities that are not seen on conventional magnetic resonance imaging. The quantitative approaches also offer the possibility of correlating structural abnormality with neurocognitive functioning.

8.2 Principles of MRI

MRI signals of human tissues are predominantly from water and fat, which have abundant hydrogen atoms. The positively charged protons in hydrogen nuclei spin about their axis producing a small magnetic field. MRI is based on the interaction between an applied magnetic field and the magnetic spin properties of the hydrogen atoms in body tissues.

In the absence of a magnetic field the spinning protons are randomly oriented. But in the presence of an external magnetic field (B_0), the hydrogen protons predominantly align along the direction of the field, so that the net magnetisation vector is along the direction of the magnetic field.

The protons in a magnetic field precess (oscillate) at a frequency that is proportional to the magnitude of the field. When a radiofrequency pulse (RF) is applied at the same frequency as the precessing protons, a phenomenon known as resonance occurs, whereby the magnetisation of the protons tilts towards the plane perpendicular to B_0. A 90^0 RF pulse flips the net magnetisation vector into the plane perpendicular to B_0.

Once the RF pulse is switched off, the protons continue to precess for a while at their resonance frequency, producing a detectable MR signal, and they return to their original energy state along the direction of the magnetic field.

The return of magnetisation to its original equilibrium can be described by two relaxation times T1 and T2. T1 denotes the longitudinal relaxation or recovery along the direction of B_0, while T2 denotes transverse relaxation or recovery perpendicular to B_0.

The T1 and T2 relaxation times vary between different tissues (T1 CSF> WM > GM, T2 CSF>GM & WM) and also between diseased and normal tissues. Thus in T1-weighted images, fluids (CSF) appear dark, GM (water-based tissue) is grey and WM (fat-based) tissues are bright. T1-weighted images provide good contrast between different tissues and are known as 'anatomy scans'.

In T2-weighted images fluids appear bright, while WM and GM appear grey. T2-weighted images are better for detecting pathology as abnormal collections of fluid (edema) appear brighter against normal tissue.

The predominant pattern of brain injury in hypoglycaemia (of varying underlying etiology) is bilateral cortical and subcortical injury to occipito parietal lobes (Alkalay et al., 2005). More recently greater involvement of white matter has been described (Burns et al., 2008). Most MR studies report findings following neonatal hypoglycaemia. MR studies in childhood in HH are lacking and till date, there are no literature reports of MR studies in KH children.

A more detailed discussion of MR findings in children with hypoglycaemia is given in chapter 5.1

8.3 Specific Aims :

To evaluate the presence and pattern of brain injury in HH and KH.

To ascertain if specific changes of hippocampus and white matter are discernible in children with HH or KH.

It is envisaged that children with HH will have an increased frequency of brain injury in comparison to KH group. Increased likelihood of white matter injury is also expected.

8.4 Methods:

8.4.1 Participants:

Scans from 21 children with HH and 14 children with KH were available for analysis.

Patient characteristics are presented in Table 16, Table 17, Table 18

8.4.2 Data acquisition

All the children were scanned unsedated on a 1.5 T Siemans Avanto scanner. The scan time was a total of 35 mins. The imaging sequences obtained are described in Table 14. Analysis of DTI and 3D FLASH data are described in chapter 10.

Table 14 Neuroimaging protocol

Sequence	TE (ms)	TR (ms)	Other
T2 Axial	101	4920	25 slices, 4 mm thick
T1 Coronal	13	561	25 slices, 4.5 mm thick
T2 FLAIR Coronal	115	9060	26 slices, 4.5 mm thick
T1 Sagittal	13	561	25 slices, 4 mm thick
DWI	96	2700	19 slices, 5 mm thick
3D FLASH	4.94	11	176 slices, 1 mm thick
DTI 60	81	7300	Voxel size 2.5× 2.5× 2.5 mm

FLASH= fast low angle shot, DWI = diffusion weighted imaging, FLAIR = fluid attenuated inversion recovery, DTI = diffusion tensor imaging.

8.4.3 Data analysis

The T1- weighted (coronal and sagittal) and T2-weighted (coronal and axial) images were assessed by an experienced paediatric neuroradiologist, blind to disease diagnosis. All the anatomic features were examined for abnormalities in morphology and the presence of abnormal signal intensity. Special attention was paid to the hippocampus and white matter (WM). Any WM lesions were further classified as focal or diffuse. High signal intensity changes on T2-weighted images were described as focal lesions. Unilateral or global reduction of white matter with ventricular dilation was described as diffuse lesions.

Statistical analysis: Normality of data was determined using the Shapiro-Wilk test. If the distribution was normal, independent sample t tests were used for continuous variables and the Mann-Whitney U was used if the distribution was skewed. For categorical variables Chi-Square (χ2) tests were performed.

8.5 Results

Abnormal scans were observed in both HH and KH groups. 8/21 (38%) children in the HH group and 5/14 (35.7%) children in the KH group had abnormal scans. Accordingly, the incidence of abnormal scans between HH and KH patients was not significantly different (χ2= 3.71 df=2 p=0.156). Gross structural changes of the hippocampus and the white matter were noted in both groups (Table 15)

Abnormalities of the hippocampus

The hippocampus was affected more frequently in children with HH (28.5%) compared to KH (7%), however, this did not reach significance (χ2= 2.41, df= 1, p value= 0.121). Bilaterally reduced hippocampus (n=4) was more common than unilateral reduction (n=2) in HH, while only 1 patient in KH group had hippocampal involvement (bilateral).

Abnormalities of white matter

Focal WM lesions were reported in 3/21 (14%) with HH and 2/14 (14%) children with KH. The lesions were visible in the periventricular, peritrigonal and frontal regions in the HH group and peritrigonal and parietal regions in KH group.

Diffuse WM lesions were seen in 7/21 (33%) in HH and 4/14 (28.5%) of the KH group. Mild global reduction of WM and unilateral (right) WM reduction were seen in both groups. Moderate and severe global WM reduction was seen only in HH group.

Of the children with MRI abnormalities, the majority in HH group had involvement of hippocampus and white matter, while the majority in KH group had involvement of WM only.

Table 15 Gross MR changes in children with HH and KH

Abnormality on scan	HH (n=21)	KH (n=14)
B/L small hippocampus	4	1
Small Right hippocampus	1	0
Small Left hippocampus	1	0
Bilateral periventricular WM lesions	1	0
Bilateral peritrigonal WM lesions	1	1
Bilateral parietal WM lesions	0	1
Right focal frontal WM lesion	1	0
Mild global reduction in WM	4	2
Moderate global reduction in WM	1	0
Severe global reduction in WM	1	0
Reduced WM right hemisphere	1	2

Relationship between abnormal scans and clinical features

The presence of an abnormal scan in children with HH, was not related to age at diagnosis (t= 0.33; p= 0.74), age at scan (t=1.64; p=0.11), birth weight (t=1.22, p=0.27), gestational age (p= 0.238), presence of mutation (p=0.86), treatment type (p=0.60), histology (p=0.51), disease course (p=0.60), seizures at diagnosis (p=0.24), ongoing epilepsy (p=0.28) or delay in diagnosis (p=0.056) (Table 16,Table 17)

In children with KH, the presence of an abnormal scan was not related to age at imaging, age at diagnosis, birth weight or gestational age (Table 18)

Other abnormal findings

1 child in HH group had mild cerebellar tonsillar descent, while 1 child in KH group had evidence of mild cerebellar atrophy. These were reported as incidental findings by the neuroradiologist.

8.6 Discussion

Incidence of abnormal scans in HH and KH

In this study, abnormal MRI scans were noted in 38% of children in HH group and 35.7% of the children in the KH group. There was no significant difference between groups in the incidence of abnormal scans, type or distribution of lesions.

The incidence of abnormal scans in children with HH or KH is not well known. This study suggests that more than a quarter of children with HH or KH are likely to have radiological evidence of brain injury.This study did not contain children with gross motor abnormalities or visual impairment in the HH group. It is likely that inclusion of such children would be associated with a higher frequency of radiological abnormalities.

It is important to note that a likely selection bias (parents with concerns about neurocognition in HH and KH group were more likely to participate) might have contributed to the high frquency of abnormal MRI scans in both groups. Observer bias is another likely contributor, and focus on hippocampus and WM could have increased the likelihood of detecting abnormalities in these regions. Ideally interobserver and intraobserver variability needs to be estimated, to determine the reliability of these results. However due to time restraints and clinical commitments, it was difficult to estimate the strength of the interobserver and intraobserver variability. Qualitative radiological reporting in this chapter will be followed by quantitative examination of the hippocampus and WM. If abnormalities of hippocampus and WM are detected in the quantitative methods, this will lend credence to the conclusions of the qualitative reporting.

Contrary to traditional perception, this study found a high incidence of brain injury (BI) in children with KH. This seems to suggest that ketotic hypoglycaemia is not necessarily neuroprotective. Neverthless, due to the possibility of selection and observer bias, larger groups of KH children recruited from District General Hospitals need to be investigated, to confirm these findings.

It is important to note that KH is a diagnosis of exclusion and although the KH children in this group have a typical clinical history, they might represent children with subtle as yet undiagnosed metabolic defects that can contribute to these MRI findings.

Pattern of abnormality on scan

This is the only report of MR findings in a homogenous group of children with HH and KH. In this study specific attention was paid to the hippocampus and white matter that may have increased the likelihood of detecting abnormalities in these regions.

Occipito-parietal cortical and subcortical involvement following neonatal hypoglycaemia has been supported by both MRI (Alkalay et al., 2005) and autopsy findings (Anderson et al., 1967) following neonatal hypoglycaemia. This typical pattern was not observed in our study group. It is likely that these reports, with similar results, reflect the type of patient's selected (prolonged severe hypoglycaemia with seizures and encephalopathy in the neonatal period).

The predominance of white matter injury noted in this study is consistent with the recent report by Burns et al (Burns et al., 2008). They looked at neurodevelopmental sequelae in 35 term neonates following symptomatic hypoglycaemia. MRI scans were performed within six weeks of life. The severity and duration of hypoglycaemia was not associated with specific distinguishing features on the MRI scan. They reported WM abnormalities in 94% of children (39% global, 36% periventricular), only 29% demonstrated a posterior pattern of brain injury.

The etiology and duration of hypoglycaemia in the children participating in the Burns study was diverse (transient, prolonged, metabolic and endocrine). This complicates the determination of the contribution of the underlying etiology and its associated biochemical milieu on the susceptibility to hypoglycaemic brain injury. Involvement of basal ganglia and posterior limb of internal capsule, ischaemic and haemorrhagic lesions commonly seen in perinatal hypoxia and not previously described in hypoglycaemia have also been reported by Burns et al (Burns et al., 2008). As 43% of the children in the Burns study required minor or major resuscitation at birth. it questions the possibility of associated comorbid condition such as perinatal hypoxia contributing to the brain injury.

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Periventricular white matter (PVM) injury has been reported frequently in children born preterm and children with Hypoxic Ischaemic Encephalopathy (HIE). The PVM is a watershed zone (boundary of major arteries) and susceptible to pertubations in perfusion. Thus it is surprising that PVM injury is seen in 1 HH patient in this study. However, neuropathological reports by Anderson et al (Anderson et al.,1967) has described periventricular leucomalacia in one of the three infants who died following profound untreated neonatal hypoglycaemia.

The underlying mechanisms causing PVM damage in premature and mature infant has been attributed to ischaemia alone or when combined with inflammation, which damages the premyelinating oligodendrocytes, by excitotoxic or free radical injury (Khwaja & Volpe et al., 2008).

Co-morbid conditions

Co- morbid conditions such as seizures and hypoxia can potentiate HBI (Basu et al., 2009; Yager et al., 2002). Seizures at presentation are common in children with HH and KH. Three HH children with abnormal scans have ongoing epilepsy, while 2 HH children with normal scans have ongoing epilepsy. 1 child with a low birth weight (1800 g) had mild global reduction of white matter. Children born SGA are reported to have white matter reduction and corpus callosum thinning on conventional MRI (Skranes et al., 2005), hence WM changes in these children cannot be attributed to hypoglycaemia alone.

Clinical details were collected retrospectively in this study. Although none of the children in this study suffered from postnatal or perinatal hypoxia, sepsis was quite common in children admitted for prolonged duration and with central venous access.

Relationship between mutations in HH children and abnormal scans

HH is a genetically heterogeneous disorder. Apart from the hypoglycaemia and the lack of alternate fuels, there is a concern that the underlying genetic causes of hyperinsulinism, may contribute to neurodevelopmental impairment.

This is certainly true of children with *GLUD1* mutations, who are at a high risk of epilepsy and developmental delay (Bahi-Buisson et al., 2008) although MRI scans have been reported to be normal (Bahi-Buisson et al., 2008). The cause of brain injury has been attributed to a combination of recurrent hypoglycaemia, hyperammonemia and reduced concentrations of neurotransmitters glutamine and GABA (gamma-aminobutyric acid) due to increased GDH activity in brain.
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In keeping with the literature, both children with *GLUD1* mutations in this study have epilepsy (generalised). Serum ammonia concentrations in 1 patient was 133 and 150 (patients 2 and 3 in Table 17) in another. Both these children display bilateral small hippocampus and global reduction of white matter. This study suggests that children with *GLUD1* mutations are more likely to have widespread radiological evidence of brain injury.

Several regions of the brain express high levels of Kir 6.2 mRNA (Dunn-Meynell et al., 1998) and developmental delay, dyspraxia and epilepsy have been associated with *KCNJ11* and *ABCC8* mutations in neonatal diabetes (Gloyn et al., 2006).

In this study 2 children with K_{ATP} channel mutations (1 KCNJ11, 1 ABCC8) had abnormal MRI, while 4 children with K_{ATP} channel mutations (1 KCNJ11, 3 ABCC8) have normal MRI. Thus the presence of K_{ATP} mutation did not increase the likelihood of abnormal scan in this study, although the the small sample size limits the interpretation of these findings.

Relationship between abnormal scans and clinical features/presentation

In children with HH, there is evidence suggesting poor neurodevelopmental outcome in children with neonatal onset (Menni et al., 2001; Steinkrauss et al., 2005) disease, persistence of hypoglycaemia beyond 35 days (Jack et al., 2003) and medically unresponsive patients requiring surgery (Steinkrauss et al., 2005).

This study failed to find any significant difference in the clinical features between children with abnormal and normal scans, in both HH and KH group , possibly due to sample size.

Other abnormalities

In this study 2 children were reported to have incidental findings. 1 HH child had mild tonsillar descent (no other abnormalities on scan) and 1 child with KH had cerebellar atrophy (small bilateral WM lesion in parietal region also noted). Neither of the 2 children have any gross neurological symptoms of concern.

Mild descent of the cerebellar tonsils can be present without neurological symptoms. Kim et al (Kim et al., 2002) reviewed findings in 225 healthy children (age 1 month – 18 years) participating in neuroimaging research and reported incidental findings in 21%. Tonsillar descent was seen in 1/225 patients. In this patient, the mild tonsillar descent may be an incidental finding, however in the absence of premorbid neuroimaging data, it is difficult to ascertain this with certainty.

Hypoglycaemia is thought to spare the cerebellum (Auer et al., 1989). A greater concentration of vascular transport sites for glucose was thought to attribute to this resistance to hypoglycaemia. In addition, animal studies have shown unchanged or slightly reduced local cerebral glucose utilisation in the cerebellum compared to cerebral cortex, during severe hypoglycaemia (Abdul-Rahman et al., 1980).

Burns et al (Burns et al., 2008) reported involvement of cerebellum in their cohort with neonatal hypoglycaemia, scanned < 6 weeks postnatal age. The main findings were increased signal intensity in one child and haemorrhage in another. The clinical features of this child are typical of any with KH and based on evidence mentioned above, it is debatable if the cerebellar atrophy in this child could be attributed to hypoglycaemia

8.7 Conclusion

Conventional MRI has shown abnormalities in both children with KH and HH suggestive of HBI. While children with HH are known to be at high risk of brain injury, this is a completely new and unexpected finding in children with KH. These findings question the perception that the presence of ketone bodies during hypoglycaemia confers protection against HBI.

Furthermore, this study suggests that HBI is not restricted to the occipito parietal lobes. The focal and diffuse WM abnormalities found in this study are consistent with recent evidence of varied WM involvement following symptomatic neonatal hypoglycaemia.

Succeeding chapters aim to probe further into the hippocampal and white matter abnormalities using quantitative MRI.

Table 16 Comparison of clinical features in HH children with normal and abnormal scans

Patient no	Abnormality on neuroimaging	Gender	Age at scan in yrs	Age at diagnosis in yrs	Birthweight in grams	Gestational age in weeks
1	B/L small hipocampus. Small periventricular WM scars. Severe global reduction in WM	M	7.9	0.04	4173	41
2	B/L small hippocampus. B/L peritrigonal WM changes. Mild global reduction in WM	F	7.31	0.37	3636	39
3	B/L small hippocampus.Moderate global reduction in WM	M	5.63	0.16	3960	40
4	Bilaterally small hippocampus. Mild global reduction in WM	M	7.46	0.25	4200	41
5	Left small hippocampus. Small right frontal WM scars	M	12.68	1.6	2860	36
6	Mild global reduction in WM	M	8.71	0.01	3880	36
7	Mild global reduction in WM	M	13.28	1	1800	32
8	Small right hippocampus, reduced wm r hemisphere	M	8.79	0.04	4290	40
9	Normal	F	9.89	0.59	4160	40
10	Normal	F	7.13	0.01	3990	40

11	Normal	M	11	0.58	3500	42
12	Normal	M	14.13	0.26	3600	40
13	Normal	F	9.41	1	4500	41
14	Normal	F	8.18	0.36	3450	40
15	Normal	M	11.27	0.01	4602	40
16	Normal	F	7.22	0.01	4160	40
17	Normal	F	13.56	0.44	3100	38
18	Normal	M	10.08	0.01	6100	39
19	Normal	F	11.31	0.01	3000	40
20	Normal	M	14.04	4	3340	40
21	Normal	M	14.47	0.01	5100	41
	HH with abnormal MR	**Mean**	**8.9**	**0.42**	**3599.8**	**40**
		SD	**2.6**	**0.57**	**858.6**	
	HH with normal MR	**Mean**	**10.8**	**0.56**	**4046.3**	**39.5**
		SD	**2.5**	**1.1**	**876.2**	
	Comparison between HH with normal and abnormal MR	**t test**	**1.64**	**0.33**	**1.12**	
		p value	**0.11**	**0.74**	**0.27**	**0.238**

Table 17 Continuation of comparison of clinical features in HH children with normal and abnormal scans

Patient no	Abnormality on MR Imaging	Mutation	Treatment	Histology	Disease course	seizures at diagnosis	Ongoing epilepsy	Delay in diagnosis
1	B/L small hipocampus. Small periventricular WM scars. Severe global reduction in WM	no	Surg	Focal	resolved	*no*	no	yes
2	B/L small hippocampus. B/L peritrigonal WM changes. Mild global reduction in WM	*GLUD1*	Med	NK	ongoing	*yes*	yes	yes
3	B/L small hippocampus.Moderate global reduction in WM	*GLUD1*	Med	NK	ongoing	*no*	yes	yes
4	Bilaterally small hippocampus. Mild global reduction in WM	no	Med	NK	ongoing	*yes*	no	yes
5	Left small hippocampus. Small right frontal WM scars	no	Surg	NK	ongoing	*yes*	no	no

6	Mild global reduction in WM	KCNJ11	Surg	Focal	resolved	*no*	no	no
7	Mild global reduction in WM	no	Med	NK	resolved	*yes*	yes	yes
8	Small right hippocampus, reduced wm right hemisphere	*ABCC8*	Med	Focal	resolved	*yes*	no	no
9	Normal	no	Med	NK	resolved	*yes*	no	no
10	Normal	no	Med	NK	ongoing	*yes*	yes	no
11	Normal	no	Med	NK	ongoing	*yes*	no	no
12	Normal	*ABCC8*	Med	Focal	resolved	*no*	no	yes
13	Normal	no	Surg	Diffuse	resolved	*yes*	no	no
14	Normal	*HADH*	Med	NK	ongoing	*yes*	no	no
15	Normal	*KCNJ11*	Surg	Focal	resolved	*no*	no	no
16	Normal	*ABCC8*	Med	NK	resolved	*no*	no	no
17	Normal	*HADH*	Med	NK	ongoing	*no*	yes	no
18	Normal	*ABCC8*	Med	NK	ongoing	*no*	no	no
19	Normal	no	Med	NK	ongoing	*no*	no	no

20	Normal	no	Med	NK	ongoing	*no*	no	yes
21	Normal	*ABCC8*	Surg	atypical	ongoing	*no*	no	no
	ChiSquare	*0.29*	*1.01*	*2.28*	*0.27*	*1.14*	*1.33*	*4.9*
	df	*1*	*2*	*3*	*1*	*1*	*1*	*1*
	p value	*0.86*	*0.6*	*0.51*	*0.6*	*0.28*	*0.24*	*0.056*

Table 18 Comparison of clinical features in KH children with normal and abnormal scans

Patient no	Abnormality on MR imaging	Gender	Age at scan	Age at diagnosis (in yrs)	Birth weight (in grams)	disease course	seizures at diagnosis
1	Reduced WM right hemisphere	M	12.08	1.92	3050	resolved	no
2	Mild global reduction in WM	F	8.07	1.54	3818	resolved	no
3	Mild global reduction in WM. B/L small hippocampus. Peritrigonal WM lesions	F	11.55	2.4	3946	resolved	no
4	Reduced WM right hemisphere	M	10.4	1.19	2860	resolved	no
5	B/L parietal WM lesions. Cerebellar atrophy	M	6.09	2.5	4270	resolved	yes
6	Normal	M	8.75	1.6	3270	resolved	no
7	Normal	F	6.04	1	2680	resolved	yes
8	Normal	M	9.02	3	3493	ongoing	no
9	Normal	M	5.2	1.36	4080	ongoing	yes
10	Normal	M	8.5	1.54	1849	ongoing	no
11	Normal	M	10.94	7	2700	ongoing	no
12	Normal	M	7.53	2	3266	ongoing	no
13	Normal	F	7.45	5	2840	ongoing	no
14	Normal	F	11	2.5	4600	resolved	no
	KH with abnormal MR	*Mean*	*9.6*	*1.91*	*3588.8*		
		SD	*2.5*	*0.5*	*605.3*		
	KH with normal MR	*Mean*	*8.2*	*2.7*	*3197.5*		

	SD	1.9	1.9	814.2
Comparison between KH with normal and abnormal MR	t test	-1.13	0.94	-0.93
	p value	0.28	0.36	0.36

Figure 16 Patient 1- T2- weighted MRI in transverse plane. Severe global WM reduction with ventricular dilation (arrow)
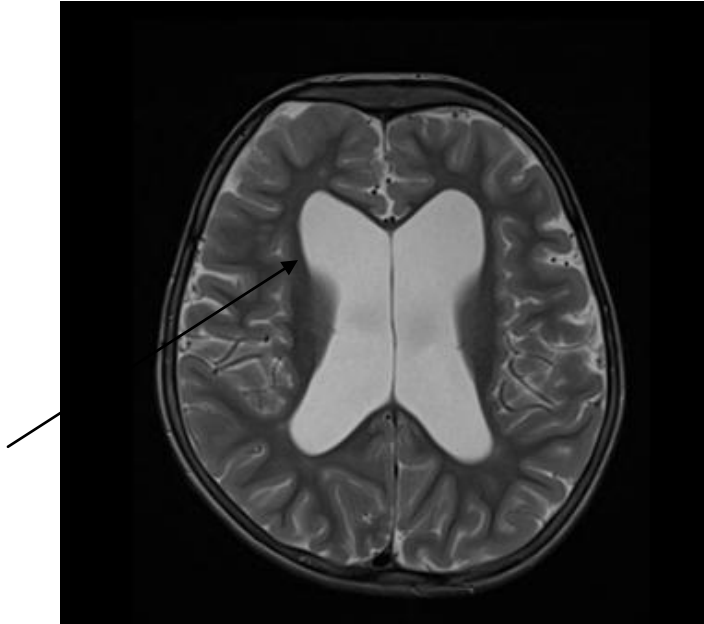
Figure 17 Patient 2- T2-weighted MRI axial plane. B/L peritrigonal WM lesions (arrows)
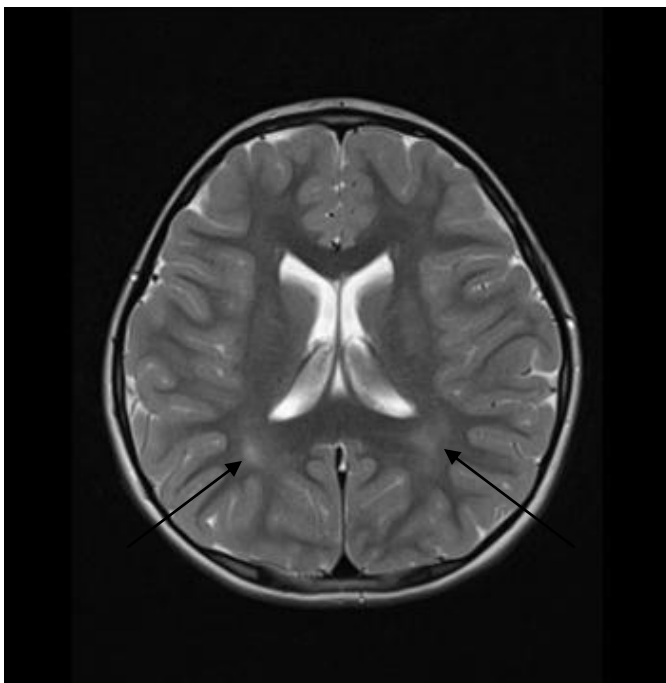
Figure 18 Patient 3- T2- weighted MRI in transverse plane. Moderate global WM reduction.
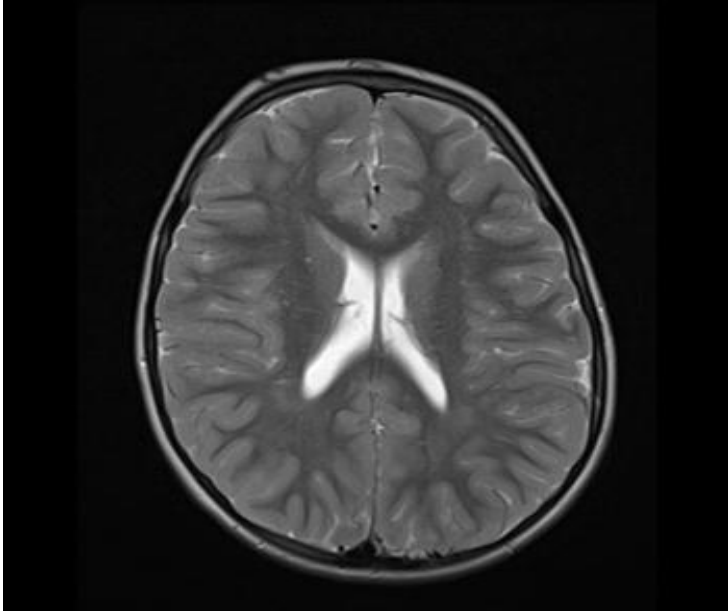
Figure 19 Patient 5 - FLAIR sequence coronal plane. Right frontal WM scar (arrow)
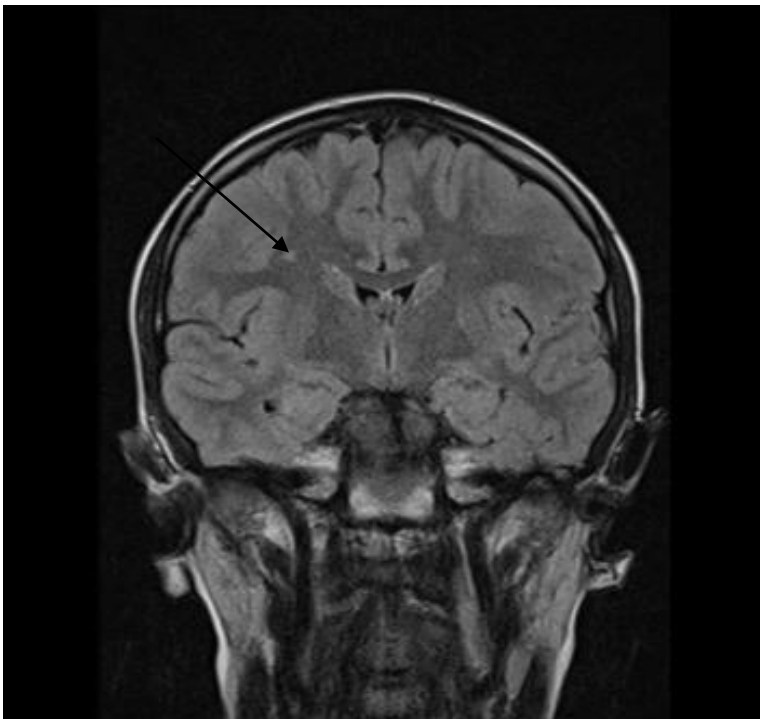
9 Neuropsychological profile of children with HH and KH

This chapter describes the neuropsychological outcomes in children with HH and KH. A battery of age appropriate tests was used to assess cognitive domains such as intelligence, memory and attention. Academic attainment and motor skills were also assessed. Emotion and behaviour were assessed using standardised questionnaires.

9.1 Introduction

A detailed overview of the neurocognitive outcomes in hypoglycaemia and HH are presented in 5.3. A brief summary is presented below.

In children with HH a high incidence of neurocognitive impairment has been reported as these children experience persistent and recurrent hypoglycaemic episodes. In addition the inappropriate secretion of insulin during hypoglycaemia, inhibits lipolysis and ketogenesis, depriving the brain of alternate fuels in the form of ketone bodies. Two large studies (n=90 and 114) have reported impaired neurodevelopment in 26-44% of patients with HH (Menni et al., 2001; Meissner et al., 2003). While developmental problems such as cerebral palsy, epilepsy, and visual disturbances have been commonly reported, it is not clear if subtle cognitive and motor deficits exist in the absence of overt neurological impairment.

In contrast children with KH have alternate fuels (KB) during hypoglycaemia and are believed to be neurologically protected. However, reports of neurocognitive outcomes in children with ketotic hypoglycaemia are few and show conflicting results. Kogut et al (Kogut et al., 1969) reported an IQ <80 in three out of thirteen children with ketotic hypoglycaemia, while Colle et al (Colle et al., 1964) reported normal intellectual outcomes in 30 children described with ketotic hypoglycaemia. Some authors (Pershad et al., 1998) even suggest that an evidence of developmental delay would exclude the diagnosis of KH.

A more recent study by Daly et al (Daly et al., 2003), reported developmental delay in two of the twenty-four children described with KH. However these reports were based on retrospective review of case notes that can be susceptible to subjective bias and did not include or relate neurological outcome to neuroimaging findings. Thus, based on available evidence it is not clear if recurrent episodes of hypoglycaemia in children with KH can result in brain injury and subtle neurocognitive deficits.

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9.2 Specific aims and predictions:

To describe the neuropsychological outcomes in HH and KH.

To ascertain if children with HH have impaired outcome relative to children with KH.

It is expected that children with KH will perform similar to standard population and HH children will be impaired in some aspects of cognition such as IQ and attention (Rother et al., 2001).

9.3 Methods:

9.3.1 Participants:

Data was collected from 21 children with HH and 14 children with KH. One child (patient 11, Table 16) in the HH group was included only in the analyses of IQ and academic attainment as memory and attention tests were not completed. Another child (patient 2, Table 16) in HH group scored very low in all cognitive domains, and excluded from all analysis as an outlier. Finally data from 20 children with HH and 14 with KH were analysed.

9.3.2 Neuropsychological protocols

All the tests were administered by a Clinical Psychologist or by an Assistant Psychologist under supervision and scored in accordance with the appropriate test manual. An overview of the assessments and questionnaires used are presented below. I have observed the performance of these assessments and understand the methodology and principles underlying them.

A detailed description of the test components are presented in Table 36.

1 – Intelligence

Wechsler Intelligence Scale for Children- Fourth UK Edition (WISC-IV UK); (Wechsler et al., 2003)

The Wechsler Intelligence Scale for Children – Fourth Edition (WISC-IV) is a standardised assessment of general cognitive ability for children between the ages of 6 to 17 years old. The WISC-IV comprises 10 core subtests and 5 supplemental subtests, which measure different aspects of intellectual functioning, such as Verbal Comprehension, Perceptual Reasoning, Working Memory and Processing Speed.

These subtests are marked using scaled scores, which allow a comparison of individual children against a large group of children of the same age. Administration time can range from 60 to 80 minutes. Only the 10 core subtests were administered.

3 – Attention

Tests of everyday attention in children (TEA-ch); (MANLY et al., 1998)

This test provides multidimensional assessment of attention in children aged 6-16 years. It consists of 9 subtests, of which the initial 4 were administered (recommended as an inital screen for attention difficulties). The subtests provide information on selective attention, sustained attention and attentional control/switching and take approximately 20 minutes to administer.

4 – Memory

The Children's Memory Scale (CMS);(Cohen et al., 1997)

This assessment is designed to evaluate learning and memory functioning in children 5 to 16 years old. It specifically assesses three main areas involved in learning and memory; visual and verbal memory, immediate and delayed memory, and attention/concentration. It contains 6 core subtests and 4 supplemental subtests. Core subtests and one supplemental subtest (Word Pairs) were administered (time taken 40-45 minutes).

5 – Academic Attainment

Wechsler Individual Achievement Test – Second UK Edition (WIAT-II UK); (Wechsler et al., 2002)

This assessment is a test of language, numerical and reading abilities for children aged 4 to 16 years 11 months. Full administration time takes approximately 45 minutes for children aged 4 to 5 years old, and 90 minutes for children 6 to 11 years old. Four of nine subtests administered. These include Word Reading, Numerical Operations, Mathematical Reasoning and Spelling.

6 – Motor

Movement ABC -2 (MABC -2); (Henderson S & Sugden D et al., 1992)

This assessment evaluates fine and gross motor skills. It contains 8 tasks that cover 3 areas, namely manual dexterity, aiming and catching and balance, for children aged 3-16 years. Number of correct responses along with qualitative description of act noted. It requires 30 minutes to administer.

7 – Emotional and Behavioural Function

Strengths & Difficulties Questionnaire (SDQ); (Goodman et al., 1997)

This 25-item questionnaire screens for emotional and behavioural problems in children. Items are grouped onto 5 sub-scales; prosocial, hyperactivity, emotional, conduct problems, and peer problems. This scale has parent, child and teacher versions.

The information provided is used to predict the possibility of emotional, behavioural and concentration problems (for each diagnosis 3 possible predictions given – low risk, medium risk, high risk). Validity and reliability has been established and this instrument is a widely used clinical tool. Self-report measure is only used for children over 10 years old. This is a brief measure which takes 10 minutes to complete.

The Paediatric Quality of Life Inventory (Peds-QL); (Varni et al., 2003)

This 23-item questionnaire examines the health related quality of life of children and adolescents. The inventory comprises four core scales; physical functioning, emotional functioning, social functioning, school functioning. Psychosocial health summary score is then computed as a mean of emotional, social and school functioning scales and total scaled scores are derived from all four core scales.

Scores range from 0-100. Higher scores indicate better quality of life. Parent reports are used for children 2 to 18 years old and the child/adolescent report covers 5 to 18 years of age. Validity and reliability have been established. It is estimated that the Peds-QL takes around 5 minutes to administer.

Conners 3; (Conners et al., 2008)

This is a 108-item multi-informant questionnaire with parent; teacher (6-18 years) and self- report (8-18 years) versions. It is a well validated tool used for identification of Attention- Deficit Hyperactivity Disorder (ADHD) and other related problems such as Oppositional Defiant Disorder (ODD) and Conduct Disorder (CD) that can impact on learning and behaviour.

Global index score and separate scores for inattention, hyperactivity/impulsivity, learning problems, executive functioning, and aggression and peer relations can be calculated. Scores above 60 are considered high average and scores above 65 as elevated DSM-IV symptom counts can also be computed for ADHD, ODD and CD to see if child has enough symptoms of that disorder to consider a diagnosis.

<u>Social Communication Questionnaire (SCQ); (Rutter, Bailey, & Lord et al., 2003)</u>

This 40-item questionnaire (requires yes or no responses) helps assess communication skills and social functioning. It is often used as an initial screen for autistic spectrum disorders (ASD). It is a parent report questionnaire, for children >4 years and requires 10 minutes to complete. Scores range from 0-39. Validity and reliability have been established. Scores >15 are suggestive of social and communication disorders like ASD.

9.3.3 Data Acquisition

Overall, depending on the chronological age of the child, their developmental level, ability to attend and motivation factors, the full assessment battery could take 8-10 hours. Often the assessment is broken down over a couple of sessions over 2 days to ensure the child is able to perform to the best of his or her ability.

Thirteen patients with HH were assessed by an Assistant Psychologist under supervision (Jessica Jackson) and more recent data from 8 HH patients were collected as part of a clinical protocol for children with HH by the team's Clinical Psychologist (Jemima Bullock). Patients with KH were assessed by 2 Assistant Psychologists under supervision (Lisa Walker and Holly Clisby). The psychologists were not blinded to the underlying diagnosis.

Data Analysis

Statistical analysis: Normality of data was determined using Shapiro-Wilk test. If distribution was normal, independent sample t-tests were used for continuous variables, and Mann-Whitney U was used as a non-parametric test for ranked data. For contingency tables Chi-Square (χ2) tests or Fishers exact tests were performed.

9.4 Results

Baseline characteristics of patients

No significant differences between groups were found in the baseline characteristics of age at test, birth weight, gestational age, gender, handedness or number of patients with seizures at diagnosis (Table 19). Children with HH presented (or were diagnosed) at a significantly younger age relative to KH group (0.16 vs 1.9 yrs).

Table 19 Comparison of baseline characteristics between HH and KH groups

Patient characteristics	HH	KH	p-value
Number of patients	20	14	
Age at test in years (mean ± SEM)	9 (0.4)	8.8 (0.5)	0.802
Age at diagnosis in years (mean)	0.16	1.9	<0.001
Gender (Male/female)	13M/8F	9M/5F	1.000
Birth weight in grams (mean ± SEM)	3876.2 (191.2)	3337.2 (199.7)	0.068
Gestational age in weeks (median)	40	39	0.145
Handedness (Right/Left)	16R/5L	11R/3L	1.000
Seizures at diagnosis (patient no)	10	3	0.162

There was no significant difference between socioeconomic status (p = 0.642, Fisher's exact test (FET)) or maternal education (p = 0.609, FET) or paternal education (p = 0.104, FET) between the HH and KH groups.

Psychological test scores

Standard scores have a mean of 100 and a Standard deviation of 15. The scores are qualitatively interpreted according to Table 20.

Table 20 Qualitative Interpretation of neurocognitive scores

Scores	Classification
130 and above	Very Superior
120–129	Superior
110–119	High Average
90–109	Average
80–89	Low Average
70–79	Borderline
69 and below	Extremely Low

Intelligence

Comparison of test scores of patient groups with the standard population means

The mean scores in the HH group differed significantly from those of the standard population mean on all the subtests of the intelligence scale. Performance in Verbal Comprehension (VC), Perceptual Reasoning (PR) and Working Memory (WM) was in the average range, and in the low average range in Processing Speed (PS) and Full Scale IQ (FSIQ) (see Table 21**)**.

The mean scores of KH group did not differ significantly from the standard population means

Table 21 WISC IV: IQ and index scores in HH and KH

	WISC IV	Mean (Range)	SEM	test	p value
HH	Verbal Comprehension	92.5 (75-119)	2.5	-3.0	0.007
n=19	Perceptual Reasoning	91.9 (69-117)	2.7	-2.9	0.008
	Working Memory	91.8 (68-116)	2.8	-2.9	0.009
	Processing Speed	87.2 (59-115)	3.2	-3.8	0.001
	Full Scale IQ	89.3 (70-112)	2.5	-4.1	0.001
KH	Verbal Comprehension	101.2 (73-130)	3.9	0.3	0.748
n=14	Perceptual Reasoning	105.8 (88-127)	4	1.4	0.174
	Working Memory	97.5 (62-126)	4.8	-0.5	0.614
	Processing Speed	93.7 (68-121)	4.1	-1.5	0.156
	Full Scale IQ	100.5 (77-127)	4.5	0.1	0.914

Comparison between KH and HH groups: Perceptual reasoning (p = 0.006) and Full scale IQ (p = 0.026) was significantly lower in HH group relative to KH group (Table 22)
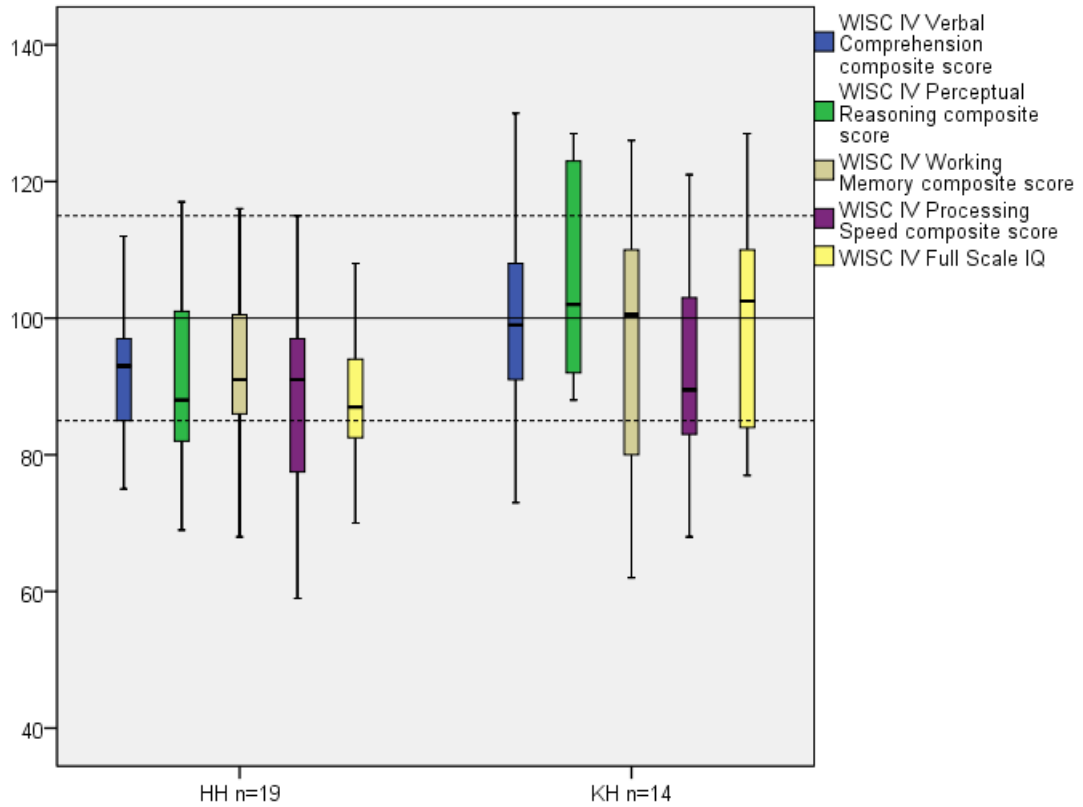
Figure 20 WISC IV: IQ and index scores in HH and KH. The scores are plotted along the y axis. The continuous horizontal reference line at score of 100 denotes the standard population mean and the interrupted horizontal reference line at scores 85 and 115 denote 1 standard deviation below and above the mean respectively. Full scale IQ and perceptual reasoning are significantly lower in the HH group.

Table 22 WISC- IV: Comparison between HH and KH

WISC - IV	HH (n=20). Mean (SEM)	KH (n=14) Mean (SEM)	t test	p value
Verbal Comprehension	92.5(2.4)	101.2 (3.9)	-1.9	0.056
Perceptual Reasoning	91.9 (2.7)	105.8 (4.0)	-2.9	0.006
Working Memory	91.8 (2.8)	97.5 (4.8)	-1	0.289
Processing Speed	87.2 (3.2)	93.7 (4.1)	-1.2	0.221
Full Scale IQ	89.3 (2.5)	100.5 (4.5)	-2.3	0.028

Academic attainment

Comparison of test scores of patient groups with the standard population means The mean scores in HH group differed significantly from those of the standard population mean on all the subtests of the academic attainment scale. Performance in word reading was in the average range and numerical operations, mathematical reasoning and spelling were in the low average range. The mean scores in KH group did not differ significantly from the standard population means (Table 23).

Table 23 WIAT II: Academic attainment in HH and KH

Group	WIAT II	Mean	SEM	t test	p value
HH n=20	Word Reading	89	3.6	-2.3	0.028
	Numerical Operations	86.8	2.4	-5.1	<0.001
	Mathematical Reasoning	83.6	4	-3.6	0.004
	Spelling standard	86	2.6	-4.4	<0.001
	Mathematics Composite	83	4	-3.7	0.004
KH n=14	Word Reading	101.7	4.1	0.4	0.674
	Numerical Operations	99.7	4.8	-0.1	0.954
	Mathematical Reasoning	100.1	4.3	0.0	0.974
	Spelling standard	99.2	4.2	-0.1	0.857
	Mathematics Composite	99.9	4.8	-0.0	0.989

SEM: Standard Error of Mean

Comparison between KH and HH: Performance of HH group was impaired in all aspects of academic attainment in comparison to KH group (Table 24, Figure 21)
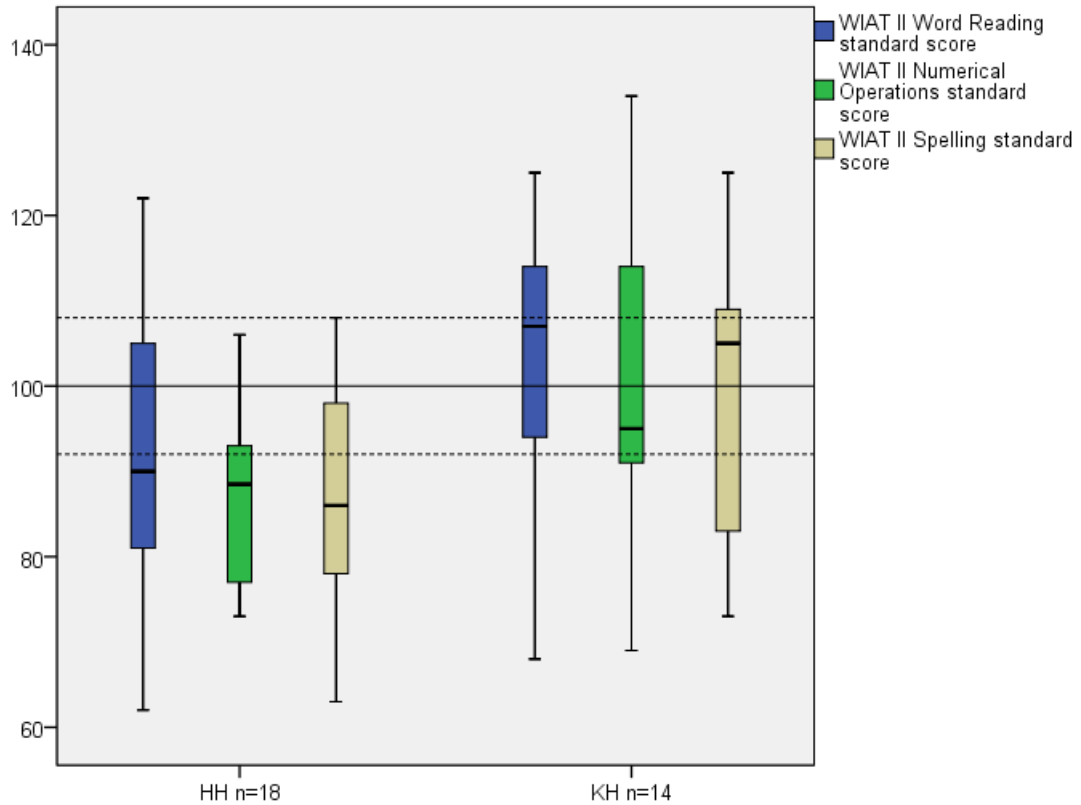
Figure 21 WIAT II: Comparison of academic attainment between HH and KH. The scores are plotted along the y axis. The continuous horizontal reference line at score of 100 denotes the standard population mean and the interrupted horizontal reference line at scores 85 and 115 denote 1 standard deviation below and above the mean respectively. HH group was impaired in all aspects of academic attainment in comparison to KH group.

Table 24 WIAT II: Comparison of academic attainment between HH and KH

WIAT II	HH (n=20). Mean (SEM)	KH (n=14) Mean (SEM)	t test HH vs KH	p value HHvs KH
Word Reading	89(3.6)	101.7 (4.1)	-2.2	0.03
Numerical Operations	86.8(2.4)	99.7 (4.8)	-2.6	0.014
Mathematical Reasoning	83.6(4)	100.1 (4.3)	-2.7	0.01
Spelling standard	86(2.6)	99 (4.2)	-2.6	0.012
Mathematics Composite	83 (4)	99(4.8)	-2.5	0.016

HH and KH group did not significantly differ in the discrepancy between their actual and predicted performance on WIAT based on their intellectual performance on WISC-IV.

Table 25 Discrepancy between actual and predicted performance on WIAT

Actual vs Predicted	Group	Word reading	Numerical operations	Spelling
Significantly worse performance	HH n=19	8	6	9
	KH n=14	3	4	3
Significantly better performance	HH n=19	2	0	1
	KH n=14	5	2	3
Chi-square test	χ^2	3.3	2.7	3.3
	p value	0.241	0.215	0.494

Attention

Comparison of test scores in patient groups with the standard population means:

HH children performed (Table 26) significantly worse in measures of sustained attention (score and sky search DT). Time taken to complete sky search (tests selective attention) was significantly longer in HH and KH children. KH children required significantly longer time to complete creature counting task (tests attention control/switching).

Table 26 TEA-Ch: Attention scores in HH and KH

Group	TEA-ch Subtests	Standard scores	Mean (range)	SEM	t	p value
HH n=19	Sky Search	Total correct	98.1 (65-130)	3.9	-0.4	0.646
		Time	92.8 (70-115)	2.8	-2.4	0.025
		Attention	96.5 (75-110)	2.4	-1.3	0.185
	Score!		89.4 (60-125)	3.7	-2.8	0.011
	Creature counting	Total correct	92.1 (65-120)	4.2	-1.8	0.081
		Time n=14	95.3 (80-120)	2.5	-1.8	0.09
	Sky search DT		81.8 (55-125)	4.5	-4.0	0.001
KH n=14	Sky Search	Total correct	107.5 (65-130)	4.6	1.6	0.133
		Time	93.9 (80-115)	2.4	-2.4	0.029
		Attention	95.7 (80-130)	3.4	-1.2	0.234
	Score!		95 (80-125)	4.1	-1.2	0.247
	Creature counting	Total correct	100 (70-135)	4.0	0	1.000
		Time n=13	91.5 (75-115)	3.1	-2.7	0.019
	Sky search DT		91.7 (55-120)	4.5	-1.8	0.095

Comparison between KH and HH: Performance of HH children was comparable to KH children in all measures of attention (Table 27, Figure 22)
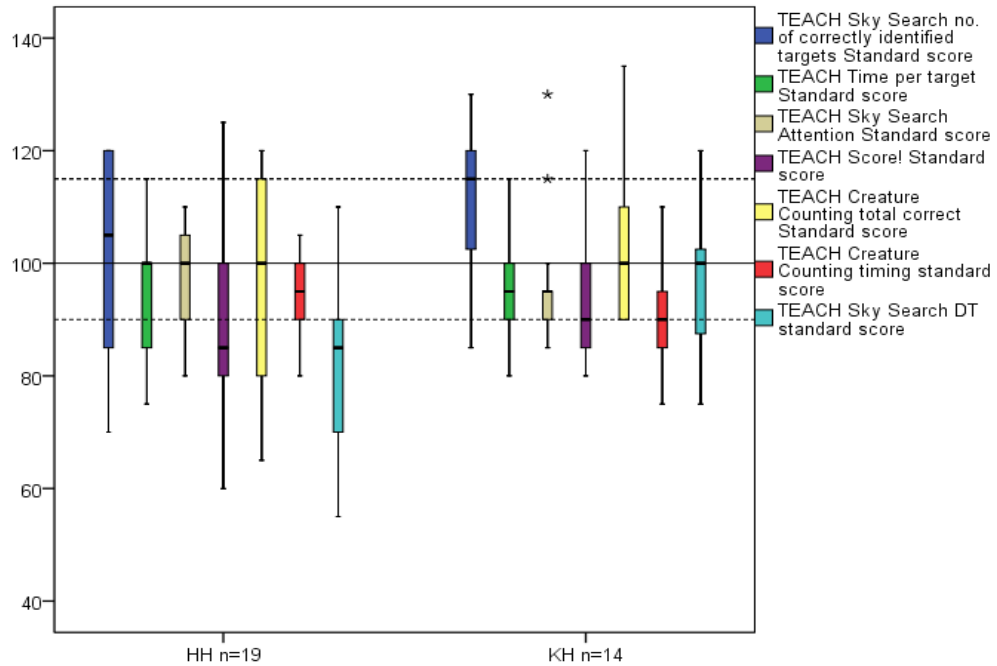
Figure 22 TEA-ch scores; Comparison between HH and KH. The scores are plotted along the y axis. The continuous horizontal reference line at score of 100 denotes the standard population mean and the interrupted horizontal reference line at scores 85 and 115 denote 1 standard deviation below and above the mean respectively. Performance of HH group was comparable to the KH group.

Table 27 TEA-Ch scores: Comparison between HH and KH

TEA-Ch Subtests	Standard scores	HH n=19	KH n=14	t test HHvsKH	p value HH vs KH
Sky Search	Total correct	98.1	107.5	-1.5	0.136
	Time per target	92.8	93.9	-0.2	0.797
	Attention	96.5	95.7	0.2	0.835
Score!	Score!	89.4	95	-0.9	0.330
Creature counting	Total correct	92.1	100	-1.3	0.230
	Timing n=14	95.3	91.5	-0.9	0.349
Sky search DT	SkySearch DT	81.8	91.7	-1.5	0.140

Memory

Comparison of test scores in patient groups with the standard population means:
The mean scores for general memory and learning were significantly lower (although within the average range) than the reference mean in HH group. The mean scores for verbal immediate, general memory, learning index and delayed recognition were significantly better than the reference mean in KH children (Table 28).

Table 28 CMS: Children Memory Scale scores in HH and KH

Group	CMS Index scores	Mean (Range)	SEM	t	p value
HH n=19	Visual Immediate	94.8 (69-122)	3.4	-1.5	0.147
	Visual Delayed	97.5 (75-118)	2.5	-0.9	0.353
	Verbal Immediate	94.9 (66-115)	3.0	-1.6	0.117
	Verbal Delayed	97.2 (69-125)	3.5	-0.7	0.455
	General Memory	93.1 (68-116)	3.1	-2.1	0.044
	Attention/Concentration	99.2 (66-122)	4.0	-0.1	0.858
	Learning index score	91.3 (60-125)	4.1	-2.1	0.049
	Delayed Recognition	101.7 (60-131)	3.8	0.4	0.646
KH n=14	Visual Immediate	106.3 (91-128)	3.0	2.0	0.059
	Visual Delayed	104.5 (88-128)	3.1	1.4	0.172
	Verbal Immediate	110.5 (72-131)	4.5	2.2	0.039
	Verbal Delayed	108.7 (75-134)	4.5	1.8	0.081
	General Memory	110.0 (77-131)	3.9	2.5	0.024
	Attention/Concentration	99.3 (88-134)	8.2	-0.08	0.939
	Learning index score	107.0 (85-125)	2.1	3.2	0.006
	Delayed Recognition	109.1 (78-131)	3.4	2.6	0.021

Comparison between KH and HH: Relative to KH group, the HH group scored significantly lower in general memory, especially visual and verbal immediate and verbal delayed memory. Learning index, that reflects the ability to learn based on repetition, was also impaired in HH group (Table 29, Figure 23)

Table 29 Children Memory scale scores: Comparison between HH and KH

CMS Index scores	HH n=19	KH n=14	T test	p value
Visual Immediate	94.8	106.3	-2.4	0.022
Visual Delayed	97.5	104.5	-1.7	0.093
Verbal Immediate	94.9	110.5	-2.9	0.006
Verbal Delayed	97.2	108.7	-1.9	0.055
General Memory	93.1	110.0	-3.3	0.002
Attention/Concentration	99.2	99.3	-1.1	0.256
Learning index score	91.3	107.0	-3.0	0.005
Delayed Recognition	101.7	109.1	-1.3	0.182
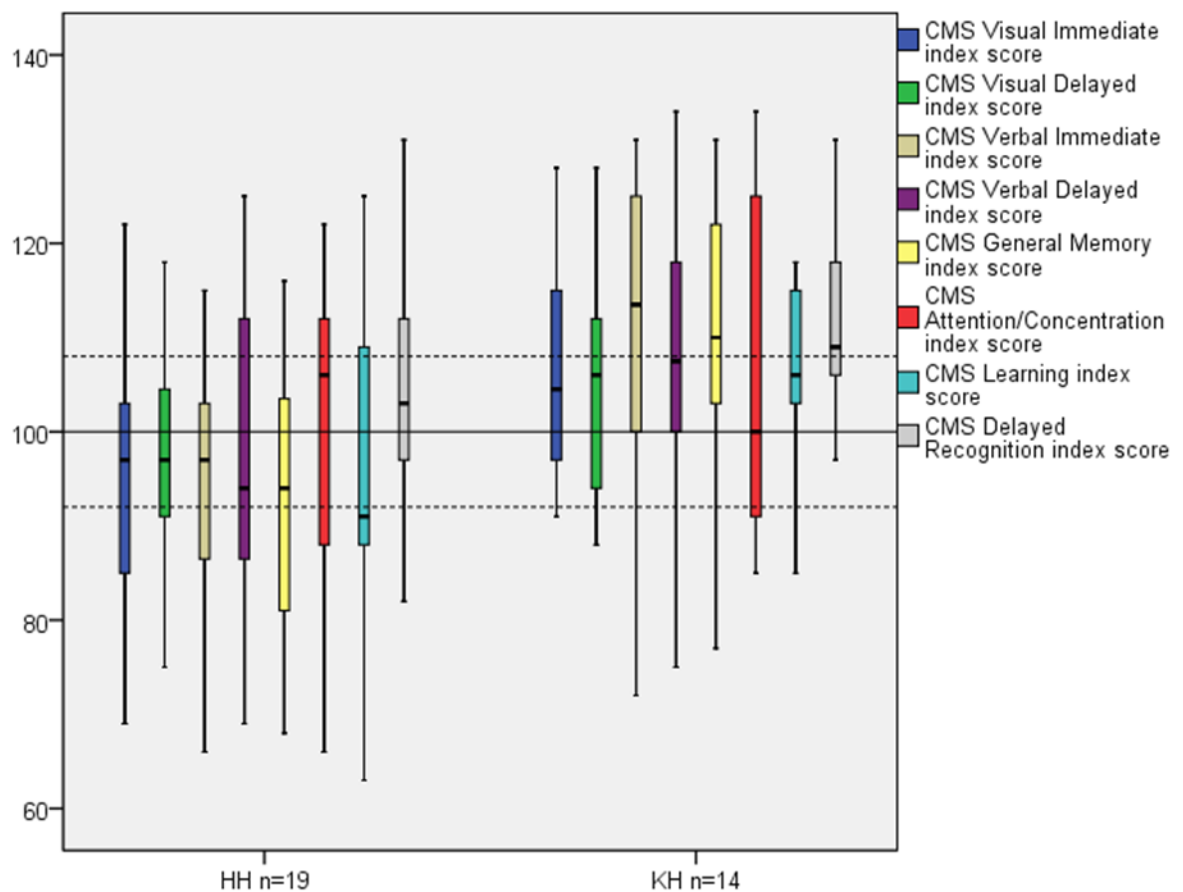
Figure 23 Children Memory Scales: Comparison between HH and KH. Horizontal reference line at score of 100 denotes the standard population mean and the interrupted horizontal reference line at scores 85 and 115 denote 1 standard deviation below and above the mean respectively. HH group scored significantly lower in general memory, especially visual and verbal immediate and verbal delayed memory.

Discrepancy between the actual CMS scores and predicted CMS (based on WISC-IV) scores was calculated using the WISC-IV technical report 5. The actual scores were then subtracted from predicted scores, to estimate the discrepancy.

Negative scores indicate better performance than predicted and positive scores indicate worse performance than predicted. Discrepancy scores were computed for verbal immediate, verbal delayed and general memory. The frequency of these discrepancy scores in the test population and any statistical significance was calculated.

Results: The mean discrepancy scores for both children with HH and KH was not significantly different from the reference population (Table 30). However 4 children from the HH group (patients 4,7,10, 15 in Table 16) and 1 child from KH group (patient 2 inTable 18) performed significantly worse than predicted (discrepancy scores 13 - 35 in 4 HH patients and 19 in 1 KH child)

Table 30 Discrepancy between predicted and actual CMS scores

Predicted-Actual CMS scores	Group	Mean	SEM
Verbal Immediate	HH	1.2	2.9
	KH	-9.8	3.4
Verbal Delayed	HH	1.2	3.3
	KH	-7.7	3.5
General Memory	HH	1.2	3.4
	KH	-10.1	3.3

Motor Skills

Comparison between HH and KH group: Children with HH scored significantly lower than KH children in the tasks of manual dexterity (Table 31, Figure 24)**.** Manual dexterity tasks included placing pegs on a pegboard and drawing a trail. Total scores (sum of manual dexterity, aiming and catching and balance scores) were comparable between groups.

Table 31 Movement ABC-2 scores: Comparison between HH and KH

Movement ABC Standard scores	Group	n	Mean	Std. Deviation	p value
Manual Dexterity	HH	17	81.1	13.7	
	KH	13	95.3	16.1	0.015
Aiming and Catching	HH I	17	93.2	21.8	
	KH	14	87.5	12.5	0.392
Balance	HH	17	90	16.6	
	KH	14	97.8	12.6	0.158
Total Test	HH	17	84.1	18.2	
	KH	14	91.7	9.1	0.163
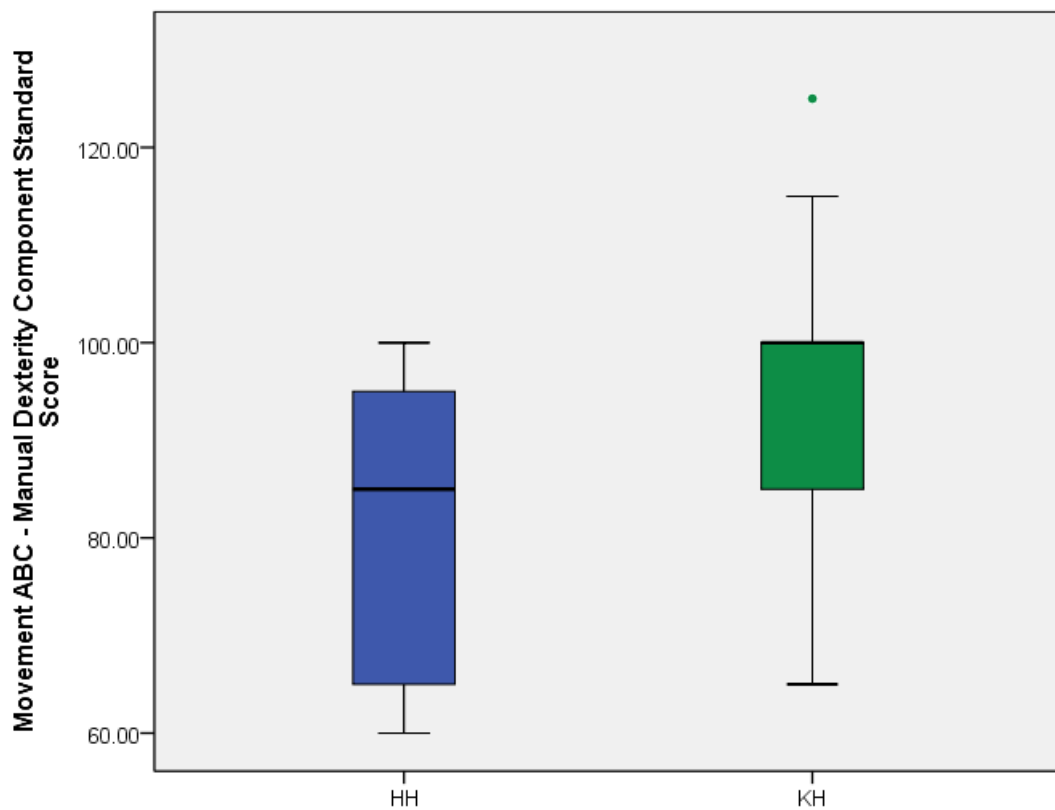
Figure 24 Manual Dexterity scores: comparison between HH and KH. Children with HH scored significantly lower (p=0.015) than KH group in tasks of manual dexterity.

Emotion and Behaviour

Social Communication questionnaire: The mean score in HH group was 5.3 (SD 2.5, range 0-9), and KH group was 4.5 (SD 3.5, range 1-12). No significant difference in mean scores between groups detected (t =0.7, df = 30, p = 0.484). 2 children in KH group had scores of 11 and 12.

Conners-3: Both the parent and teacher reports indicate high average-elevated scores in inattention, learning and executive functioning in HH group relative to KH group (Table 32, Table 33).

Table 32 Conner-3 Parent report

CONNER 3-Parent Tscore	HH	KH	p-value
Inattention	67.4	56.4	0.009
Hyperactivity/Impulsivity	60.5	55.4	0.298
Learning Problems	66.2	46.9	0.001
Executive Fucntioning	60.7	47.6	0.014
Aggression	55.9	50.5	0.204
Peer Relations	56.2	50.0	0.242
Conners 3 Global Index Total	64.1	56.9	0.149
DSM-IV-TR ADHD Inattentive	63.8	53.6	0.021
DSM-IV-TR ADHD Hyperactivity/Impulsivity	60.3	55.5	0.346
DSM-IV-TR Conduct Disorder	53.1	49.0	0.153
DSM-IV-TR Oppositional Defiant Disorder	56.4	53.1	0.451

DSM = Diagnostic and Statistic Manual, ADHD = Attention Deficit Hyperactivity Disorder

Table 33 CONNERS 3 - Teacher's report

CONNERS 3 - Teacher T score	HH	KH	p value
Inattention	62.4	52.2	0.046
Hyperactivity/Impulsivity	56.5	56.0	0.955
Learning Problems/Executive Functioning T score	62.9	51.3	0.030
Aggression	59.7	56.1	0.617
Peer Relations	59.4	51.0	0.226
Conners 3 Global Index Total	64.1	55.1	0.193
DSM-IV-TR ADHD Inattentive	63.1	50.2	0.020
DSM-IV-TR ADHD Hyperactive-Impulsive	60.0	55.5	0.590
DSM-IV-TR Conduct Disorder	53.0	48.7	0.227
DSM-IV-TR Oppositional Defiant Disorder	62.7	56.8	0.413

DSM = Diagnostic and Statistic Manual, ADHD = Attention Deficit Hyperactivity Disorder

Strength and Difficulties Questionnaire: No significant difference between groups was identified in emotional, behavioural or hyperactivity problems.

Table 34 Strength and Difficulties Questionnaire Diagnostic Predictions

SDQ Diagnostic predictions	HH	KH	p-value
Any diagnosis (medium/high risk)	7M/6H	1M/2H	0.053
Emotional Disorder (medium/high risk)	3M/3H	1M/1H	0.596
Behavioural Disorder (medium/high risk)	3M/2H	0M/2H	0.460
Hyperactivity (medium/high risk)	7M/1H	1M/1H	0.155

PedsQL - Impact on health related quality of life: Overall both HH and KH groups experience comparable quality of life. Parent rating of school functioning was significantly lower in HH group (Table 35)

Table 35 PedsQL Paediatric Quality of Life: Parent and Child scores

PEDS-QL Pediatric Quality of Life Inventory Scores	Group	Mean	Std. Deviation	p value
Physical Functioning Child	HH	75.4	19.2	
	KH	69.7	21.3	0.424
Emotional Functioning Child	HH	67.1	21.4	
	KH	60.3	19.6	0.363
Social Functioning Child	HH	68.1	20.7	
	KH	75.7	24.2	0.343
School Functioning Child	HH	52.8	25.9	
	KH	58.9	24.6	0.506
Psychosocial Health Child	HH	62.8	19.6	
	KH	62.0	23.7	0.907
Total Child Score	HH	66.9	17.5	
	KH	60.8	23.3	0.396
Physical Functioning Parent	HH	78.3	13.8	
	KH	79.1	22.0	0.903
Emotional Functioning Parent	HH	61.5	17.0	
	KH	71.9	19.4	0.122
Social Functioning Parent	HH	76.8	19.3	
	KH	82.6	19.8	0.412
School Functioning Parent	HH	53.6	17.1	
	KH	69.6	17.4	0.016
Psychosocial Health Parent	HH	64.8	12.5	
	KH	68.0	21.7	0.604
Total Parent Score	HH	68.4	12.3	
	KH	70.3	22.0	0.753

Relationship between neurocognitive scores and clinical features in HH group

Neonatal presentation, presence of mutation and type of treatment (medical or surgical), was not related to lower IQ or memory or attention scores. However children with parental perception of delay in diagnosis had significantly lower perceptual reasoning scores on WISC-IV (81 vs 95, p = 0.005). Children with epilepsy also had lower perceptual reasoning (76.6 vs 94.8, p = 0.005), IQ (76 vs 96, p = 0.044) and verbal immediate memory scores (82 vs 98, p=0.017)

The relationship between cognitive profile and quantitative neuroimaging findings are described in chapter 10

9.5 Discussion

Results of this study suggest that widespread cognitive and motor deficits exist in children with HH relative to the standard population, and to the KH group. Significant differences were noted in all domains assessed (IQ, memory, attention, and fine motor skills) and were reflected in the academic acheivement. The performance of the KH children as a group was functionally equivalent to the standard population means, although large inter - individual variations exist.

HH group

Intelligence: Children with HH demonstrate an overall low average IQ (mean 89.3), relative to the standard population, with no discrepancy among index scores. Relative to KH group mean IQ was 10 points lower and perceptual reasoning component of IQ, was nearly 15 points lower.

This is consistent with the report by Rother et al (Rother et al., 2001) of 8 children with HH, although no correlation with neuroimaging was available in this group. They used WISC-III and reported low verbal comprehension (53-80) and perceptual organisation scores (50-83) in 4 of 8 children with HH that had undergone subtotal pancreatectomy within 2 months of diagnosis. Similar to the HH group they report a wide variation in the scores, with few children performing > 2SD below the mean.

In this study, the diffuse white matter reduction detected by conventional neuroradiological assessment (discussed in chapter 8) may explain the overall low average IQ in the HH group, as white matter volume is considered to be one of the neuroanatomical correlates of intelligence. In healthy adults greater full scale IQ scores were positively associated with grey matter and white matter volumes (Narr et al., 2007).

Total white matter volume was found to be the major determinant of IQ in adolescents born preterm (Northam et al., 2011). A study of 79 adolescents born preterm (<32 weeks gestation) in comparison to 30 controls born term, showed WM reduction in the preterm group that accounted for 70% variance in the IQ scores.

Increased white matter volume may indicate an increase in the number of axons and degree of myelination, contributing to increased efficiency of interneuronal communication, reflected in the IQ scores. However objective quantitative assessments of white matter would be required to confirm this and will be explored in a subsequent chapter on diffusion tensor imaging.

Attention: 3 aspects of attention were assessed in this study, namely selective attention (sky search), sustained attention (score! and sky search DT) and attention control/switching (creature counting).

HH children show impairment of sustained attention but sparing of selective attention and attention control. Sustained attention is the ability to maintain a particular processing function over time. Score! tests sustained auditory attention while sky search DT tests sustained divided attention (children have to attend to 2 tasks at the same time).

This is consistent with a report by Rust et al (Rust et al., 2008) on young children with HH, suggesting impaired sustained attention and suggests selective damage to neuroanatomical networks that subserve different attentional processes.

Functional neuroimaging studies in healthy adults, has shown right frontal activation using measures similar to Score! (Lewin et al., 1996). Children with ADHD, assessed using TEA-Ch have shown impaired sustained attention and attention control with sparing of selective attention (Manly et al., 2001) and neuroimaging studies of ADHD children, have shown a delay in cortical maturation especially in the prefontal region that subserves attention and motor planning (Shaw et al., 2007). Based on the above it is reasonable to speculate that injury to the frontal regions of the brain in HH children may explain the finding of impaired sustained attention.

The presence of focal and diffuse white matter lesions (including frontal regions) on conventional radiological assessments in the HH group lends some support to this hypothesis. However these lesions were not specific or greater in the frontal regions and does not fully explain the pattern of attention impairment. Specific studies investigating cortical thickness and the characteristics of specific white matter tracts (tractography) in the frontal region that subserve attention (such as the frontostriatal network) would be required to confirm this hypothesis.

2 other aspects of attention were assessed in this study, namely selective attention (sky search), and attention control/switching (creature counting).

The time taken to complete the sky search task (selective attention) was significantly prolonged in both HH and KH groups. This reflects the way both groups approach the task. According to clinical observation both groups were cautious in performing this task, sacrificing speed for accuracy, thus taking a longer time than normal. Alternatively this could reflect impairments in processing speed or manual dexterity.

KH children took significantly longer time to complete creature counting task correctly. Although time taken by HH children does not seem to differ relative to reference, it is important to note that nearly 5 children struggled to produce correct responses in the creature counting task and they were not included in timing analysis. It's also important to note the normative data for TEA-Ch is derived from australian children and any difference in the study group may reflect cultural influences.

Memory: Children with HH scored significantly lower in general memory, relative to standard population (7 points lower). This discrepancy was greater when compared to KH group (HH group 17 points lower). Learning index that reflects ability to improve scores with repetition was also impaired in HH group.

Although memory scores were impaired in HH, there was no discrepancy as a group between memory score predictions based on IQ and the actual memory scores to suggest specific memory impairment.

However 4 HH children showed a discrepancy of 13-35 from their predicted general memory scores. While 2 of these children were noted to have normal scans, 1 was reported to have bilateral hippocampal and diffuse white matter reduction and another was reported to have only white matter reduction on conventional neuroradiological assessment.

The pattern of memory deficit was also inconsistent between these children. 1 child had impaired visual immediate memory (69), 2 children had impaired visual (75, 78) and verbal delayed memory (69, 85), and 1 child had impaired visual and verbal immdiate score (66).

In children with childhood onset amnesia, bilateral hippocampal pathology is associated with impaired delayed memory (verbal and visual) with intact performance on immediate memory measures (Gadian et al., 2000; Vargha-Khadem et al., 1997).

Unilateral lesions in the MTL circuit can result in impaired verbal memory in left sided lesions and deficits in nonverbal memory in right sided lesions (Milner et al., 1971). In 16 healthy adults, using event related fMRI, Rosazza et al (Rosazza et al., 2008) were able to demonstrate left lateralised activation (of hippocampus and parahippocampus) with words and right lateralised activation with faces. Thus based on neuroimaging findings some prediction can be made regarding pattern of memory deficit.

However the lack of a specific pattern of memory impairment and a combination of normal and abnormal scans on conventional neuroradiological assessments make it difficult to conclusively establish or exclude memory impairment in this group.
Further exploration utilising quantitative assessment of hippocampal volumes and relationship to memory indices are discussed in chapter 10.

Academic attainment: Children with HH underperformed (12 -16 points lower) in all 3 academic areas assessed (reading, mathematics and spelling), relative to the standard population and the KH group. This seems consistent with their overall low average IQ.

Motor skills: The MABC-2 is a screening tool used to assess motor impairment. It has been particularly useful in detecting developmental coordination disorders (Mon-Williams et al., 1994), and is increasingly used in children, without any gross neurological abnormality to detect subtle movement problems (Webb et al., 2012).

In this study, although the total MABC scores were comparable between groups, children with HH (relative to KH) performed significantly poorly in skills of manual dexterity. Outcome reports in children with HH have documented gross motor (Meissner et al., 2003; Menni et al., 2001), and coordination difficulties (Cresto et al., 1998; Mazor-Aronovitch et al., 2007; Meissner et al., 2003). Mazor-Aronovitch et al (Mazor-Aronovitch et al., 2007) reported motor coordination difficulties as the most prevalent motor problem, although improvement was noted by school age.

Deficits in white matter microstructural integrity of the corpus callosum, corticospinal tract, anterior corona radiata and internal capsule have all been associated with impaired performance on MABC-2.

Emotion and behaviour

Several studies have reported emotional and behavioural problems in children with hypoglycaemia.

High scores on Achenbach Child Behaviour checklist suggestive of aggression or oppositionality was reported by Rother et al (Rother et al., 2001). Bahi-Buisson et al (Bahi-Buisson et al., 2008) have reported mild behavioural problems (social withdrawal, attention difficulties) in children with HIHA syndrome.

In contrast Mazor-Aronovitch et al (Mazor-Aronovitch et al., 2007) compared 21 children with medically treated HH with sibling controls and found no increased susceptibility to behavioural problems in HH group. However, the outcome was based on telephone or personal interview with parents that can underestimate the presence of any emotional or behavioural difficulties. In addition, this study consisted of only medically treated patients who may represent a milder group with regard to disease severity

Elevated scores for inattention were obtained from both parent and teacher reports in HH children, using Conners-3. Along with their poor performance in sustained attention tests on TEA-Ch, this strongly suggests attention difficulties in children with HH in this study.

In this study poor communication skills suggestive of autism were not seen in either HH or KH group. Overall perceived health related quality of life did not differ between groups

Relationship between neurocognitive scores and clinical features in HH group

In children with HH, the evidence suggests, poor neurodevelopmental outcome in children with neonatal onset (Menni et al., 2005) disease, persistence of hypoglycaemia beyond 35 days (Jack et al., 2003) and medically unresponsive patients requiring surgery (Menni et al., 2001; Steinkrauss et al., 2005).

In this study impaired cognitive outcomes were not related to neonatal onset or medically unresponsive diasease, possibly owing to small numbers. However presence of delay in diagnosis and epilepsy were associated with impaired outcomes. This is consistent with evidence suggesting co- morbid conditions such as seizures, and hypoxia can potentiate HBI (Basu et al., 2009; Das, 2009; Yager, 2002)

The widespread cognitive deficits in HH children in this study cannot be fully explained by the current neuroimaging findings. Within the HH group, there are inter-individual variations, with some children performing well below the mean scores. This variation could be due to the marked clinical heterogeneity of this disorder, duration and severity of hypoglycaemia in each individual. However the contribution of additional factors such as presence of epilepsy, seizures at diagnosis, infection, genetic mechanisms (increased likelihood of impairment in children with *GLUD1* mutations), age at injury, may all have influenced the outcome.

KH group

Overall the performance of the KH children as a group is consistent with the clinical observation, that these children are functionally unimpaired.

However, 4 children in KH group (age 7.45-11.5 years), scored FSIQ in the borderline (77, 79- patient 3 and 14 in Table 18) and low average range (81, 84 - patient 12 and 13 in Table 18). Discrepancy in the index scores were also noted in these children. 3 scored lower in processing speed (PS) and working memory (WM) relative to verbal comprehension (VC) and perceptual reasoning (PR), while 1 scored lower in VC and PS relative to WM and PR.

Apart from one child (patient 13, Table 18) that presented at 5 years of age with irritable behaviour inbetween meals, the clinical features documented in the other 3 children are typical of KH and do not account for the neurological compromise. The 2 children that performed in the borderline range had additional MRI features of mild global white matter reduction and 1 in addition had bilateral hippocampal volume reduction.

White matter reduction was also seen in 2 additional children without IQ impairment (patient 1 and 4 in Table 18). However, IQ impairment in the KH group was not restricted to children with abnormal scans. Although the neuroradiologist was blinded to patient diagnosis, reporting of scans involved specific inspection of white matter and may have lead to a bias in the perception white matter compromise. However the association of low IQ in some of them tends to indicate neurological impairment in those children with KH.

Further quantitative investigation of white matter structural integrity and hippocampal volumes are required comparing KH group to a healthy paediatric population to corroborate these findings.

9.6 Conclusion

A comprehensive assessment of IQ, memory, attention, academic attainment, motor skills, and emotion and behaviour was conducted in HH and KH children. Overall assessments indicate widespread cognitive and motor deficits, academic underachievement and behaviour difficulties in the HH group.

Specific differences observed between HH and KH group, include impaired PR and FSIQ scores, general memory (consistent with low IQ) and learning index scores in HH group. Academic performance in reading, spelling and mathematics was impaired in HH group. In addition, HH group showed impaired sustained attention scores relative to test standard population. Furthermore, performance of HH group was impaired in tasks of manual dexterity relative to KH group. Presence of epilepsy and delay in diagnosis was associated with impaired performance in PR score in the HH group.

Neuroimaging studies have revealed white matter lesions in children with HH (discussed in chapter 8). This may partly explain the widespread neurocognitive deficits noted in this group. Further exploration utilising quantitative assessments of neuroimaging studies are discussed in chapter 10.

Although children with KH are used in this study as a control group, the unexpected finding of impaired cognitive performance in some children and the presence of abnormal neuroimaging scans may indicate that KH can result in brain injury and warrants further research.

Table 36 Description of psychometric assessments

Neurocognitive Tests	Index scores	Subtests	Description of subtests
WISC IV	Verbal Comprehension	Similarities	2 related (as objects or concepts) words are presented and child needs to describe similarity, ex: How are cat and mouse alike
		Vocabulary	Name pictures presented in a stimulus book and define words read aloud by examiner
		Comprehension	Child questioned on general principles and social situations
	Perceptual Reasoning	Block Design	Child needs to recreate a displayed design with red and white bricks (time limited)
		Picture Concepts	2 or 3 rows of pictures displayed and child needs to select one from each row with a common theme
	Working Memory	Digit Span	A series of numbers are read aloud by examiner and child repeats it in the same order heard or in the reverse order when prompted
		Letter-Number Seq	Intermixed numbers and letters read aloud by examiner and childs needs to recall numbers in ascending and letters in alphabetical order

	Processing Speed	Coding	A key of shapes with symbols inside are provided and child needs to copy the symbol into a corresponding shape (time limited)
		Symbol Search	A target group of symbols are given and the child need to specify if a corresponding search group has any matching target group symbols
WIAT II	Reading	Word Reading	Child is asked to read aloud from a list of familiar words. Accuracy and speed of response are noted
	Mathematics	Numerical operations	Child is asked to recognise and reproduced dictated numerals and solve written maths problems involving addition, subtraction,multiplication and division
		Mathematical reasoning	Maths problems are presented verbally and non-verbally to evaluate reasoning skills
	Written language	Spelling	Child is asked to write dictated letters and words
TEA-ch	Sky Search	selective attention	An A3 paper with randomly distributed craft pairs provided. Task required children to circle identical pairs quickly. An attention score was also derived that removed the influence of motor speed on this test.
	Score	sustained attention	Children were asked to silently count the number of tones produced by a media player in a trial and announce them at the end of the trial. Number of trials with correct responses recorded (total 10 trials)

	Creature counting	attentional control/switching	A map of creatures are displayed and children are expected to count and switch to count up or down based on cues. Correct responses and timing noted
	Sky Search DT	sustained attention	This is a combination of sky search and score. Children were expected to circle identical crafts while observing the number of tones in each trial
CMS	Dot locations		3 array of dots presented, after a distractor array, children need to reproduce the original array. Tested again after 30 min delay
	Stories		Children are read 2 stories and asked to repeat as much as they remember immediately and again after 30 mins. The child is aslo asked yes or no questions about the story.
	Faces		A list of faces presented in series, following this child asked to recall if a series of presented faces are from the initial list. Repeated after 30 min delay
	Word pairs		The child is provided with a list of word pairs. 3 trials where examiner prompts child with one of the words in a pair. After trials child asked to remember as many word pairs as possible. After 30 mins child asked to recollect word pairs again with prompts.Finally examiner reads word pairs and child asked to indentify if they were present in original list

	Numbers	series of numbers presented and child asked to repeat them forward and backward
	Sequences	Tests mental capacity for verbal manipulation of data in a sequence. For example recollecting months in a year backwards or alternating counting numbers with alphabets
MABC-2	Manual dexterity	This is assessed by asking child to place pegs in a pegboard using preferred hand and non preferred hand, threading lace (or 'placing coins in a slot', this task varied depending on the age of the child) and drawing a trail
	Aiming and catching	This includes catching with 2 hands and throwing a beanbag onto a mat at a specified distance
	Balance	This includes balancing on best leg and the other leg, walking heel to toe and hoping on mats on best leg followed by the other leg

10 Quantitative MRI studies

10.1 Hippocampal Volumetry

10.1.1 Introduction

The hippocampus is a component of the medial temporal lobe and plays an important role in learning and memory (Eichenbaum et al., 1997). Structurally the hippocampus contains the cornu ammonis subfields CA1-4, subiculum and the dentate gyrus. The medial temporal lobe-cortical circuit contains the hippocampus, parahippocampal cortex, mammillary bodies, medial thalamus and the prefrontal cortex. This complex interconnected neural network forms the neuroanatomical basis of declarative (explicit) memory.

Neuropathological studies of prolonged human neonatal hypoglycaemia have shown the hippocampus to be susceptible to hypoglycaemic brain injury (Auer et al., 1989). The dentate gyrus is especially vulnerable to damage following prolonged hypoglycaemia. Hypoglycaemic coma associated with electrocerebral silence, in adult animal studies has been shown to selectively damage cerebral cortices, hippocampus and striatum, with dense necrosis in the dentate gyrus of the hippocampus (Auer et al., 1984).

Recurrent severe hypoglycaemia in the setting of diabetes has also been shown to affect the hippocampus. Hershey et al (Hershey et al., 2010) have described hippocampal volumes in young individuals (7-17 years) with type 1 diabetes. Children with increased episodes of severe hypoglycaemia were found to have significantly larger hippocampal volumes. This contrasts with most studies, where hippocampal volume reduction is implicated in pathology and associated with impaired memory. The authors speculated that the increased hippocampal volumes could be secondary to reactive neurogenesis or disruption of normal developmental pruning.

Diffusion-weighted imaging in a diabetic adult following hypoglycaemia coma has demonstrated bilateral high intensity signal in the hippocampi and associated anterograde amnesia for 2 months (Ko et al., 2008).

Decreased hippocampal volumes have been reported in children with temporal lobe epilepsy, preterm children with low birth weight and children with hypoxic-ischaemic injury (HIE) (Isaacs et al., 2000; Gadian et al., 2000). Reduction of hippocampal volumes in these groups of children has been associated with significantly reduced performance on memory scales. A specific impairment of episodic memory (memory for events of personal significance) with relative preservation of semantic memory (memory for general knowledge and facts) has been described in some of these children. This condition has been termed developmental amnesia (Gadian et al., 2000).

Isaacs et al compared 2 groups of children with reduced hippocampal volumes (preterm with low birth weight, children with developmental amnesia secondary to HIE) to ascertain the degree of hippocampal atrophy associated with developmental amnesia (Isaacs et al., 2003). They concluded that a 20-30% bilateral reduction of hippocampal volume is necessary for manifestation of developmental amnesia.

Unpublished data from a group of 17 children with HH from GOSH described general memory scores in the low average range (mean 81.62, range 66-103, SD 12.28). As the hippocampus is one of the key structures in the memory system and is susceptible to hypoglycaemia, we decided to investigate these further using quantitative methods to ascertain hippocampal volume. The relationship between hippocampal volume and memory was also examined

Quantitative assessment of hippocampal volumes (HV) includes manual and automated methods. Manual hippocampal volumetry is considered the gold standard method to assess HV (Tae et al., 2008), although it is labour intensive and relies on rater experience. The ability to discern and define the boundaries of the hippocampus is a crucial determinant of hippocampal volume accuracy. This greatly depends on the expertise of the rater and other technical aspects of neuroimaging, such as image resolution of scanner, acquisition parameters and slice orientation.

10.1.2 Specific Aims

To investigate if children with HH have reduced hippocampal volumes in comparison to children with KH

To investigate the relationship between hippocampal volumes and memory indices

10.1.3 Methods

10.1.3.1 Participants

21 children with HH and 14 children with KH underwent MRI scans. 1 child in HH group (Patient 10 in Table 16) did not complete the 3D FLASH sequence required to assess HV, hence data from 20 children in HH group and 14 children in KH group were included in hippocampal volumetry analysis.

10.1.3.2 Image Acquisition

Fast low angle shot (FLASH) three-dimensional MRI scans were acquired on a Siemens 1.5T avanto scanner. The 3D FLASH parameters were: repetition time, TR = 11 ms; echo time, TE = 4.94 ms; flip angle = 15°; matrix size 224*256; and 176 slices with a slice thickness of 1 mm. The scan time was 5.34 minutes.

10.1.3.3 Image processing and analysis

The hippocampal volumes were measured by Professor David Gadian, blind to patient diagnosis. After acquisition, the dataset was transferred to a PC, and analysed using MEDx, version 3.3 (Sensor Systems Inc., VA). The data were reformatted in the tilted coronal plane perpendicular to the long axis of the hippocampus. Each hippocampal slice, 1 mm in thickness, was then manually traced. Manual tracing was guided by anatomical references (H.M.Duvernoy., 1991).

The volume was then derived from the summed slices. Intracranial volume was calculated from the sagittal images. A random slice and every 10th slice after that was systematically measured and the volume calculated by summation of the slices. The hippocampal volumes then were corrected for intracranial volume and presented in the corrected form (Gadian et al., 2000; Van Paesschen et al., 1997). I learnt the principles of the HV measurement technique and performed HV measurements on some control samples, under the supervision of Prof Gadian to gain a better understanding of the methodology involved and its complexity.

10.1.3.4 Statistical tests

The distribution of hippocampal volumes was analysed using the Shapiro-Wilk test. As the distribution of the right hippocampal volume was skewed in the HH group, non-parametric statistical tests were performed. Differences between groups (unrelated samples) were assessed using the Mann-Whitney U test. Within group difference between sides (related samples) was assessed using the Wilcoxon signed rank test. Partial correlation was used to examine the relationship between hippocampal volumes and memory indices controlling for age. Intra-rater intraclass coefficient of variation was calculated for right and left hippocampal volumes and intracranial volume. All statistical analysis was performed in IBM SPSS version 20.

10.1.4 Results

Baseline characteristics

The groups did not exhibit significant differences in baseline characteristics.

Table 37 Baseline characteristics of HH and KH group in hippocampal volumetry analysis

Patient characteristics	HH	KH	p-value
Number of patients (no)	20	14	
Age at scan in yrs (mean ± SEM)	10.3 (2.6)	8.7 (2.1)	0.073
Gender (Male/female)	13M/7F	9M/5F	0.960
Gestational age in weeks	39.3	38.7	0.420
Birth weight in grams (mean ± SEM)	3870.5 (898.7)	3337.2 (747.4)	0.069
Handedness (Right/Left)	15R/5L	11R/3L	1.000
Height sds (median)	-0.06 (0.8)	-0.05 (0.6)	0.703
Weight sds (median)	0.09	-0.17	0.287
Seizures at diagnosis (patient no)	9	3	0.270

No significant difference in maternal education (p=0.597, FET) or socioeconomic status (p=0.677, FET) was seen between groups.

Comparison of hippocampal volumes between HH and KH (Table 38, Figure 25)

The intrarater intraclass correlation coefficient (ICC) was calculated from volumetry data of 20 patients (KH and HH), that were assessed 1 week apart. The ICC was 0.908 for right hippocampus, 0.929 for left hippocampus and 0.996 for intracranial volumes.

Between groups: No significant differences in hippocampal volumes were seen in the right (p = 0.959) or left (p = 0.877) hippocampus between the KH and HH groups.

Within groups: The left hippocampus was significantly smaller than the right hippocampus in both HH (p = 0.023) and KH (p = 0.002) children.
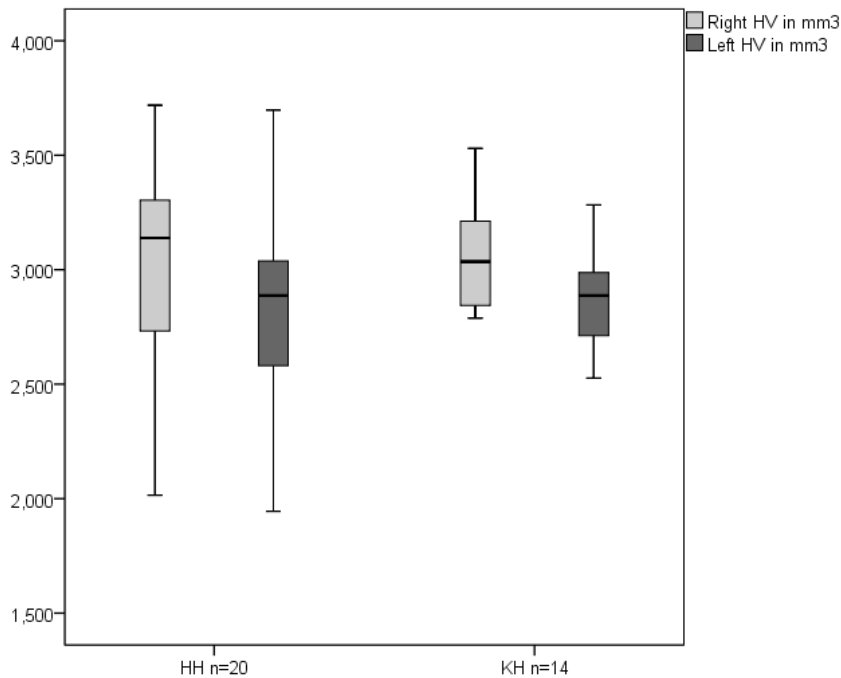
Figure 25 Comparison of hippocampal volumes (HV) between HH and KH. The HV in cu mm is plotted along the y axis. There was no significant difference in HV between HH and KH group.

Table 38 Comparison of hippocampal volumes (HV) between HH and KH

Hippocampus Volume	KH (n=20)	HH (n=14)	Mann-Whitney U (KH vs HH) p-value
Right (median) mm^3	3035.19	3138.36	0.959
Left (median) mm^3	2887.09	2887.22	0.877
Wilcoxon signed rank test (right vs left) p-value	0.002	0.023	

Percentage hippocampal volume reduction relative to the mean hippocampal volumes of the KH group

The mean right (3068.76 mm^3) and left hippocampal volumes (2905.55 mm^3) were calculated for the KH group. For each individual in the KH and HH groups, the hippocampal volumes were compared with the mean hippocampal volumes of the KH group. The differences were expressed as a percentage hippocampal volume reduction (Table 39), negative values indicating a larger hippocampal volume relative to the KH group mean, and positive values indicating a smaller hippocampal volume relative to the KH group mean.

In the KH children, the percentage difference ranged from 10.1% to -15.0% for the right hippocampus and 13.0% to -17.4% for the left hippocampus. In the HH children, the percentage difference ranged from 34.3% to 21.2% for the right hippocampus and 33.1% to 27.2% for the left hippocampus. 3 children in the HH group (patient 1, 3 and 4) have > 20-25% reduction in bilateral hippocampal volumes. Of these only patient 4 showed a significant discrepancy between general memory score achieved and predicted by IQ (13 points) and more specifically delayed memory was impaired (verbal and visual).

Of these 3 children (patient 1: 7.9 yr old male, patient 4: 7.46 yr old male, patient 3: 5.63 yr old male). One patient was diagnosed within 2 weeks of life, while the other 2 were diagnosed in the 2nd month of life. Parents report a delay in diagnosis in all 3 patients with symptom manifestations within 48 hrs of life. All 3 were born at term with birth weights ranging between 3960 – 4173 g. Co-morbid conditions such as hypoxia and seizures at diagnosis were not present; however patient 3 has a specific type of HH known as HIHA and has developed generalised epilepsy.

Patient 3 has a mutation in the *GLUD1* gene that results in hyperinsulinism associated with hyperammonemia (HIHA). The other 2 do not have mutations in any of the genes associated with hyperinsulinism. Patient 1 is cured following surgical resection of a focal lesion in tail of pancreas, while the other 2 are managed medically (diazoxide responsive). All 3 children are neurologically normal, although patient 4 had broad based gait at a younger age that has improved with time.

Conventional MRI in these 3 children was also abnormal. All 3 showed varying degrees (patient 1 – severe, patient 3 – moderate, patient 4 – mild) of global white matter reduction.

Table 39 Hippocampal volumes and percentage reduction in comparison to mean hippocampal volume of KH group. Highlighted in bold are children with >20% reduction in bilateral hippocampal volume.

Group	Patient no	Right HPV	Left HPV	Right % difference	Left % difference
HH (n=20)	1	**2094.88**	**1944.88**	**31.74**	**33.06**
	2	3112.64	2902.64	-1.43	0.10
	3	**2014.86**	**2042.86**	**34.34**	**29.69**
	4	**2028.50**	**2278.50**	**33.90**	**21.58**
	5	3326.04	3043.04	-8.38	-4.73
	6	2783.16	2604.16	9.31	10.37
	7	2739.28	2463.28	10.74	15.22
	8	2726.16	2558.16	11.16	11.96
	9	3193.78	3032.78	-4.07	-4.38
	11	3281.36	2614.36	-6.93	10.02
	12	3718.39	3530.89	-21.17	-21.52
	13	2452.80	2871.80	20.07	1.16
	14	3474.29	3017.29	-13.21	-3.85
	15	3164.08	2850.08	-3.11	1.91
	16	3013.01	3032.51	1.82	-4.37
	17	3464.80	3697.30	-12.91	-27.25
	18	3265.64	3059.64	-6.42	-5.3
	19	3199.94	3017.94	-4.27	-3.87
	20	3388.10	3063.10	-10.41	-5.42
	21	3109.04	2845.04	-1.31	2.08
KH (n=14)	1	2788.84	2609.84	9.12	10.18
	2	2788.42	2526.42	9.14	13.05
	3	3038.91	2697.41	0.97	7.16
	4	3304.23	3255.23	-7.67	-12.03
	5	3189.22	2935.22	-3.93	-1.02
	6	3530.10	3283.10	-15.03	-12.99
	7	3212.00	2969.00	-4.67	-2.18

8	3384.14	3412.14	-10.28	-17.44
9	2925.06	2712.06	4.68	6.66
10	2805.77	2858.77	8.57	1.61
11	2989.42	2915.42	2.59	-0.34
12	2844.34	2784.34	7.31	4.17
13	3130.82	2987.82	-2.02	-2.83
14	3031.47	2730.97	1.22	6.01

In the KH group one child (patient 1, 12 yr old male), with bilateral hippocampal volume reduction of 9-10% did not display any memory impairment. However another child (patient 2, 8yr old female) with bilateral hippocampal volume reduction of 9-13%, had an IQ- memory discrepancy of 19 points. Verbal memory (immediate (72) and delayed (75) was more impaired than visual memory (immediate and delayed (both 94)) in this child

Patient 1 attends mainstream school without any support, although parents note that he is a little bit behind his peers, while Patient 2 attends mainstream school with support for learning. Academic attainment was normal in both children. Conventional MRI in both these children was also abnormal, patient 1 had white matter reduction in the right hemisphere and patient 2 had mild global white matter reduction and. Full scale IQ assessed using WISC-IV was 105 in patient 1 and 89 in patient 2 (impaired perceptual reasoning and working memory).

These 2 children did not display any differences with regards to clinical features, disease onset was in the 2nd year of life in both (22 months in patient 1 and 18 months in patient 2) and seizures at onset was not present in either child.

Concurrence between hippocampal volumetry and conventional MRI

In all 3 HH patients with >20-25% bilateral hippocampal volume reduction, the conventional MRI report concurred. In 2 children with hippocampal volume equivalent or greater (HH patient 5 and KH patient 3) than mean KH hippocampal volume, conventional MRI reported an asymmetrical decrease in hippocampal volume. In 1 HH child with 11% bilateral reduction in hippocampal volume (patient 8), conventional MRI reported an asymmetrical change in hippocampus and in 1 HH child (patient 2) with asymmetrical reduction in hippocampal volume; conventional MRI reported bilaterally small hippocampi.

Table 40 Hippocampal volumes in children with abnormal hippocampus report on conventional neuroimaging

Type	Patient no	Hippocampus on conventional MRI	Rt HPV	Lt HPV	Right % difference	Left % difference
HH	1	Bilateral small	2094.88	1944.88	31.74	33.06
	2	Bilateral small	3112.64	2902.64	-1.43	0.10
	3	Bilateral small	2014.86	2042.86	34.34	29.69
	4	Bilateral small	2028.5	2278.5	33.9	21.58
	5	Left small	3326.04	3043.04	-8.38	-4.73
	8	Right small	2726.16	2558.16	11.16	11.96
KH	3	Bilateral small	3038.91	2697.41	0.97	7.16

Relationship between hippocampal volumes and memory indices

The relationship between hippocampal volumes and memory indices such as visual immediate memory, verbal immediate memory, visual delayed memory, verbal delayed memory, general memory and delayed recognition scores were analysed using partial correlations, controlling for age at scan.

No significant relationship was seen between memory indices and the hippocampal volumes in the HH group.

In the KH group significant strong positive correlations were seen between corrected left hippocampal volume and verbal immediate memory, verbal delayed memory and delayed recognition, while strong significant positive correlations were seen between corrected right hippocampal volume and verbal immediate memory (Table 41).

Table 41 Relationship between hippocampal volumes and memory indices in KH group

Hippocampus	Children memory scale	partial correlation r	p-value
Left	Delayed recognition	0.641	0.025
	Verbal delayed memory	0.729	0.007
	Verbal immediate memory	0.667	0.018
Right	Delayed recognition	0.544	0.068
	Verbal delayed memory	0.558	0.059
	Verbal immediate memory	0.592	0.043

Relationship between age and hippocampal volumes (Figure 26, Figure 27)

There is a significant positive correlation between age at imaging and right (r =0.628, p = .003) and left (r = 0.570, p=0.009) hippocampal volumes in children with HH. No such relationship was seen in children with KH (right hippocampus r =-.082, p = 0.780. left hippocampus r = -0.064, p = 0.827).
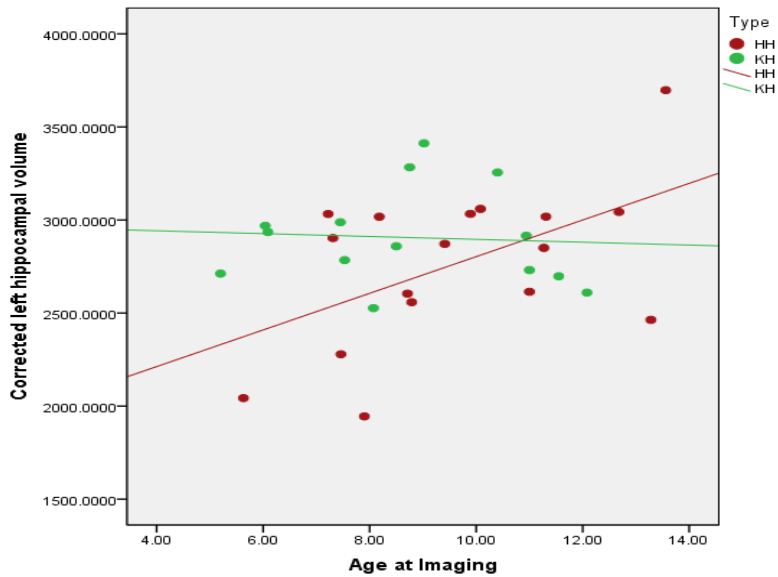
Figure 26 Relationship between age and corrected left hippocampal volume in children with HH and KH
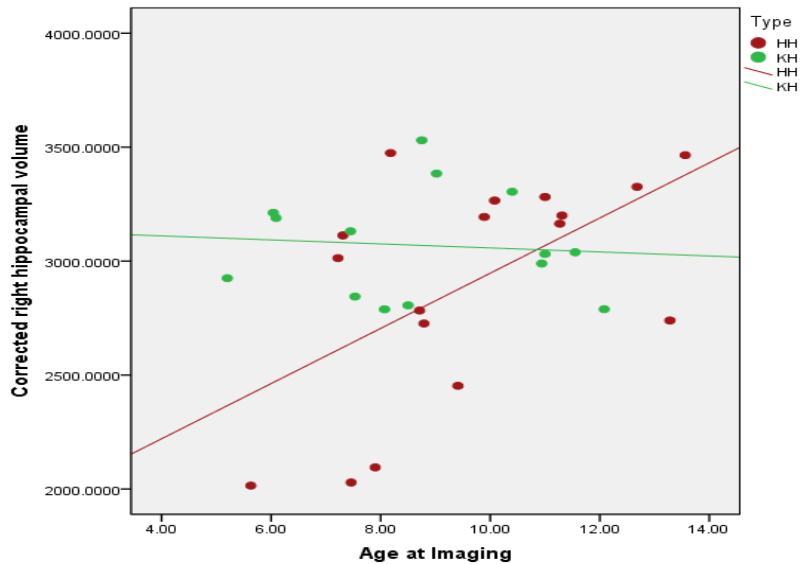
Figure 27 Relationship between age and corrected right hippocampal volume in children with HH and KH

10.1.5 Discussion:

In this study, using manual hippocampal volumetry, group differences in hippocampal volumes have not been demonstrated between HH and KH group.

However, 3 patients (all males) in HH group had bilateral volume reduction of more than 20- 25%. Although these children were among the youngest in the HH group, similar volumes were not seen in age matched children within HH group or in the KH group, suggesting that the reduction may reflect actual hippocampal volume loss. In one child (patient 4, HH group) memory indices showed low general memory scores, especially verbal and visual delayed memory and the discrepancy between actual and predicted memory scores was 13 points. The other 2 children (patient 1 and 3) did not manifest specific memory impairments.

Hippocampus dependent memory functions mature around 5-7 years of age in humans, and deficits associated with hippocampal damage sustained early in life, are known to emerge later in childhood (Bachevalier et al., 2005; Gadian et al., 2000). Thus it will be important to re-evaluate these children at a later age (10-12 years), with specific focus on hippocampus-dependent memory functioning, to understand the impact of the hippocampal damage.

Following hypoxic ischaemic encephalopathy, bilateral hippocampal volume reduction of 40% (29%-55%), in comparison to age matched controls, has been associated with specific memory impairments, such as impairment of episodic memory with preservation of semantic memory (Isaacs et al., 2003). The maximal hippocampal reduction in our HH group was lower 20-25%, and may explain the lack of similar memory impairment as reported by Isaacs et al (Isaacs et al., 2003)

Everyday memory problems and semantic memory (vocabulary, information and comprehension subtests in WISC-IV) were not assessed in this study, and they need to be ascertained in order to gain a complete understanding of the impact of severe hippocampal volume reduction on memory.

This study suggests that HH is associated with a risk of severe bilateral hippocampal reduction, although the clinical or functional impact of the damage and features that increase susceptibility to injury needs to be investigated further. The impact of KH on hippocampal volumes is less clear. One child in the KH group (patient 2) had bilateral reduction of hippocampal volumes, albeit to a lesser degree (9-13%) and this child also displayed impaired verbal memory

Conventional MRI concurred with hippocampal volumetry in only 3 patients. Studies in adults with temporal lobe epilepsy (Cendes et al., 1993; Jack et al., 1990) have shown volumetric studies to be more sensitive than conventional MRI in ascertaining hippocampal pathology. Cendes et al (Cendes et al., 1993) have shown 92% concordance between volumetric and EEG in lateralising epileptogenic areas in TLE in adults, in comparison to a concordance of 56% between qualitative visual assessment of MRI and EEG. Qualitative assessment of hippocampal pathology can be affected by several factors such as image orientation; slice thickness and type of sequences used (spin echo T2 and Fluid Attenuation Inversion Recovery sequence enable better detection of pathology).

In KH children, no relationship was demonstrated between hippocampal volume and age. This is consistent with longitudinal MRI studies of hippocampal volumes in children, that indicate a stable hippocampal volume between the ages of 4 – 25 yrs (Gogtay et al., 2006). In contrast HH children show a strong positive correlation of bilateral hippocampal volumes with age. However, exclusion of the 3 younger children with severe bilateral reduction in hippocampal volume renders this relationship insignificant (right hippocampus vs age $r = 0.398$, $p = 0.113$ and left hippocampus vs age $r = 0.323$, $p = 0.205$).

In the KH group, left hippocampal volumes correlated with verbal memory. This is in accordance with the study by Frisk et al (Frisk et al., 1990), where adults with left hippocampal resection showed impairment in long term maintenance of verbal information. No such correlation was noted in the HH group. An explanation for this could be the significant age discrepancy between neurocognitive assessment and age at neuroimaging in this group. 13 children with HH had previously participated in a pilot project investigating neurocognition, so the neurocognitive data from these patients predate the neuroimaging acquisition by 1.5-3.3 years.

Thus it is difficult to conclude with certainty if children with HH and KH have hippocampal involvement or a specific memory impairment. It is likely that some children may be affected, and a larger study would be required to discern the risk factors associated with such an involvement.

An important limitation of note in this study is the lack of healthy population. This may underestimate the effect of HH on the hippocampus.

10.1.6 Conclusion

Hippocampal volumetry has not shown any significant group differences between HH and KH children. The absence of specific memory impairment as a group on neurocognitive assessment also supports this finding.

However, increased risk of hippocampal injury following HH cannot be completely excluded, as 3 children in the HH group have severe bilateral reduction of hippocampal volumes (> 20%), and 3 children with IQ- memory discrepancy also had significantly lower hippocampal volumes.

Hippocampal injury in children with KH also cannot be excluded as 1 child in the KH group had 9-13% reduction in hippocampal volume and an IQ-memory discrepancy of 19 points.

However, additional white matter injury in both groups (seen in conventional MRI) may have contributed to the cognitive deficit.

10.2 Diffusion tensor imaging

10.2.1 Introduction

Diffusion tensor imaging is a unique MRI technology to assess white matter microstructure by utilising the properties of water diffusion in brain tissue. Water molecules within the body tissue are in a state of constant random motion (Brownian movement). The magnitude and direction of diffusion of water molecules within the brain can be used to understand the integrity of the underlying structure (Basser et al., 2002; Le Bihan et al., 1986).

Water diffusion is uniform and equal in all directions (isotropic diffusion Figure 28) when cellular barriers are not coherently organised as in the grey matter or when no barriers are present as in cerebrospinal fluid.
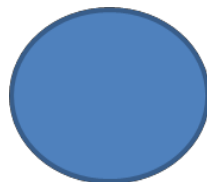
Figure 28 Isotropic diffusion

The white matter (WM) in contrast, is coherently organised due to axons, with a cylindrical structure that is bundled into tracts, with additional cellular barriers in the form of the myelin sheath. Thus in WM water diffusion is greater along one direction

(parallel to tract), but restricted perpendicular to the tract, resulting in anisotropic diffusion (Figure 29) (Le Bihan et al., 2001).
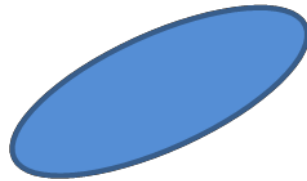
Figure 29 Anisotropic diffusion

The diffusion within each voxel of the brain image is described mathematically by a diffusion tensor that has magnitude and direction. To best characterise the diffusion tensor in each voxel, DWI is obtained by applying diffusion gradients in at least 6 non-collinear directions (Le Bihan et al., 2001)

The diffusion tensor is expressed by 3 eigenvectors (V1-V3) and their respective 3 eigenvalues (λ 1-3). The eigenvalues represent the magnitude of diffusion in each of the 3 dimensions and the eigenvectors represent the orientation of the 3 eigenvalues. In isotropic diffusion all eigenvalues are equal. In anisotropic diffusion the principal eigenvalue λ 1 is greater than λ 2 and λ 3.

An MRI voxel can also contain crossing fibre tracts that produce a more complex pattern of diffusion. This is usually characterised by 2 eigenvalues that are larger than the third (λ 1= λ 2 > λ 3)

The degree of anisotropic diffusion is summarised by a quantitative measure known as fractional anisotropy (FA), which reflects how strongly structured the underlying white matter is. In very coherent structures like WM tracts the FA value approaches unity and in less coherent structures such as CSF and grey matter the FA value approaches 0.

Three other indices of significance, obtained from DTI are mean diffusivity, radial diffusivity (λ_{radial}) and axial diffusivity (λ_{axial}). Mean diffusivity (MD) is an indicator of the total amount of diffusion in a given voxel, irrespective of the direction of diffusion. Axial diffusivity, quantifies diffusion in the principal direction of diffusion and radial diffusivity quantifies diffusion perpendicular to the principal direction of diffusion. (Pierpaoli et al., 1996). Thus changes in one or both (λ_{radial}, λ_{axial}) could underlie changes in FA and MD.

The two most commonly used DTI parameters in neuroimaging research are FA and MD (Pierpaoli et al., 1996). Diffusion within WM is influenced by density of axons, axonal diameter and integrity and degree of myelination.

Longitudinal quantitative MRI studies in paediatric and adults populations have revealed a linear increase in white matter volume until 20 yrs of age (Giedd et al., 1999a). DTI studies of white matter development in healthy paediatric populations have shown increased FA and decreased MD values with increasing age and maturity (Schmithorst et al., 2002) (Cascio et al., 2007). Thus, increased diffusion within the WM, indicated by low FA levels, reflect underdeveloped or compromised WM (Beaulieu et al., 2002; Cascio et al., 2007).

In a healthy paediatric population, Schmithorst et al have demonstrated that FA correlates positively and MD negatively with full scale IQ and reduced FA levels has been shown to correlate with neurocognitive and emotional and behavioural dysfunction in children with prematurity (van Kooij et al., 2012), diabetes (Kodl et al., 2008) and autism (Ameis et al., 2011), amongst several other conditions.

Thus DTI enables detection of subtle WM changes, unrecognised by conventional neuroimaging and can be used to investigate the relationship between structural deficit and neurocognitive impairment.

Children with HH are at a high risk of motor and neurocognitive development (Menni et al., 2001). Unpublished data from a pilot study of HH children (n=17) from our centre, highlighted cognitive deficits in the form of low average memory and attention scores and average IQ scores. As white matter involvement has been implicated in cognitive impairments in children with hypoglycaemia (Burns et al., 2008), we hypothesised that children with HH and cognitive deficits may have underlying white matter damage.

10.2.2 Specific Aims

To detect if children with HH have white matter microstructural abnormalities/deficits (reflected by reduced FA) in comparison to children with KH

To detect if FA values correlate to neurocognitive measures

10.2.3 Method

10.2.3.1 Participants

HH group: 21 children recruited into study. One child with gross structural abnormality (patient 1, Table 16) was excluded to prevent alignment issues in group analysis and 5 children >13yrs (patients 7,12,17, 20 and 21 in Table 16) were excluded to perform age matched comparison with KH group. Thus, 15 children were included in the TBSS (Tract Based Spatial Statistics) analysis.

KH group: 15 children recruited into study. DTI images were not completed for 2: patients 2 and 3 inTable 18 (due to compliance with scanning and motion) and 1 patient excluded from study as parent has learning difficulties. Thus, 12 children were included in TBSS analysis.

10.2.3.2 Image acquisition

All the children were scanned unsedated on a 1.5 T Siemans Avanto MRI system. DTI was obtained using diffusion-weighted spin-echo echoplanar imaging sequence (TR 7300 ms, TE 81 ms, field of view 240 mm, and acquisition matrix 96x96). 60 slices of 2.5 mm thickness and zero gaps were collected encompassing the whole brain. Data was collected in 60 non-collinear directions, by applying diffusion gradients at b=1000 s mm^{-2}. Four non- diffusion weighted datasets (b=0) were also collected in an interleaved fashion during the sequence. Final image resolution was 2.5×2.5×2.5 mm.

Images were then transferred to a separate workstation for processing. The processing and statistical analysis of the data is outlined below.

10.2.3.3 Image processing and analysis

Diffusion-weighted images were processed using tools from the Functional Magnetic Resonance Imaging of the Brain Software Library (FSL; www.fmrib.ox.ac.uk/fsl). Initial processing of data involved inspection for movement artefacts, removal of eddy-current induced distortions and brain extraction by skull stripping. The diffusion tensor model was then fitted in each voxel and FA, MD, λ_{radial} and λ_{axial} maps were generated.

FA maps were then processed using Tract-based spatial statistics (TBSS), part of FSL (Smith et al. 2004). TBSS is an automated, observer independent, statistical approach to compare diffusion tensor imaging data between groups (Smith et al., 2006).

In TBSS, the FA image of each subject is non-linearly aligned to a common space. A group mean FA map is created and thinned to form a group mean FA skeleton by projecting the FA data of all subjects onto it. A projection step that searches for the point of maximal FA perpendicular to the FA skeleton ensures that the FA measurements projected are from the centre of each individual's white matter tract. This is a crucial step in TBSS that overcomes alignment issues faced by other automated whole brain voxel-wise comparison methods such as voxel-based morphometry (VBM).

The FA threshold is then set > 0.20 to ensure inclusion of major central white matter tracts in the analysis and exclusion of grey matter and peripheral white matter tracts that may be highly variable between subjects. This is followed by voxel-by-voxel analysis between groups, of mean FA WM skeleton, which is fully corrected for multiple comparisons across space. Similar transformations were applied to MD, λ_{radial} and λ_{axial} maps.

All preprocessing of images were performed by myself and the final TBSS analysis and extraction of DTI indices were performed by Dr.Kiran Seunarine (Institute of Child Health).

Statistical analysis

Analysis of covariance was used to compare the DTI indices between groups, using age and gender, (that influence brain development and myelination) as covariates. The relationship between cognitive measures and DTI indices (FA, MD, λ_{radial}, λ_{axial}) was investigated using partial correlations, controlling for age at scan and gender.

10.2.4 Results

Baseline characteristics

DTI data from 15 children with HH and 12 children with KH were compared using TBSS.

As children in the KH group were <13 years, in order to perform an age matched comparison, children >13 years were excluded from HH group.

Baseline characteristics are summarised in Table 42.

Table 42 Baseline characteristics of HH and KH group in TBSS analysis

Patient characteristics	HH	KH	p-value
Number of patients (no)	15	12	
Age at scan in yrs (mean ± SEM)	9 (0.5)	8.5 (0.6)	0.549
Age at neuroimaging in yrs (mean± SEM)	8.2 (0.4)	8.5 (0.6)	0.673
Age at diagnosis in yrs (mean ± SEM)	0.32	2.5	<0.0001
Gender (Male/female)	8M/7F	9M/3F	0.424
Birth weight in grams (mean ± SEM)	4019.2 (197.5)	3246.2 (222.9)	0.016
Handedness (Right/Left)	10R/5L	9R/3L	0.696
Height SDS (median)	0.08	-0.24	0.674
Weight SDS (median)	0.37	-0.19	0.14
Seizures at diagnosis (patient no)	9	3	0.121

The mean age of presentation in the HH group is significantly lower than the mean age of presentation in the KH group, which is consistent with underlying diagnosis. Birthweight is also significantly different between groups, primarily due to an increase in weight of hyperinsulinaemic patients. Although the KH group has a significantly lower birth weight, this is clinically within normal limits.

No significant difference in maternal education (p=0.692, FET) or socioeconomic status (p=1.000, FET) was found between groups.

Results of TBSS analysis (Table 43)

Global White Matter: WM skeleton mean FA, MD, λ_{radial} and λ_{axial} values were compared (ANCOVA) between groups, controlling for age and gender. Results indicate significantly lower FA (p = 0.018, Figure 30) and greater λ_{radial} (0.022, Figure 32) in HH group, relative to KH group. This suggests that decrease in FA is secondary to increase in radial diffusivity. Mean diffusivity (p = 0.070, Figure 31) and axial diffusivity (p= 0.77) were not significantly different between groups.
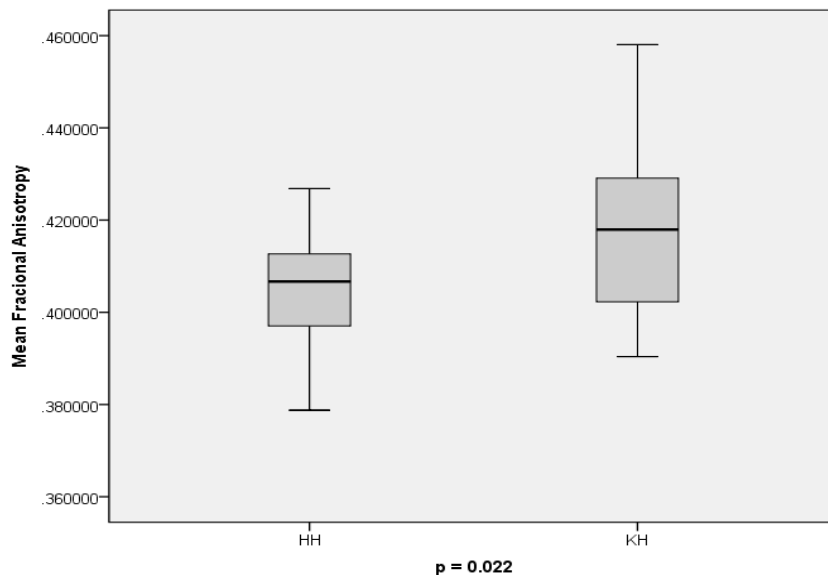
Figure 30 Comparison of WM skeleton fractional anisotropy between HH and KH group
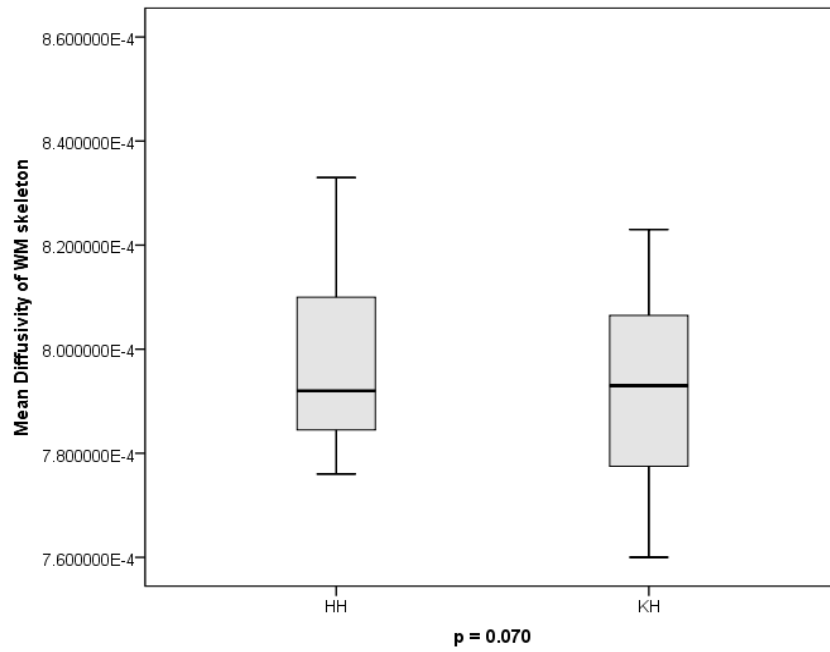
Figure 31 Comparison of WM skeleton mean diffusivity between HH and KH
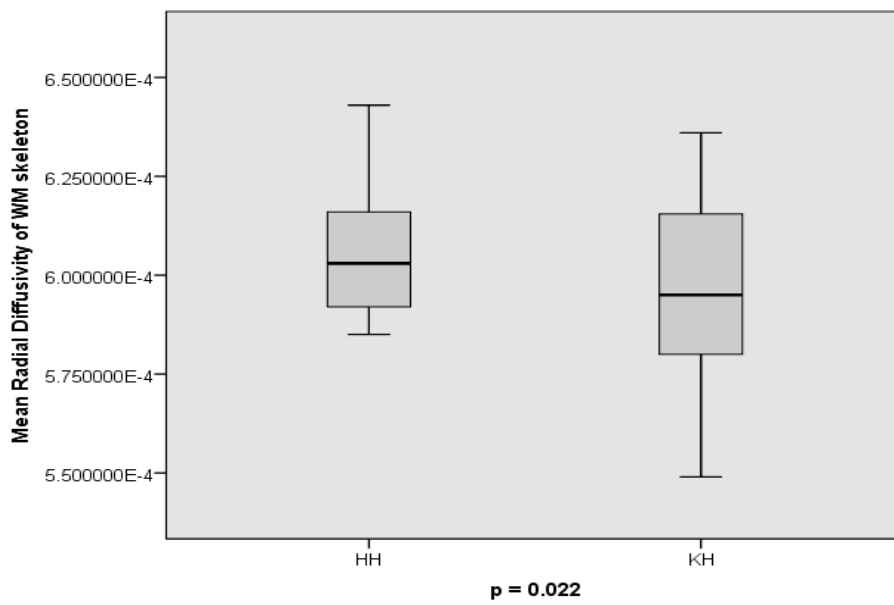
Figure 32 Comparison of WM skeleton radial diffusivity between HH and KH

Regional WM: Results of TBSS comparing HH and age- matched KH groups are shown in Figure 33. The mean FA skeleton is overlaid on mean FA image. Green areas denote regions with no significant differences in FA values between HH and KH, areas of red and yellow denote significantly lower FA values in HH group.

Significantly low FA values (p=0.05) were seen in genu, splenium and body of corpus callosum in the HH group.
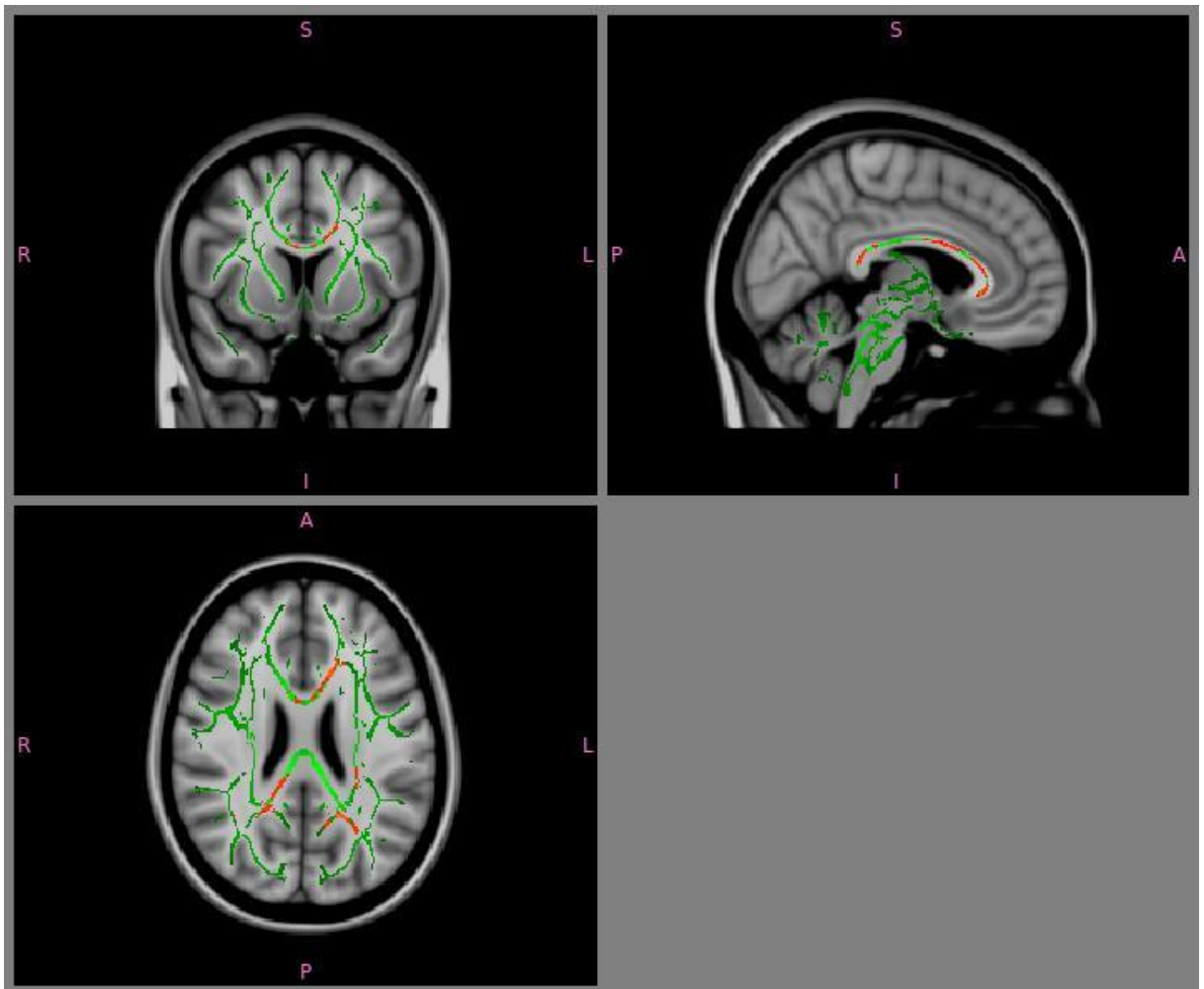
Figure 33 Results of TBSS analysis comparing fractional anisotropy (FA) between HH and KH group. Green areas denote regions with no significant differences in FA values between HH and KH, areas of red and yellow denote significantly lower FA values in HH group. Significantly low FA values (p=0.05) were seen in genu, splenium and body of corpus callosum in HH group.

Corpus Callosum (CC): FA values were significantly lower in CC (p = 0.022), especially in genu (p = 0.021) and splenium (p = 0.043). Radial diffusivity was significantly higher in CC (p = 0.018), including body (p = 0.041), genu (p = 0.035) and splenium (p = 0.053). Mean Diffusivity was significantly greater in CC (p = 0.050) and body of CC (p = 0.039) (Table 43).

Table 43 Comparison of DTI indices between HH and KH group

DTI indices	Type	Mean	p-value
Mean Fractional Anisotropy of WM skeleton	HH	0.405760	
	KH	0.418433	0.018
Mean Diffusivity of WM skeleton	HH	0.000800	
	KH	0.000793	0.07
Mean Radial Diffusivity (RAD) of WM skeleton	HH	0.000610	
	KH	0.000596	0.022
Mean Axial Diffusivity (AD) of WM skeleton	HH	0.001182	
	KH	0.001186	0.77
Corpus Callosum FA	HH	0.676470	
	KH	0.699510	0.022
Body of Corpus Callosum FA	HH	0.639028	
	KH	0.660683	0.081
Genu of Corpus Callosum FA	HH	0.686601	
	KH	0.718064	0.021
Splenium of Corpus Callosum FA	HH	0.718047	
	KH	0.736817	0.043
Corpus Callosum AD	HH	0.001611	
	KH	0.001617	0.729
Body of Corpus Callosum AD	HH	0.001626	
	KH	0.001623	0.363
Genu of Corpus Callosum AD	HH	0.001591	
	KH	0.001598	0.786
Splenium of Corpus Callosum AD	HH	0.001607	

	KH	0.001624	0.681
Corpus Callosum RAD	HH	0.000438	
	KH	0.000406	0.018
Body of Corpus Callosum RAD	HH	0.000491	
	KH	0.000458	0.041
Genu of Corpus Callosum RAD	HH	0.000419	
	KH	0.000374	0.035
Splenium of Corpus Callosum RAD	HH	0.000384	
	KH	0.000362	0.053
Corpus Callosum MD	HH	0.000829	
	KH	0.000810	0.05
Body of Corpus Callosum MD	HH	0.000869	
	KH	0.000846	0.039
Genu of Corpus Callosum MD	HH	0.000810	
	KH	0.000782	0.106
Splenium of Corpus Callosum MD	HH	0.000792	
	KH	0.000783	0.253

Relationship between DTI indices and cognitive function: In children with HH mean WM FA correlated significantly with full scale IQ (r = 0.586, p = 0.035) and perceptual reasoning index (r = 0.691, p = 0.009). FA values of body of corpus callosum also correlated positively to full scale IQ (r = 0.675, p = 0.011).
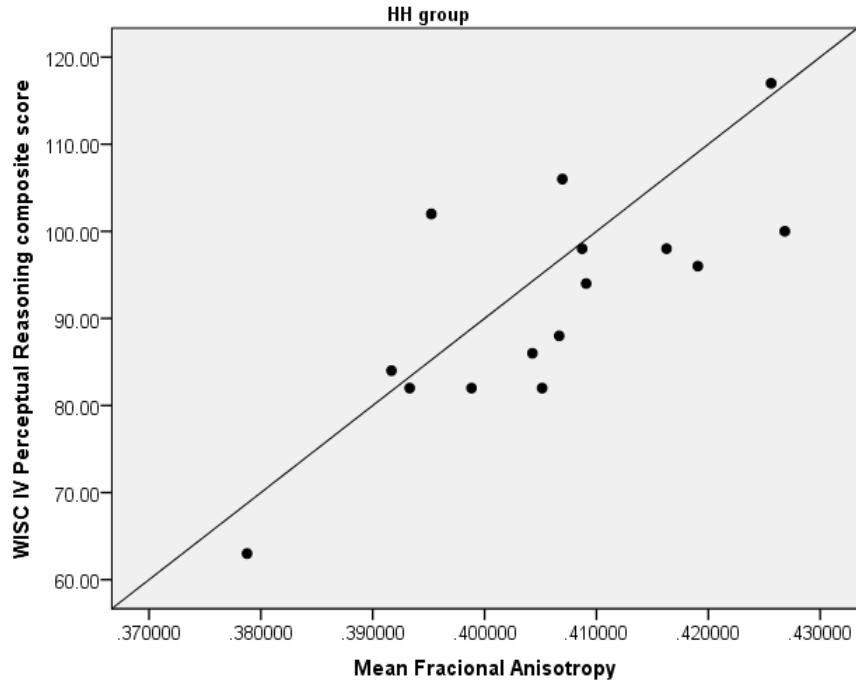
Figure 34 Relationship between mean fractional anisotropy and perceptual reasoning index in HH group
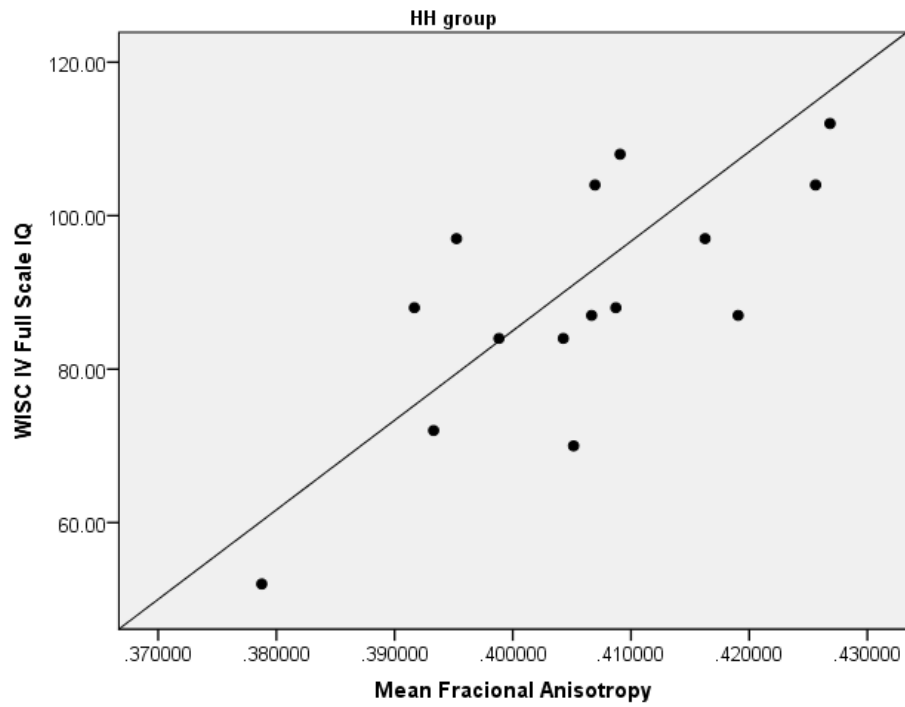
Figure 35 Relationship between mean fractional anisotropy and full scale IQ in HH
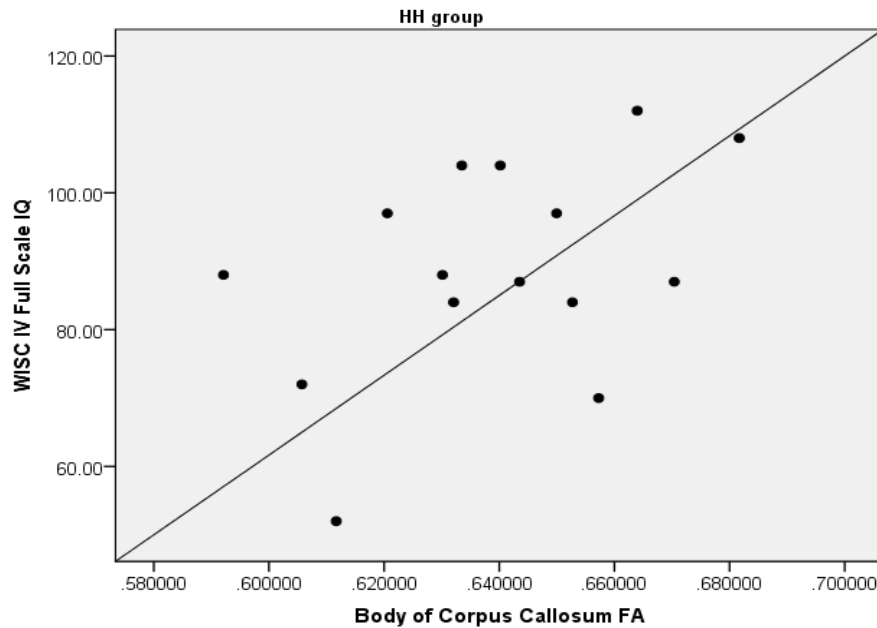
Figure 36 Relationship between body of corpus callosum fractional anisotropy and full scale IQ in HH group

10.2.5 Discussion

In this study FA, MD, axial and radial diffusivity were estimated for the whole brain in HH and KH patients and TBSS was used to look for significant differences in WM between groups

Results of this study suggest that white matter microstructural deficits exist in children with HH. Relative to to the KH group, significant differences were noted in whole white matter skeleton and more specifically in genu, splenium and body of corpus callosum. FA values (WM skeleton and body of corpus callosum) positively correlated to perceptual reasoning and full scale IQ measures in the HH group, suggesting white matter deficits in HH partly underlie these cognitive impairments.

Global WM: To date, no DTI studies investigating white matter changes in HH have been performed in children. The global reduction of WM suggested by the decreased FA of WM skeleton corresponds with the conventional MRI report of reduced white matter in 33% of HH children described in an earlier chapter (chapter 8).

This global reduction of WM is consistent with the report by Burns et al (Burns et al., 2008), describing a predominant white matter involvement in early MRI scans (< 6 weeks) of children with symptomatic hypoglycaemia that was associated with impaired neurocognitive development at 18-24 months of age. This study as an extension has shown predominant WM deficit following HH, persisting in late scans that correlated to neurocognitive measures.

A comprehensive assessment of the neurocognitive profile of the HH and KH group (described in chapter 9), has highlighted significantly impaired perceptual reasoning and full scale IQ scores in children with HH. The positive correlation of PR and FSIQ scores with mean FA in this study, suggests that impaired white matter structural integrity most likely underlies the IQ impairment.

The KH group in this study also has a high incidence of white matter abnormalities on conventional neuroimaging (28.5%, chapter 8). This probably underestimates the white matter differences detected by TBSS. It's likely that larger differences may have been detected if HH group had been compared to healthy controls.

White matter is composed of glial cells (microglia, radial glia, astrocytes and oligodendrocytes) and myelinated axons. The axons are organised into projection (between brain and spinal cord), association (between different regions within a hemisphere) and commissural (between right and left hemispheres) fibres that enable communication between different neuronal cortical regions. These pathways are a crucial component of the complex integrated neural networks that underlie various cognitive processes.

Human and animal studies have indicated, a peak period of synaptogenesis during the last trimester and first postnatal year of life (De Graaf-Peters et al., 2006), and both synapse formation and elimination continues on during childhood and adolescence.

MRI studies of human brain development have shown that myelination begins as early as 29 weeks of gestation (Inder et al., 2000), and occurs in a predefined spatial and temporal sequence with most tracts myelinated by early childhood, although some tracts (eg: arcuate fasciculus and corpus callosum) can continue to myelinate through adolescence (Lenroot & Giedd et al., 2006; Pujol et al., 1993).

Thus it is reasonable to speculate that recurrent episodes of HH during this crucial period, maybe detrimental to this process and affect white matter structure and function.

Rivkees et al (Yan & Rivkees et al., 2006) have shown that hypoglycemia in newborn rats can affect oligodendrocyte precursor cell differentiation, maturation, migration and viability in vitro. Oligodendrocytes are cells that contribute to myelin formation in the central nervous system. This may be one of the pathogenetic mechanisms of white matter injury in HH. Excitotoxic mechanisms and free radical generation can also cause injury to glia, myelin and axons and cause cytoskeletal degradation of these structures (Stys et al., 2004)

Regional WM: This study has identified the CC as particularly vulnerable to injury in children with HH.

The CC is a major WM tract connecting the cerebral hemispheres. It connects homologous regions between the right and left hemispheres. Thus fibres within the anterior part of CC, the genu, are mostly derived from the frontal lobes, while the fibres in the posterior CC, the splenium are derived from the temporal and occipital cortices.

Quantitative MRI studies in children and adults has shown that the corpus callosum continues to mature (fiber redirection, myelination and pruning) into adulthood, with maximal growth during childhood and adolescence (Pujol et al., 1993) and with more marked changes occurring in the splenium (Giedd et al., 1999)

Diffusion weighted imaging in adults with hypoglycaemic encephalopathy, has demonstrated restricted diffusion in the splenium of the corpus callosum and posterior limb of internal capsule, in the acute phase (Atay et al., 2012). Diffusion weighted imaging in neonatal hypoglycaemia has shown restricted diffusion in OP lobes and splenium of the corpus callosum in the acute phase and increased diffusion on DWI and atrophy of these regions on conventional imaging in chronic phase (Kim et al., 2006)

Thus the vulnerability of the CC to HH demonstrated in this study is consistent with case reports of diffusion weighted imaging in adults and neonates with hypoglycaemia Children in this study were specifically chosen above 5 yrs of age, to allow for maximal myelination (Nakagawa et al., 1998) and brain growth (Lenroot & Giedd et al., 2006).

Of the 6 children in the HH group that were excluded, 2 had abnormal scans on conventional neuroimaging. One had bilateral hippocampal volume reduction and severe global reduction of white matter and one had mild global reduction of WM. Of the 3 children with KH excluded from the analysis, 2 had abnormal scans on conventional neuroimaging, one had bilateral hippocampal volume reduction and mild global reduction of white matter and one had mild global reduction of WM. As the distribution of WM involvement was equivalent in groups, it's unlikely that exclusion of these subjects has adversely influenced outcome.

10.2.6 Conclusion

In this study, TBSS analysis of DTI, comparing HH to KH has shown white matter microstructural deficits in the HH group (significantly reduced diffusion anisotropy). The corpus callosum, especially the genu and splenium are highlighted as specific white matter regions vulnerable to injury in HH. Mean FA in the WM skeleton in the HH group correlated positively with IQ and perceptual reasoning, indicating that structural white matter deficits may underlie the neurocognitive impairment relative to the KH group. This study indicates that WM is sensitive to hypoglycaemic brain injury and could result in impaired neurocognition.

This is a novel finding in HH and consistent with the recent evidence of a predominant pattern of white matter injury on conventional neuroimaging associated with impaired neurocognition following symptomatic neonatal hypoglycaemia (Burns et al., 2008).

11 General Discussion

This chapter summarises the findings of this study and relates the findings to the current knowledge regarding pathophysiological and biochemical mechanisms of the underlying disease. Areas requiring future research are also highlighted.

11.1 Review of neuropsychological and neuroimaging findings and their implications for HH group

White matter pathology and relation to cognitive indices

In the HH group (n=21) conventional neuroradiological assessments (chapter 8) revealed abnormal scans in 8/21 children. Diffuse white matter lesions and reduced hippocampus were the predominant pattern of brain injury along with some focal white matter lesions. The presence of diffuse white matter injury was corroborated by significantly low fractional anisotropy values in the mean white matter skeleton and corpus callosum analysed using novel quantitative MRI techniques (chapter 10). Consistent with the presence of diffuse white matter injury, neuropsychological tests (chapter 9) revealed widespread neurocognitive deficits in the domains of intelligence (perceptual reasoning), memory, attention (sustained attention) and manual dexterity A clear relationship between IQ scores and DTI indices, suggests that white matter structural deficits partly explain the neurocognitive deficits noted in the HH group. However despite the reports of reduced hipppocampal volumes on conventional neuroradiological assessments, hippocampal volumetry did not reveal any significant differences in comparison to children with KH.

Cognitive profiles with correlation to quantitative and qualitative neuroradiological measurements have not been reported previously in HH children. The widespread neurocognitive deficits are consistent with the knowledge that HH children are at a higher risk of brain injury compared to other forms of hypoglycaemia.

Bilateral parieto-occipital cortical and subcortical injury is considered the hallmark of hypoglycaemic brain injury in children (Alkalay et al., 2005). The patients with this pattern of brain injury reported in the literature are invariably neonates with prolonged severe hypoglycaemia associated with seizures or coma. The predominat pattern of global WM injury reported in this thesis most likely reflects the pattern of injury following recurrent ongoing moderate hypoglycaemia although some of these children have also experienced severe hypoglycaemia during the neonatal period. Burns et al (Burns et al., 2008) found a similar pattern of predominant WM injury and cognitive impairment following symptomatic neonatal hypoglycaemia. A high incidence of seizures at diagnosis, ongoing epilepsy and prematurity were all noted in the HH group that could have potentiated the hypoglycaemic brain injury, however these features are common in children with HH.

In accordance with the hypothesis of increased white matter vulnerability to hypoglycaemia and higher risk of injury in HH group, this study has clearly established the presence of white matter microstructural deficits in children with HH utilising qualitative and quantitative MRI techniques with correlation to cognitive indices.

Hippocampal pathology and memory indices

This study was unable to conclusively establish the presence of hippocampal damage or a specific memory impairment in children with HH.

The absence of significant differences in hippocampal volumes (hippocampal volumetry studies) between HH and KH group may be explained by the presence of hippocampal pathology in the KH group as suggested by the conventional neuroradiological assessments. Thus the ability to detect a significant change in HV was limited by comparison with a group that has also experienced hypoglycaemia and may have consequently developed hippocampal damage. Comparison with a healthy paediatric population would be required to establish the presence of hippocampal damage in either group.

However memory scores were significantly lower in HH group relative to the KH group (mean general memory scores HH vs KH 93 vs 110) but the results of this study failed to show any correlation between memory impairments in HH group and HV. Thus it is difficult to attribute the memory impairment to hippocampal pathology. A likely explanation would be that injury to MTL-cortical system (and not specifically the hippocampus) underlies this memory impairment. Injury to cortical structures subserving memory other than the hippocampus, such as the parahippocampal cortex may contribute to the memory impairment and were not explored in this study.

Another explanation for the low memory scores could be the relationship between intelligence and memory functioning. Concurrent administration of WISC-IV and CMS to 126 children aged 6-16 yrs have demonstrated low to high correlations (0.21 to 0.74) between the WISC-IV composite and CMS index scores. These correlations have been utilised to predict memory scores based on intellectual functioning (Drozdick et al., 2008). In this study, HH children, as a group did not demonstrate a disassociation between the predicted and achieved memory scores. Thus the memory impairments in HH may just be a reflection of their intellectual functioning.

Four children with HH achieved lower memory scores than predicted by 13-35 points. Thus some children seem to exhibit a specific memory impairment (disassociation between IQ and memory functioning). These children had lower left hippocampal volumes relative to children with IQ-memory discrepancy of <13 points, implicating hippocampal pathology as a cause of specific memory impairment in this group. Nonethless contribution from other regions subserving memory (medial temporal cortical circuit) cannot be excluded.

Three children in HH group demonstrated a bilateral reduction (21-35%) in hippocampal volume. Surprisingly apart from 1 child, specific memory impairment was not seen in the other two children. As hippocampus- dependent memory functions mature by 9-10 years (Bachevalier et al., 2005), these children may manifest impairments at a later date.

The findings of this study have important implications for the interpretation of neuroimaging findings in children with HH and counseling regarding neurocognitive prognosis.   It is recommended that children with HH undergo comprehensive neurocognitive assessment at various key stages of development to ascertain the pattern of cognitive impairment and to implement targeted strategies that will optimise neurocognitive outcomes.

11.2 Review of biochemical, neuropsychological and neuroimaging findings and their implications for KH group

Fasting studies

The KB response in children with KH is qualitatively and quantitatively different from control children. A novel finding is the increased rate of KB response in KH group (87% increase/hour) in comparison to controls, within the physiological limits of fasting tolerance.

The ability of the brain to utilise KB as an alternate fuel during fast depends on the concentration of KB in the blood, permeability of the blood brain barrier to KB transport and the activity of ketolytic enzymes (Morrisl et al., 2005).

Owen et al (Owen ., 1967) demonstrated an increase in KB concentrations to 5.8 - 9.7 mmol/l following a 5-6 week fast in 3 obese adults and cerebral vascular catheterisation, at the end of the fast demonstrated that KB had replaced glucose as the predominant fuel for metabolism. Measurement of cerebral arteriovenous difference of ketone bodies in children anasthetized for elective surgery (n=70, age 11-15 years) has demostrated a cerebral uptake of KB greater than adults after comparable periods of fasting (Settergren et al., 1980)

It is well known that ketonemia following a fast is greater and earlier in children compared to adults (Haymond et al., 1982). The greater cerebral metabolic demand and larger brain/body surface ratio in children in comparison to adults is thought to necessitate such a response to spare glucose while maintaining cerebral metabolism.

Thus the significantly increased magnitude and rate of KB response in KH children in this study relative to the control group is an indirect evidence of greater KB utilisation by the brain.

This study also suggests that KH group have an altered epinephrine response to fasting. The baseline adrenaline conentrations were elevated. Children are diagnosed with KH after exclusion of metabolic or endocrine causes of hypoglycaemia; however adrenaline concentrations during fasting are not routinely measured. The role of adrenaline in fasting metabolism in children and its response in KH needs to be explored further before the findings of this study can be extrapolated to the general population.

Studies investigating glucose kinetics in children with KH need to incorporate measurement of hormones (including adrenaline and glucagon) and gluconeogenic substrates to substantiate these findings.

Cortisol concentrations were found to be a significant predictor of KB concentrations at the end of the fast, with poor correlation to NEFA concentrations at the end of the fast. This suggests that cortisol regulates KB response during a fast in children with a possible direct role of cortisol on hepatic ketogenesis.

As adult reports of KH do not exist, follow up of the KH children to understand the nature of this epinephrine resonse (transient or persistent) and the mechanism of symptom resolution (increased gluconeogenic substrates or behavioural modification) may further the knowledge regarding the underlying mechanisms of KH

Neuroimaging studies and neurocognitive outcomes in KH

The presence of hippocampal pathology and focal and diffuse white matter lesions on conventional neuroradiological assessments in children with KH was unexpected. Five of 14 children had abnormal scans. Bilateral reduction of hippocampus was seen in one child who had additional focal and diffuse WM lesions, 1 child had focal WM lesion and 3 chidren had diffuse WM lesions. The clinical phenotype of KH children with abnormal scans was no different from the children with normal scans with respect to age at diagnosis, gestational age, birthweight and presence of seizures at diagnosis

The similarity of findings in HH and KH group suggest that changes may reflect injury due to hypoglycaemia. As a healthy control group was not studied in parallel, the occurrence of these lesions in the general population is not known. However the presence of associated neurocognitive impairment in two of these children with abnormal scans (Full scale IQ 77 (patient 3, Table 18) and 89 (patient 2, Table 18) suggest that these lesions may represent true injury as a consequence of hypoglycaemia. In addition 3 other children with normal scans had IQ scores (IQ 79-84: patient 12, 13 and 14 in Table 18) below 1 SD (i.e < 85). The above observations challenge the perception that ketotic hypoglycaemia is not associated with neurological injury and subsequent functional impairment.

Although children with KH have recurrent episodes of hypoglycaemia, they do not tend to be prolonged or as frequent as in children with HH. In addition fasting tests have shown that these children have higher concentration of KB during the entire catabolic state even prior to the episode of hypoglycaemia. Thus the evidence of neurological injury and impairment despite the presence of alternate fuels is surprising.

As premorbid cognitive and neuroimaging data are not available for this group, it is difficult to conclude with certainity that the impairment is a consequence of hypoglycaemia. However in the absence of any other associated pathology and similarity of findings to HH group it is highly likely that these impairments indicate hypoglycaemic brain injury. These finding would also support inconsistent reports in the literature regarding neurological impairment in KH.

The presence of injury in this group also questions the ability of KB to act as a neuroprotective fuel in the absence of glucose. The mechanism of injury and role of KB is discussed in the following section.

At present children with KH do not undergo routine neurodevelopmental follow up. While the findings of this study need to be interpreted with caution in the absence of comparison to a normal healthy population, it is recommended that children with KH undergo neuropsychological assessment at 5 years or at diagnosis (if age at diagnosis is > 5 years) to enable detection of neurocognitive deficits and institute appropriate management.

11.3 Mechanism of white matter injury and the role of ketone bodies

Immunohistochemical evidence of axonal injury following hypoglycaemic coma has been demonstrated by post-mortem adult studies (Dolinak et al., 200). In addition in vitro animal studies have demonstrated the susceptibility of oligodendrocytes, that form myelin, to hypoglycaemia (Yan and Rivkees., 2006). Thus white matter injury in hypoglycemia could be secondary to a combinaltion of axonal and oligodendrocyte injury and degeneration.

Experimental studies in adult rats have indicated that in the absence of glucose KB can maintain high energy phosphates but cannot maintain neural activity, however in immature rats in addition to maintenence of high energy phophates, neural activity also decayed more slowly, the time of decay increasing with decreasing age (Izumi et al., 1998; Wada et al., 1997; Arakawa et al., 1991).

A recent study that sheds light on the mechanism of ketotic hypoglycemic brain injury was performed by Schutz et al (Schutz et al., 2011). They investigated the ability of KB to compensate for glucose deprivation in developing rat brain. Neuronal injury was not noticed in this study, however KB treated hypoglycaemic rats manifested WM injury with a 20 fold increase in apoptosis of mature oligodendrocytes. As KB were maintained in high concentrations during hypoglycaemia and infant rats have high rate for cerebral KB utilization, the presence of injury was attributed to biochemical factors (explained below) resulting in oxidative injury to WM rather than insufficient transport or enzyme insufficiency.

Glucose via glycolysis and entry into Krebs cycle ensures oxidative energy production to maintain neuronal integrity and activity. In addition glycolytic substrates (glucose-6-phosphate) maintain the pentose phosphate pathway that generates nicotinamide adenine dinucleotide phosphate (NADPH) and 5-carbon sugars that have an anabolic role. The NADPH helps prevent oxidative stress and enables fatty acid synthesis, while the 5-carbon sugars enable nucleotides, nucleic acids and amino acids .While KB can enter krebs cycle and maintain oxidative phosphorylation, it is unable to compensate for the role of glucose in maintaining the pentose phosphate pathway. Schutz et al hypothesized that the lack of NADPH increases the susceptibility for oxidative stress injury to WM ketotic hypoglycaemia.

11.4 Limitations of study

Absence of a healthy control group was the main limitation of the study. Ethical approval was refused to recruit a healthy group. In addition limited resources (psychologist's time) did not permit recruitment of a greater number of children that may have influenced detection of subtle group differences. Absence of a healthy control group may lead to over estimation or under estimation of the effects of HBI. Healthy control group is especially essential in the quantitative imaging studies, as these newer techniques rely on semiautomated methods of brain segmentation and analysis and it would be ideal that in the absence of a gold standard, the validity of the method is assessed with a control group.

Another limitation was the use of multiple comparisons or statistical tests with a small data set. Multiple comparisons without correction increase the likelihood of false positive (type I error) or significant results. But correction (such as bonferroni correction) increases the likelihood of type II error or false negative results. The use of bonferroni correction for multiple comparisons is much debated in medical statistics (Perneger et al., 1998).

The biochemical tests and the neurocognitive tests used in this study, in clinical practice would be interpreted independently with reference to normal ranges. Hence it was felt appropriate to present findings without correction for multiple comparisons to enable interpretation that would be clinically relevant. The statistical tests for hippocampal volumetry were not significantly different between groups, hence adjustment for multiple comparisons would not have affected outcome. The TBSS technique used in the white matter studies enables correction for multiple comparisons within the statistical package.

Alternate quantitative methods to detect hippocampal pathology, such as T_2 relaxometry was not estimated in this study. This method involves obtaining images at different echo times and calculating the T_2 values for each pixel within the region of interest (hippocampus) and constructing a T_2 map for comparison with the control group. This

technique enables detection of pathology that is too subtle to be discerned by convenventional T_2 imaging and better at detecting bilateral pathology.

Due to the retrospective nature of data collection, additional clinical features such as frequency of hypoglycaemia, degree and duration of hypoglycaemia, management with glucose reperfusion, premorbid cognitive concerns were not available for this cohort that may have influenced the neurological outcome. Although neurological examination was normal in these children, head circumference measurements are not available to detect microcephaly.

11.5 Conclusion

In accordance with the hypothesis this study was able to establish that children with HH show evidence of widespread cognitive deficits despite the absence of overt neurological impairment. A novel finding is the pattern of brain injury. While previous studies have noted a specific pattern of occipito parietal injury following hypoglycaemia of different aetiologies, brain injury in this group of HH children, is predominantly in the form of white matter injury. The presence of WM injury has been corroborated by the use of qualitative (conventional neuroradiological assessments) and quantitative assessments (TBSS analysis of DTI). A clear relationship between cognitive deficits (IQ) and DTI indices, suggests that white matter structural deficits partly explain the neurocognitive deficits noted in the HH group. However, this study was unable to conclusively establish the presence of hippocampal damage or specific memory impairments in children with HH.

The biochemical studies have demonstrated novel biochemical changes in KH. Fasting studies have shown an increased rate and magnitude of KB response in KH children during hypoglycaemia relative to controls within physiological fasting time periods and also implicated cortisol as a possible regulator of ketogenesis in children. The increased KB response during fasting also supports the availability of KB's as alternate fuels during KH.

However, contrary to the hypothesis, this study was unable to conclusively establish KH children as a neurologically unimpaired group. The presence of abnormalities on conventional radiological assessment and hippocampal volumetry and the wide variation in the neurocognitive scores indicating the presence of neurological impairment in some of these children was unexpected and warrants further research.

11.6 Future research

The result of this study has highlighted several aspects that require further exploration.

The findings of this study in the HH and KH group need to be replicated in a larger cohort of children recruited prospectively and with neuroimaging and neuropsychological tests in comparison to a healthy control group. In KH children this would help confirm or refute the findings of this study. While in HH children the modulating effects of co-morbid conditions, treatment regimes (medical vs surgical) and clinical phenotype (early vs late onset) could to be explored further. Owing to the rarity of the condition (HH), this could be implemented as a multicentre national or international study.

The hippocampal volumes in both children with HH and KH require further exploration, in comparison with a healthy paediatric population Concomitant administration of neuropsychological tests that evaluate hippocampus dependent memory functions, such as the Rivermead behavioural memory test and Rey-Osterrieth complex figure test are also recommended to test everyday memory and visuospatial abilities respectively

Restricted by the small number of study participants, no attempt was made in this study to relate the neurocognitive or neuroimaging findings to the underlying genetic mechanism of HH. While it is clear that children with *GLUD*1 mutations, as reported in literature and in this study are more likely to sustain neurological injury, the impact of the other genetic mechanisms are less obvious and need to be explored further in larger cohorts with known genetics mechanisms of HH.

The white matter changes in the HH group also deserve further exploration. Advanced fibre tracking techniques (tractography) can be utilised to study specific white matter tracts in conjuction with appropriate neurocognitive tests. For example corpus callosum was vulnerable to injury in HH group in this study. Integrity of the the corpus callosum is necessary for bimanual coordination of hands and fingers; this can be explored further with tractography along with tests that assess bimanual coordination.

Grey matter changes were not explored in this study and this can be performed using voxel based morphometry (VBM) analysis of 3D structural (3D FLASH) data. Group specific regional changes in grey and white matter can be detected using VBM. This method could also be utilised to detect bilateral hippocampal pathology (Gadian et al., 2000).

The fasting studies in KH and control children have revealed that cortisol plays a role in the regulation of ketogenesis in children independent of lipolysis. This can be explored further by investigating KB response following an overnight fast in children suspected of cortisol insufficiency. In addition adrenaline response and its impact on KH children is not clear and this response needs to be investigated in larger studies, with additional investigation of glucose kinetics.

12 Appendix

12.1 Appendix 1- Consent form

Institute of Child Health

and Great Ormond Street Hospital for Children NHS Trust

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CONSENT FORM

Title of Project: **Hypoglycaemia and Brain Injury**

Name of Researcher: **Dr Khalid Hussain,** Consultant Paediatric Endocrinologist (02079052128 or K.Hussain@ich.ucl.ac.uk) , **Dr Anitha Kumaran**, Clinical Research Fellow (07930314414,0207 2429789/ Extn 2245, kumarananitha@hotmail.com)

Please fill in as fully as possible and circle Yes/ No where appropriate

1. I confirm that I have read and understand the information sheet dated....................
(version............) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. Yes/No

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. Yes/No

3. I understand that relevant sections of my medical notes and data collected during the study,

may be looked at by individuals from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. Yes/No

4. I agree for the psychologist to contact my child's school teacher to get details of progress at school if there are any concerns. Yes/No

5. I agree to take part in the above study.   Yes/No

_____   _____   _____

Name of Patient/Parent   Date   Signature

_____   _____   _____

Name of Person   Date   Signature

taking consent

When completed, 1copy for patient; 1 for researcher site file; 1 (original) to be kept in medical notes

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The
child
first
and
always

Participant Information Sheet (Parent information)

Study title: Hypoglycaemia and Brain Injury

You and your child are invited to take part in a research study, which is being carried out by Dr. Anitha Kumaran (Clinical Research Fellow) with the assistance of Dr. Khalid Hussain, Dr. Matthew Pitt, Professor David Gadian and Professor FarenehVargha-Khadem at Great Ormond Street

Hospital and Institute of Child Health. Before you decide to take part, please take time to read the following information which explains why we are performing this research and what it would involve for you. After reading the leaflet, Dr.Kumaran will contact you to explain the research in greater detail and answer any queries and obtain your written consent if you wish to take part.

What is the purpose of the study?

Hypoglycaemia (low blood glucose level) is one of the most common problems seen in children. As glucose is a major fuel for brain function, anything that leads to hypoglycaemia can cause brain damage, epilepsy, mental retardation. There are many reasons why children become hypoglycaemic, and can vary from severe forms like congenital hyperinsulinism (too much insulin is produced that lowers blood glucose level) to milder forms like fasting ketotic hypoglycaemia (low blood glucose accompanied by presence of ketone bodies which are by products of fat breakdown and serve as an alternate fuel for the brain). Though hypoglycaemia in children is common, we do not know the "safe" blood glucose level for normal brain function.

This study will help us to understand the blood glucose levels at which changes occur in brain electrical activity and understand the connection between brain injury, development and if the presence of alternative fuels (ex ketone bodies) protect against brain injury.

What will happen during the study?

All the tests explained below will be arranged around your routine clinic visits or according to your convenience. If you have to make any extra trips to the hospital for this study we will reimburse your travel costs.

1. <u>Fasting study</u>

When your doctor admits your child for a routine fasting investigation to Kingfisher /Rainforest ward, a cannula will be inserted as routine and some bloods taken at regular intervals as specified by your doctor based on your child's age and clinical condition. Most of the bloods we require would already be requested by your doctor as part of your fast **(ex cortisol, growth hormone, carnitine, amino acids, Insulin, ketone bodies, lactate). Of the blood sample taken from your child, we will take a tiny amount (2 mls) extra, at the start and end of fast, to measure chemical substances called intermediary metabolites and hormones (ex: glycerol, adrenaline, noradrenaline).** Our study will not involve any additional pain or cannula insertion for your child.

Additionally we would also like to study electrical signals of your child's brain during hypoglycaemia. To enable us to do this we will attach some wires with sensors to the scalp (head) only for the duration of the fast or towards end of fast. This procedure is commonly known as EEG (electro encephalogram). It is a painless (does not involve any needles) and safe procedure. **Changes in attention and arousal will be assessed by**

providing sound stimulus (ex clicks) via headphones and recording any changes in EEG. These investigations do not emit any rays which could harm your child.

2. <u>MRI Scan of Brain</u>

We will also perform an MRI scan of brain to help us understand the impact of hypoglycaemia on brain size and shape. As we need detailed information of the brain we have to use special imaging techniques which are not part of routine clinical MRI. This procedure is safe as it does not involve ionising radiation like CT or Xray or **use of dyes called contrast medium**. The MRI scanner consists of a large doughnut-shaped magnet that has a tunnel in the center. Your child will be placed on a table that slides into the tunnel. Your child has to lie still during this procedure to prevent blurring of images and the scanner may be noisy.

Children less than 6 years: Your child might be undergoing routine MRI scan of brain under general anaesthesia or sedation, if so we would like to add 15-20 mins extra scan time to obtain better pictures. The decision for MRI under general anaesthesia will be done by your doctor and is independent of participation in this project.

Children more than 6 years: No sedation will be required. The scan will last 40 – 50 mins.

3. <u>Developmental assessment</u>

We would like to asses your child's social, emotional, intellectual and physical development. This will help us understand areas of development that are affected by hypoglycaemia. Tests will be conducted by psychologists who are specially trained to examine children. Most children

enjoy this, but if your child gets tired we will take a break or stop altogether. We will need to see your child once for 5-6 hrs (with breaks) or we can split them into separate sessions if that is more convenient for you. These tests will give us a very clear idea of your child's developmental progress and we will also be able to find any delay or problems if present. We will ask your child's teacher how they are doing in school **only if you and** your child are happy for us to contact them.

We will also ask you and your child to fill out a questionnaire to explain the impact of managing hypoglycaemia on your day to day quality of life.

How long will the study take?

This study will run for 3 years but your child is required for only 3 visits. The first visit for the fast will be part of routine hospital visit and will not last longer that the duration of the fast. We will need to see your child two more times for the MRI (1 hour) and developmental assessment (5-6 hours).

Does my child have to take part?

If you decide not to take part in this study that is entirely your right. If you agree to take part we will ask you to sign a consent form. You can also withdraw at any time during the study, without giving a reason. This will not affect your child's present or future treatment.

Will this affect my child's clinical treatment?

No treatment or investigation will be withheld from your child as a result of this study.

What will happen to my child's treatment during and after this period?

Our study will not affect or influence your child's ongoing and future clinical care.

What are the risks or discomforts of taking part in the study?
None of the above mentioned procedures involve any additional risk as they are part of routine investigation and additional investigations are non invasive. If you child becomes uncomfortable or distressed by the sensors of the EEG we will discontinue the investigation.

The MRI scan will require your child to stay still for 40-50 mins and may be noisy, we will try to minimise discomfort by providing head phones and videos to watch while in scanner.

What are the benefits of taking part in the study?
This study might not be of any direct benefit to you but will help us understand the level of hypoglycaemia that affects the brain. We will also be able to define the areas of the brain affected and correlate this with development. This will improve our management of children with hypoglycaemia in the future**.**

What about my results?
Once the results are available we will discuss them with you and your child.

Will information about my child be confidential?
All information collected about your child will be kept strictly confidential, only the research team and a representative of the research ethics committee

will have access to them. The results of the study will be published or presented in meetings, but no personal information will be given to any report or publication.

Who has approved the study and who is funding it?
The Great Ormond Street Hospital Children's Charity is funding this study. 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.

Who do I speak to if problems arise?
If you have any concerns or questions about any aspect of the study, please contact Dr.Kumaran who will do her best to answer your question. If still unresolved then you can discuss them with the Principal Investigator, Dr. Khalid Hussain by post at Institute of Child Health, 30 Guilford Street, London WC1N 1EH, or if urgent, you can contact the switchboard on 02074059200, and they will put you in contact with him. If you wish to complain formally, you can do this through the NHS complaints procedure (details can be obtained from the hospital)

Dr.Anitha Kumaran
Clinical Research Fellow in Paediatric Endocrinology
Clinical and Molecular Genetics Unit, Institute of Child Health

30 Guilford Street, London.WC1N 1EH

Telephone: 07930314414. Email: kumarananitha@hotmail.com.

Thank you for taking the time to read this leaflet and considering taking part in this study

**Institute of Child Health
and Great Ormond Street Hospital for Children NHS Trust
UNIVERSITY COLLEGE LONDON**

Participant Information Sheet (Children information leaflet age 5-12 years)

Study title: Hypoglycaemia and Brain Injury

You are invited to take part in a research study. The study will look at how low blood sugar levels affect your brain. Before you decide to say YES or NO, you need to understand why this study is being done. So please go through this leaflet. Your parents have also been told about this, so you can also discuss with them about taking part.

Introduction

Glucose (sugar) levels in your body need to be kept steady so your brain can function well, as it needs sugar for it to run smoothly (like petrol for a car). When you have low blood sugar this can affect the signals from your brain. We want to understand the level of blood sugar at which these changes happen. When you have been having low blood sugars for some time this can change the way your brain looks and also the way you act and learn things.

Why do you want me to help?

We want to look at your brain signals when your blood sugar drops and also see if you behave or learn differently. This can help us take better care of children with your condition. We ask all children who have a condition like yours to take part.

What will happen if I take part?

When you come to the hospital for your regular blood tests you will have a canula inserted as routine. From the routine blood taken in the hospital we will take a small amount extra from the samples (**2 mls – half a teaspoonful**) without any additional pain for you. **We will also attach some wires to your head to look at your brain signals. You will also hear some sounds through a headphone that will change the brain signals that we record.** This will not hurt you. We would also use some game like tests to see how you learn new things, use words and play. We will have breaks so you don't get tired. We will also take pictures of your brain with a special machine called an MRI scanner. This is like a big doughnut shaped machine with a tunnel in the middle and you will be on a table that slides into the tunnel (look at picture). You have to lie still when the pictures are taken and the scanner can make a lot of noises. We will give you headphones and you can even watch a video while the pictures are being taken.

Is there any danger?

No, all these tests are very safe. Many children routinely have these tests without a problem.

What about my results and who will see information about me?

The doctors looking after you will talk to you and your parents about the results of these tests. Only your doctors will have all information about you. We may print the results in a magazine, so everybody can learn more about this condition, but your name will not be revealed.

Do I have to take part?

No it's up to you and your parents to decide. It's fine if you do not want to take part. The doctors and nurses will look after you the same as before.

Who can I speak to if I have questions?

You can speak to your parents as they have also been given information about the study. You can also speak to the doctors and nurses who look after you in the hospital.

Dr.Anitha Kumaran

Clinical Research Fellow in Paediatric Endocrinology

Clinical and Molecular Genetics Unit, Institute of Child Health

30 Guilford Street, London

WC1N 1EH. Telephone: 07930314414

Email: kumarananitha@hotmail.com

Institute of Child Health

and Great Ormond Street Hospital for Children NHS Trust

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Participant Information Sheet (Children information leaflet age 12-16yrs)

Study title: Hypoglycaemia and Brain Injury

You are invited to take part in a research study. The study will look at how low blood sugar levels affect your brain. Before you decide to take part, you need to understand why this study is being done. So please go through this leaflet carefully. Your parents have also been told about this, so you can discuss with them about taking part.

Introduction

Glucose (sugar) levels in your body need to be kept steady so your brain can function well, as it needs sugar for it to run smoothly (like petrol for a car). When you have low blood sugar this can affect the signals from your brain. We want to understand the level of blood sugar at which these changes happen. When you have been having low blood sugars for some time this can change the way your brain looks and also the way you act and learn things.

Why do you want me to help?

We want to look at your brain signals when your blood sugar drops and also see if you behave or learn differently. This can help us take better care of children with your condition. We ask all children who have a condition like yours to take part.

What will happen if I take part?

When you come to the hospital for your regular fasting blood tests you will be canulated as routine. Of the routine blood sample taken from you, we will take a small amount extra **(2 mls – half a teaspoonful) without any additional pain for you, to measure certain chemical substances . We will also attach some wires to your head to look at your brain signals. This is called an EEG (Electroencephalogram). You will also hear some sounds through a headphone that will change the brain signals that we record.** This will not cause you any pain, but can be slightly uncomfortable. We would also use some game like tests to see how you learn new things, use words. We will have breaks so you don't get tired. We will also take pictures of your brain with a special machine called an MRI scanner. This is like a big doughnut shaped machine with a tunnel in the middle and you will be on a table that slides into the tunnel (look at picture). You have to lie still when the pictures are taken and the scanner can make a lot of noises. We will give

you headphones and you can even watch a video while the pictures are being taken.

Is there any danger?

No, all these tests are very safe. Many children routinely have these tests without a problem.

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The doctors looking after you will talk to you and your parents about the results of these tests. Only your doctors will have all information about you. We may print the results in a magazine, so everybody can learn more about this condition, but your name will not be revealed.

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No it's up to you and your parents to decide. It's fine if you do not want to take part. The doctors and nurses will look after you the same as before.

Who can I speak to if I have questions?

You can speak to your parents as they have also been given information about the study. You can also speak to the doctors and nurses who look after you in the hospital.

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