The University Of Sheffield.

Exploring the Relationship between Impaired Awareness of Hypoglycaemia and Autonomic Neuropathy in Type One Diabetes Mellitus.

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Abstract

Background: Severe hypoglycaemia is a much feared complication of type one diabetes mellitus (T1DM) and historically diabetic autonomic neuropathy (DAN) was described as the underlying mechanism. The aim of these studies was to clarify if there is a relationship between DAN and impaired awareness of hypoglycaemia (IAH), the role DAN has in preventing improvement in IAH and cardiac autonomic response to hypoglycaemia.

Methods: Participants with T1DM and IAH (Gold score ≥4), and participants with T1DM and normal awareness of hypoglycaemia (Gold score <4) were recruited. They underwent cardiac autonomic function testing and were invited to undergo a stepped hyperinsulinaemic hypoglycaemic clamp study. The participants with IAH underwent a treatment programme aimed at improving awareness of hypoglycaemia.

Results: The prevalence of DAN and IAH was associated with a longer duration of disease. Awareness of hypoglycaemia improved in all patient groups (mean improvement Gold score ≥1) independent of cardiac autonomic function status but younger participants and those with a shorter duration of disease showed greater improvements. During hypoglycaemic clamp studies baseline heart rate was higher and vagal tone lower, in participants with diabetes compared to participants without diabetes. During hypoglycaemia heart rate increased with vagal withdrawal in participants with T1DM and normal awareness of hypoglycaemia. Little change in heart rate or heart rate variability was seen in participants with IAH.

Conclusions: The presence of cardiac autonomic neuropathy is not associated with IAH. Heart rate response to hypoglycaemia was time dependent and differed according to self reported awareness of hypoglycaemia. We suspect this is due to a failure of autonomic responses to hypoglycaemia associated with long duration of disease and reduced autonomic responses following repeated episodes of hypoglycaemia. Future work would be aimed at epidemiological studies to document an accurate prevalence of DAN and examining the possible associations with other complications of diabetes.

Abstract word count: 300

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Declaration

This thesis is original, unpublished and independent work by the author Emma Walkinshaw. The candidate confirms that the work submitted is her own and that appropriate credit has been given where work which has formed part of joint-authored publications. The contribution of the candidate and the other authors to this work has been explicitly indicated below.

Statement of Contribution

The hypoCOMPaSS trial was a multicentre trial and done in collaboration with a large team. None of the text of this thesis is taken directly from previously published or collaborative articles. The full hypoCOMPaSS trial protocol has been published (Little et al., 2012). The candidate, Emma Walkinshaw (EW), contributed to development of the hypoCOMPaSS protocol and the aforementioned publication. EW designed visits 4 and 21 where detailed cardiac autonomic function testing was carried out and completed the standard operating procedure for these visits.

The hypoCOMPaSS trial was run by a clinical research fellow (Olivia Chappel, Royal Bournemouth Hospital, Bournemouth; Lalantha Leelarathna, Addenbrooke's Hospital, Cambridge; Stuart Little, Newcastle Diabetes Centre, Newcastle Upon Tyne; Horng K Tan, Derriford Hospital, Plymouth and EW, Sheffield Teaching Hospitals, Sheffield) in each site overseen by the principal investigator in each unit. EW visited the other hospital sites to ensure autonomic function equipment was adequate and that the raw ECG data could be exported and sent to Sheffield University for analysis. EW co-ordinated the export of data from each site and prepared it for analysis.

The sub-study of the hypoCOMPaSS trial described in section 2.1 was designed and executed by EW. EW wrote the protocol and was responsible for recruitment, collection of data, conduct of ambulatory monitoring and hyperinsulinaemic clamp studies. Professor Simon Heller advised on study design and analysis of data, supervised conduct of hypersulinaemic clamp studies and has reviewed this

thesis. Alan Bernjak (postdoctoral fellow) analysed ECG recordings for heart rate variability using customised software.

Publications Arising from the HypoCOMPaSS Trial

1) HK TAN, H.K., LITLLE, S.A., LEELARATHNA, L., WALKINSHAW, E., LUBINA SOLOMON, A., KERR, D., HELLER, S.R., EVANS, M.L., SHAW, J.A., FLANAGAN, D., AND SPEIGHT, J. Hypoglycaemia avoidance training improves glucose variability in Type 1 diabetes (hypoCOMPaSS Study). Diabetic medicine 2016; 32:7.
2) Response to Comment on Little et al. Recovery of Hypoglycemia Awareness in Long-standing Type 1 Diabetes: A Multicenter 2× 2 Factorial Randomized Controlled Trial Comparing Insulin Pump With Multiple Daily Injections and Continuous With Conventional Glucose Self-monitoring (HypoCOMPaSS). *Diabetes Care* 2014; 37: 2114–2122
3) LITTLE, S.A., LEELARATHNA, L., WALKINSHAW, E., TAN, H.K., CHAPPLE, O., LUBINA-SOLOMON, A., CHADWICK, C., BARENDSE, S. STOCKEN, D.D., BRENNAND, C. MARSHALL, S., WOOD, R., SPEIGHT, J., KERR, D., FLANAGAN, D., HELLER, S.R., EVANS, M.L. AND SHAW, J.A.M. 2014. Recovery of Hypoglycaemia Awareness in Long-Standing Type 1 Diabetes: A Multicenter 2x2 Factorial Randomized Controlled Trial Comparing Insulin Pump with Multiple Daily Injections and Continuous With Convential Glucose Self Monitoring (HypoCOMPaSS). *Diabetes Care*, published online before print May 22, 2014 1935-5548.
4) LITTLE, S., LEELARATHNA, L., BARENDESE, S.M., WALKINSHAW, E., TAN, H.K., LUBINA SOLOMON, A., DE ZOYSA, N., ROGERS, H., CHOUDHARY, P., AMIEL, S.A., HELLER, S.R., EVANS, M. FLANAGAN, D., SPEIGHT, J., AND SHAW, J.A.M. 2014. Severe Hypoglycaemia in type 1 diabetes mellitus: underlying drivers and potential straegies for successful prevention. *Diabetes/Metabolism Research and Reviews*, 30, 175-190.
5) LEELARATHNA, L., LITTLE, S., WALKINSHAW, E., KAI TAN, H., LUBINA-SOLOMON, A., KUMARESWARAN, K., LANE, A., CHADWICK, T., MARSHALL, S., SPEIGHT, J., FLANAGAN, D., HELLER, S. R., SHAW, J. & EVANS, M. 2013. Restoration of Self-Awareness of Hypoglycemia in Adults With Long-Standing Type 1 Diabetes. *Diabetes Care*, 36**,** 4063-4070.
6) LITTLE, S., CHADWICK, T., CHOUDHARY, P., BRENNAND, C., STICKLAND, J., BARENDSE, S., OLATEJU, T., LEELARATHNA, L., WALKINSHAW, E., TAN, H., MARSHALL, S., THOMAS, R., HELLER, S. R., EVANS, M., KERR, D., FLANAGAN, D., SPEIGHT, J. & SHAW, J. 2012. Comparison of Optimised MDI versus Pumps with or without Sensors in Severe Hypoglycaemia (the Hypo COMPaSS trial). *BMC Endocrine Disorders*, 12**,** 1-14.

Presentations Arising from the Work in this Thesis

1) WALKINSHAW, E., LITTLE, S.A., BERNJAK, A., LUBINA SOLOMON, A., CHOW, E., SHAW, J.A.M., and HELLER, S. Cardiac autonomic regulation during experimental hypoglycaemia in type 1 diabetes with impaired awareness of hypoglycaemia. Oral presentation, *EASD annual meeting*, 2015.
2) WALKINSHAW, E., LITTLE, S.A., BERNJAK, A., LUBINA SOLOMON, A., CHOW, E., SHAW, J.A.M., and HELLER, S. Cardiac autonomic regulation during experimental hypoglycaemia in type 1 diabetes with impaired awareness of hypoglycaemia. Poster presentation, *Diabetes UK annual conference*, 2014.

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List of Abbreviations

ACR	Albumin creatinine ratio
ADA	American Diabetes Association
BH	Biochemical hypoglycaemia
BRS	Baroreflex sensitivity
CGM	Continuous glucose monitoring
CONSORT	Consolidated Standards of Reporting Trials
CSII	Continuous subcutaneous insulin infusion
DAN	Diabetic autonomic neuropathy
DCCT	Diabetes control and complications trial
EDIC	Epidemiology of diabetes interventions and complications
HF	High frequency
HRV	Heart rate variability
HypoCOMPaSS	Comparison of Optimised MDI versus Pumps with or without Sensors in Severe Hypoglycaemia
IAH	Impaired awareness of hypoglycaemia
LF	Low frequency
LF norm	Normalised low frequency
MDI	Multiple daily injections
NN	Normal to normal or RR interval
QTc	Corrected QT interval
RCT	Randomised controlled trial
RMSSD intervals	Root mean square of the difference of successive RR
RT-CGM	Real time continuous glucose monitoring
SD	Standard deviation

SDNN	Standard deviation of the average RR intervals
SH	Severe hypoglycaemia
SMBG	Self monitoring of blood glucose
T1DM	Type one diabetes mellitus
WHO	World Health Organisation

Chapter 1: Background

1.1 Type One Diabetes Mellitus

Diabetes mellitus is a disorder of glucose metabolism that leads to high blood glucose levels (hyperglycaemia). There are two main types; type one (T1DM) and type two (T2DM). T1DM usually presents in childhood or adolescence and is an autoimmune condition. Destruction of the insulin producing beta cells of the pancreas results in insulin deficiency and hyperglycaemia.

T1DM is treated with subcutaneous insulin either via injection or by continuous infusion. Intensive insulin therapy is a term used to describe the tight management of blood glucose levels with the aim of avoiding the long term complications of diabetes (Group, 1993). It involves multiple daily injections of insulin, regular blood glucose testing and carbohydrate counting to calculate insulin doses.

The aim of intensive insulin therapy in T1DM is to mimic physiological endogenous insulin release. When blood glucose levels rise, the beta cells of the pancreas are stimulated to release insulin. The insulin is secreted into the portal vein, which enters the liver. Endogenous glucose production is inhibited and there is significant portal extraction of insulin before the blood enters the systemic circulation (Song et al., 2000).

The limiting factor in subcutaneous insulin delivery is the systemic utilisation of insulin before hepatic extraction. 'The periphery is over insulinised and the liver is underinsulinised' (Russell-Jones and Khan, 2006). This leads to a disproportionate systemic effect of insulin with increased peripheral glucose utilisation, a reduction in lipolysis and reduced hepatic glucose production suppression.

The clinical effects of over insulinisation are weight gain and hypoglycaemia. Despite the advances made in insulin formulations and delivery, subcutaneous insulin does not match the physiological response of the pancreatic beta cells. As a consequence, when aiming for normal blood glucose (normoglycaemia), there is

at times, an excess of insulin within the blood stream, which can cause low blood glucose (hypoglycaemia).

Hypoglycaemia is the main limiting factor preventing good glycaemic control in T1DM. It is a major cause of physical and psychological morbidity and can in rare cases be fatal (Cryer et al., 2003). The Diabetes Control and Complications Trial (DCCT) showed that intensive insulin therapy leads to an increase in episodes of severe hypoglycaemia (SH) (Group, 1991) (Figure 1). If it were not for hypoglycaemia patients could aim for tight management of blood glucose levels without concern (Cryer, 2002).

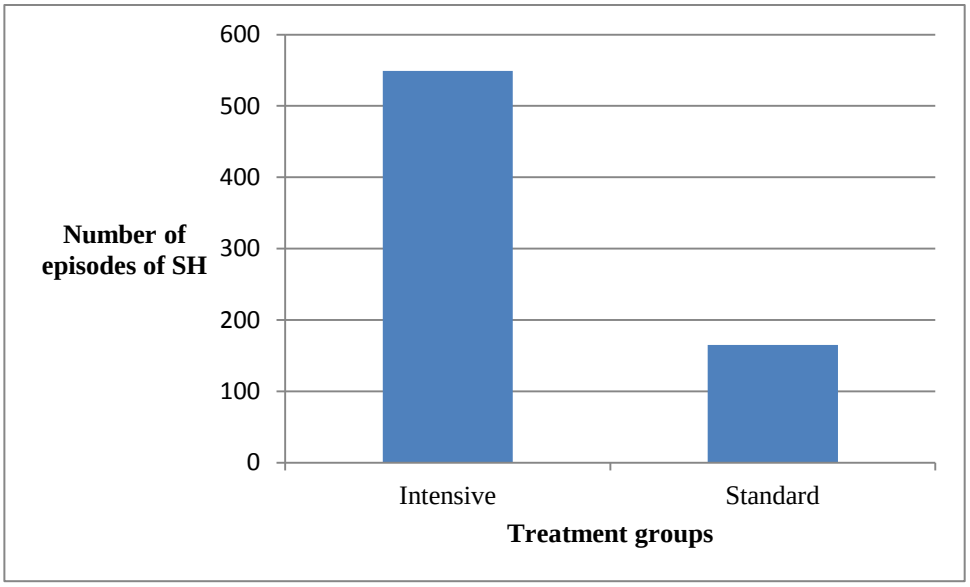

Figure 1 Occurrence of SH in treatment groups in the DCCT (Adapted from (Group, 1991)).

1.2 Hypoglycaemia

"*The price of normoglycaemia is often hypoglycaemia"* (Hillson, 2008).

Hypoglycaemia is a recognised side effect of treatment with insulin. It was first described by Banting et al (Banting et al., 1923) during his early work regarding insulin administration in diabetes. Early symptoms of hunger, tremor, sweating and pallor were followed by confusion and disorientation and finally coma. The episodes were relieved by administration of carbohydrate.

Hypoglycaemia in T1DM is common. Patients report mild (self-treated) episodes occurring once to twice a week (Leese et al., 2003). Severe episodes (defined as an episode requiring assistance from another person) affect 30% of patients with T1DM and occur on average once a year (Leese et al., 2003) (Cryer et al., 2003). Nocturnal hypoglycaemia is also common and often asymptomatic (Beregszaszi et al., 1997) (Kaufman et al., 2002). The incidence of nocturnal hypoglycaemia in young patients with T1DM has been reported to be as high as 47% with almost half being asymptomatic (Beregszaszi et al., 1997).

Hypoglycaemia affects all aspects of life in a patient with T1DM (Leelarathna et al., 2013) including employment and leisure time. Alongside the physical effects, psychological effects include anxiety and fear of hypoglycaemia (Cryer et al., 2003). Episodes of SH are associated with an increased risk of death with a reported 3.4 fold increased risk at five years (McCoy et al., 2012)

1.2.1 Glucose Counterregulation

Glucose counterregulation is the normal physiological response that prevents and corrects hypoglycaemia. In an individual without diabetes, endogenous insulin secretion is reduced and then completely inhibited as blood glucose levels decrease (Cryer, 2002) preventing hypoglycaemia from occurring (McAulay et al., 2001). In experimental studies where insulin is given to induce hypoglycaemia, the counterregulatory hormones glucagon, adrenaline, growth hormone and cortisol are released. This stimulates the liver to produce glucose and reduce utilisation by the peripheral tissues. The release of the counterregulatory hormones occurs before the individual experiences any symptoms (Schwartz et al., 1987) (Figure 2).

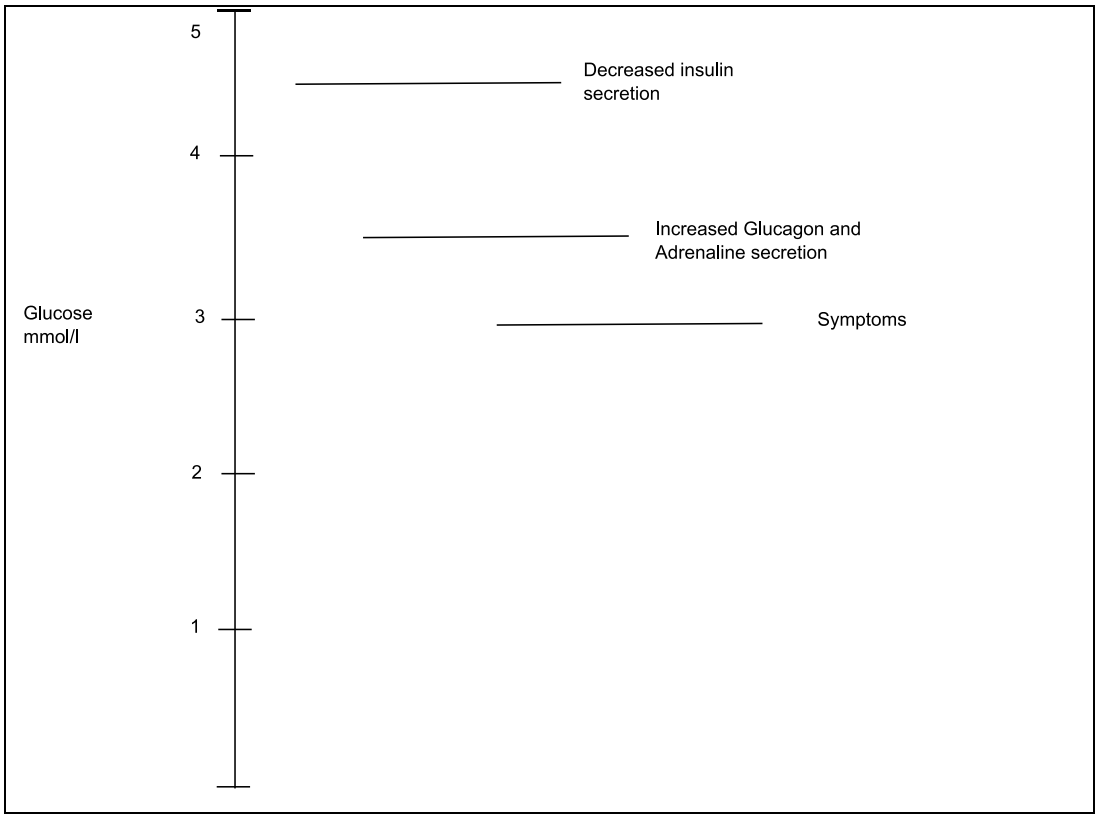

Figure 2 As plasma glucose is reduced insulin secretion is reduced and glucagon and adrenaline are released before the occurrence of symptoms (Adapted from (Cryer et al., 2003)).

Symptoms of hypoglycaemia are divided into two groups; autonomic symptoms and neuroglycopenic symptoms (McAulay et al., 2001). Autonomic warning symptoms of hypoglycaemia include palpitations, sweating, tremor and hunger. They are mediated by the autonomic nervous system and are thought to be mainly a result of sympathoadrenal activation and the release of noradrenaline, adrenaline and acetylcholine (McAulay et al., 2001) (Cryer et al., 2003). The neuroglycopenic or cognitive symptoms of hypoglycaemia include confusion, poor concentration, blurred vision and, more rarely, seizures and coma. They usually occur at lower blood glucose levels than the autonomic symptoms (Mitrakou et al., 1991) and are caused by a lack of glucose available for utilisation in the brain.

1.2.2 Glucose Counterregulation in T1DM

Normal glucose counterregulation is impaired in T1DM patients. Firstly, the exogenous insulin used to treat T1DM, is injected into the subcutaneous tissues, and will continue to be absorbed irrespective of blood glucose levels (Cryer, 2001). The second line of defence is the release of glucagon which stimulates the

release of glucose from the liver. This response has been shown to be blunted in comparison to adults without diabetes (Bolli et al., 1982) after five years of treatment of T1DM. As these first two defences against hypoglycaemia are impaired in T1DM, adrenaline release becomes increasingly important. Growth hormone and cortisol levels also increase in hypoglycaemia but they seem to play a minor role in glucose counterregulation (Cryer, 2002).

T1DM patients often have an attenuated adrenaline response to hypoglycaemia. Amiel et al (Amiel et al., 1988) showed that after a period of intensified insulin therapy, with improved glycaemic control and a reduction in HbA1c, patients required a lower blood glucose level to stimulate adrenaline release. The concentration of adrenaline produced was also lower and symptom awareness of hypoglycaemia was reduced.

1.2.3. Antecedent hypoglycaemia reduces awareness of subsequent episodes

A previous episode of hypoglycaemia will reduce both plasma adrenaline and symptomatic responses to future episodes. Heller and Cryer (Heller and Cryer, 1991) studied nine subjects without diabetes that underwent morning hypoglycaemic (glucose 2.8 mmol/l) clamp studies following afternoon hypoglycaemia (glucose 3mmol/l) and afternoon euglycaemia (5mmol/l). Plasma adrenaline, glucagon, cortisol and symptomatic responses in the hypoglycaemic clamp studies were reduced after a single episode of afternoon hypoglycaemia. This was confirmed by a similar study by Dagogo-Jack et al in patients with T1DM (Dagogo-Jack et al., 1993)(Table 1).

Time (Minutes)	After Hyperglycaemia	After Hypoglycaemia	Glucose (mmol/l)
0	14	13	5.6
60	16	15	5.0
120	18	15	4.4
180	19	18	3.9
240	22	19	3.3
300	42	22	2.8

Table 1: Total symptom score in response to morning hypoglycaemia following either afternoon hyperglycaemia or hypoglycaemia (Adapted from (Dagogo-Jack et al., 1993)).

The term 'Hypoglycaemia Associated Autonomic Failure', or HAAF, is used by some groups to describe the reduction in adrenaline release and symptoms to hypoglycaemia following a previous episode (Dagogo-Jack et al., 1993) (Vinik et al., 2003). HAAF reduces the defence against hypoglycaemia, leading to further episodes, which then result in further reductions in symptomatic and hormonal response to low blood glucose levels. This cycle of repeated hypoglycaemia reducing the defence against further episodes is particularly common in patients with tight glycaemic control (Vinik et al., 2003).

The term HAAF can be misinterpreted to suggest that classical diabetic autonomic neuropathy (DAN) is the underlying cause. Classical DAN is a complication of diabetes. The pathogenesis is still unclear but it has been shown to be a structural disorder with degenerative changes of the distal axons and nerve terminals (R.E.Schmidt, 2002) (Adamson et al., 1984). DAN is mostly irreversible; some improvement has been shown in mild cases with improved glycaemic control but treatment is mainly aimed at symptomatic relief if advanced (Vinik et al., 2003). The mechanism underlying HAAF is also unclear. It occurs after one episode of hypoglycaemia in both subjects with and without diabetes and can be reversed by complete avoidance of hypoglycaemia. This suggests a functional disorder rather than a structural one.

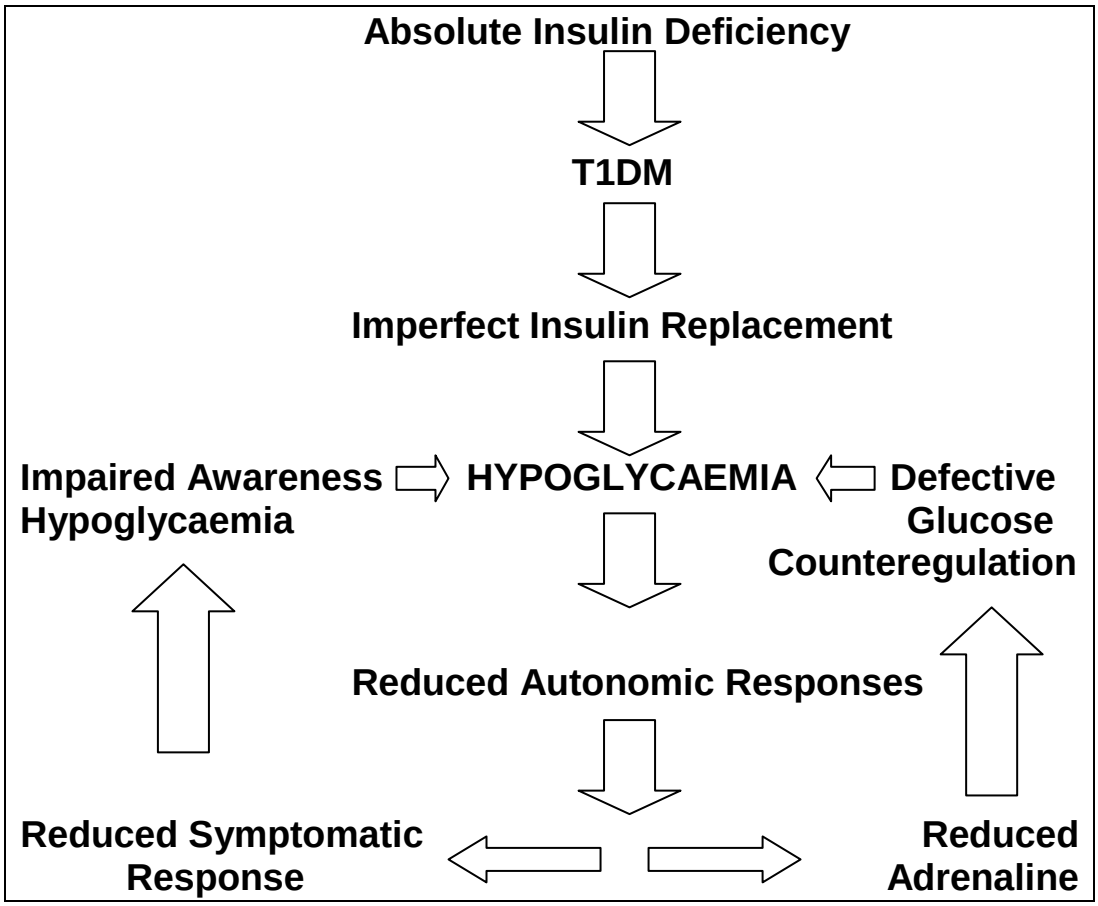

Figure 3: HAAF. Recent antecedent hypoglycaemia reduces adrenaline release and symptomatic awareness of further episodes of hypoglycaemia (Adapted from (Cryer, 2002)).

Dagogo-Jack et al (Dagogo-Jack et al., 1993) showed that in subjects, with and without DAN, lower glucose levels were required to elicit symptomatic responses to morning hypoglycaemia following afternoon hypoglycaemia compared to afternoon hyperglycaemia. Twenty six participants were recruited including 11 patients with DAN. Adrenaline levels were also significantly lower (P=0.0060) in response to hypoglycaemia following afternoon hypoglycaemia in participants with diabetes. The group found that adrenaline responses to hypoglycaemia did not differ in participants with and without DAN.

Although DAN and HAAF are separate entities other groups have challenged the findings by Dagogo-Jack and colleagues. It has been reported by several groups that a further reduction in adrenaline secretion is seen in patients with DAN compared to patients without neuropathy (Hilsted et al., 1981) (Bottini et al., 1997) (Polinsky et al., 1980, Hoeldtke et al., 1982). Patients in the Dagogo-Jack study (Dagogo-Jack et al., 1993) were diagnosed with DAN on the basis of one

abnormal test and self reported symptoms. Recommendations now state that definite DAN should only be diagnosed in those patients with two or more abnormal tests (O'Brien et al., 1991). This may explain the discrepancy between studies.

1.2.4 Impaired Awareness of Hypoglycaemia

Impaired awareness of hypoglycaemia (IAH) refers to the absence of the early, autonomic, warning symptoms of hypoglycaemia before neuroglycopenia occurs (Boyle et al., 1995) and is associated with an increased risk of SH (Gold et al., 1994). A prospective study carried out by Gold et al (Gold et al., 1994) showed that patients with IAH had an average annual incidence of severe hypoglycaemia of 2.8 compared to 0.5 episodes per year in patients with normal symptomatic responses to hypoglycaemia. Patients were clinically divided into two groups; those with reversible unawareness related to tight glycaemic control and those with irreversible unawareness related to a long duration of disease (Gold et al., 1994).

Experimental studies indicate that IAH is a result of an abnormal response of the sympathoadrenal system. Berlin et al (Berlin et al., 1987) challenged this by showing that in patients with reduced symptomatic awareness of hypoglycaemia there was also a reduced sensitivity to Isoproterenol, a Beta-adrenergic agonist. However, catecholamines (adrenaline and noradrenaline) were not measured during the Isoproterenol sensitivity test and Hilsted et al (Hilsted et al., 1987) have showed a normal response to adrenaline infusions in patients with diabetes. Other studies have consistently shown a reduction in adrenaline levels in response to experimental hypoglycaemia in patients with IAH (Heller et al., 1987, Adamson et al., 1984).

IAH is a well established phenomenon (Lawrence, 1941) and has a prevalence of 25% in patients with T1DM (Geddes et al., 2007). Studies regarding IAH are difficult to interpret due to numerous confounding factors including duration of diabetes, glycaemic control and HbA1c (Mokan et al., 1994). Hepburn et al. (Hepburn et al., 1990) studied a group of 226 patients with insulin dependent diabetes selected at random from a clinic in Edinburgh. Forty eight (16%)

patients reported partial awareness and twenty one patients (7%) had absent awareness of hypoglycaemia. The group with absent awareness was divided by duration of diabetes and only four patients (19%) had diabetes of less than 15 years duration.

The relationship between IAH, duration of diabetes and tight glycaemic control was corroborated by a study by Mokan et al (Mokan et al., 1994). They studied 43 patients with T1DM. Patients were admitted for a hypoglycaemic clamp study in which plasma glucose was clamped at 4.3, 3.6, 3.0 and 2.3 mmol/l. Patients completed symptom questionnaires every 15 minutes and underwent cognitive testing at each glucose plateau. Eleven patients (26%) were classified as having IAH which was defined as the development of autonomic symptoms at plasma glucose levels more than 2 SD below normal. Those patients with IAH had a longer duration of diabetes and a lower HbA1c (Table 2).

	IAH	P values
Duration of diabetes <10 years	5/30 (17%)	
Duration of diabetes >10 years	6/13 (46%)	<0.04
HbA1c <9%	8/13 (62%)	
HbA1c >9%	3/30 (10%)	<0.001
Duration >10 years and HbA1c <9%	5/6 (83%)	
Duration <10 years and HbA1c >9%	6/37 (16%)	<0.001

Table 2 Effect of duration of diabetes and HbA1c on frequency of IAH (Adapted from (Mokan et al., 1994)).

Impaired adrenergic and symptomatic responses to hypoglycaemia are common in patients with T1DM with established disease even in those who claim to have normal awareness of hypoglycaemia (Heller et al., 1987) (Olsen et al., 2014). Heller et al (Heller et al., 1987)found that only 4/15 subjects with diabetes recognised hypoglycaemia of 2.5mmol/l during a hypoglycaemic clamp study despite 10/15 subjects claiming to have intact awareness. They also established that adrenaline secretion was more pronounced in subjects without diabetes and the 4/15 subjects with diabetes that recognised hypoglycaemia from their symptoms. Olson et al (Olsen et al., 2014) found that subjective symptoms of hypoglycaemia change over time. Participants reported a lower intensity of

autonomic symptoms of hypoglycaemia as duration of diabetes increased and the mean neuroglycopenic/autonomic symptom ratio increased.

IAH can potentially be reversed. A group of patients with insulinomas (insulin secreting tumour) and frequent hypoglycaemia showed reduced symptomatic and counterregulatory responses to hypoglycaemia. This recovered following surgical cure (Mitrakou et al., 1993). Some recovery of symptomatic and adrenaline response to hypoglycaemia is also seen in T1DM patients following meticulous avoidance of low blood glucose levels (Fanelli et al., 1993a) (Cranston et al., 1994). However, patients with a longer duration of diabetes only showed a partial recovery of adrenaline response (Fannelli et al., 1994). This may explain the conflicting findings in a further study by Dagogo-Jack and colleagues that studied a small number of patients with a long duration of diabetes (Dagogo-Jack et al., 1994).

Treatment of IAH requires complete avoidance of hypoglycaemia (Zoysa et al., 2013). To achieve this patients undergo time consuming and expensive educational treatment programmes (Zoysa et al., 2013). Ideally patients that will not or will only partially respond to these treatment programmes would be identified to allow these resources to be used appropriately.

1.2.5 Summary

Hypoglycaemia prevents good glycaemic control in T1DM. Patients are vulnerable because of imperfect insulin replacement, the need for tight glycaemic control and impaired counterregulatory responses. Warning symptoms of a low blood glucose reduce after a single episode of hypoglycaemia and change with increased duration of disease. This increases the risk of SH although patients are often unaware of their diminished symptomatology.

IAH is a clinical entity where the autonomic symptoms of hypoglycaemia are reduced or absent increasing the risk of neuroglycopenia and SH. It is more prevalent in patients with a long duration of disease and those with tight glycaemic control. Traditionally it has been thought to be due to DAN but this is a structural, irreversible complication of diabetes where IAH is a functional and

reversible disorder. Treatment of IAH is expensive due to the requirement of intensive support from skilled and specially trained medical staff. Despite this, there are a proportion of patients who will not respond to treatment but currently these patients can't be identified before commencing treatment.

1.3 Diabetic Autonomic Neuropathy

'DAN is among the least known and understood complications of diabetes'(Vinik et al., 2003). It is a peripheral neuropathy that affects the autonomic nervous system, in patients with diabetes, after exclusion of other causes (Tesfaye et al., 2010). It can manifest with abnormalities in the cardiovascular, gastrointestinal, genitourinary, sudomotor or ocular systems (Consensus, 1988) and can be a clinical or subclinical finding (Vinik et al., 2003).

The reported prevalence of DAN in patients with T1DM ranges between 0 to 90% depending upon the group of patients investigated and the diagnostic criteria used (Vinik et al., 2003). The pathogenesis of DAN is unclear and hypotheses include vascular disease, hyperglycaemia and autoimmune dysfunction (Vinik et al., 2003) (Clarke et al., 1979). There is an increased risk for patients with a longer duration of diabetes (Dyrberg et al., 1981), increasing age (Boulton et al., 2005) and poor glycaemic control (Group, 1993).

Cardiac autonomic dysfunction is the most studied subgroup of the autonomic neuropathies (Boulton et al., 2005) because of the association with an increased risk of death (O'Brien et al., 1991). The diagnosis is made by examining changes in blood pressure and heart rate variability (HRV) at rest and during different manoeuvres. The tests are simple, non-invasive and can be completed at the bedside (Fisher, 2002).

Prognosis of DAN is poor and, once established, mostly irreversible. Ewing et al. (Ewing et al., 1980) studied a population of 73 participants with diabetes participants. Forty had abnormal cardiac autonomic function tests (AFTs) of which 21 (53%) died in comparison with 5 (15%) in the group of 33 patients with normal tests. Repeat testing during the five year follow up period showed that some tests that were normal became abnormal but abnormal tests did not normalise. A later review by Vinik and Ziegler (Vinik and Ziegler, 2007) did

report the potential to improve heart rate variability (HRV) in patients with minimal abnormalities. Regular exercise can improve HRV in patients with diabetes with no or early DAN (Howorka et al., 1997) and some improvement has also been seen with the use of angiotensin converting enzyme inhibition and angiotension receptor blockade (Didangelos et al., 2006).

The clinical symptoms of DAN are varied (Freeman, 2005) and depend upon the organ affected. Clinically, cardiac autonomic neuropathy, can cause a resting tachycardia and postural hypotension (Boulton et al., 2005) as the condition progresses. Traditionally DAN has been described as the underlying cause of IAH. This is because adrenaline release seen in response to hypoglycaemia is a function of the autonomic nervous system and adrenaline release is attenuated in DAN (Hilsted et al., 1981) (Bottini et al., 1997) (Polinsky et al., 1980, Hoeldtke et al., 1982) but the evidence in the literature is limited and contradictory.

The EURODIAB IDDM Complications Study (Stephenson et al., 1996) reported that DAN increases the risk for severe hypoglycaemia in T1DM. 3248 patients with insulin dependent diabetes were recruited and 1046 (32%) reported an episode of severe hypoglycaemia in the year prior to recruitment to the study. Cardiac autonomic function was assessed by measuring HRV and blood pressure response to standing. In those patients that reported severe hypoglycaemia 126 (13%) were diagnosed with autonomic neuropathy compared to 157 (8%) without episodes of severe hypoglycaemia ((p=0.002), unpaired t test). This was a large study but only two measures of autonomic function were undertaken in comparison to the standard five tests usually completed risking under diagnosis of neuropathy. The study also relied upon patients to report episodes of severe hypoglycaemia rather than measuring response to experimental hypoglycaemia.

Meyer and colleagues (Meyer et al., 1998) explored the claim that DAN was an independent risk factor for severe hypoglycaemia with a study published in 1998. Twenty two participants with T1DM, including eight with autonomic neuropathy, were recruited with thirty three participants without diabetes. All participants underwent a stepwise hypoglycaemic clamp. The threshold for the release of

adrenaline was higher (required a lower blood glucose) and levels of adrenaline lower in participants with diabetes. The impairment of adrenaline release was further attenuated in the participants with autonomic neuropathy. The reduction in adrenaline response to hypoglycaemia in patients with DAN has been well documented in other studies (Bottini et al., 1997) (Polinsky et al., 1980) (Hoeldtke et al., 1982) although duration of diabetes, a well established confounding factor, is not always controlled for.

In their analysis, Meyer and colleagues found that symptomatic responses to hypoglycaemia correlated with the magnitude of adrenaline released in the participants without diabetes but not among the participants with diabetes. They compared responses in the thirty three participants without diabetes, four patients with diabetes with DAN and five patients with diabetes and normal autonomic function. The lack of correlation in the populations with diabetes may be due to the small number of participants included in the analysis.

Ryder and colleagues (Ryder et al., 1990) reported a lack of correlation between symptomatic awareness of hypoglycaemia and the presence or absence of DAN in 1990. They hypothesized that if there is an association between DAN and IAH then patients with autonomic neuropathy will have IAH and patients with poor warning symptoms for hypoglycaemia will have autonomic neuropathy. To examine this hypothesis they studied four groups of patients; seven T1DM patients with symptomatic autonomic neuropathy, nine T1DM patients with frequent episodes of severe hypoglycaemia, seven T1DM patients without the concerns of the first two groups and ten healthy controls. A thorough assessment of autonomic function was completed followed by an insulin infusion to assess the counterregulatory response to hypoglycaemia. An inadequate response to hypoglycaemia was defined as neurological manifestations of hypoglycaemia or a blood glucose that dropped to below 1.9mmol/l. Counterregulatory hormones were not measured.

Of the seven patients with DAN there were no patients with an inadequate response to hypoglycaemia and of the seven patients with an inadequate response to hypoglycaemia none had autonomic neuropathy. Ryder et al (Ryder et al., 1990) reported that there was no association between unawareness of hypoglycaemia and DAN. However, the validity of their conclusions is threatened by the small number of participants recruited and lack of measurement of catecholamines.

Hepburn et al (Hepburn et al., 1990) supported the hypothesis made by Ryder and colleagues (Ryder et al., 1990) that IAH is not invariably associated with autonomic dysfunction. They studied 306 insulin dependent diabetics. Twenty one (7%) patients reported absence of symptomatic awareness of hypoglycaemia. AFTs were carried out on 226 patients and eighty five (38%) had abnormal tests. Sixty two (37%) patients with normal awareness had abnormal AFTs compared with 14 (67%) in the absent awareness group. The groups were then analysed depending on duration of diabetes. For patients that had had diabetes for more than 15 years, thirty nine (54%) with normal awareness had abnormal autonomic function tests compared to ten (59%) in the absent awareness group. They concluded that 'the precise relationship between DAN and loss of hypoglycaemia awareness remains undefined'

Studies investigating reversibility of IAH usually exclude patients with DAN to rule out its potential impact on the response to treatment. Fanelli and colleagues (Fanelli et al., 1997) recruited 21 patients with IDDM with a history of recurrent hypoglycaemia without warning symptoms and 15 subjects without diabetes. Thirteen of the participants with diabetes had DAN and eight had normal autonomic function tests. Participants with autonomic neuropathy were older but the long duration of diabetes and HbA1c levels were comparable between the groups. The participants underwent a stepped hypoglycaemic clamp before and after 6 months of meticulous avoidance of hypoglycaemia. After 6 months of hypoglycaemia avoidance all patients with diabetes had an improvement in autonomic symptoms and adrenaline response to a low blood glucose but the improvement was lower in participants with DAN than those with normal

autonomic function. This suggests that DAN does impede the reversal of hypoglycaemia unawareness to a degree.

Interestingly the participants with diabetes did not improve their symptomatic and adrenaline responses to hypoglycaemia to the level of the subjects without diabetes (Figure 4) confirming the findings in other studies with patients with a long duration of diabetes. This conflicts with the complete recovery from hypoglycaemia unawareness seen in patients with a short duration of T1DM. It may be that patients with a long duration of diabetes have a degree of autonomic neuropathy that cannot be detected clinically, that causes a lack of complete recovery of IAH. Or a long duration of disease could cause structural changes, potentially in the glucose sensing areas of the brain, preventing a normal counterregulatory response.

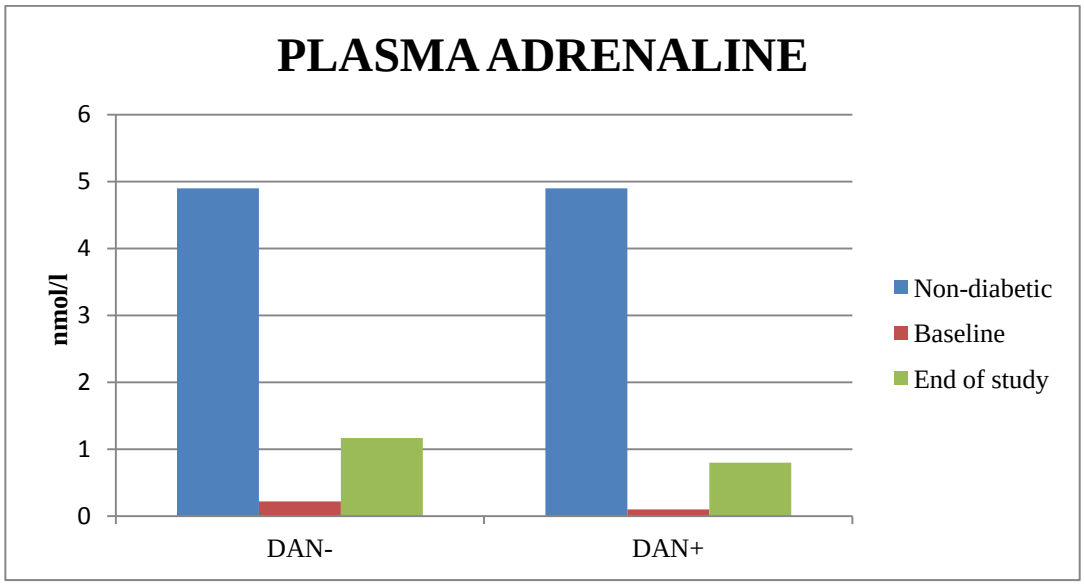

Figure 4 Maximal adrenaline responses to hypoglycaemia in subjects without diabetes, patients with diabetes without autonomic neuropathy (DAN-) and those with autonomic neuropathy (DAN+) (Adapted from Fanelli et al (Fanelli et al., 1997)).

In summary exploring the contribution of DAN alone to IAH is challenging. Patients with established DAN have longstanding diabetes which confounds investigation. Currently there are no studies in the literature that have measured the responses of DAN affected patients to hypoglycaemia while controlling for duration of disease.

1.4 T1DM and the Heart

1.4.1 Pathophysiology of Hypoglycaemia

Acute hypoglycaemia stimulates the sympathetic nervous system with the release of catecholamines (Schwartz et al., 1987). This is crucial in T1DM as the first two mechanisms of defence, reduction in circulating insulin and glucagon release, are impaired. Activation of sympathetic nervous system has a well documented effect on the cardiovascular system which includes an increase in heart rate and cardiac output (Triposkiadis et al., 2009).

Experimental hypoglycaemia has been shown to cause an increase in heart rate, blood pressure and cardiac output (Hilsted et al., 1984) (Fisher et al., 1987) (Fisher et al., 1990). The effects of hypoglycaemia on the cardiovascular system are a direct effect of the increase in heart rate. Some of these effects are due to the direct neural sympathetic innervations to the heart, rather than to counterregulatory hormonal response, as maximal heart rate has been shown to precede maximal catecholamine rise (Fisher et al., 1987).

The cardiovascular response in participants with diabetes has been shown to be slower and blunted in comparison to the people without diabetes (Russell et al., 2001). This coincides with a blunted catecholamine response to hypoglycaemia in subjects with diabetes. The cardiovascular parameters also change during hyperinsulinaemic euglycaemia but to a lesser extent than during hypoglycaemia.

Hyperinsulinaemic hypoglycaemia is associated with specific and well documented ECG changes. Studies have shown that prolonged cardiac repolarisation or heart rate corrected QT interval (QTc) occurs during hypoglycaemia (Laitinen et al., 2003, Marques et al., 1997). The mechanism is thought to be due to a combination of adrenaline and hypokalaemia (Robinson et al., 2003a). This effect is seen in spontaneous hypoglycaemia, although to a lesser degree, as well as in experimental clamp studies (Christensen et al., 2014).

1.4.2 Hypoglycaemia Associated Mortality

Intensive treatment of T1DM improves glycaemic control and prevents the long term complications of diabetes (Group, 1993). In the DCCT patients in the intensive treatment arm had delayed onset of diabetic retinopathy, nephropathy and neuropathy (Group, 1993). The delay in complications was sustained for at least 8 years despite a deterioration in glycaemic control once the trial had finished (Study, 2003).

However, despite this, concern has been raised that intensive treatment of diabetes could be linked to an increase in mortality (Group, 2008). The intensive treatment arm of the ACCORD study, a randomised controlled trial, to assess the effect of intensive glycaemic management versus standard therapy on cardiovascular events, had to be prematurely stopped due to high mortality rates. Of the participants in the intensive treatment group 1.42% of patients died each year compared to 1.14% in the standard treatment arm (HR 1.22 CI 1.01 to 1.46 P=0.04) (Bonds et al., 2010).

It was initially suggested that this increase in mortality was related to hypoglycaemia. Participants that had experienced an episode of SH were at an increased risk of death. Further analysis, however, showed that this increased risk was regardless of which treatment arm the participant was in (Table 3 p=0.009). Also, the increased risk of death in the intensive treatment group could not be solely attributed to SH as the risk of death was lower in the intensive arm of the study than in the standard arm in participants that had experienced an episode of SH (HR 0.55, 95% CI 0.31-0.99). Other studies confirm that episodes of SH are an independent risk factor for death (Duckworth et al., 2009) (Zoungas et al., 2010) but the link is not direct and it is unclear if hypoglycaemia is a result of or contributing factor of vulnerability to morbidity and mortality.

A recent meta analysis by Goto et al (Goto et al., 2013) concluded that SH was associated with increased cardiovascular death. The risk of cardiovascular disease was found to double in those patients experiencing SH. A bias analysis found the presence of co-morbidities could not explain this increased risk.

Treatment Arm	Zero episodes of SH requiring medical assistance	At least one episode of SH requiring medical assistance	Hazard Ratio for no previous events vs. at least one event (95% CI)
Intensive	1.3% 220 deaths per 17031 person years	2.8% 34 deaths per 1208 person years	Unadj : 1.72 (1.19 – 2.47) Adj: 1.28 (0.88-1.85)
Standard	1.0% a year 180 deaths per 17 516 person years	4.9% a year 17 deaths per 345 person years	Unadj: 3.88 (2.35 to 6.40) Adj: 2.87 (1.73 to 4.76)

Adjusted hazard ratios are adjusted for age, gender, smoking status, history of cardiovascular disease, history of heart failure, peripheral neuropathy, albumin to creatinine ratio, heart rate, QT score, visual acuity score, statin use, sulphonuria use, glycaemia intervention, enrolled in lipid v blood pressure trial, intensive blood pressure control group, and fibrate group.

Table 3 Mortality rates within treatment groups by occurrence of episodes of SH requiring medical intervention in the ACCORD Study (Adapted from (Bonds et al., 2010).

Tattersall and Gill (Tattersall and Gill, 1991) described sudden and unexpected deaths in patients with T1DM and hypothesised that hypoglycaemia was the underlying cause. The majority of patients studied had a previous history of nocturnal hypoglycaemia and there was some circumstantial evidence indicating hypoglycaemia as a cause of death. However, nocturnal hypoglycaemia in T1DM is common (Amin et al., 2003) and death from this is relatively rare (Klatt et al., 1988) suggesting a possible predisposing condition that puts a small proportion of T1DM patients at risk for sudden death. One possible predisposing condition is DAN.

DAN is also associated with an increased risk of death. O'Brien et al (O'Brien et al., 1991) studied 506 participants with insulin dependent diabetes mellitus (IDDM) randomly selected from clinic. DAN was diagnosed in 84 (16.6%) of patients and these patients were matched with a case control group to control for sex and duration of diabetes (Table 4). Five year survival in patients with DAN (72.6%) was lower than in those patients in the case control group (91.7%).

	Subjects with diabetes and DAN (84)	Case control group (84)
Sex	36M,48 F	38M.46F
Age (years)	46±15	46±16
Duration of diabetes (years)	20 + 9	19±9
Insulin (u/day)	50 ±22	46±I6
BMI	24±3	24±3
Vascular disease	11%	2%
Retinopathy	75%	30%
Proteinuria	25%	4%
Sensory neuropathy	57%	24%

Table 4 Clinical characteristics of patients (Adapted from (O'Brien et al., 1991)).

	Subjects with diabetes and DAN	Subjects with diabetes without DAN
Vascular disease	8	7
Infection	2	3
Renal failure	10	1
Malignant disease	2	3
Trauma/suicide	0	2
Other	1	1
Unknown	0	4

Table 5 Causes of death (Adapted from (O'Brien et al., 1991)).

It is difficult to establish the cause of the increase in mortality and a proportion of patients with DAN have reportedly died suddenly and unexpectedly with no cause of death found on post-mortem (O'Brien et al., 1991) (Ewing et al., 1980). The relationship is complicated by the tendency of diabetic complications (table 4) to occur together and the increased of risk of death caused by renal failure (table 5) confirmed by other studies (Ewing et al., 1980). Ewing studied a group of 73 patients including 40 patients with proven DAN. Over a five year follow up period 21 subjects with DAN died (53%) compared to 5 (15%) in the control group. Of those that died, 5 died suddenly and unexpectedly with no cause of death found at post mortem (Ewing et al., 1980).

1.4.3 Hypoglycaemia and Autonomic Nervous System Function

Hypoglycaemia stimulates a specific response by the autonomic nervous system which is well established, but less is known about the wider effects of this response, particularly on cardiac autonomic function. It is plausible that either clinical or subclinical DAN could predispose patients to life threatening arrhythmias that occur during hypoglycaemia causing sudden death in patients. This has started to be considered and recently heart rate variability (HRV) and baroreceptor sensitivity (BRS) have been measured during experimental hypoglycaemia. These measurements have sought to establish the effect of hypoglycaemia on cardiac autonomic function but the results have been conflicting (Laitinen et al., 2003, Schachinger et al., 2004, Koivikko et al., 2005)

Power spectral analysis was introduced by Akselrod et al as a way to understand how the different aspects of the nervous system affect HRV (Electrophysiology, 1996). HRV in the frequency domain is now commonly used to investigate cardiac autonomic function (Gustavo A. Reyes Del Paso et al., 2013). HRV describes the variation in the RR interval, or time between consecutive heart beats, on the ECG. This oscillates continuously around the mean.
Two oscillatory components are typically defined: the high frequency band (0.04 to 0.15Hz) which reflects the effect of respiration on the heart rate and the low frequency band (0.1Hz) related to blood pressure and vasomotor control (Gustavo A. Reyes Del Paso et al., 2013). These components have then been used to demonstrate the balance between the two different aspects of the autonomic nervous system; the parasympathetic and sympathetic branches (Malliani et al., 1991). The sympathetic influence on the RR interval is mediated by adrenaline and noradrenaline release and parasympathetic influence is maintained by acetylcholine release and the vagus nerve (Electrophysiology, 1996).

It is accepted that the high frequency band represents parasympathetic or vagal activity (Malliani et al., 1994). The low frequency band is often assumed to represent sympathetic activity and then the HF/LF ratio is used to represent the balance between the parasympathetic and sympathetic input. This relies upon the belief that the autonomic nervous system is thought to be antagonistic therefore with increased activity in the sympathetic system there is reduced activity in the

parasympathetic system (Malliani et al., 1994). More recently the assumption that the HF/LF ratio represents the sympatho-vagal balance has been challenged (Billman, 2013). The LF peak is now thought to have a combined vagal and sympathetic input and there are certain circumstances when a change in one component of the autonomic nervous system does not cause a reciprocal change in the other (Billman, 2013).

BRS measures changes to the RR interval or heart rate in response to changes in systolic blood pressure (Bernardi et al., 2011). In a healthy individual increases in systolic blood pressure reduces sympathetic nervous system activity and increases parasympathetic, vagal activity causing an immediate reduction in heart rate and consequently blood pressure. Impaired BRS causes a reduction of parasympathetic activity and a reciprocal increase in sympathetic activity.

The clinical use and implications of BRS in diabetes is still unclear. In cardiology abnormal BRS in cardiovascular disease has been shown to be a predictor for death and an important prognostic marker (Rovere et al., 2012). Abnormal BRS in patients with diabetes is common in patients with and without autonomic neuropathy (Bernardi, 2000). Some studies have suggested that their data shows that BRS can detect DAN at an earlier stage before the classical tests become abnormal (Frattola et al., 1997) but to date there hasn't been the completion of a prospective study to provide the evidence for this assumption. Some studies have also suggested that BRS has a functional component by showing normalisation with deep breathing (Rosengard-Barlund et al., 2011). The review undertaken by Tesfaye et al (Tesfaye et al., 2010) in 2010 recommended the use of BRS in research studies but not for diagnostic purposes in routine clinical practice.

1.4.4 The Effect of Experimental Hypoglycaemia on Cardiac Autonomic Function

Laitinen and colleagues (Laitinen et al., 2003) hypothesised that the increased sympathetic activity during an episode of hypoglycaemia would result in reduced cardiac parasympathetic activation based on the concept of the antagonistic autonomic nervous system. Eighteen healthy volunteers were recruited and participated in a hyperinsulinaemic euglycaemic clamp (glucose 5.0 mmol/l) followed by a hyperinsulinaemic hypoglycaemic clamp (glucose 3.0 mmol/l).

Five minute ECG and continuous blood pressure recordings were made at baseline, during euglycaemia and hypoglycaemia.

During hyperinsulinaemic hypoglycaemia there was sympathetic activation with secretion of adrenaline and noradrenaline but no evidence of parasympathetic response with no significant changes in HRV or BRS. The authors concluded that this challenges the antagonistic nature of the autonomic nervous system but agreed that although there was pronounced adrenaline secretion they did not demonstrate muscle sympathetic nerve activity or sympathetic stimulation to the heart. The other concern is that whether the level of hypoglycaemia (3.0mmol/l) is significant enough to provoke a response.

These results were contradicted in a similar study by Schachiger and colleagues (Schachinger et al., 2004). Fifteen healthy adults underwent normoglycaemic (4.7mmol/l) and hypoglycaemic (2.7mmol/l) clamps that were carried out four weeks apart in a single blinded cross over study. As in the study by Laitinen et al (Laitinen et al., 2003) adrenaline and noradrenaline levels increased during hypoglycaemia without an increase in heart rate. However, there was an increase in HF HRV indicating an increase in parasympathetic activity. They concluded that the lack of increase in heart rate during hypoglycaemia was due to the increased parasympathetic activity.

The differences may be due to the different clamp protocols. Laitinen et al (Laitinen et al., 2003) carried out both clamps on the same day, used paced breathing during the measurement of heart rate variability, and used a higher level of hypoglycaemia. Schachiger et al (Schachinger et al., 2004) separated their clamps by four weeks to reduce variation caused by the tests being done at different times of day and used a lower level of hypoglycaemia of 2.7 mmol/l. They didn't ask subjects to pace their breathing as they felt this could prove difficult to standardise while patients were cognitively impaired during hypoglycaemia. Respiratory rate doesn't affect the heart rate but has a significant effect on HRV. Slower respiratory rates increase HRV and the increase is seen at the frequency band at which the patient was breathing in. For example LF peak is

higher at respiratory rates of 4 and the high frequency HRV is higher at respiratory rates of 10, 12 and 14 breaths a minute (Song and Lehrer, 2003).

Koivikko et al (Koivikko et al., 2005) went on to explore the effect of hypoglycaemia on cardiac autonomic function in patients with diabetes. Sixteen patients with diabetes with normal baseline cardiac autonomic function tests were recruited with eight subjects without diabetes. HRV was measured during euglycaemic (glucose between 4.5 and 5.5 mmol/l) and hypoglycaemic clamp studies (glucose 3-3.5 and then 2-2.5 mmol/l) carried out one week apart. Heart rate increased and the HF HRV was reduced indicating reduced parasympathetic activity in both the patients with and without diabetes during moderate (glucose 2-2.5mmol/l) but not during mild hypoglycaemia (glucose 3-3.5mmol/l).

This supports the work by Laitinen et al (Laitinen et al., 2003) in finding no changes in heart rate variability in mild hypoglycaemia but contradicts the findings by Schachiger et al (Schachinger et al., 2004) that suggested increased parasympathetic output during hypoglycaemia. The differences may be explained by the severity and length of time that participants were hypoglycaemic. Participants had a more prolonged episode of hypoglycaemia in the Koivikko study.

The studies by Laitinen, Schachiger and Koivikko et al looked at the contemporaneous effect of experimental hypoglycaemia on cardiovascular autonomic responses. Adler et al (Adler et al., 2009) explored the downstream effect of hypoglycaemia on cardiac AFTs. Twenty subjects without diabetes were recruited for two three-day inpatient visits, separated by a minimum of one month. AFTs were carried out on days one and three and a hyperinsulinaemic hypoglycaemic or euglycaemic clamp was carried out on day 2 (Figure 5).

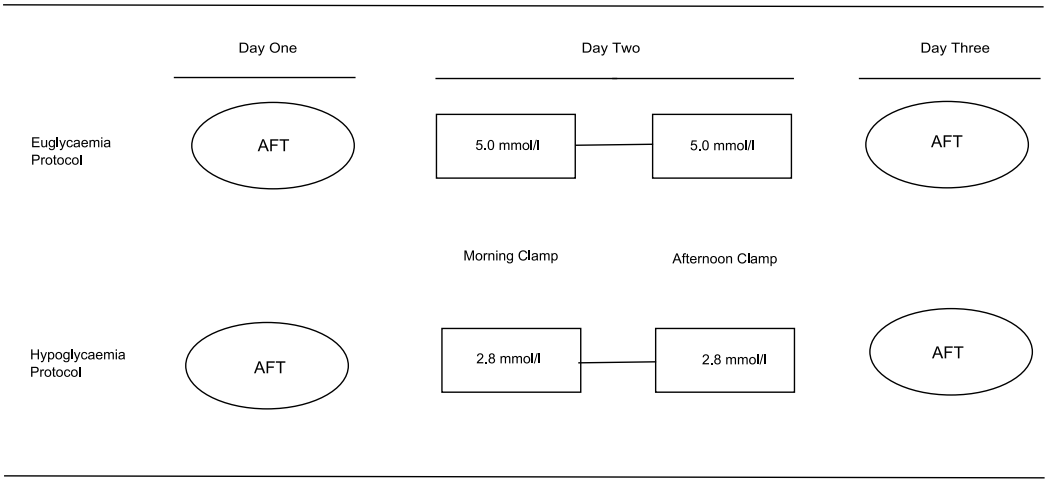

Figure 5 Two protocols were undertaken by each subject separated in time by at least one month. Autonomic function tests were completed on days one and three and a euglycaemic or hypoglycaemic clamp study was undertaken on day two (Adapted from Adler et al (Adler et al., 2009)).

Cardiac vagal BRS was significantly reduced following the hypoglycaemic clamps in comparison to the euglycaemic clamps. There was also a reduction in HF HRV following hypoglycaemia. The authors expressed concern about this reduction in parasympathetic activity due to the association of a reduced BRS with increased mortality in myocardial infarction patients (Bigger et al., 1993). This response may be more severely impaired in patients with diabetes with clinical and subclinical autonomic neuropathy but these patients were not included in this study.

Summary

An increased risk of death has been seen in patients suffering repeated episodes of SH and those with DAN. It is plausible that clinical or subclinical DAN could predispose patients to life threatening arrhythmias during hypoglycaemia causing sudden death. Experimental work has been done to assess the effect of hypoglycaemia on cardiac autonomic function but results have been inconsistent and have to date only included patients without classical autonomic neuropathy. The inconsistencies seen are most likely due to the different experimental protocols undertaken and characteristics of the study participants.

1.5 Aims of the Thesis

IAH and DAN are important clinical entities in diabetes. They cause an increase in episodes of SH and death respectively. Classically DAN has been described as the underlying mechanism causing IAH but this has been challenged in the literature. To date studies have been unable to clarify the relationship successfully due to the complicating and confounding factors of age and duration of diabetes.

It is important to understand the relationship between IAH and DAN. IAH can be at least partially reversed by completely avoiding hypoglycaemia for a period of time. Treatment is often difficult. It requires intensive input from specially trained staff and is expensive.

Patients do not always respond to treatment of IAH. Two clinical scenarios are generally seen: patients with a shorter duration of disease and tight glycaemic control and those with a long duration of disease. It is those patients with a longer duration of disease that do not always respond to treatment but it is difficult to predict those that will reverse and those who will not improve.

Ideally a safe and inexpensive test would be available to identify responders and non-responders to treatment. If clinical or subclinical DAN is a factor contributing to the potential to reverse IAH then AFTs could be used as a predictor to responsiveness to treatment as the tests are simple and non –invasive.

The increased risk of death seen in patients suffering multiple episodes of SH is a current concern. Potential mechanisms include cardiovascular disease, vulnerability because of co morbidities and DAN. The potential link between these entities needs clarification as the currently the literature is inconsistent.

It is plausible that DAN could be a predisposing condition that would increase the risk of death in patients experiencing episodes of SH. The effect of experimental hypoglycaemia on cardiac autonomic function has started to be investigated. Differing patient populations and clinical protocols in studies have given inconsistent results and the effect of hypoglycaemia on parasympathetic output remains undefined.

The aim of this research is to investigate the relationship and potential association between IAH and DAN. I have proposed three hypotheses, which have been tested in this work and are listed below:

1) DAN is more prevalent in people with T1DM and IAH compared to people with T1DM and normal awareness of hypoglycaemia.

2) The presence of clinical or subclinical DAN impedes the ability to reverse IAH in people with T1DM.

3) Experimental hypoglycaemia provokes different cardiac autonomic responses in people with T1DM and IAH in comparison to individuals with T1DM and normal awareness of hypoglycaemia.

The structure of this thesis reflects the three hypotheses to be investigated. Chapter 2 gives a detailed description of the methods used. The three results chapters concentrate on the individual hypotheses with an appropriate introduction, method and discussion. Chapter 6 completes the thesis with a summary of the conclusions made and plans for future work.

Chapter 2: Methodology

The purpose of this chapter is to describe the methods I used to investigate the relationship between impaired awareness of hypoglycaemia (IAH) and diabetic autonomic neuropathy (DAN). The main hypoCOMPaSS (Comparison of Optimised MDI versus Pumps with or without Sensors in Severe Hypoglycaemia) trial and subsequent autonomic neuropathy sub-study will be described in detail including participant inclusion and exclusion criteria. The hyperinsulinaemic hypoglycaemic clamp study protocols including the use of cardiovascular and biochemical markers will also be presented.

2.1 Overview

The hypoCOMPaSS Trial (Little et al., 2012) was a UK-based, multicentred, prospective randomised controlled trial (RCT). It aimed to optimise diabetes treatment in patients with IAH to prevent biochemical hypoglycaemia (BH) and restore symptomatic awareness. The five UK sites involved in the study were tertiary referral and academic hypoglycaemia/continuous subcutaneous insulin infusion (CSII) centres, and included, Royal Bournemouth Hospital, Bournemouth; Addenbrooke's Hospital, Cambridge; Newcastle Diabetes Centre, Newcastle Upon Tyne; Derriford Hospital, Plymouth and Sheffield Teaching Hospitals, Sheffield. The protocol has been published (Little et al., 2012) in full but the key features have been described in this chapter.

Detailed cardiac autonomic function testing was carried out during the 'wash-in period' of the HypoCOMPaSS trial prior to randomisation and intervention. Participants were randomised to one of four treatment arms; multiple daily injections (MDI) with self monitoring of blood glucose (SMBG), MDI with SMBG and real time continuous glucose monitoring (RT-CGM), CSII with SMBG and CSII with SMBG and RT-CGM and underwent a six month treatment period. The aim of treatment was to completely avoid BH (glucose less than 4mmol/l) with an aim of restoring awareness. Participants had at least weekly contact with a member of the hypoCOMPaSS team and were seen face to face every 4 weeks to offer support and maximise treatment. At the end of the treatment period cardiac autonomic function testing was repeated (figure 6).

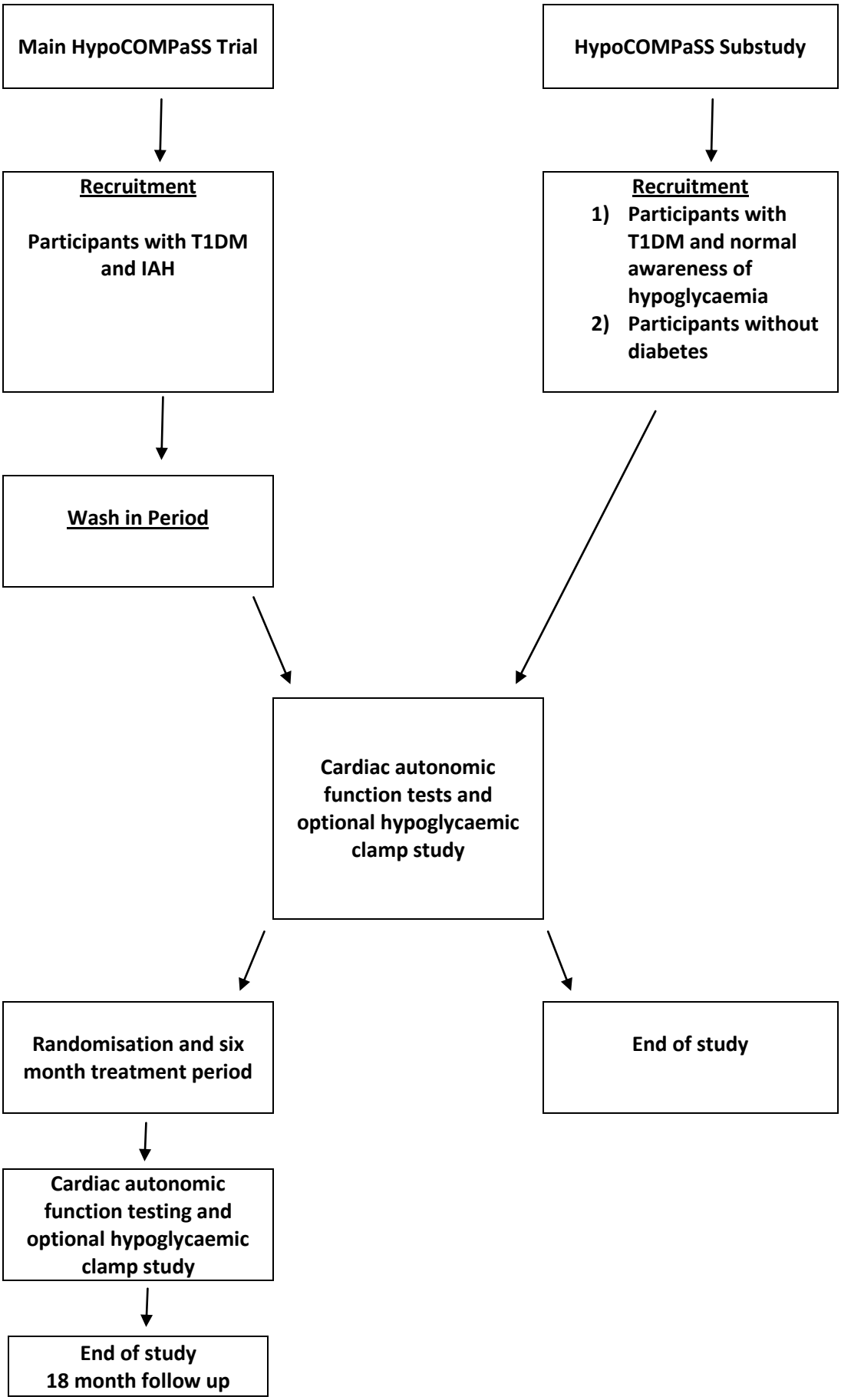

Figure 6 Overview of studies.

Eligible participants were invited to undergo an optional stepped hyperinsulinaemic hypoglycaemic clamp study at the start and end of the hypoCOMPaSS trial. A five minute ECG recording with continuous blood pressure readings were made during each glucose step of the clamp. The recordings were analysed to measure heart rate variability (HRV) and baroreflex sensitivity (BRS) to investigate cardiovascular and autonomic responses to hypoglycaemia.

Following completion of the main hypoCOMPaSS trial I designed a sub-study (figure 6) to investigate the relationship between IAH and DAN. The sub-study recruited participants with T1DM and normal awareness of hypoglycaemia to match and compare to the hypoCOMPaSS population. To ascertain if there was an association between IAH and DAN, the sub-study participants underwent cardiac autonomic function testing, and were invited to undergo a hypoglycaemic clamp study if eligible. I also recruited ten participants without diabetes as a control group. The data from the main hypoCOMPaSS trial and sub-study have been used to investigate the three hypotheses listed below.

Hypothesis one: *DAN is more prevalent in people with T1DM and IAH compared to people with T1DM and normal awareness of hypoglycaemia.* The hypoCOMPaSS participants, and participants in the sub-study, underwent detailed cardiac autonomic function tests to ascertain the presence or absence of DAN. The prevalence of DAN in the two study populations were compared to ascertain if there was a difference and an association between IAH and DAN.

Hypothesis two: *The presence of clinical or subclinical DAN impedes the ability to reverse IAH in people with T1DM.* Following the six month treatment period of the hypoCOMPaSS trial participants were labelled as responders to treatment of IAH or non-responders. The prevalence of DAN in the responder group was compared to the prevalence of DAN in the non-responder group.

Hypothesis three: *Experimental hypoglycaemia provokes different cardiac autonomic responses in people with T1DM and IAH in comparison to individuals with T1DM and normal awareness of hypoglycaemia.* The effect of

hypoglycaemia on heart rate variability (HRV) and baroreflex sensitivity (BRS) during the clamp studies was compared between the two participant groups.

2.2 The HypoCOMPaSS Trial

The aim of the HypoCOMPaSS study was to optimise diabetes treatment in patients with IAH to prevent BH and restore awareness. Patients were randomised to either optimised MDI or CSII with or without RT-CGM in a 2x2 factorial design (figure 7). The study design complied with the CONSORT statement.

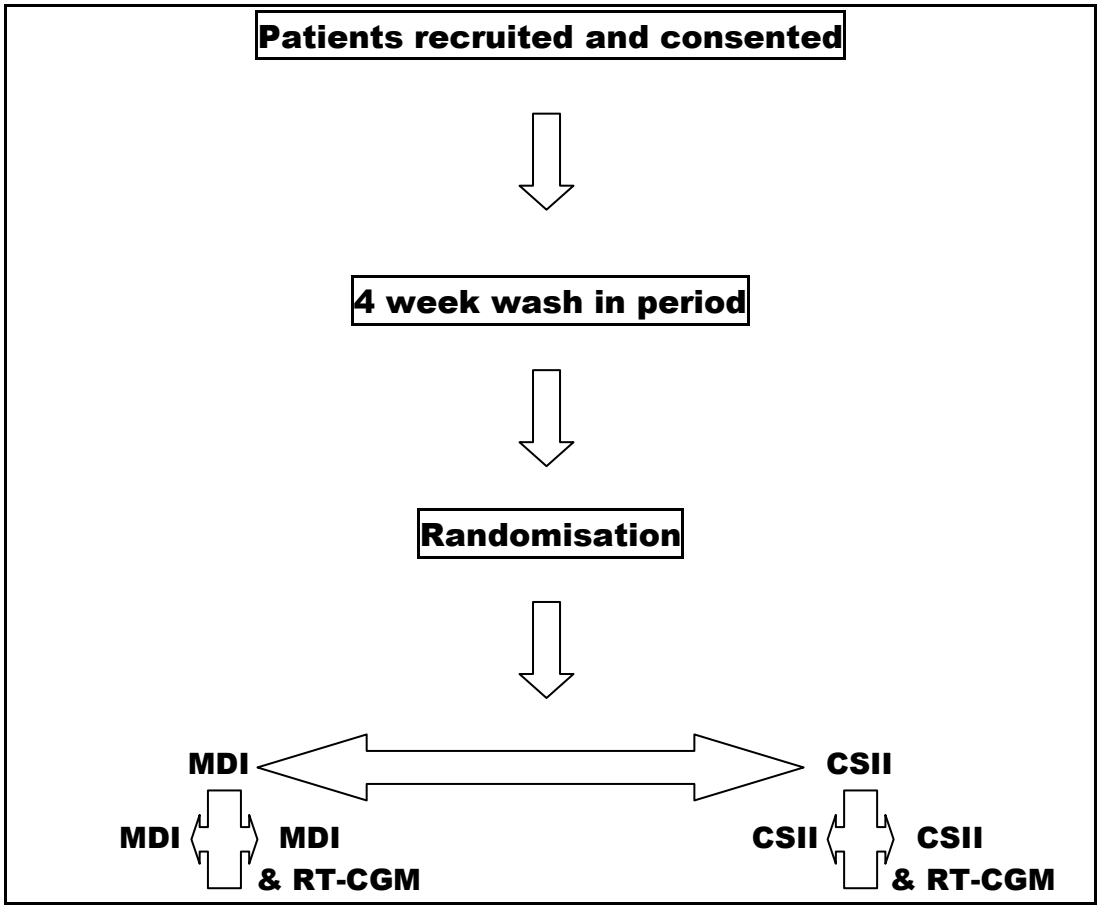

Figure 7 HypoCOMPaSS RCT, a 2x2 factorial design (Adapted from (Little et al., 2012)).

Participants underwent detailed cardiac autonomic function testing during the 4 week wash-in period of the study, and after the six month treatment period. All eligible participants were also invited to undergo an optional hyperinsulinaemic, hypoglycaemic clamp study before, and after the trial, with an aim to objectively demonstrate reversal of IAH (Leelarathna et al., 2013). Cardiac autonomic

function was measured during the different glucose steps of the clamp studies in centres with suitable recording equipment.

Research Governance

Ethical approval for this study was sought and gained from Sunderland Research Ethics Committee. Clinical Trial Authorisation was given by the Medicines and Healthcare products Regulatory Agency (17136/0246/001-0001). Site Specific Approval was granted by all participating Acute Hospital Trust Research and Development Departments (Little et al., 2012).

Study Participants

Participants were recruited from five UK tertiary referral and academic hypoglycaemia/CSII centres: Royal Bournemouth Hospital, Bournemouth; Addenbrooke's Hospital, Cambridge; Newcastle Diabetes Centre, Newcastle upon Tyne; Derriford Hospital, Plymouth; Sheffield Teaching Hospitals, Sheffield. Individuals with T1DM and IAH were referred by members of the participant's usual clinical team and then assessed to ascertain eligibility for the study. Those that were considered eligible were approached and provided with written information about the trial before written consent was obtained.

<u>Inclusion criteria for hypoCOMPaSS trial</u>

- Male or female aged 18-74 years inclusive at the start of the trial.
- Diagnosis of diabetes mellitus according to American Diabetes Association (ADA) and World Health Organisation (WHO) criteria (Appendix 1) and consistent with a clinical diagnosis of T1DM.
- Serum C-peptide below the quality assured limit of detection for the assay with simultaneous exclusion of biochemical hypoglycaemia (glucose <4.0 mmol/L) by laboratory glucose level analysis on a sample taken at the same time point.
- History of severe hypoglycaemia in the preceding one year (as defined by the ADA (Workgroup, 2005) and / or impaired awareness of hypoglycaemia as confirmed by a score of ≥ 4 in the Gold score (Appendix 2)

Exclusion criteria for hypoCOMPaSS trial

- Any condition that in the investigator's judgement is likely to cause the subject to be unable to understand the information in the Informed Consent Document or provide informed consent.
- A level of English below that to enable the participant to understand both verbal and written information required by the study.
- Unwilling to undertake intensive insulin therapy including the use of CSII, optimised MDI regimen and use of RT-CGM.
- Unwilling to undertake glucose profiles using continuous glucose monitoring (CGM).
- Unwilling to monitor home blood glucose levels at least 4 times daily.
- Unwilling to monitor and record signs and symptoms of hypoglycaemia.
- A history of intolerance to insulin Glargine.

Baseline visits and 4 week 'wash in period'

Visit one; screening and consent

Informed consent was taken before participants entered a four week wash in period (Table 6) prior to the 24 week RCT. Participants were provided with a handheld glucometer and were asked to measure a 4 point daily glucose profile and a weekly 8 point profile. Participants were also asked to record all glucose levels less than 4mmol/l. Blood was taken for HbA1c with a paired C-peptide and glucose to confirm eligibility.

Visit two; blinded CGM

All participants attended for the placement of a blinded CGM sensor (Medtronic iPro) which was worn for 7 days. Retinal photography was carried out if not completed within the last six months and a urine sample for albumin creatinine ratio (ACR) was collected.

4 week wash in period ⟷ 24 week RCT

Visit number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Informed consent	x																				
Eligibility screen	x																				
Information about clamp	x																				
C peptide	x																				
Glucose	x																				
HbA1c	x		x							x		x		x		x		x		x	
CGM sensor Placement		x							x		x		x		x		x		x		
4 week glucose diary	x		x							x		x		x		x		x			
Retinal screening		x																			
ACR		x																			
Clinical history & demographics			x																		
Medication																					
Detailed hypoglycaemia history			x							x		x		x		x		x		x	
Physical examination			x																		
Blood pressure			x							x		x		x		x		x		x	x
Weight			x							x		x		x		x		x		x	
Height			x																		
DAN questionnaire				x																	x
Clamp study				x																x	
AFTs				x																	x
AI screening					x																
Education programme						x	x	x													

Table 6 HypoCOMPaSS trial visit schedule (Adapted from (Little et al., 2012)).

Visit three; baseline assessment and hypoglycaemic clamp study

A full clinical history and examination was undertaken and blood was taken for HbA1c, urea and electrolytes, liver function tests and a lipid profile. Hypoglycaemia unawareness was reassessed by the completion of the Gold score (Appendix 1). If the participant was willing to proceed with, and eligible for, the hyperinsulinaemic hypoglycaemia clamp study separate consent was obtained.

Hyperinsulinaemic hypoglycaemic clamp studies

Hyperinsulinaemic hypoglycaemic clamp studies were carried out before and after the 24 week intervention period (Table 6) in eligible participants willing to undergo the procedure. Separate written consent was obtained for each study. Additional exclusion criteria were applied to ensure patient safety.

- Age > 60 years
- History of epilepsy
- History of ischaemic heart disease
- Other significant disease which in the judgement of the investigator precludes participation. This included cardiac arrhythmia and heart failure, peripheral vascular disease or stroke and a history of nephropathy as demonstrated by elevated serum creatinine accompanied by proteinuria (microalbuminuria alone was not an exclusion criterion).

Prior to the clamp study commencing, the CGM sensor fitted at visit two was reviewed with their blood glucose diary, to determine if any antecedent BH had occurred over the preceding 24 hour period. This was undertaken to ensure that counter-regulatory responses to hypoglycaemia were not attenuated by antecedent hypoglycaemia. Studies were postponed if any CGM and or self monitored capillary glucose below 3.0mmol/l were detected for greater than 20 minutes in the 12 hours prior to the study.

Participants were admitted to a dedicated clinical research facility at 8am, within the respective hospitals on the study day, after fasting from 10pm the previous day. An intravenous cannula was inserted in the antecubital vein on the non-dominant arm. Blood glucose levels were stabilised using a sliding scale Actrapid

(Novo Nordisk, Baegsvard, Denmark) insulin infusion aiming initially for a blood glucose reading of between 6.0 to 7.0 mmol/l reducing to between 5.0 and 6.0 mmol/l between 10:30am and 11am for the start of the clamp.

A retrograde cannula was inserted in a vein on the dorsum of the hand of the non-dominant arm using local anaesthetic. The hand was heated to 50-60 degrees using a hotbox, starting at least 30 minutes prior to the start of the clamp, to arterialise the blood. The retrograde cannula was used for sampling and a slow intravenous infusion of saline was used to keep the line patent.

At 11am, a primed infusion of 60mU/m2/min soluble human Actrapid insulin in a 4% solution of autologous blood in 0.9% saline was started via the non-dominant antecubital vein catheter. A simultaneous infusion of 20% dextrose, via the same antecubital catheter, was adjusted as required, aiming to stabilise the blood glucose at 5.0mmol/l at 40 minutes. Blood glucose was then lowered in a step wise manner to 3.8mmol/l, 3.4mmol/l, 2.8mmol/l and 2.4mmol/l. Plasma glucose was sampled from the retrograde cannula every 5 minutes, spun rapidly and assayed using Yellow Springs glucose analyser (YSI STAT Plus, Farnborough, U.K.). Each step consisted of 40 minutes, allowing 20 minutes to fall to target and 20 minutes for stabilisation. Participants were blinded to glucose levels throughout the study.

In addition to samples for plasma glucose, additional arterialised, venous blood samples for insulin, metanephrines, cortisol, growth hormone and electrolytes were drawn at the start of the clamp and at least every 40 minutes thereafter. Heart rate and blood pressure measures were taken with a 2 minute ECG recording every twenty minutes. At the end of each clamp stage participants completed the Edinburgh Hypoglycaemia Score (Appendix 3 (Deary et al., 1993)).

At each glucose step a five minute ECG recording with continuous blood pressure monitoring was completed to assess HRV and BRS. This allowed a non-invasive assessment of cardiac autonomic responses during euglycaemia and hypoglycaemia. The methodology of recording and interpreting HRV and BRS

are discussed in detail below. During the clamp studies recordings were undertaken with the participants at rest with spontaneous breathing. Current recommendations state that HRV should be assessed while the patient's breathing is paced (Tesfaye et al., 2010) or measured to ensure accurate interpretation of HRV. During the hypoglycaemic clamp the respiratory rate was measured rather than attempting to pace breathing due to concerns about consistency because of potential cognitive impairment during hypoglycaemia. Recordings of the respiratory rate ensured that the rate was between 10 and 15 breaths per minute in all participants during each recording (Guzik et al., 2007).

At the end of the clamp study the dextrose infusion was increased to raise blood sugar to euglycaemia and then tapered off gradually during a carbohydrate rich meal. The insulin infusion was reduced to basal requirements. Frequent blood glucose measurements were made until glucose levels were stable. Participants were discharged with advice about their subsequent insulin doses.

HRV

Heart rate is constantly changing under the influence of parasympathetic and sympathetic input (Vander et al., 1998) and HRV describes this variation in the RR interval, or time between consecutive heart beats, on the ECG. Stimulation of the vagus nerve will reduce heart rate which has been shown in animal studies in healthy anaesthetised dogs (Hamlin and Smith, 1968). Human studies have confirmed this with stimulation of the vagal nerve during radical neck dissection (Carlsten et al., 1957).

HRV oscillates continuously around the mean. Time domain measurements of the RR interval explore parasympathetic activity (Bernardi et al., 2011). These include the difference between the longest and shortest RR intervals, the standard deviation of the average RR intervals in a five minute recording (SDRR) and the root mean square of the difference of successive RR intervals (RMSSD)

Further information about heart rate variability can be gained by measuring the fluctuation in the frequency domain (Bilchick and Berger, 2006). The RR intervals are converted to a power spectral density function by a computational algorithm called the fast Fourier transform. The power spectral analysis is usually

integrated into specific frequency bands as fluctuations in each band are mediated by specific physiological mechanisms (Bilchick and Berger, 2006).

Two frequency bands are commonly defined; the high frequency (HF) band (0.15-0.40Hz) which reflects the effect of respiration on heart rate and the low frequency band (LF) (0.04-0.15Hz) reflecting variation related to blood pressure and vasomotor tone (Paso et al., 2013). It is widely accepted that the HF component of the spectrum represents vagal or parasympathetic activity (Bernardi et al., 2011, Berntson et al., 1997). The evidence for this was first shown in dogs in 1981 (Akselrod et al., 1981) and confirmed in human studies in 1985 (Pomeranz et al., 1985). The Toronto Diabetic Neuropathy Group recommended that heart rate spectral power in the high frequency region can be used as a measure of parasympathetic modulation in their most recent consensus statement (Tesfaye et al., 2010). The effect of respiration must be considered either by adjusting for the respiratory rate or by controlling respiratory rate when interpreting HF heart rate variability (Berntson et al., 1997).

The interpretation of the LF band is more controversial (Gustavo A. Reyes Del Paso et al., 2013). The initial evidence for the LF band being linked to sympathetic activity came from studies examining the effect of passive orthostatic tilt on HRV (Montano et al., 1994) when passive tilt was shown to increase LF and decrease HF. This work has been challenged (Berntson et al., 1997, Electrophysiology, 1996) and it has been suggested that LF represents both sympathetic and parasympathetic input. As a result the LF/HF ratio is used by some groups to illustrate sympathovagal balance (Electrophysiology, 1996). HRV measures indicate fluctuations in autonomic inputs and therefore parasympathetic withdrawal and sympathetic increases can lead to reduced HRV.

Normalised LF (LF norm) was suggested as an alternative marker of sympathetic activation and units are obtained by dividing LF by the total power (Gustavo A. Reyes Del Paso et al., 2013). This has been accepted as a marker of sympathetic activity by the Toronto Diabetic Neuropathy Expert Group (Tesfaye et al., 2010). It however should be interpreted with care and some groups still recommend Baroreflex control of heart rate as a more accurate measure of sympathetic activity (Paso et al., 2013).

The most recent recommendations from the Toronto Diabetic Expert Group (Tesfaye et al., 2010) state that both time domain and frequency domain measures of HRV can be used in clinical trials and research. Recordings should be 5-7 minutes in length. HF power can be used as a measure of parasympathetic activity and LF power can be used as a measure of both parasympathetic and sympathetic input. Respiratory rate should be measured or controlled to ensure accurate interpretation of power spectral analysis measures.

HRV Analysis

In these studies HRV analysis was carried out in accordance with the recommendations by the Toronto Diabetic Neuropathy Expert Group in the ADA update statement published in 2010 (Tesfaye et al., 2010). HRV was calculated on five minute ECG recordings where the respiratory rate was measured during spontaneous breathing with normal tidal volumes. Time domain measures were calculated including SDNN and RMSSD and power spectral analysis was performed using the Fast Fourier Transformation. The HF band was defined as 0.15 to 0.4 Hz and the LF band between 0.04 to 0.15 and LF norm was defined as the ratio between LF and total power. Ectopic beats were excluded to ensure reliability of the measures presented.

BRS

BRS is a measure of the changes in RR intervals produced by changes in blood pressure (Fisher, 2003). An increase in arterial blood pressure causes stretch in the arterial walls stimulating the baroreceptors in the carotid sinus. Inhibition of the sympathetic system and stimulation of parasympathetic system follows, causing a reduction in peripheral resistance and lowering of the heart rate, resulting in a reduction in blood pressure (Swenne, 2013).

Previously, measurement of BRS was an invasive procedure requiring arterial cannulation, but this can now be done by non-invasive measurement of beat to beat blood pressures using the Finapres. The Finapres is based on the Penaz technique (Wesseling, 1990) of measuring blood volume in the artery photoelectrically and changing the pressure of an inflatable cuff to keep the blood volume constant. With this apparent fixing of the volume in the artery there is an

assumption that the extra-arterial pressure is equal to, and can be used as, an indirect measure of intra-arterial pressure. The technique requires application of a small finger cuff and the intra-arterial pressure equates to the cuff pressure which can be measured with a manometer. This method has been validated against intra-arterial recordings (Omboni et al., 1993).

During these studies BRS was measured non-invasively using the portapres device; a portable Finapress (TNO Biomedical Instrumentation Amsterdam, Netherlands) with simultaneous ECG monitoring using an Ivy Cardiac Trigger Monitor 3000 (Ivy Biomedical Systems Inc, USA). A finger cuff of the correct size was selected and applied to the middle finger of the right hand with the frontend unit attached to the wrist. The Height Correction Unit was nulled and then attached to the upper arm at the height of the heart. Initially on starting the device the internal calibration system was switched on until consistent readings were obtained and then it was turned off. Blood pressure data was digitised with a sampling frequency of 1000 Hz and synchronised with the ECG data by WR TestWorks^{TM} software. Recordings were made for five minutes with the study participant at rest and lying flat.

BRS was calculated and analysed using Nevrokard^{TM} BRS version 5.1.3 (Intellectual Services Slovenia) using the sequence method (Omboni et al., 1993). The sequence method measures BRS as the slope of the relationship between changes in systolic blood pressure and subsequent changes in the RR interval (Kardos et al., 2001). An 'UP' sequence was generated with an increase in systolic blood pressure of at least 0.5 mmHg associated with an increase in the R-R interval of at least 5 ms for three or more consecutive beats. A 'DOWN' sequence was identified where there was a reduction in systolic blood pressure of at least 0.5 mmHg with a corresponding decrease in the R-R interval of at least 5 ms for three or more consecutive beats. The total BRS (ms/mmHg) was calculated using the average of the regression coefficients for UP and DOWN sequences.

Visit four; cardiac autonomic function testing

Cardiovascular reflex tests were used to assess cardiac autonomic function. The tests are widely accepted to provide an objective diagnosis of abnormalities in the autonomic nervous system (Ewing et al., 1985) (Tesfaye et al., 2010). The testing gives an indication of severity and are safe, non-invasive and can be done at the bedside (Neurology, 1996).

Autonomic testing evaluates the physiologic responses to various stimuli (Gibbons et al., 2014). Heart rate response to deep breathing, standing and to the valsalva manoeuvre depend upon vagal, parasympathetic innervations (Vinik et al., 2003). Blood pressure response to standing is mediated by sympathetic nerve fibres (Vinik et al., 2003).

Tests were carried out as initially described by Ewing in 1982 (Ewing and Clarke, 1982) with the modifications recommended by O'Brien published in 1986 (O'Brien et al., 1986). Results were compared against the O'Brien age adjusted normal ranges (O'Brien et al., 1986) with the results below the 95% confidence limits regarded as abnormal. Based on the most recent consensus (Tesfaye et al., 2010) the presence of one abnormal cardiac reflex tests identifies possible cardiac autonomic neuropathy and at least two abnormal heart rate tests are required for a definite diagnosis. Orthostatic hypotension in addition to heart rate reflex test abnormalities identifies more severe or advanced disease (Tesfaye et al., 2010). A further five minute ECG recording was made with continuous blood pressure monitoring to ascertain HRV and BRS at rest.

To ensure the tests were not influenced by external factors participants were asked to refrain from smoking and caffeine on the day of the tests. If the participant was taking a beta blocker this was discontinued 48 hours prior to autonomic function testing. A period of 3 minutes of rest preceded each test and all tests were carried out in a darkened room where noise was kept to a minimum.

Cardiac autonomic function testing was completed at each site using local equipment. The raw ECG recordings were exported and analysed at Sheffield

Teaching Hospitals, Sheffield, UK. Custom built semi-automatic software was developed to detect QRS complexes from the ECG recordings and the RR intervals were used for further analysis. The algorithm automatically detected QRS complexes in the ECG recordings based on the threshold method. In the first stage, the ECG recordings were smoothed using a low-pass filter with a 40Hz cut-off frequency. R peaks were then detected as peaks above a predefined threshold value which was initially calculated as 3 times the standard deviation above the mean of the ECG recording. The detection of R peaks was visually inspected and the threshold was adjusted if necessary. All peaks were visually inspected and manually corrected to exclude movement artefacts and ectopic beats.

Spectral analysis of HRV was performed based on 5 minute resting ECG recordings with the subject supine with spontaneous breathing in accordance with recommendations of the Taskforce on Heart Rate Variability (Electrophysiology, 1996). Normal RR intervals (NN) were extracted as described in the previous paragraph for heart rate variability analysis. The time domain measures of HRV included standard deviation of NN (SDNN) and root mean square of standard deviation (RMSSD). Fast Fourier transform was applied to NN time series for spectral analysis and the power was calculated within two frequency bands: low frequency (LF: 0.04-0.15 Hz) and high frequency (HF: 0.15-0.4 Hz) (Electrophysiology, 1996). The ratio between the LF power and total power (sum of low and high frequency power) was also calculated (LFnorm), which has been associated with sympathetic modulation in heart rate variability (Bernardi et al., 2011).

In Sheffield ECG signals were obtained using a three lead ECG monitor (Ivy Cardiac Trigger Monitor 3000, Ivy Biomedical Systems Inc., Branford CT, USA), which generates a synchronised pulse at the peak of each R wave. This was connected to the WR TestWorks™ Analogue Interface (WR Medical Electronics Company, Maplewood, USA) and ECG signals were digitised using a sampling frequency of 1000 Hz via a data acquisition device (NI-DAQCard-6062E, National Instruments™, UK) connected to a laptop computer (Toshiba Satellite 1800-S274). Data were recorded and analysed using the WR-TestWorks™ software (version 2.4.0, WR Medical Electronics Co., Maplewood, USA). This

software contained dedicated modules for the heart rate response to deep breathing, Valsalva, and standing. A marker was placed on the ECG recording denoting the beginning of the manoeuvre and R-R intervals (measured in ms) were generated within the programme, with the tests results calculated according to definitions below.

Heart Rate Response to Deep Breathing

Participants were asked to remain supine and breathe deeply at a rate of one breath per 10 seconds (six breaths per minute) for one minute while an ECG was recorded continuously. A light box was used to indicate when the participant should inspire and expire to improve the reproducibility of this test. It was expected that the heart rate would increase on inspiration and fall with expiration. The Expiration: Inspiration ratio (E:I) was calculated by taking the mean of the longest R-R intervals during deep expirations to the mean of the shortest R-R intervals during deep inspirations.

Heart Rate Response to the Valsalva Manoeuvre

The participant was asked to forcibly exhale into a modified sphygmomanometer to a pressure of 40mmHg for 15 seconds. Once the valsalva is released subjects were asked to lie still without talking and the ECG recording was continued for a further 45 seconds. The expected reflex response to the Valsalva manoeuvre includes tachycardia and peripheral vasoconstriction during strain, followed by an overshoot in blood pressure and bradycardia after release of strain. The Valsalva R/R ratio was calculated as the highest RR interval after the manoeuvre divided by the lowest RR interval during the manoeuvre. The Valsalva manoeuvre was not undertaken by participants with proliferative retinopathy to prevent retinal haemorrhages that can be precipitated by sudden increases in intrathoracic pressure (Duane, 1972).

Heart Rate Response to Standing

The participant was connected to the ECG while lying down and then stood to a full upright position. In a normal response there is a characteristic and rapid increase in heart rate in response to standing that is maximal at approximately the

15th beat after standing. This is followed by a relative bradycardia that is maximal at approximately the 30th beat after standing. The 30:15 ratio was calculated as the ratio of the longest R-R interval to the shortest R-R interval. If the maximum and minimum R-R intervals did not occur at exactly the 15th or 30th beats after standing the 30:15 ratio was redefined as the longest R-R interval during beats 20–40 divided by the shortest R-R interval during beats 5–25 (Ziegler et al., 1992).

Systolic Blood Pressure Response to Standing

Blood pressure was measured using an automatic sphygmomanometer with the patient having remained supine for greater than 5 minutes and then repeated one minute after standing. A drop of systolic blood pressure >20mmHg is classified as abnormal (Witte et al., 2005).

Visit 5; Autoimmune disease screening

Participants attended for a short synacthen test to exclude adrenocortical insufficiency, blood sampling for thyroid stimulating hormone assay to exclude thyroid disease and anti-endomysial antibody analysis to exclude coeliac disease. Participants with a new diagnosis or uncontrolled concomitant autoimmune disease were referred for assessment and treatment before continuing in the study. A new diagnosis did not preclude ongoing participation.

Visit 6; Education session

All participants attended an education session to complete the 'My Hypo COMPaSS tool', as described in the pilot study and main study protocols (Thomas et al., 2007, Little et al., 2012). The session lasted up to 3 hours and participants attended individually or in small groups facilitated by a clinical research fellow, diabetes specialist nurse or a specialised dietician. Discussions and exercises explained the importance of completely avoiding hypoglycaemia while continuing to maintain good glycaemic control.

The Intervention

Randomisation

Randomisation was administered by Newcastle Clinical Trials Unit using a secure web based system (Little et al., 2012). Treatment intervention was stratified by centre and baseline HbA1c (cut off 64 mmol/mol or 8%). Participants were allocated to one of four interventions by a third party:

- MDI with SMBG
- MDI with SMBG and RT-CGM
- CSII with SMBG
- CSII with SMBG and RT-CGM

Following randomisation all participants attended an educational session (Little et al., 2012) regarding the technical aspects of their treatment arm. All participants were provided with a Medtronic Veo insulin pump and instructed how to use the bolus calculator whether or not insulin was being delivered by CSII. All participants were provided with a Contour link SMBG meter with direct transmission of blood glucose values to the insulin pump bolus calculator.

The common goal of each intervention was to completely avoid blood glucose levels of less than 4mmol/l determined by SMBG and RT-CGM. Participants were instructed to treat all blood glucose levels of less than 4 mmol/l with 15g of carbohydrate with repeat testing 15 minutes after treatment and repeated treatment if required. Following treatment insulin dose reduction was then to be considered.

a) MDI

Participants randomised to MDI were provided with insulin aspart, 3ml cartridge with 100 Units/mL, in a disposable pen (Flexpen) and insulin glargine, 3ml cartridge with 100 Units/mL, in a disposable pen (SoloStar). Insulin lispo, 3ml cartridge with 100 Units/mL in a disposable pen (Kwikpen), was offered if participants had a previous negative experience or adverse reaction to insulin aspart. Participants were taught how to use the pens and received information about injection site care. Participants were also provided with a Medtronic Veo insulin pump and were taught how to use the bolus calculator.

Insulin glargine was advised to be taken within 30 minutes of retiring to bed. Participants were told to aim for a stable blood glucose overnight. Doses were advised to be reduced in the event of any nocturnal hypoglycaemia or if blood glucose levels were less than 5.0mmol/l between 4am and breakfast. Doses were advised to be adjusted by 1-2 units if the fasting target was not achieved but not at the expense of hypoglycaemia.

If blood glucose levels were greater than 7.0mmol/l pre-evening meal or very variable before breakfast and evening meal then a second dose of glargine was introduced at 4 units. The evening dose was reduced by 2-4 units if there was evidence of a reducing blood glucose overnight preceding the introduction of the second dose. Participants taking glargine twice a day on entering the trial were advised to continue if randomised to MDI.

The second dose of glargine was advised to be taken within 30 minute of rising. Stable blood glucose levels were aimed for during the afternoon. Doses were reduced if any hypoglycaemia occurred during the period of time between 2 hours post lunch and the evening meal. Doses were advised to be adjusted by 1-2 units if the pre-evening meal target was not achieved but not at the expense of hypoglycaemia.

b) CSII

All participants randomised to CSII were provided with insulin aspart in 10ml vials with 100 Units/mL. Insulin lispo was offered to participants with a previous negative experience or adverse reaction to insulin aspart. Participants received information about the technical aspects of the pump including the changing of infusion sets during the education session.

The basal rates were titrated according to fasting, pre-meal, pre-bed and 4am targets. If within targets no changes were made. If above target the basal insulin was increased by 0.1units/hour from the previous checkpoint. If below target the basal insulin was reduced by 0.1units/hour.

c) SMBG

All participants received information about the technical aspects of using the Contour link meter which was able to transmit data automatically to the bolus calculator on the pump. All participants were asked to check blood glucose levels four times a day and then undertake a weekly 8 point profile including those using RT-CGM.

d) RT-CGM

Participants randomised to RT-CGM were provided with a MiniLink Real-Time Transmitter which was connected to the participant's insulin pump to allow the display of CGM data. Participants were informed about how to use the hypoglycaemic and hyperglycaemic alarms and trend analysis. Participants were shown how to use the data to alter their insulin doses during real time and retrospectively.

Meal time insulin (CSII and MDI)

Insulin aspart or lispro were given either by subcutaneous injection or as a pump bolus before meals or snacks with a carbohydrate content of greater than 10g. Carbohydrate counting skills were evaluated in each individual during the education sessions. Individualised insulin carbohydrate ratios and insulin sensitivity factors were calculated. Insulin: carbohydrate ratios were calculated using the '500 rule': 500 divided by the total daily dose of insulin (pre-randomisation) equals the grams of carbohydrate covered by one unit of aspart or lispro insulin.

Corrective doses were recommended to be given with the meal time bolus if pre-prandial blood glucose levels were above target. This was calculated by the '100 rule': 100 divided by the total daily dose of insulin equals the glucose reduction in mmol/l per 1 unit of insulin aspart or lispro. The insulin: carbohydrate ratio and insulin sensitivity factor would be adjusted if an unexplained episode of hypoglycaemia occurs within 2 hours of a meal or if glucose levels are consistently above or below blood glucose target 2 hours after a meal.

Blood Glucose Targets

- Fasting blood glucose: 5.0 to 7.0mmol/l
- Pre-prandial blood glucose: 4.5-7mmol/l
- Post-prandial (2 hours) blood glucose 6.0-8.0mmol/l
- Pre-bed blood glucose (within 30minutes of retiring): 6.0-8.0mmol/l
- 4am blood glucose: 5.0-7.0mmol/l

Follow up and support

Participants were telephoned daily for the first week after commencement of the study intervention and at least weekly there after throughout the six month RCT. Participants attended for a study visit every 4 weeks for the collection of their diaries and for a HbA1c. A blinded CGM device (Medtronic iPro) (Tan et al., 2016) was fitted and worn during the week before this visit. A further hypoglycaemic clamp study was carried out during the last visit in willing and eligible participants. Cardiac autonomic function testing was completed at the end of the RCT during a separate visit. Participants were invited to attend three follow up visits at 6months, 12 months and 18 months after the RCT was completed.

2.3 The HypoCOMPaSS Sub-study

Following the completion of the HypoCOMPaSS trial I designed a sub-study to recruit a control group to compare to the main hypoCOMPaSS population. Participants with T1DM and normal awareness of hypoglycaemia were recruited. The control population underwent cardiac autonomic function testing to ascertain the presence or absence of autonomic neuropathy and were then invited to undergo an optional hypoglycaemic clamp study. Ten participants without diabetes were also recruited.

Research Governance

Ethical approval for this study was sought and gained from Yorkshire and Humber Research Ethics Committee (12/YH/0035). Local approval was sought

and granted by Sheffield Teaching Hospitals Foundation Trust (STH16283/CSP94410).

Study Participants

Sub-study participants were recruited from Sheffield Teaching Hospitals, Sheffield. Potential study participants were identified when attending routine clinic appointments and were provided with written information. Permission was obtained to allow a member of the research team to contact these potential participants at a later date to discuss the study further. Eligibility and written consent were then confirmed and obtained in compliance with good clinical practice at visit one. An optional hyperinsulinaemic hypoglycaemic clamp study was discussed at visit one with eligible volunteers.

Inclusion criteria for the hypoCOMPaSS sub-study

- Male or female aged 18-74 years inclusive at start of the trial.
- Diagnosis of diabetes mellitus according to ADA and WHO criteria (Appendix 1) and consistent with a clinical diagnosis of T1DM.
- Serum C-peptide below the quality assured limit of detection for the assay and laboratory with simultaneous exclusion of biochemical hypoglycaemia (glucose <4.0 mmol/L) by laboratory glucose level analysis on a sample taken at the same time point.
- Normal awareness of hypoglycaemia as confirmed by a score of < 4 in the Gold score (Appendix 2).

Exclusion criteria for the hypoCOMPaSS sub-study

- Any condition that in the investigator's judgement is likely to cause the subject to be unable to understand the information in the Informed Consent Document or provide informed consent.
- Patients that are taking beta blocking medication which cannot be discontinued safely for 48 hours.

Relatives or spouses of the main hypoCOMPaSS trial study participants and members of staff from the diabetes department of Sheffield Teaching Hospitals,

Sheffield were invited to take part in the hypoCOMPaSS sub-study as participants without diabetes. All participants underwent a hyperinsulinaemic, hypoglycaemic clamp study. The exclusion criteria for participants without diabetes are more extensive than that of the T1DM participants to ensure safety during the clamp study.

Inclusion criteria

- Male or female aged 18-60 years inclusive at start of the trial.

Exclusion criteria

- Any condition that in the investigator's judgement is likely to cause the subject to be unable to understand the information in the Informed Consent Document or provide informed consent.
- Patients that are taking beta blocking medication which cannot be discontinued safely for 48 hours.
- Random glucose > 11.1mmol/l or HbA1c > 48mmol/mol
- History of epilepsy
- History of ischaemic heart disease, cardiac arrhythmia or heart failure
- History of peripheral vascular disease or stroke
- History of nephropathy as demonstrated by elevated serum creatinine accompanied by proteinuria.
- Pregnancy

Baseline visit

After informed consent a full clinical history and physical examination was completed and recorded in accordance with the main study. Blood was taken for HbA1c, C-peptide (in participants with T1DM), glucose and urea and electrolytes with an ECG. The hypoglycaemic clamp study was discussed with eligible T1DM participants and all participants without diabetes.

Cardiac autonomic function testing

All participants underwent detailed cardiac autonomic function testing as described in the main hypoCOMPaSS study visit 4.

Hyperinsulinaemic hypoglycaemic clamp study

The willing and eligible participants with diabetes and all participants without diabetes underwent a hyperinsulinaemic hypoglycaemic clamp study. The procedure was carried out as described in the main hypoCOMPaSS trial. Participants with T1DM were fitted with a blinded CGM sensor (Medtronic iPro) five to seven days prior to the clamp study. This was reviewed with their blood glucose diary to determine if any antecedent BH had occurred over the preceding 24 hour time period to ensure that counter-regulatory responses to hypoglycaemia were not attenuated by antecedent hypoglycaemia. Studies were postponed if any CGM and or self monitored capillary glucose below 3.0mmol/l were detected for greater than 20 minutes in the 12 hours prior to the study.

2.4 Statistical Analysis

Power and sample size considerations

Based upon a previous study with a not dissimilar age and duration of diabetes expected in these studies (Hepburn et al., 1990) a reasonable estimate of prevalence of autonomic neuropathy in patients with normal awareness of hypoglycaemia was 37% and in patients with IAH 63%. Hepburn and colleagues compared the prevalence of DAN depending upon symptomatic awareness of hypoglycaemia and duration of disease. Participants underwent cardiac autonomic function testing. Each test was scored:0 for normal, 1 for borderline and 2 for abnormal. Participants were then given an autonomic score. A score of above 3 were considered to have definite autonomic dysfunction. They found that IAH was associated with a long duration of disease but not invariably associated with DAN.

The expected dropout rate of the hypoCOMPaSS studies was expected to be low. An allowance was made for a 5% withdrawal rate. To have 90% power, to detect a difference in prevalence of autonomic neuropathy between the groups, based on the figures from the Hepburn study, 80 patients with IAH and 80 patients with intact awareness would need to be recruited and undergo AFTs. .

Statistical tests

Data that followed an approximate normal distribution was summarised using mean (standard deviation (SD)) unless otherwise stated, whilst skewed data were summarized using the median (interquartile range). Spectral HRV parameters were logarithmically transformed to approximate a normal distribution. Statistical analysis was performed with SPSS (version 22.0, IBM, Chicago, Illinois). A p-value ≤ 0.05 was deemed statistically significant.

Comparison of the prevalence of DAN between the two groups of participants defined by awareness of hypoglycaemia was completed with the chi square test (for independence). Potential confounding factors of age, duration of diabetes, sex and pre-existing micro- and macrovascular complications were examined using an independent t test. The impact of duration of diabetes was explored further using a model of logistic regression; awareness of hypoglycaemia was defined as the categorical variable and duration of disease as a predictor variable.

Following the 24 week RCT a comparison of baseline and end HbA1c, insulin dose, gold score and number of episodes of SH were compared using a paired t test. Participants were divided by cardiac autonomic status, and change in gold score between groups, was compared using chi square test. Potential confounding factors of age, duration of diabetes, sex and pre-existing micro- and macrovascular complications were examined using one way analysis of covariance (ANOVA).

During the clamp studies changes in metanephrines were analysed using ANCOVA. To compare symptom scores during the clamp, area under the curve was calculated for each person, and then ANOVA was used to test for a difference in the mean area under the curve. Differences between mean heart rates of each group were calculated using a mixed effects repeated measures model. Heart rate at 60 minutes, 100 minutes, 140 minutes and 180 minutes were defined as dependent variables and heart rate at 20 minutes independent with a fixed factor for time and for patient group with an interaction term for time and group. Post hoc analysis was completed with the Holm procedure (Aickin and Gensler, 2011).

Glucose thresholds for the release of metanephrine were defined as the measured glucose at the time of onset of a sustained (≥2 successive time points) increase in hormone concentrations ≥ 2SD above the mean baseline measurements. Thresholds for the increase in total, autonomic and neuroglycopenic symptoms were determined as the measured glucose level at which the symptom score increased ≥ 2 over baseline on two consecutive assessments (Choudhary et al., 2009). Where no defined change occurred the lowest glucose measurement recorded for the participant was used as the threshold for that individual (Choudhary et al., 2009)

2.5 Biochemical Analysis

Blood samples taken during visit one including glucose, c-peptide and HbA1c were analysed in local laboratories. Autoimmune screening and assessment of renal function were also analysed in each individual centre. During the clamp studies arterialised plasma glucose was analysed in real time using Yellow Springs analyzer (YSI STAT Plus, Farnborough, U.K.) (intraassay coefficient of variation (CV) 1.5% and interassay CV 2.8%). Plasma insulin was measured by ELISA (Dako, Glostrup, Denmark) (intra-assay CV 1.8% and interassay CV 7.8%). Glucagon was measured by ELISA (Alpco Diagnostics) (intra-assay CV 1.6% and interassay CV 2.4%). Cortisol levels were measured using a two-step sandwich immunoassay (Roche Modular E-170 platform, Elecsyscortisol reagents) (intra-assay CV 1.4% (based on a mean value of 593 nmol/L) and interassay CV 4.7% (based on a mean value of 535 nmol/L). Plasma metanephrine (separate from normetanephrine) was measured using ELISA (Alpco Diagnostics) (intra-assay CV 12% (based on a mean value of 652 pmol/L) and interassay CV 12.2% (based on a mean value of 350 pmol/L). Growth hormone was measured using ELISA (Alpco Diagnostics) (intra-assay CV 1.4% and interassay CV 4.5%).

Chapter 3: The Association between Impaired Awareness of Hypoglycaemia and Diabetic Autonomic Neuropathy in Type One Diabetes Mellitus.

3.1 Abstract

Background: Impaired awareness of hypoglycaemia (IAH) affects 20-25% of people with type 1 diabetes (T1DM) (Geddes et al., 2008) and increases the risk of severe hypoglycaemia (SH) (Gold et al., 1994). Traditionally, diabetic autonomic neuropathy (DAN) has been described as the underlying mechanism causing IAH (Vinik et al., 2003) but the literature is conflicting and the relationship in confounded by duration of disease (Stephenson et al., 1996, Ryder et al., 1990). This study investigates the relationship between DAN and IAH by comparing the prevalence of cardiac autonomic neuropathy in patients with T1DM and IAH and a control group of patients with T1DM and intact awareness of hypoglycaemia.

Methods: Participants with impaired awareness of hypoglycaemia (Gold score ≥4), recruited to the hypoCOMPaSS trial (Little et al., 2012), underwent detailed cardiac autonomic function testing prior to study intervention. Participants with normal awareness of hypoglycaemia (Gold score <4), matched for glycaemic control and duration of disease underwent the same testing protocol. The prevalence of cardiac autonomic neuropathy in both populations was compared.

Results: A total of 93 participants with IAH and 51 participants with intact awareness of hypoglycaemia underwent detailed cardiac autonomic function testing. Participants with IAH had a higher gold score (IAH gold score 5, intact awareness gold score 2, p=<0.005) and an increased incidence of episodes of severe hypoglycaemia (IAH median of 4 (7) episodes per year, intact awareness 0 (0) episodes per year). Both participant groups had a long duration of disease (intact awareness 21 (11) years, IAH 29 (12) years), and despite attempting to match the duration of disease the participants with IAH had a significantly longer duration of disease (p=0.001). The overall prevalence of DAN, defined as an abnormality in at least two cardiac reflex tests, was low in both participant groups (intact awareness 2/51 (4%) and IAH 5/93 (5%)). There was no statistical difference in cardiac autonomic status between the two groups (p=0.37).

Conclusions: The prevalence of cardiac autonomic neuropathy, defined as an abnormality in two or more cardiac reflex tests, was low in these patients with

T1DM and was associated with a long duration of disease. The presence of cardiac autonomic dysfunction does not appear to be a major influencing factor on the development of IAH supporting other recent studies investigating this association (Olsen et al., 2016, Kamel et al., 2015). Further epidemiological studies are required to establish the natural history of DAN and to identify patients at risk.

3.2 Background

Studies previously carried out to determine the relationship between IAH and DAN have been inconsistent and contradictory (Stephenson et al., 1996, Meyer et al., 1998, Ryder et al., 1990, Hepburn et al., 1990). This is due to a previous lack of a standard accepted definition of DAN and the confounding factors of age, glycaemic control and duration of diabetes. Baroreflex sensitivity (BRS), a measurement of how much control the baroreflex has on the heart, has not been considered in these earlier studies.

The aim of this chapter is to investigate the possible association between IAH and DAN by comparing the prevalence of DAN in a group of subjects with T1DM and IAH, and a group with T1DM and normal awareness of hypoglycaemia. DAN will be defined, as described in the consensus statement of 2010 (Tesfaye et al., 2010), which provided an update on definitions and diagnostic criteria for DAN. Spectral analysis of HRV and BRS will be used to identify cardiac dysfunction or subclinical neuropathy and confounding factors of age, glycaemic control and duration of disease will also be assessed and compared.

3.3 Methods

The HypoCOMPaSS trial was a UK-based, multicentred, prospective randomised control trial (RCT). The study aimed to optimise diabetes treatment in patients with IAH (Gold score $\geq$4) to prevent biochemical hypoglycaemia (BH) and restore awareness. Each participant underwent detailed cardiac autonomic function testing during a pre-randomisation, 4 week run in period, completed before any intervention was undertaken.

A sub-study of the hypoCOMPaSS trial was then designed. The aim of this sub-study was to recruit a population of adults with T1DM and normal awareness of hypoglycaemia (Gold score <4) and compare the prevalence of cardiac autonomic neuropathy in the two populations. The sub-study population were matched for age and duration of disease. Detailed cardiac autonomic function testing was completed following the same protocol as in the main trial.

The protocol for the hypoCOMPaSS trial and the hypoCOMPaSS sub-study are described in full in chapter two but the salient points are reported below. The study protocol, participant information sheets and consent forms for both studies were approved by an independent research ethics committee. Written informed consent was obtained from each participant.

Recruitment

Participants were recruited to the main hypoCOMPaSS trial between July 2010 and June 2011 and to the sub-study between August 2012 and August 2014. Subjects were recruited to the main HypoCOMPaSS trial from five UK tertiary referral and academic hypoglycaemia centres: Bournemouth Diabetes and Endocrine Centre, Bournemouth; Adenbrooke's Hospital, Cambridge; Newcastle Diabetes Centre, Newcastle upon Tyne; Derriford Hospital, Plymouth and Sheffield Teaching Hospitals, Sheffield. Sub-study participants were recruited from Sheffield Teaching Hospitals.

Cardiac autonomic function testing

All participants underwent detailed screening cardiac autonomic function testing as described by Ewing (Ewing and Clarke, 1982) including the modifications suggested by O'Brien (O'Brien et al., 1986) in accordance with the latest consensus on the diagnosis of cardiac autonomic neuropathy (Tesfaye et al., 2010). Tests included heart rate response during deep breathing, one valsalva manoeuvre and heart rate and blood pressure response to standing. Participants were asked to refrain from smoking and caffeine on the day of the tests and beta blockers were discontinued 48 hours prior to testing. A period of three minutes of

rest preceded each test and testing was carried out in a darkened room where noise was kept to a minimum.

Two five minute simultaneous ECG and continuous blood pressure recordings, using a Portapres device, were made to measure BRS and allow spectral analysis of heart rate variability (HRV). During the first five minute recording, participants remained at rest, with spontaneous breathing and monitoring of the respiratory rate. During the second recording participants were asked to pace their breathing at a rate of 15 breaths per minute whilst maintaining a normal tidal volume. Results were compared to the O'Brien age adjusted normal ranges (O'Brien et al., 1986).

Statistical analysis

Data that followed an approximate normal distribution were summarized using mean (standard deviation (SD)) unless otherwise stated, whilst skewed data were summarized using the median (interquartile range). Spectral HRV parameters were logarithmically transformed to approximate a normal distribution. Prevalence of DAN was compared using chi square test for independence. Potential confounding factors of age, duration of diabetes, sex and pre-existing micro- and macrovascular complications were examined using independent t test. The impact of duration of diabetes and the presence of DAN on symptomatic awareness of hypoglycaemia were further explored using a model of logistic regression. Awareness of hypoglycaemia was defined as the categorical outcome and DAN and duration of diabetes as predictor variables. Statistical analysis was performed with SPSS (version 22.0, IBM, Chicago, Illinois). A p-value ≤ 0.05 was deemed statistically significant.

3.4 Results

Participants

A total of 110 participants with IAH (Gold score ≥4) were recruited to the main hypoCOMPaSS trial; six were excluded due to elevated C-peptide levels and eight withdrew from the study before randomisation. Ninety six were randomised, all with c-peptide negative, (<50pmol/L in all except two:87; 103 pmol/L) T1DM.

Three participants were excluded from this sub-analysis due to incomplete autonomic function testing.

A total of sixty four participants with normal awareness of hypoglycaemia (Gold score<4) were recruited to the hypoCOMPaSS sub-study. Ten were excluded due to elevated C-peptide levels and three withdrew before completion of the study. Of the fifty one subjects included in this analysis, thirty nine were c-peptide negative (<50pmol/L) and eleven participants had a c-peptide above 50pmol/L but below the lower end of the local reference range (298pmol).

Baseline characteristics for all participants are shown in table seven. The mean gold score for participants with intact awareness of hypoglycaemia was two and for participants with IAH the mean gold score was five (independent t test p=<0.005). The incidence of SH was higher in participants with IAH (median 4 (7) in 12 months vs. 0 (0) p=<0.005).

Participants with IAH had a significantly longer duration of disease compared to those with intact awareness (independent t test p=0.001). The prevalence of retinopathy and neuropathy was similar between the two groups but participants with intact awareness of hypoglycaemia had a higher incidence of nephropathy. Participants with IAH had a higher incidence of macrovascular complications. There was no statistical difference in age between the two groups (p=0.06) and there were a higher number of female participants in both groups.

Baseline characteristics	Intact awareness n=51	IAH n=93	P value
Age (years)	45 (11)	49 (12)	0.066
Sex (M/F)	22/29	35/58	0.64
BMI	26 (4)	27 (1)	0.303
Duration of diabetes (years)	22 (11)	29 (12)	0.001
HbA1c	69 (13)	66 (13)	0.182
Insulin dose (units/kg)	0.7 (0.3)	0.6 (0.2)	0.073
Gold score	2 (1)	5 (1)	<0.005
SH in the last 12 months	0 (0)	4 (7)	<0.005
HR (bpm)	71 (11)	71 (10)	0.206
Systolic blood pressure (mmHg)	131 (16)	131 (18)	0.989
Diastolic blood pressure (mmHg)	76 (9)	76 (11)	0.913
Retinopathy	35 (69%)	58 (62%)	0.264
Neuropathy	9 (17%)	16 (17%)	1
Nephropathy	5 (10%)	4 (4%)	0.345
Macrovascular disease	3 (6%)	12 (13%)	0.301

Results given as mean (SD) except episode of SH where median (IQR) is given and for retinopathy, neuropathy, nephropathy and macrovascular disease prevalence which is given as actual number (%).
Table 7 Baseline characteristics.

The prevalence of definite DAN, defined as the presence of two or more abnormal cardiac reflex tests (Tesfaye et al., 2010) was similar in both participant groups, affecting four percent of participants with normal awareness of hypoglycaemia and five percent of those with IAH. (Figure 8 and Table 8). Seventy one percent of participants with intact awareness of hypoglycaemia had normal cardiac autonomic function tests compared to 80% of participants with IAH. The prevalence of DAN was lower than expected in both participant groups. A Chi-square test for independence indicated no significant association between awareness of hypoglycaemia and cardiac autonomic function (p=0.37). The

prevalence of abnormal BRS was higher in participants with IAH (Figure 9 and Table 8) but a Chi-square test for independence indicated no significant association between awareness of hypoglycaemia and abnormal BRS (p=0.64). Heart rate and measures of HRV were similar between the two participant groups.

Cardiac autonomic function

	Intact awareness n=51	**IAH n=93**	**p value**
Heart rate	71 (11)	71 (10)	0.206
SDRR	33 (18)	35 (20)	0.795
RMSSD	24 (16)	23 (19)	0.955
Log HF	2.0 (0.62)	1.9 (0.62)	0.081
LF norm	0.63 (0.16)	0.66 (0.20)	0.001
BRS	12 (6)	13 (16)	0.956
Abnormal BRS	11/44 (25%)	11/34 (32%)	0.217
Cardiac autonomic function status			
Normal	36 (71%)	74 (80%)	0.397
Possible/early DAN	13 (25%)	14 (15%)	
Definite DAN	1 (2%)	4 (4%)	
Definite DAN with orthostatic hypotension	1 (2%)	1 (1%)	

Results given as mean (SD) except BRS and cardiac autonomic function status which is given as actual number (%).

Table 8 Results of cardiac autonomic function tests.

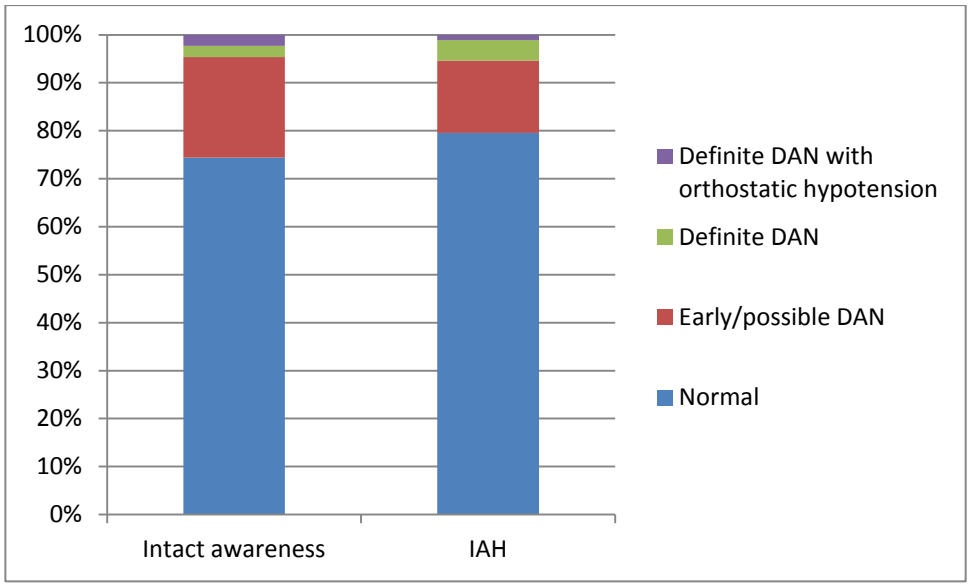

Figure 8 Prevalence of cardiac autonomic neuropathy in participants with intact and impaired awareness of hypoglycaemia.

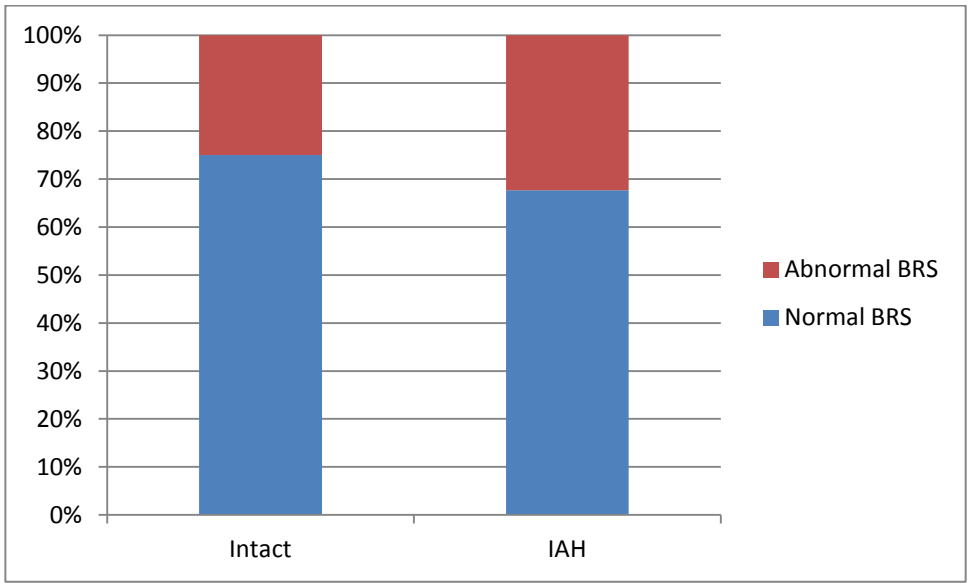

Figure 9 Prevalence of abnormal BRS in participants with intact and impaired awareness of hypoglycaemia.

Figure ten shows cardiac autonomic status results when participants are divided into categories based on duration of diabetes. This indicates that the prevalence of DAN increases with duration of diabetes. However, chi-square test for independence, indicated no significant association between duration of disease and cardiac autonomic status (p=0.44).

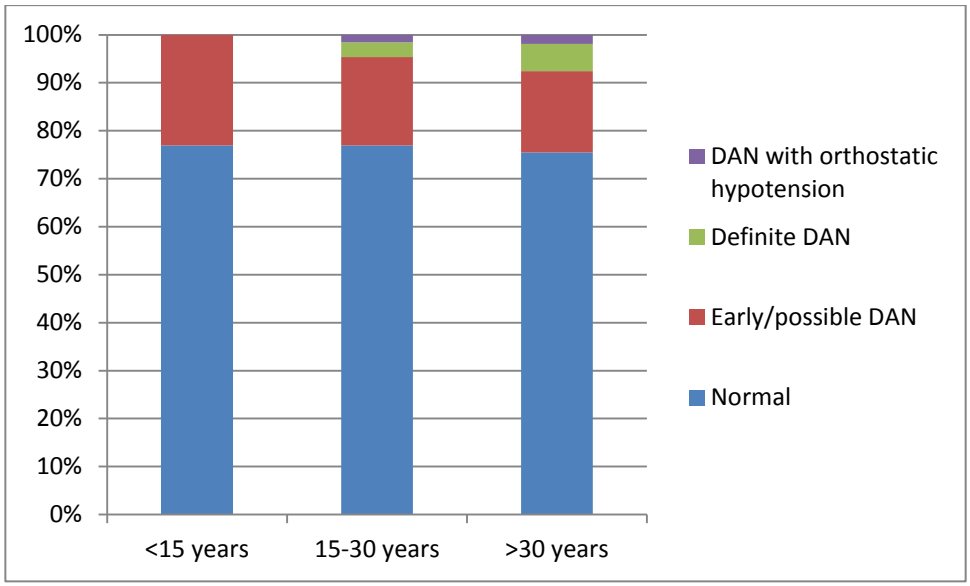

Figure 10 Prevalence of cardiac autonomic neuropathy in participants with short, medium and long duration of disease.

Direct logistic regression was performed to assess the impact of both cardiac autonomic function status and duration of diabetes on reported awareness of hypoglycaemia. The model was statistically significant, $\chi 2$ (2, N=144) = 12.10, p=0.002, indicating that the model was able to distinguish between participants that reported IAH and those that did not. The model explained between 8.1% (Cox and Snell R square) and 11.1% (Nagelkerke R squared) of the variance in awareness of hypoglycaemia and correctly classified 66% of cases. Duration of diabetes made a statistically significant contribution to the model with an odds ratio of 1.06. This indicates that for every year of disease the participants were 1.06 times more likely to develop IAH or for each additional year of diabetes increases the odds of developing IAH by six percent (table 9). The presence of DAN did not make a statistically significant contribution (table 9).

	B	S.E.	Wald	Df	P value	Odds Ratio	95.0% C.I for Odds Ratio	
							Lower	Upper
Presence of DAN	0.06	0.89	0.005	1	0.945	1.06	0.19	6.05
Duration of diabetes (years)	0.05	0.02	10.59	1	0.01	1.06	1.02	1.09
Constant	-0.75	0.44	2.97	1	0.09	0.47		

Table 9 Logistic regression predicting the development of IAH.

3.5 Conclusions

The prevalence of DAN was low in participants with impaired (5%) and normal awareness of hypoglycaemia (4%). The most commonly reported data evaluating the prevalence of DAN is from the Diabetes Control and Complications Trial (DCCT) and the follow up study, Epidemiology of Diabetes Interventions and Complications (EDIC). In the DCCT (Pop-Busui et al., 2016), studying patients with T1DM of duration of 1-15years, prevalence of DAN was 9%. This rose to 31% in the EDIC, when researchers followed up 90% participants of the DCCT with duration of diabetes greater than 15 years.

The reported prevalence of DAN varies depending on the cohort of patients recruited, tests used and the definition of DAN in each study. In these studies cardiac autonomic neuropathy was evaluated by heart rate variability during deep breathing and the valsalva manoeuvre and diastolic blood pressure on standing. Cardiac autonomic neuropathy was defined as an abnormality in any one of these tests. Applying the same diagnostic criteria to participants in this study 24% of participants would be classified as having cardiac autonomic neuropathy. The DCCT and EDIC did not use age-related normal ranges for the cardiac reflex tests which were used in this study which may account for the reduction in prevalence of DAN in the subjects in the hypoCOMPaSS studies.

The cardiac autonomic neuropathy seen in the hypoCOMPaSS studies was more advanced in participants with a longer duration of disease. In 2010 Tesfaye et al (Tesfaye et al., 2010) reported on the updates regarding classification, definitions and diagnostic criteria of DAN. The diagnostic criteria and staging of DAN are still being debated but the panel recommended that at least two abnormal HRV tests are required to confirm DAN and orthostatic hypotension would indicate more advanced cardiac autonomic neuropathy. Based on these diagnostic criteria only 10% (14/144) of the total study population have cardiac autonomic neuropathy and all these participants had a duration of diabetes of greater than 15 years. Only two participants had cardiac autonomic neuropathy with orthostatic hypotension; one subject with IAH and a diabetes duration of 44 years and one subject with normal awareness of hypoglycaemia with a duration of diabetes of 23 years.

IAH is associated with a long duration of disease and an increased incidence of SH. Both participant groups had a mean duration of diabetes of greater than 20 years but the participants with IAH had a significantly longer duration of disease despite efforts made to match participant groups (Table 8 p=0.001). Direct logistic regression was performed to assess the impact of duration of diabetes on reported awareness of hypoglycaemia. Duration of diabetes made a statistically significant contribution to the model and indicated that for each additional year of diabetes the odds of developing IAH increased by six percent (table 9). Previous studies (Mokan et al., 1994) (Hepburn et al., 1990) have also consistently reported the association of IAH with a long duration of disease. The study also confirms previous findings (Schopman et al., 2011) that participants with IAH (median 4(7)) have significantly more episodes of SH than those participants with normal awareness of hypoglycaemia (0(0) p=<0.005).

This study shows that IAH is not associated with an increased prevalence of DAN or abnormal BRS. Seventy one percent of participants with normal awareness of hypoglycaemia had completely normal cardiac autonomic function tests in comparison to eighty percent of the impaired awareness group. There was a slightly higher prevalence of abnormal BRS in the impaired awareness group but this did not reach statistical significance. Historically DAN was described as the mechanism causing IAH but the data in the literature is conflicting (Stephenson et al., 1996, Meyer et al., 1998, Ryder et al., 1990, Hepburn et al., 1990). More recently a study by Olsen and colleagues (Olsen et al., 2016) also concluded that IAH was not associated with autonomic neuropathy and a further study by Kamel et al (Kamel et al., 2015) has showed that reversal of hypoglycaemia unawareness following islet cell/pancreatic transplant was not hampered by the presence of autonomic dysfunction.

Limitations

The unexpected low prevalence of DAN in the study participants means that this study is underpowered, but increasing participant numbers, even significantly, is unlikely to change the study conclusions. Power calculations for this study were based on a study by Hepburn and colleagues in 1990 (Hepburn et al., 1990) with a

prevalence of DAN of 37% in participants with normal awareness of hypoglycaemia and 67% in subjects with IAH. The prevalence of cardiac autonomic neuropathy only differed by one percent between participants with IAH and normal awareness of hypoglycaemia, and therefore, it would be unlikely that increasing participant numbers would change the conclusions made.

Other potential limitations of this study include the use of spontaneous breathing during ECG recordings and differences in geographical distributions between the two participant groups. It is recommended that heart rate variability should be measuring when participants are undergoing paced breathing at a rate of 15 breaths per minute. As participants were being investigated by different clinical teams in different parts of the country it was felt that spontaneous breathing would limit inter-investigator differences. All participants had their respiratory rate measured to ensure that this was not fewer than 10 breaths per minute.

Further consideration should be given to other measures of autonomic dysfunction. This study used cardiac reflex tests to assess autonomic function and did not utilise other tests of the autonomic nervous system including tests of papupillomotor or sudomotor function. Tests for cardiac autonomic function are simple, quick, non-invasive and can be done at the bedside. Testing was limited to these tests to allow the tests to be done in each centre and then analysed in one site to improve reliability.

Final conclusions

The presence of cardiac autonomic neuropathy is not associated with or an influencing factor in the development of IAH. IAH is associated with a long duration of diabetes and this is an independent risk factor for the development of reduced symptomatic awareness of hypoglycaemia. The prevalence of DAN is lower than previously reported and illustrates the need for further epidemiological studies to investigate DAN in patients with T1DM. It is an important complication of diabetes because of the increased risk of death but it is one of the least studied. Further work is also required to document the natural history of the autonomic neuropathy particularly the potential progressive nature of the condition in order to develop potential treatments in the future.

Chapter 4: The Effect of Diabetic Autonomic Neuropathy on the Ability to Improve Awareness of Hypoglycaemia in Type One Diabetes Mellitus.

4.1 Abstract

Objectives: Impaired awareness of hypoglycaemia (IAH), affects 25% of patients with type one diabetes (T1DM), and increases the risk of episodes of severe hypoglycaemia (SH). IAH can potentially be reversed by complete avoidance of biochemical hypoglycaemia (BH), but treatment programmes are time consuming, expensive and a proportion of patients will not respond. Traditionally diabetic autonomic neuropathy (DAN) was described as the underlying mechanism causing IAH. Studies examining the relationship have shown it is unlikely to be the primary cause but the relationship between the two entities remains undefined. The aim of this study was to examine the effect of DAN on the ability to reverse IAH.

Methods: Eighty three participants with T1DM and IAH (Gold score ≥ 4) were recruited to the hypoCOMPaSS trial; a 24 week 2x2 factorial randomised controlled trial. All participants underwent screening cardiac autonomic function testing prior to study intervention to ascertain the presence or absence of DAN. All received comparable education and support, aimed at avoiding BH, and improving symptomatic awareness of hypoglycaemia. Participants were grouped depending on their cardiac autonomic status, and changes in gold score in the different groups were compared.

Results: The prevalence of DAN was low with only 5/83 (6%) participants having definite cardiac autonomic neuropathy, defined as the presence of two or more abnormal cardiac reflex tests. All patient groups including those with normal cardiac autonomic function, possible or early cardiac autonomic dysfunction and definite cardiac autonomic dysfunction showed a mean improvement in Gold score ≥1. There was a greater reduction in gold score in participants that were younger and had a shorter duration of disease.

Conclusion: IAH can be improved in patients with T1DM, and a long duration of disease, with and without the presence of cardiac autonomic dysfunction. This suggests that autonomic neuropathy is not a prime driver of the syndrome of IAH. The lower than expected prevalence of DAN in this study indicate a need for up to

date epidemiological studies to establish the prevalence of DAN in T1DM with a short and long duration of disease.

4.2 Background

Impaired awareness of hypoglycaemia (IAH) is the loss of the autonomic symptoms of hypoglycaemia before neuroglycopenia occurs. There is evidence that the clinical syndrome of IAH can improve, and can at least be partially reversed, by complete avoidance of biochemical hypoglycaemia (BH) (Fanelli et al., 1993b, Cranston et al., 1994). Studies investigating the reversibility of IAH often exclude patients with DAN but a small study by Fanelli and colleagues (Fanelli et al., 1997) showed that DAN did impede reversibility to a degree.

The hypoCOMPaSS (Comparison of Optimised MDI versus Pumps with or without Sensors in Severe Hypoglycaemia) Trial was a UK-based, multicentred, prospective randomised controlled trial (RCT). The aim was to optimise diabetes treatment in patients with IAH, to prevent BH, and restore symptomatic awareness of hypoglycaemia. In this sub-analysis we investigate if cardiac autonomic status, and the presence of clinical or subclinical autonomic neuropathy, adversely affects a patient's ability to respond to and reverse the clinical syndrome of IAH.

Participants of the hypoCOMPaSS trial underwent detailed cardiac autonomic function testing in the wash in period, before randomisation, and before any treatment intervention. The participants underwent the six month treatment period and cardiac autonomic function testing was repeated. Participants were divided into groups depending on their cardiac autonomic function status as defined by the latest consensus published by the Toronto working group (Tesfaye et al., 2010). Changes in Gold score were compared between groups with different cardiac autonomic status.

4.3 Methods

Participants with T1DM and IAH (gold score$\geq$ 4) were recruited to the HypoCOMPaSS trial. The protocol for the hypoCOMPaSS trial is described in full in chapter two but the salient points are reported below.

Cardiac autonomic function testing

All participants underwent detailed screening cardiac autonomic function testing as described by Ewing (Ewing and Clarke, 1982) including the modifications suggested by O'Brien (O'Brien et al., 1986) in accordance with the latest consensus on the diagnosis of cardiac autonomic neuropathy (Tesfaye et al., 2010). Tests included heart rate response during deep breathing, one valsalva manoeuvre and heart rate and blood pressure response to standing. Participants were asked to refrain from smoking and caffeine on the day of the tests and beta blockers were discontinued 48 hours prior to testing. A period of three minutes of rest preceded each test and testing was carried out in a darkened room where noise was kept to a minimum.

Two five minute simultaneous ECG and continuous blood pressure recordings, using a Portapres device, were made to measure baroreflex sensitivity (BRS) and allow spectral analysis of heart rate variability (HRV). During the first five minute recording, participants remained at rest, with spontaneous breathing and monitoring of the respiratory rate. During the second recording participants were asked to pace their breathing at a rate of 15 breaths per minute whilst maintaining a normal tidal volume. Results were compared to the O'Brien age adjusted normal ranges (O'Brien et al., 1986).

HypoCOMPaSS Intervention

A web-based system was used to randomly allocate participants on an equal allocation basis, stratified by baseline HbA1c (<64mmol/L and ≥ 64mmol/L) and study centre, to one of four treatment arms: multiple daily injections (MDI) with self monitoring of blood glucose (SMBG), MDI with SMBG and RT-CGM, CSII with SMBG, and CSII with SMBG and RT-CGM. The primary goal of insulin dose titration throughout the 24 week RCT period was the absolute avoidance of all glucose levels less than 4 mmol/L as determined by RT-CGM and SMBG.

Prior to randomisation, all participants underwent a standardised education session on an individual or small group basis. Discussions were facilitated focusing on avoidance of BH and early detection of all blood glucose levels less

than 4 mmol/L. The aim of the session was prevention of progression to significant hypoglycaemia while maintaining overall glycaemic control. Participants were advised to treat all glucose levels less than 4 mmol/L with 15g of carbohydrate with repeat SMBG every 15 min until glucose rose above 4 mmol/L and to consider prospective insulin dose reduction.

During the 24 week intervention participants received at least weekly contact from a health care professional for advice regarding insulin titration. All participants received an insulin pump (Paradigm Veo insulin pump; Medtronic), whether allocated insulin via CSII (aspart) or MDI (aspart/glargine), to allow the benefit of direct transmission of SMBG levels to a bolus calculator. Participants recorded episodes of SH prospectively.

Statistical analysis

Data that followed an approximate normal distribution were summarized using mean (standard deviation (SD)) unless otherwise stated, whilst skewed data were summarized using the median (interquartile range). Spectral HRV parameters were logarithmically transformed to approximate a normal distribution. Following the 24 week RCT a comparison of baseline and end HbA1c, insulin dose, gold score and number of episodes of SH was completed using paired t test. Participants were divided by cardiac autonomic status, and change in gold score between groups, was compared using chi square test. Potential confounding factors of age, duration of diabetes, sex and pre-existing micro- and macrovascular complications were examined using one way ANOVA. Statistical analysis was performed with SPSS (version 22.0, IBM, Chicago, Illinois). A p-value ≤ 0.05 was deemed statistically significant.

4.4 Results

Participants

A total of 110 participants with IAH (Gold score ≥ 4) were recruited to the hypoCOMPaSS trial. Six participants did not meet C-peptide inclusion criteria, and 8 withdrew from the study before randomisation. Ninety six were randomised, all with long-standing (mean duration 29 years), c-peptide negative, (<50pmol/L in all except two:87; 103 pmol/L) T1DM. Six participants did not

adhere to their randomised treatment but were included in the intention-to-treat analysis.

The results from the hypoCOMPaSS trial were reported in 2014 (Little et al., 2014) and showed that hypoglycaemia awareness can be improved, and recurrent SH prevented, in long standing type 1 diabetes and IAH. This was through strategies deliverable in routine clinical practice, targeted at rigorous avoidance of BH without relaxation of overall control. When provided with equal education and attention, equivalent biochemical outcomes and reduction in fear of hypoglycaemia were attained with conventional MDI and SMBG regimens compared with CSII and CGM-RT. Treatment satisfaction was higher in CSII users.

A total of eighty three subjects are included in this sub-analysis. Of the ninety six subjects that were randomised in the hypoCOMPaSS trial, nine were excluded from this analysis, due to not completing the six month treatment period. A further two participants were excluded because of incomplete end of study questionnaires which included Gold score assessment. A further two subjects were excluded due to incomplete cardiac autonomic function testing.

Baseline characteristics are shown in table ten. The incidence of severe hypoglycaemia was high with a mean rate of episodes of 9 (13) in the twelve months prior to the study (median 4 [2.5] Range 0 to >50 episodes). Eight participants reported no severe episodes in the year prior to the study and five reported greater than 50 episodes in the previous 12 months.

Cardiac autonomic function

All participants underwent detailed cardiac autonomic function testing. Definite DAN was defined as the presence of two or more abnormal cardiac reflex tests (Tesfaye et al., 2010). The results are presented in table eleven. Eleven participants had possible or early cardiac autonomic neuropathy and five participants had definitive cardiac autonomic neuropathy.

Baseline characteristics	**n=83**
Age (years)	48 (12)
Sex (M/F)	31/52
BMI	27 (5)
Duration of diabetes (years)	29 (13)
HbA1c	66 (13)
Insulin dose (units/kg)	0.7 (0.2)
Gold score	5 (1)
SH in the last 6 months	5 (10)
SH in the last 12 months	9 (13)
HR (bpm)	72 (10)
Systolic blood pressure (mmHg)	131 (18)
Diastolic blood pressure (mmHg)	76 (11)
Retinopathy	52 (63%)
Neuropathy	14 (17%)
Nephropathy	3 (4%)
Macrovascular disease	11 (13%)

Results given as mean (SD) except for retinopathy, neuropathy, nephropathy and macrovascular disease prevalence which is given as actual number (%).

Table 10 Participant characteristics.

	n=83
Heart rate	72 (10)
SDRR	34 (20)
RMSSD	23(20)
Log HF	2.5 (1.4)
LF norm	0.61 (0.23)
BRS (n=32)	10.3 (5.4)
Cardiac autonomic function status	
Normal cardiac autonomic function	67 (78%)
Abnormal BRS	10/32 (12%)
Possible/early DAN	11 (13%)
Definite DAN	4 (5%)
Definite DAN with orthostatic hypotension	1 (1%)

Table 11 Screening cardiac autonomic function results

Study outcomes

The results of the hypoCOMPaSS trial have been published in full (Little et al., 2014). The population in this sub analysis showed similar improvements in hypoglycaemia awareness and reduction in episodes of SH (table 12). Glycaemic control, as assessed by HbA1c, did not deteriorate and insulin requirements reduced (table 13).

	Baseline	Week 24	P value*
SH			
Annualised rate	9 (13)	2 (6)	<0.0005
	4 [7]	0 [0]	
Proportion affected	92%	6%	
Gold score	5 (1)	4 (2)	<0.0005
	5 [1]	4[2]	

Data are mean (SD), Median [interquartile range]. *paired t test between week 24 end point and baseline.

Table 12 SH and hypoglycaemia awareness in study population at baseline and 24 week end point (n=78).

	Baseline	Week 24	P value*
HbA1c	66 (13)	65 (11)	0.40
Insulin (units per kg)	0.7 (0.3)	0.5 (0.2)	<0.0005

Data are mean (SD) *paired t test between week 24 end point and baseline.

Table 13 HbA1c and insulin doses in study population at baseline and 24 week endpoint.

The presence of cardiac autonomic neuropathy did not impede the ability of participants to improve hypoglycaemia awareness defined as the reduction of gold score ≥1 (figure 11). A Chi-square test for independence indicated no significant association between cardiac autonomic function status and a reduction in gold score of ≥1 (p=0.743). The mean reduction in gold score was -1.2 (1.6). The greatest reduction was seen in participants with early DAN with a mean reduction of 1.36 (1.5) and the smallest reduction was seen in participants with definite DAN (1.0 (1.2)). Participants that had normal cardiac autonomic function had a mean reduction in gold score of 1.2 (1.7). Participants that had normal BRS showed a reduction in gold score of -1.45 and those with abnormal BRS showed a greater mean reduction in gold score of- 1.8.

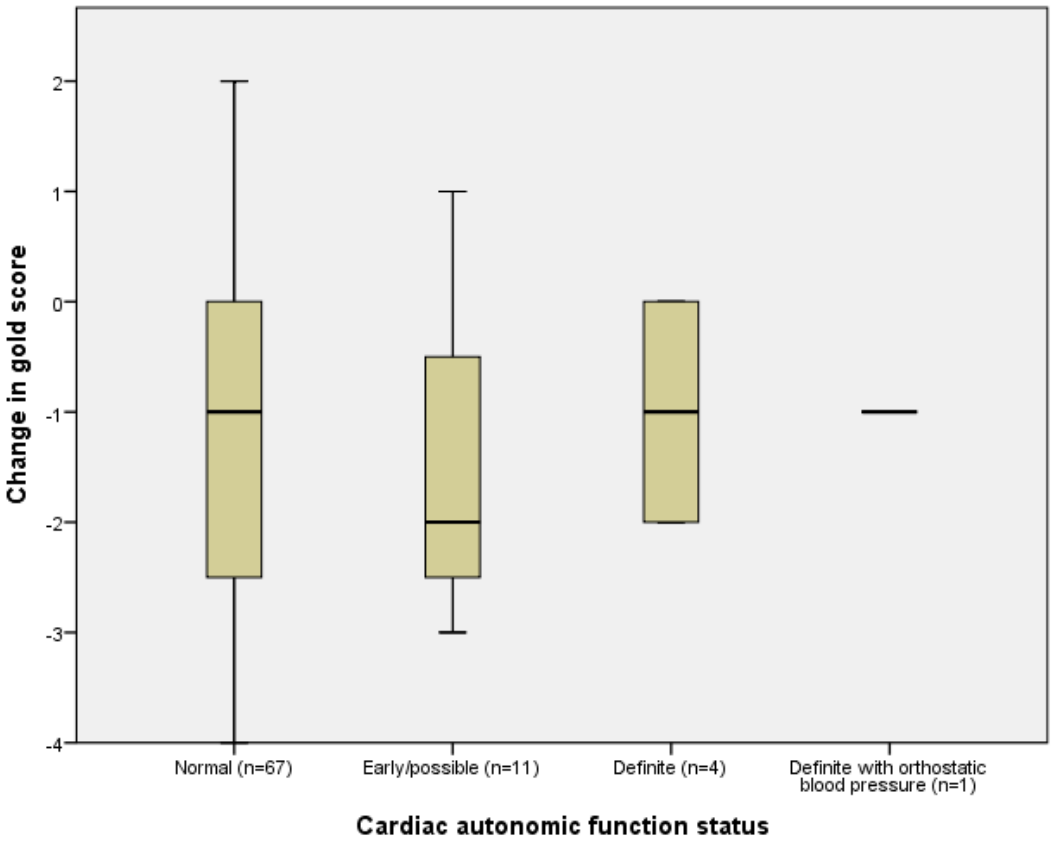

Figure 11 The effect of cardiac autonomic status on the improvement of Gold score. Chi-square test for independence indicated no significant association between cardiac autonomic function status and a reduction in gold score of ≥1 (p=0.743)

Participants aged less than 30 years had a greater improvement in gold score compared to participants aged over thirty years (p=0.017) (Figure 12). Nine participants were aged under 30 and had a mean reduction in gold score of 2 (0.9). Seventy four participants were aged over 30 years and had a mean reduction in gold score of 1.1 (1.6).

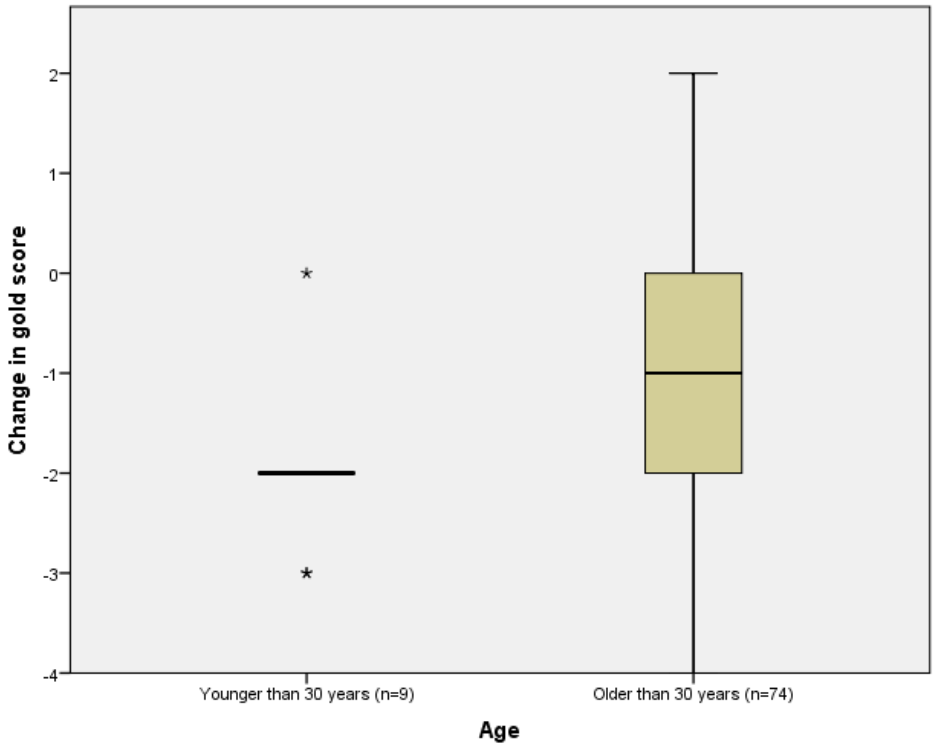

Figure 12 Effect of age on Gold score reduction (p=0.017).

Participants with shorter duration of diabetes (<30 years) had a greater improvement in gold score in comparison to those with a longer duration of disease (figure 13). Forty seven participants had a duration of diabetes of less than 30 years and had a mean reduction in gold score of -1.3 (median reduction of -2.0[2]). Participants with a duration of greater than 30 years had a mean reduction of -0.9 (1.6) and median reduction of -1.0 [2].

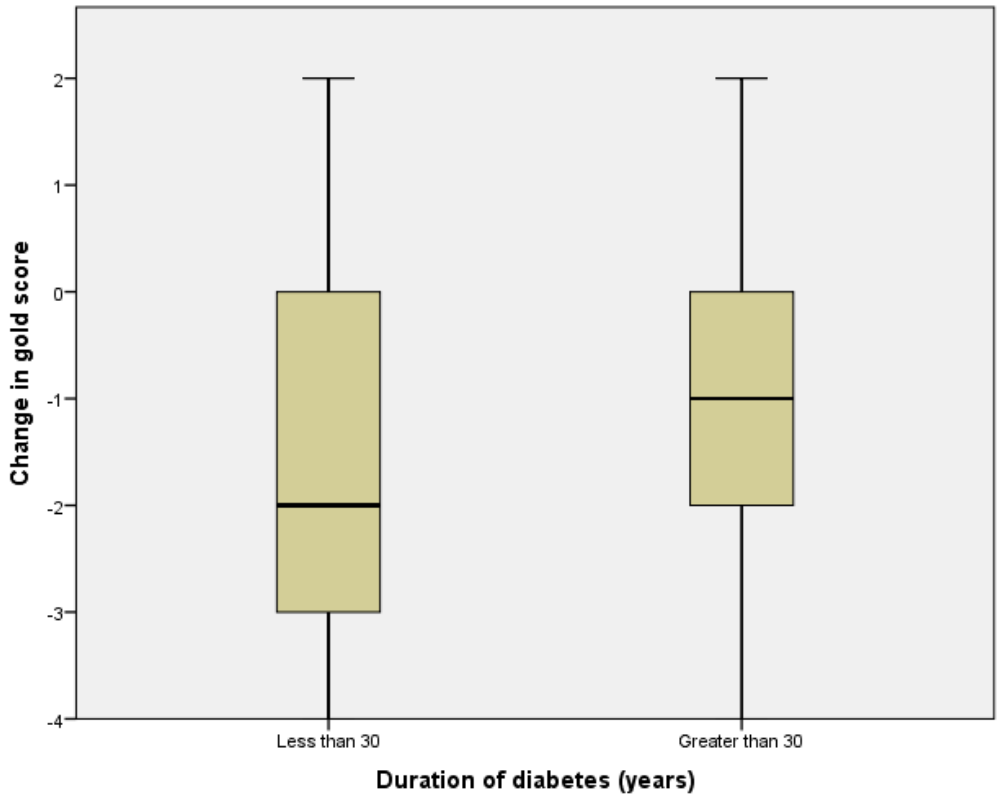

Figure 13 Effect of duration of disease on Gold score reduction.

Sex and the presence of other complications of diabetes did not impede the reduction in hypoglycaemia and the improvement in Gold score seen in participants. Median change in Gold score in both male and female participants was -1.0. There was no difference in median Gold scores in participants with or without retinopathy.

4.5 Conclusions

This study confirms previous observations that IAH in T1DM is associated with an older age and a long duration of disease (Graveling and Frier, 2010). It is also associated with a high incidence of recurrent episodes of severe hypoglycaemia (Gold et al., 1994). The prevalence of both microvascular and macrovascular complications was also similar to previously reported levels in participants with a long duration of disease (Nathan et al., 2009).

The prevalence of DAN (defined as an abnormality in two or more cardiac reflex tests) in this group of subjects with T1DM and a long duration of disease was low.

The prevalence of DAN reported in other published studies varies depending on the cohort of patients studied, the diagnostic tests used and the diagnostic criteria adhered to (Vinik et al., 2003). A study by Ziegler et al recruited from 22 diabetes centres in Germany, Austria and Switzerland and reported that 25.3% of subjects had abnormalities in two or more cardiac reflex tests (Ziegler et al., 1992). This study was reported in 1992, 19 years prior to the hypoCOMPaSS study, and before intensive insulin therapy had been fully established, and may indicate a change in the prevalence of DAN in patients with T1DM.

More recently the DCCT and, then the follow up study, Epidemiology of Diabetes Interventions and Complications (EDIC) reported the prevalence of DAN to be 9% and 31% respectively (Pop-Busui et al., 2016). In these studies DAN was defined as an abnormality in one cardiac reflex test. Applying the same diagnostic criteria in this study 19% of participants would be classified as having DAN.

The hypoCOMPaSS study has shown that improvement in symptomatic awareness of hypoglycaemia, with a reduction in episodes of SH, is possible in patients with long standing T1DM without deterioration in glycaemic control (Little et al., 2014). In this cohort of patients mean Gold score reduced from 5(1) to 4(2) and the proportion of patients affected by SH reduced from 92% to 6%. There was a greater reduction in Gold score in those participants that were younger and those with a shorter duration of disease confirming previous observations (Fannelli et al., 1994).

The presence of DAN did not impede the ability of participants to improve their awareness of hypoglycaemia in this study. The majority of previous studies investigating the ability to reverse IAH exclude participants with autonomic neuropathy with the exception of a study by Fanelli and colleagues (Fanelli et al., 1997) and more recently by Kamel et al (Kamel et al., 2015). Fanelli and colleagues designed a 6 month treatment period aimed at avoiding BH in patients with T1DM and DAN to improve their symptomatic awareness of hypoglycaemia. The participants (eight without DAN and thirteen with DAN) in this study underwent a hypoglycaemic clamp study and symptomatic and adrenergic

responses to hypoglycaemia improved in participants with and without DAN after the 6 month treatment period. The improvements observed were less in subjects with abnormal cardiac autonomic function. Kamel et al reviewed five patients with T1DM and DAN undergoing either islet cell or whole pancreas transplants. Improvement in hypoglycaemia awareness was seen all patients after transplant despite abnormal cardiac function tests.

Younger participants and those with a shorter duration of diabetes had greater improvements in gold score compared to participants that were older and had a longer duration of disease. Fanelli et al (Fanelli et al., 1993b) reported that IAH was reversible in patients with short duration diabetes in 1993 and then extended this observation to included participants with a long duration of disease (>15 years) in 1994 (Fannelli et al., 1994). They found that participants with a longer duration of disease showed improvement in hypoglycaemia awareness but the recovery of responses was inversely correlated with duration of disease. There was no clear underlying cause for the reduction in improvement in these patients and the authors specifically commented that patients did not have overt diabetic autonomic neuropathy. There is little comment in the literature regarding the impact of age in relation to the reversibility of hypoglycaemia unawareness, presumably due to the difficultly in separating this from duration of disease. Investigating the relationship between age and duration of diabetes in this group of subjects using the Pearson product-moment correlation coefficient revealed a strong positive relationship (r=0.508).

Limitations

The main limitations of this study were the unexpected low prevalence of DAN and that testing was limited to cardiac autonomic function testing. This study only used cardiac reflex tests to assess autonomic function and did not utilise other tests of the autonomic nervous system including tests of pupillomotor or sudomotor function. Tests for cardiac autonomic function are simple, quick, non-invasive and can be done at the bedside. Testing was limited to these tests to allow the tests to be done in each centre and then analysed in one site to improve reliability.

Final conclusions

IAH can be improved in participants with a long duration of disease, and in those with and without cardiac autonomic neuropathy. Since the awareness of hypoglycaemia can be improved, despite the presence of autonomic dysfunction, it suggests that autonomic neuropathy is not a prime driver of the syndrome. The prevalence of DAN was lower than previous studies and up to date epidemiological studies are required to establish the prevalence of DAN in subjects with a short and long duration of disease.

Chapter 5: Cardiac Autonomic Regulation During Experimental Hypoglycaemia in Type One Diabetes with Impaired Awareness of Hypoglycaemia.

5.1 Abstract

Aims: Impaired awareness of hypoglycaemia (IAH) and classical diabetic autonomic neuropathy (DAN) have been respectively shown to reduce the symptomatic and autonomic counter-regulatory responses to hypoglycaemia (Heller et al., 1987, Bottini et al., 1997). These exploratory studies investigated the effect of experimental hypoglycaemia on cardiac autonomic regulation in patients with type 1 diabetes (T1DM) and IAH.

Methods: Eight participants with T1DM and IAH (Gold score ≥4), ten with T1DM and intact awareness of hypoglycaemia (Gold score<4) and ten without diabetes underwent a stepped hyperinsulinaemic hypoglycaemic clamp study. Glucose was held at 5mmol/l at time 20 minutes and reduced step-wise to a nadir of 2.4mmol/l at time 180 minutes. Heart rate and heart rate variability were measured during each glucose step.

Results: Participants with IAH had a longer duration of diabetes and lower symptom scores and metanephrine responses to hypoglycaemia than participants with intact awareness of hypoglycaemia. Baseline cardiac autonomic function tests were normal in all participants but baseline heart rate was higher, and vagal tone lower, in participants with diabetes compared to participants without diabetes. During hypoglycaemia participants without diabetes showed an increase in heart rate, with associated vagal withdrawal, that recovered to baseline by the end of the study. Participants with T1DM and intact awareness of hypoglycaemia showed a gradual increase in heart rate and corresponding vagal withdrawal without evidence of recovery. Participants with IAH showed little changes in heart rate or vagal tone during hypoglycaemia.

Conclusions: Participants with diabetes had evidence of subclinical cardiac autonomic neuropathy with an elevated heart rate and reduced vagal tone despite normal cardiac reflex tests. Heart rate response to hypoglycaemia was time dependent and differed according to self reported awareness of hypoglycaemia. Participants with IAH showed smaller changes in heart rate and HRV during hypoglycaemia, possibly due to a failure of autonomic responses to hypoglycaemia rather than true classical DAN. This is likely to be due to

dysfunction associated with a long duration of disease and reduced autonomic responses following repeated episodes of hypoglycaemia.

5.2 Background

Intensive insulin therapy is used in type one diabetes mellitus (T1DM) to avoid the long term complications of the disease. Impaired awareness of hypoglycaemia (IAH) is an acquired complication of insulin therapy whereby the ability to detect the onset of hypoglycaemia is diminished or absent. The aim of these exploratory studies was to document the effect of hypoglycaemia on cardiac autonomic function in participants with T1DM and intact and impaired awareness of hypoglycaemia. These results will be compared to a group of volunteers without diabetes.

5.3 Methods

The hypoCOMPaSS (Comparison of Optimised MDI versus Pumps with or without Sensors in Severe Hypoglycaemia) Trial was a UK-based, multicentred, prospective, randomised controlled trial (RCT). All eligible participants were invited to undergo a stepped hyperinsulinaemic hypoglycaemic clamp study before and after the 6 month intervention period. A sub-study was then designed to recruit a control population to compare to the main hypoCOMPaSS population.

The protocol for the main hypoCOMPaSS trial and hypoCOMPaSS sub-study are described in full in chapter two but the salient points are reported below.

Participants

Eight participants with IAH recruited to the HypoCOMPaSS trial from Sheffield Teaching Hospitals NHS Foundation Trust and Newcastle upon Tyne NHS Foundation Trust underwent a pre-treatment hyperinsulinaemic hypoglycaemic clamp study. Ten participants with T1DM and intact awareness of hypoglycaemia and ten participants without diabetes were recruited as a control population.

Cardiac autonomic function testing

All participants underwent detailed screening cardiac autonomic function testing as described by Ewing (Ewing and Clarke, 1982) including the modifications suggested by O'Brien (O'Brien et al., 1986) in accordance with the latest consensus on the diagnosis of cardiac autonomic neuropathy (Tesfaye et al., 2010). Tests included heart rate response during deep breathing, one valsalva manoeuvre and heart rate and blood pressure response to standing. Results were compared to the O'Brien age adjusted normal ranges (O'Brien et al., 1986).

Two five minute ECG and continuous blood pressure recordings, using a Portapres device, were made to measure baroreflex sensitivity (BRS) and allow spectral analysis of heart rate variability (HRV). During the first five minute recording, participants remained at rest, with spontaneous breathing and monitoring of the respiratory rate. During the second recording participants were asked to pace their breathing at a rate of 15 breaths per minute whilst maintaining a normal tidal volume.

Statistical analysis

Data that followed an approximate normal distribution were summarized using mean (standard deviation (SD)) unless otherwise stated. Skewed data were summarized using the median (interquartile range). Spectral HRV parameters were logarithmically transformed to approximate a normal distribution.

Changes in metanephrines were tested using analysis of covariance (ANCOVA). To compare symptom scores during the clamp, area under the curve was calculated for each person, and then analysis of variance (ANOVA) was used to test for a difference in the mean area under the curve. Differences between mean heart rates of each group were calculated using a mixed effects repeated measures model. Heart rate at T60, T100, T140 and T180 were defined as dependent variables and heart rate at T20 independent with a fixed factor for time and for patient group with an interaction term for time and group. Post hoc analysis was completed with the Holm procedure.

Glucose thresholds for the release of metanephrine was defined as the measured glucose at the time of onset of a sustained (≥2 successive time points) increase in hormone concentrations ≥ 2SD above the mean baseline measurements (Choudhary et al., 2009). Thresholds for the increase in total, autonomic and neuroglycopenic symptoms were determined as the measured glucose level at which the symptom score increased ≥ 2 over baseline on two consecutive assessments (Choudhary et al., 2009). Where this threshold was not reached, the lowest measured glucose for the individual was used as the threshold for that person (Choudhary et al., 2009). Statistical analysis was performed with SPSS (version 22.0, IBM, Chicago, Illinois). A p-value ≤ 0.05 was deemed statistically significant.

5.4 Results

Participant Characteristics

A total of eight participants with T1DM and IAH (gold score ≥4), ten with T1DM and intact awareness of hypoglycaemia (gold score<4) and ten participants without diabetes are included in this analysis. Baseline participant characteristics are shown in table fourteen. Participants with IAH were older than the participants in the two control groups, although the differences did not reach statistical significance (p=0.26). There were a higher proportion of female participants in the participants without diabetes and intact awareness and the participants without diabetes had a higher BMI.

Participants with diabetes had a long duration of disease with a mean average length of disease being over twenty years in both groups. Subjects with IAH had a non-significant longer duration of disease, (p=0.09) and glycaemic control was similar within the groups with diabetes. There was an expected increased incidence of SH in participants with IAH, with an average incidence of 3.5 episodes in the six months prior to the study. There were no reported episodes of SH in the participants with intact awareness in the 12 month time period prior to enrolment in the study.

Screening cardiac autonomic function tests showed that no participants had clinically significant cardiac autonomic neuropathy (table 15). This was based on the definition of DAN, as having two or more abnormal cardiac reflex tests

(Tesfaye et al., 2010). Average heart rate was higher and HRV was lower in the participants with diabetes (table 15). The prevalence of retinopathy was high, corresponding to the increased risk with a longer duration of disease (Yau et al., 2012), and the prevalence of peripheral neuropathy was between 10 and 20%. Participants were excluded if there was any evidence of nephropathy or macrovascular disease to ensure safety during the clamp studies.

Participants	Without diabetes n=10	Intact awareness n=10	IAH n=8	P value
Age in years	38 (17)	41 (20)	48 (18)	0.26
Sex (Male: Female)	3:7	4:6	4:4	0.69
Body mass index (kg/m^2)	30.3 (8.1)	25.3 (2.1)	24.3 (2.5)	0.041
Duration of diabetes in years	N/A	21 (11)	31 (19)	0.09
HbA1c (mmol/mol)	37 (5.3)	75 (13)	74 (10)	0.86
Severe hypoglycaemia in last 6 months	N/A	0	3.5 (11.4)	0.02
Heart rate (beats per minute)	69 (11)	74 (9)	78 (10)	0.058
Systolic blood pressure (mmHg)	132 (18)	131 (13)	131 (11)	0.98
Diastolic blood pressure (mmHg)	83 (17)	78 (4)	76 (8)	0.38
Retinopathy	0	7/10 (70%)	5/8 (63%)	1
Peripheral neuropathy	0	2/10 (20%)	1/8 (13%)	1
Nephropathy	0	0	0	
Macrovascular disease	0	0	0	

Results given as mean (standard deviation) except for age, duration of diabetes and SH where median (interquartile range) are given. Retinopathy, peripheral neuropathy, nephropathy and macrovascular disease are number of patients/n (%).

Table 14 Participant characteristics at screening visit 1.

Participants	Without diabetes n=10	Intact awareness n=10	IAH n=8	P value
Heart rate (beats per minute)	62 (7)	70 (12)	76 (14)	0.058
SDRR (milliseconds)	61 (29)	34 (12)	34 (23)	0.025
RMSSD	50.6	24.9	27.2	0.017
HF power	2.7 (0.5)	2.2 (0.4)	2.3 (0.4)	0.054
LF norm	0.6 (0.1)	0.6 (0.2)	0.6 (0.3)	<0.001
BRS	22.9198	13.3149	15.2073	0.67
Cardiac autonomic function status Normal Abnormal BRS Possible/early DAN Definite DAN Definite DAN with orthostatic hypotension	 8 2 0 0 0	 7 2 1 0 0	 8 0 0 0 0	

Table 15 Cardiac autonomic function test results.

Blood glucose

Arterialised blood glucose during the clamp studies are shown in figure fourteen. Starting blood glucose levels were higher in the participants with intact awareness in comparison to the other two groups (Without diabetes 5.84mmol/l, intact awareness 8.45mmol/l, IAH 6.35mmol/l). At 200 minutes (T200) blood glucose in all three groups was under 3mmol/l (Without diabetes 2.62mmol/l, intact awareness 2.51mmol/l, IAH 2.44mmol/l).

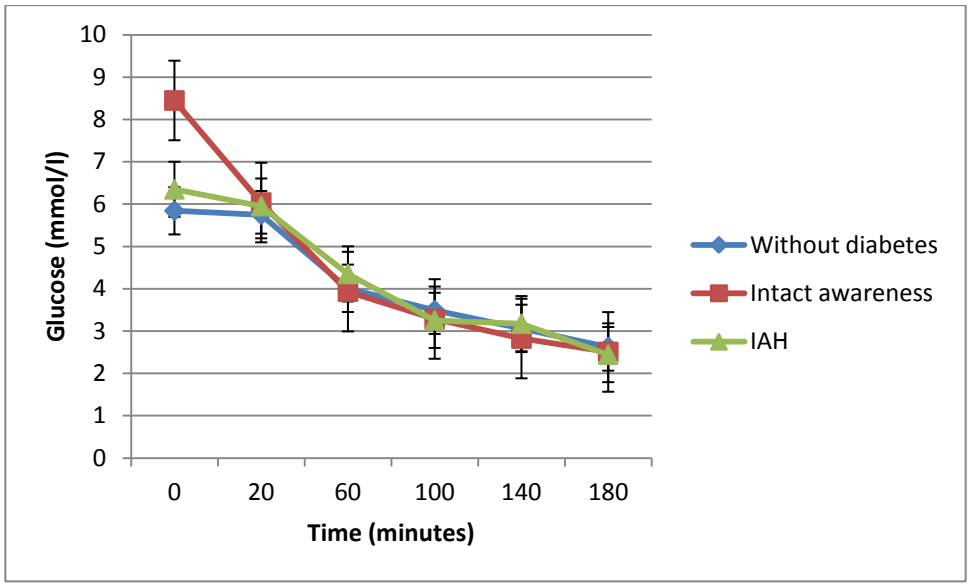

Figure 14 Arterialised blood glucose during stepped hypoglycaemic clamp studies.

Symptom scores

Participants with IAH reported lower symptom scores at the onset of hypoglycaemia (time 100 minutes (T100)) (figure 15) in comparison to participants with intact awareness. This difference was maintained until the end of the studies, although the difference between the groups did not meet statistical significance (p=0.231). Total symptom thresholds developed at similar glucose concentrations (IAH 2.74, intact awareness 2.74 and without diabetes 2.86) but thresholds were not reached in three of the eight (38%) participants with IAH. Autonomic symptoms developed at a lower glucose in participants with IAH (2.55) in comparison to participants with diabetes with intact awareness (2.73) and in the participants without diabetes (2.85). When reviewing autonomic symptom scores, glucose thresholds were only reached in three of the eight participants with IAH (38%).

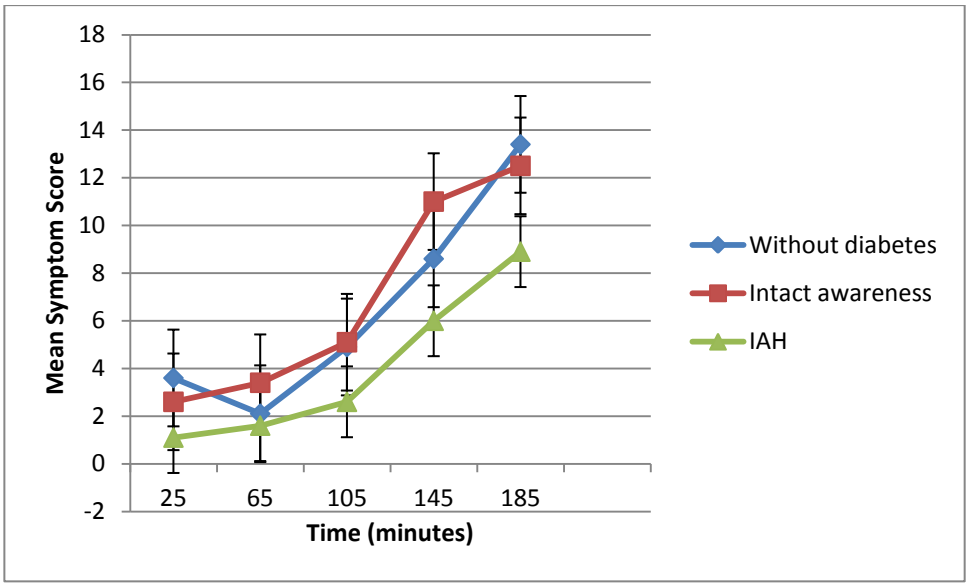

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Figure 15 Mean symptom scores during hypoglycaemic clamp studies.

Biochemical markers

Plasma metanephrine increased in all groups between baseline and at time 200 minutes, but the increase seen in participants with IAH was less in comparison to the control groups (table 16). Using analysis of co-variance, adjusting for baseline, there was a statically significant difference between the groups (p=0.004). Post hoc analysis showed the differences were statistically significant between the groups without diabetes and groups with diabetes (table 16). Mean glucose thresholds for metanephrine release was 2.67 mmol/l in participants with IAH, 2.92 in participants with intact awareness and 3.29mmol/l in participants without diabetes. Glucose threshold for the release of plasma metanephrines was not reached in five (63%) of the participants with IAH during the hypoglycaemic clamp.

	Without diabetes	Intact awareness	IAH	p value
Plasma metanephrines (pg/mL)				
Baseline (Glucose 5.0mmol/l)	175	213	200	
200 minutes (Glucose 2.4 mmol/l)	590	517	342	0.025 0.001
Cortisol (nmol/L)				
Baseline (Glucose 5.0mmol/l)	220	317	311	
200 minutes (Glucose 2.4 mmol/l)	788	767	582	
Growth Hormone (ng/mL)				
Baseline (Glucose 5.0mmol/l)	0.4	1.9	5.7	
200 minutes (Glucose 2.4 mmol/l)	15.3	19.8	21.3	
Potassium (mmol/l)				
Baseline (Glucose 5.0mmol/l)	3.8	3.8	4.2	
200 minutes (Glucose 2.4 mmol/l)	3.1	3.3	3.7	

Using analysis of co-variance, adjusting for baseline, there was a statically significant difference between the groups (p=0.004). Post hoc analysis showed the differences were statistically significant between the groups without diabetes and groups with diabetes.

Table 16 Change in biochemical markers during the clamp studies.

Heart rate

Baseline heart rate, at T20, was higher in participants with diabetes (Table 17). Participants without diabetes had the lowest mean baseline heart rate of 65bpm. The highest mean baseline heart rate was seen in participants with IAH (79bpm). Participants with T1DM and intact awareness of hypoglycaemia had a mean baseline heart rate of 72bpm.

Heart rate response to hypoglycaemia was different in the three groups (figure 16). An increase in heart rate was observed in participants without diabetes with recovery to below baseline by time 180 minutes (T180). Participants with T1DM and intact awareness showed a gradual increase in heart rate without evidence of recovery by T180. The participants with IAH showed a non-sustained brief increase in heart rate. A repeated measures model using heart rate at T20 as an

independent variable showed that the difference in heart rate changes over time. At T180 the difference between the participants without diabetes and the groups with diabetes reaches statistical significance (table 17).

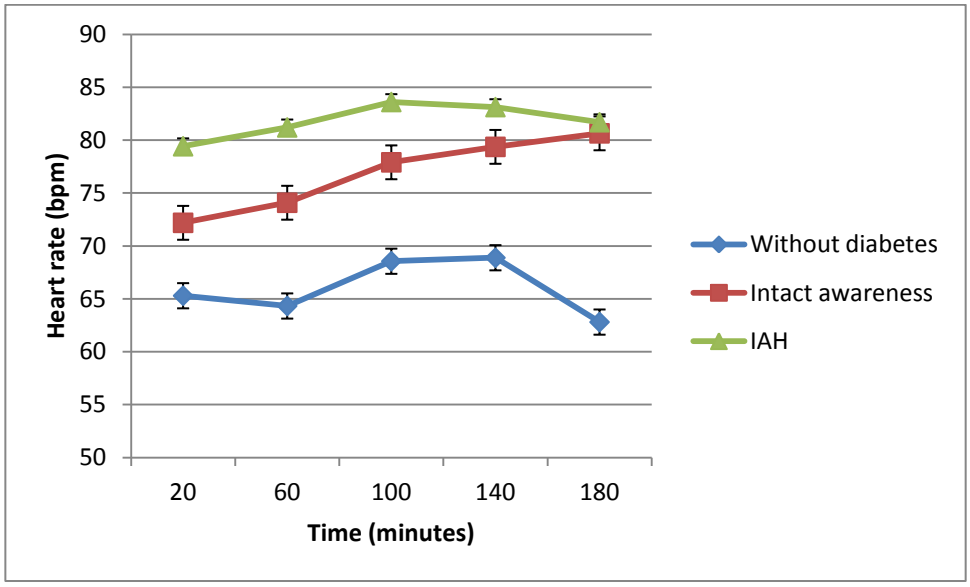

Figure 16 Heart rate response to hypoglycaemia.

Time (minutes)	Blood glucose (mmol/l)	Without diabetes	Intact awareness	IAH	P value
20	5	65 (12)	72 (11)	79 (14)	
60	3.8	64 (11)	74 (11)	81 (13)	
100	3.4	69 (10)	78 (12)	84 (16)	
140	2.8	69 (10)	79 (9)	83 (17)	
180	2.4	63 (9)	81 (8)	82 (16)	<0.001 0.003

Values given as mean (SD). A repeated measures model using heart rate at T20 as an independent variable showed that the difference in heart rate changes over time. At T180 the difference between the participants without diabetes and the groups with diabetes reaches statistical significance.

Table 17 Heart rate (bpm) during the clamp study.

Spectral analysis results

HF power, or vagal tone, at baseline (T20), was higher in the participants without diabetes compared to the groups with diabetes (figure 17). Baseline LFnorm showed the inverse of the HF results (figure 17) and as LFnorm reflects both parasympathetic and sympathetic contribution, the differences seen in LFnorm is most likely due to the influence of HF power on LFnorm.

HF power and LFnorm response to hypoglycaemia differs between the three participant groups. Participants without diabetes showed a reduction in vagal tone during early hypoglycaemia which recovered by T180. Participants with intact awareness of hypoglycaemia showed gradual vagal withdrawal without signs of recovery. Participants with IAH showed little change in HF power.

The participants with IAH showed a reduction in LFnorm indicating a possible reduction in sympathetic tone during hypoglycaemia. Participants with intact awareness and without diabetes show an increase in LF norm during the clamp. These changes show evidence of recovery by T180.

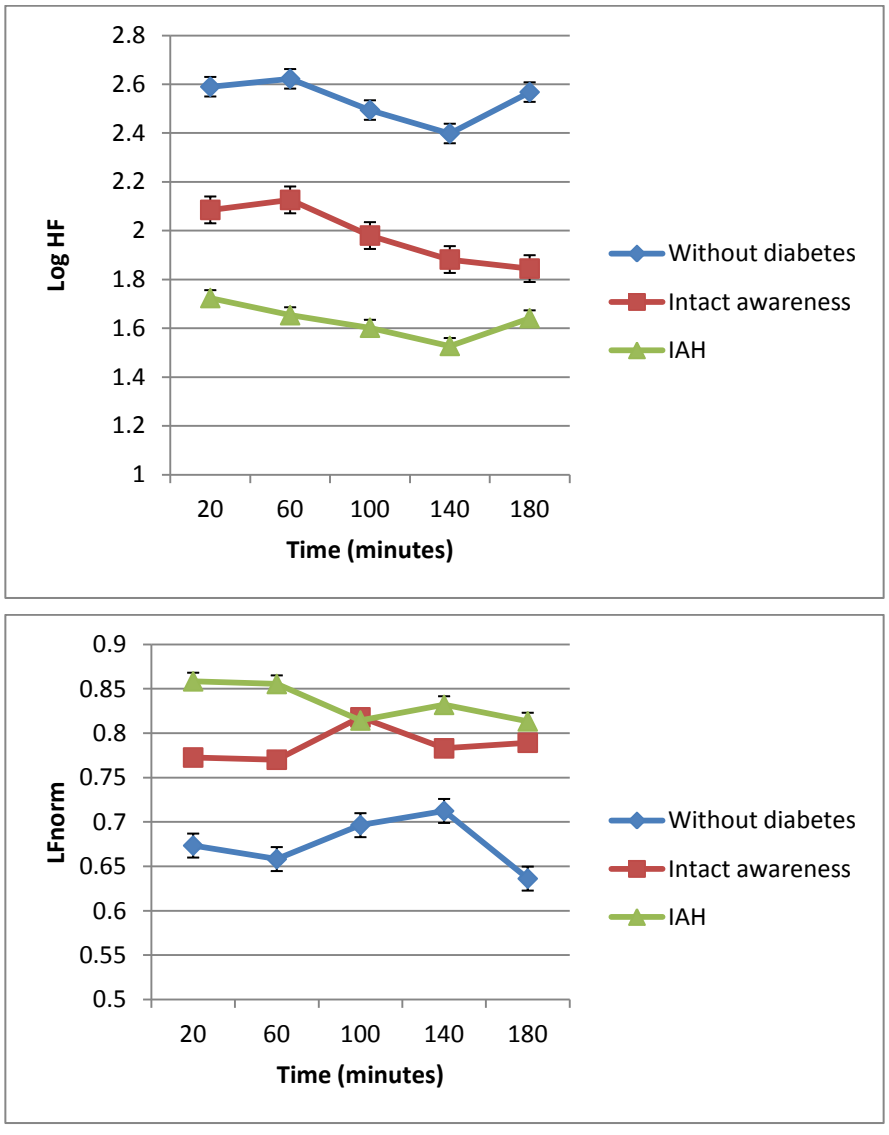

Figure 17 Changes in HF and LFnorm during the clamp studies.

Participants	Without diabetes	Intact awareness	IAH
HR			
T20	65 (12)	72 (11)	79 (14)
T180	63 (9)	81 (8)	82 (16)
SDNN			
T20	70 (30)	48 (23)	41 (20)
T180	74 (30)	41 (10)	37 (19)
HFpower			
T20	2.59 (0.69)	2.08 (0.41)	1.72 (0.66)
T180	2.56 (0.38)	1.84 (0.34)	1.64 (0.56)
LFnorm			
T20	0.67 (0.18)	0.77 (0.12)	0.86 (0.09)
T180	0.64 (0.18)	0.78 (0.11)	0.86 (0.10)
RMSSD			
T20	49 (29)	25(14)	20(19)
T180	48(22)	18(7)	16(8)

Table 18 Changes in HR and HRV between T20 and T180 (* p<0.05).

Blood pressure and baroreceptor sensitivity

Figures eighteen and nineteen illustrate the changes in systolic blood pressure and BRS during the clamp studies. There was little change in systolic blood pressure during the clamp study but slight reductions were seen in the participants without diabetes and IAH. A reduction in BRS was seen in the participants without diabetes which recovered to baseline by T180. There was little changes seen in BRS in participants with diabetes.

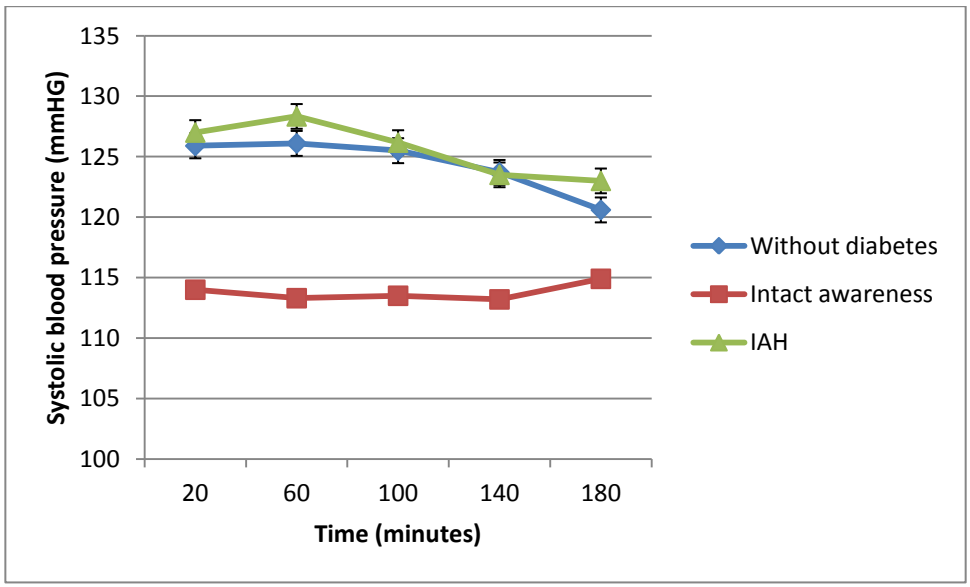

Figure 18 Change in systolic blood pressure during the clamp studies.

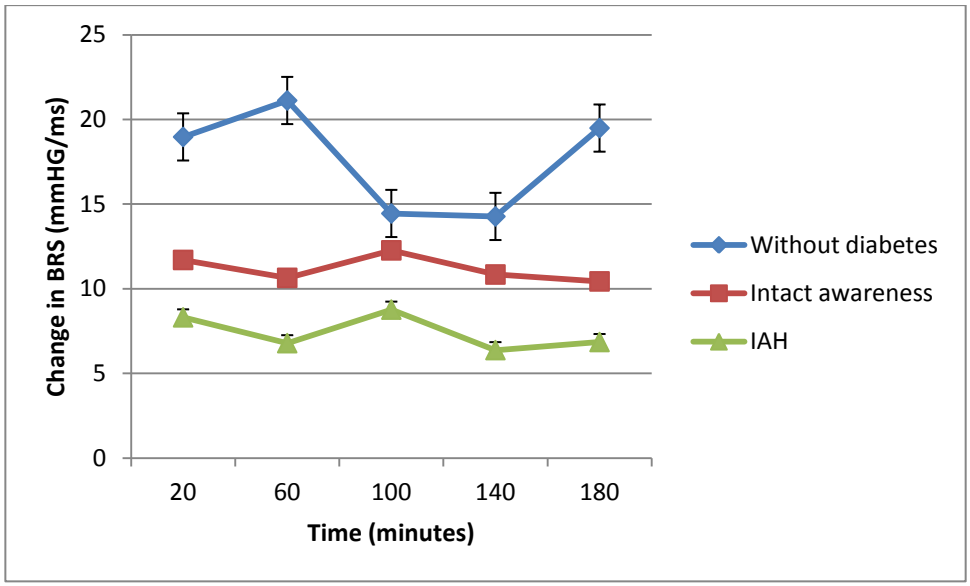

Figure 19 Change in BRS during the clamp studies.

Paired clamps

Five participants that underwent a baseline hypoglycaemic clamp study also consented to an end of study clamp. Three of these participants reported an improvement in hypoglycaemia awareness, with an improvement in Gold score to less than four, and were defined as responders to treatment. The remaining two participants did not improve their Gold score, which remained above four, and were defined as non-responders to treatment.

Figure twenty shows the changes in mean symptom scores and mean heart rate responses to hypoglycaemia in the responder group. In the end of study clamp, symptom scores increased, at the onset on hypoglycaemia (T100) but were lower than in the baseline clamps towards the end of the studies. Participants reported increases in both autonomic and neuroglycopenic symptoms but the glucose threshold for autonomic symptoms was still not reached in any of the three participants in the end of study clamp. Threshold for total symptom scores showed little change from baseline clamps to the end of study clamps (2.73 mmol/l to 2.77 mmol/l). Heart rate response to hypoglycaemia in the second clamp study showed a reduction in heart rate from the onset of hypoglycaemia to below baseline without evidence of recovery.

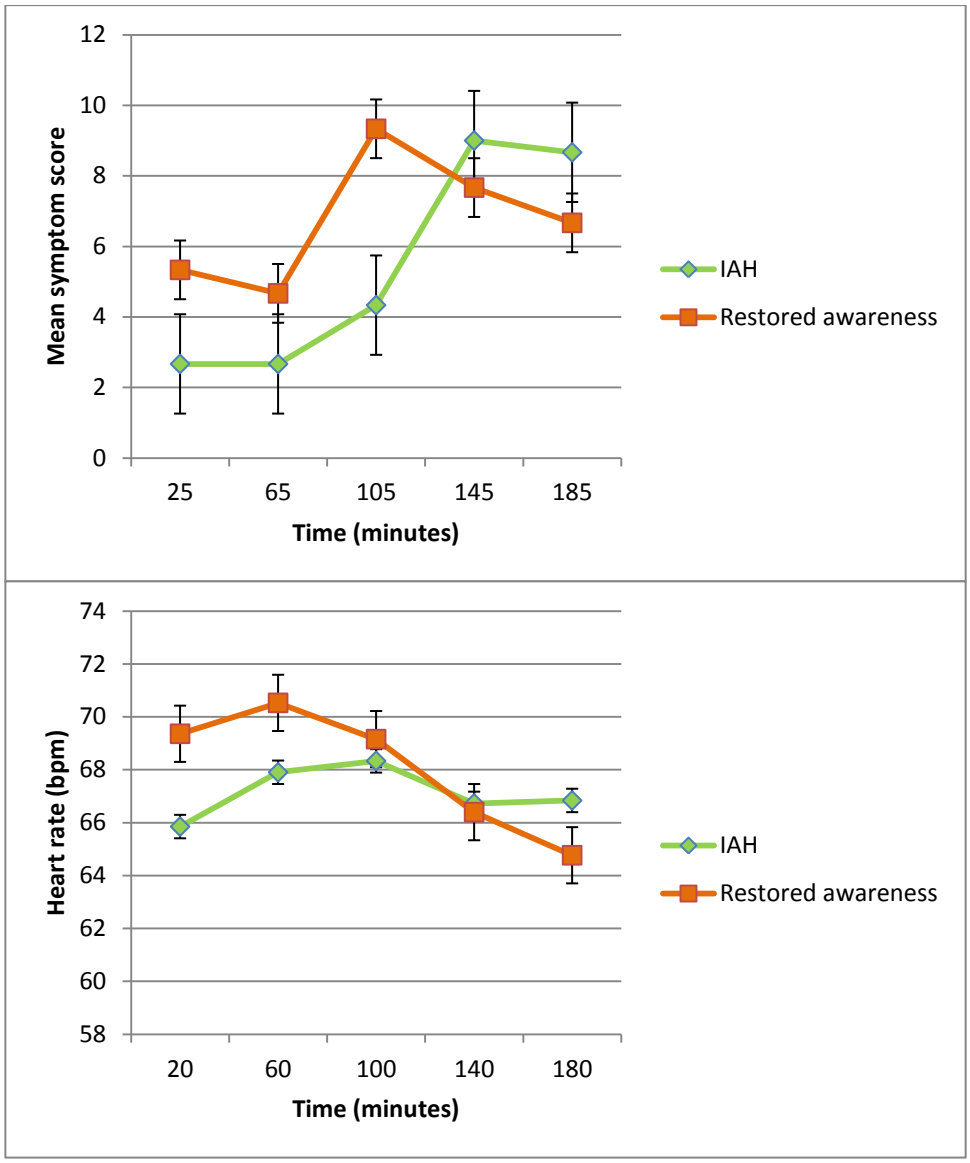

Figure 20 Paired clamp results for responders.

Despite reports of a lack of improvement of hypoglycaemia awareness in the non-responder group, symptom scores in the clamp study did improve (figure 21). The first participant improved both autonomic and neuroglycopenic symptom awareness and increased glucose thresholds from 2.4 to 2.88mmol/l in total, autonomic and neuroglycopenic symptom scores. The second participant improved neuroglycopenic scores (glucose threshold of 3.46mmol/l) but an improvement of autonomic symptoms was not seen. Heart rate increased during hypoglycaemia in the end of study clamp without recovery at T200.

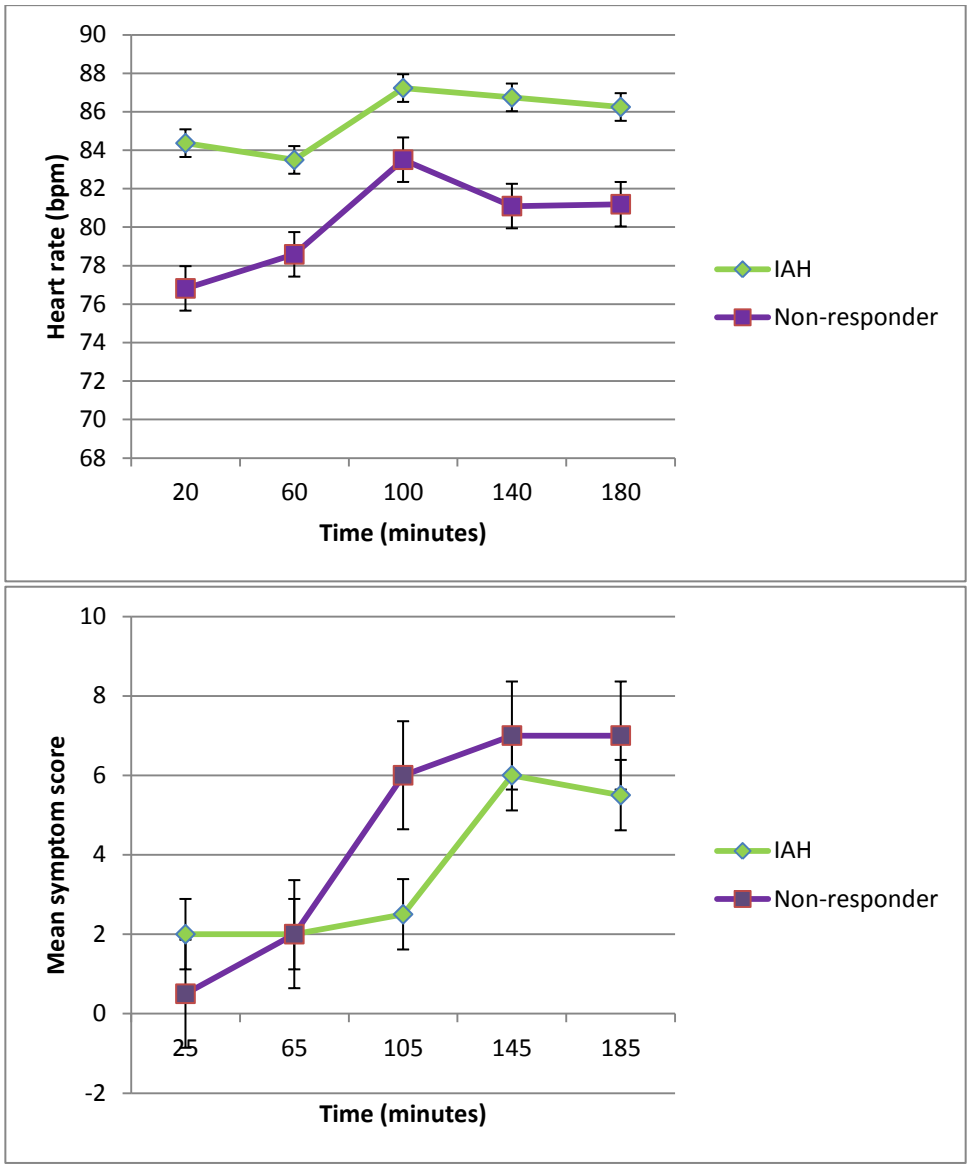

Figure 21 Paired clamp results for non-responders.

5.5 Conclusions

This study recruited participants with T1DM and IAH, participants with T1DM and intact awareness of hypoglycaemia and participants without diabetes to act as a control group. The participants with IAH had a longer duration of disease in comparison to those with intact symptomatic awareness of hypoglycaemia. Autonomic function testing showed that there was no evidence of clinically significant cardiac autonomic neuropathy in any participants, but resting heart rate was higher in participants with diabetes.

All participants underwent a hyperinsulinaemic hypoglycaemic clamp study. There was a reduction in symptom score and metanephrine response to hypoglycaemia in participants with IAH. The heart rate responses to hypoglycaemia were time dependent and differed according to self reported awareness of hypoglycaemia. There was some improvement in symptom scores in repeated clamp studies after a six month treatment period of hypoglycaemia avoidance.

IAH was associated with a long duration of disease. Participants with IAH had a mean duration of disease of 31 years, in comparison to 21years, in participants with intact awareness. This was despite attempting to match participants according to age and duration of disease. This did not reach statistical significance which is most likely due to the small participant numbers in this study. It does however support the conclusions summarised in chapter three, that participants with IAH had a significantly longer duration of disease compared to those with intact awareness.

There was no evidence of clinically significant cardiac autonomic neuropathy on screening tests but baseline heart rate was higher in participants with diabetes. This study defined cardiac autonomic neuropathy as an abnormality in two or more cardiac reflex tests, as agreed and defined by the Diabetic Neuropathy Study Group in Toronto, Canada in 2009 (Tesfaye et al., 2010). Screening autonomic function tests revealed that no participants in any of the three groups met these criteria. However, baseline heart rate (T20) was higher in the participants with IAH (79bpm), in comparison to participants with intact awareness (72bpm) and those without diabetes (65bpm). This corresponded to a reduction in Log HF or vagal tone of 1.7 in participants with IAH in comparison to 2.1 in participants with intact awareness and 2.6 in those without diabetes.

IAH was associated with reduced symptom scores and metanephrine responses to hypoglycaemia. Although glucose thresholds for symptom score and metanephrine release appeared similar between the groups these thresholds were not reached in sixty three percent of the participants with IAH. This is most likely due to the level of hypoglycaemia. The final glucose step was 2.4mmol/l which

was probably not low enough to precipitate a full autonomic response in the participants with IAH. The threshold for total symptom scores reduced in the participants that reported improved symptomatic awareness of hypoglycaemia after involvement in the hypoCOMPaSS trial. However the threshold for autonomic symptoms was still not met in any of these participants.

Heart rate response to hypoglycaemia was time dependent and differed according to self reported awareness of hypoglycaemia. Participants without diabetes showed a transient increase in heart rate with recovery to baseline, caused by transient vagal withdrawal illustrated by a reduction in high frequency power. Participants with T1DM and intact awareness of hypoglycaemia showed an increase in heart rate caused by a withdrawal in vagal tone, which did not show evidence of recovery during the clamp studies. The participants with IAH showed little changes in heart rate with minimal reductions in both vagal and sympathetic tone.

The association of a long duration of diabetes and a reduction in awareness of hypoglycaemia is fairly well documented (Hepburn et al., 1990, Mokan et al., 1994). Impaired adrenergic and symptomatic responses to hypoglycaemia are common in patients with T1DM with established disease, even in those who claim to have normal awareness of hypoglycaemia (Heller et al., 1987). More recently a study by Geddes and colleagues have confirmed that patients with IAH have a significantly longer duration of disease (Geddes et al., 2007).

The participants in this study did not have clinically significant cardiac autonomic neuropathy. The prevalence of DAN in T1DM depends upon the population studied and the criteria used to identify cardiac autonomic neuropathy (Pop-Busui, 2010). The prevalence of cardiac autonomic neuropathy reported in the Epidemiology of Diabetes Interventions and Complications (EDIC) study, with participants with duration of diabetes greater than 15 years, was 31%. In these studies cardiac autonomic neuropathy was evaluated by heart rate variability during deep breathing and the valsalva manoeuvre and diastolic blood pressure on standing. Cardiac autonomic neuropathy was defined as an abnormality in any one of these tests which significantly differs from the more recent guidance (Tesfaye et al., 2010) requiring an abnormality in two or more cardiac reflex tests.

However, if the EDIC criteria were applied to our study population only one participant would be diagnosed with DAN.

The low prevalence of cardiac autonomic neuropathy in our study population may be due to bias introduced by the exclusion criteria of the clamp studies. These excluded participants with ischaemic heart disease, peripheral vascular disease and nephropathy. Microvascular complications such as nephropathy are associated with DAN as are cardiovascular risk factors such as hypertension and high risk lipid profiles (Vinik et al., 2003). The exclusion criteria were included to ensure safety during the clamp studies but will have caused a selection bias.

Although participants did not fulfil the criteria for definitive cardiac autonomic neuropathy, resting heart rate in participants with diabetes was higher than those participants without diabetes. Ewing, Campbell and Clarke (Ewing et al., 1981) reported that patients with diabetes and parasympathetic abnormalities had higher heart rates in 1981. More recently resting tachycardia (Vinik and Ziegler, 2007, Maser and Lenhard, 2005) as a result of vagal neuropathy has been described as a clinical implication of cardiac autonomic neuropathy.

The reduction in hypoglycaemic symptoms and catecholamine responses to hypoglycaemia in patients with diabetes with impaired and intact awareness is well established (Heller et al., 1987, Amiel et al., 1988). More recently Olsen and colleagues (Olsen et al., 2014) examined the effect of duration of diabetes upon symptomatic awareness of hypoglycaemia. Questionnaires were sent to 636 patients with T1DM and researchers found that a long duration of disease was associated with a lower intensity of autonomic symptoms.

Previous experimental work has been done to assess the effect of hypoglycaemia on cardiac autonomic function but results have been inconsistent and contradictory (Laitinen et al., 2003, Schachinger et al., 2004, Koivikko et al., 2005, Chow et al., 2017). Our results are consistent with Koivikko et al (Koivikko et al., 2005) who reported HRV responses to hypoglycaemia in people with and without diabetes undergoing a hyperinsulinaemic hypoglycaemic clamp study. Average baseline heart rate was higher in participants with diabetes

(72bpm) in comparison to participants without diabetes (59bpm). The authors reported an increase in heart rate and a decrease in HF power during hypoglycaemia, indicating vagal withdrawal. Koivikko et al did not report evidence of recovery but the results in this study were reported at a single time point and we have shown that responses seen in cardiac autonomic function are time dependent.

The differences seen in the work by Laitinen and colleagues (Laitinen et al., 2003) and the Schachinger group (Schachinger et al., 2004) are also possibly due to reporting results at a single time point. Both studies recruited participants without diabetes and heart rate and HRV measures were recorded in euglycaemic and hypoglycaemic clamp studies. They reported heart rate and HRV measures at a single time point. Schachinger et al made these measures at 65 minutes following achievement of hypoglycaemia (glucose 2.7mmol/l). Participants in our study had shown recovery at this time with heart rate returning back to baseline.

Our results indicate that duration of diabetes is a major influencing factor in both symptomatic awareness of hypoglycaemia and cardiac autonomic function. The relationship between subclinical and clinical autonomic neuropathy is potentially similar to that observed in diabetic peripheral neuropathy. Diabetic peripheral neuropathy affects fifty percent of patients with diabetes, with abnormalities in nerve conduction tests, but clinical symptoms are only present in a third of patients (Tesfaye and Selvarajah, 2012). Diabetic peripheral neuropathy is also associated with a long duration of disease. We hypothesise that the increase in heart rate that is seen in the participants with diabetes at baseline, is most likely due to subclinical autonomic neuropathy, which is more pronounced in participants with IAH because of a longer duration of disease.

The presence of subclinical cardiac autonomic neuropathy, in participants with diabetes and a long duration of disease, has potential clinical implications because of previous reports of the cardioprotective role of vagal activity. The majority of this work has been observed in patients following an acute myocardial infarction. Initially animal studies indicated that increased vagal tone following myocardial

infarction gave protection from ventricular fibrillation (Schwartz et al., 1984, Hull et al., 1990). Studies in humans followed showing that reduced HRV was a predictor of increased mortality following acute myocardial infarction (Kleiger et al., 1987, Bigger et al., 1992).

Limitations

This is experimental work and therefore caution should be applied when extrapolating the results to the clinical scenario of spontaneous hypoglycaemia. Experimental work investigating the changes in cardiac repolarisation in hypoglycaemia showed a shortening of QT interval caused by adrenergic stimulation (Robinson et al., 2003b). A follow up study by the same group showed these observed changes were less in spontaneous hypoglycaemia which was thought to reflect the reduced sympathoadrenal responses in this clinical scenario (Robinson et al., 2004).

Limitations of this study include the use of spontaneous breathing during cardiac autonomic function testing, and the use of two rather than three questionnaires and catecholamine results at baseline to calculate glucose thresholds for symptoms and sympathoadrenal responses respectively. Paced breathing is recommended for the interpretation of HF power (Tesfaye et al., 2010) because HRV is affected by the respiratory rate and depth of breathing. During the clamp studies participants were instructed to breathe spontaneously during ECG recordings rather than to pace their breathing at a particular rate. This was because of the concern about cognitive impairment during hypoglycaemia which could potentially impair the ability of the participants to pace their breathing consistently. Ideally three questionnaires and three blood samples for metanephrines would have been used to calculate thresholds for participants. It was felt that this was too cumbersome in these studies and therefore two baseline samples were used instead.

Plasma metanephrine were used as a measurement of sympathoadrenal response to hypoglycaemia rather than adrenaline. This was chosen for logistical reasons in the main hypoCOMPaSS trial and was therefore also used in the sub-study to allow a direct comparison of results. Although studies (Lee et al., 2011, Whiting

and Doogue, 2009) have shown plasma metanephrine to be as effective as plasma catecholamines in measuring adrenal response, previous hypoglycaemic clamp studies (Koivikko et al., 2005, Laitinen et al., 2003) have traditionally used adrenaline. This makes it more difficult to directly compare our results to previously published work.

Summary

In summary participants with T1DM had evidence of subclinical cardiac autonomic dysfunction which is associated with a long duration of disease. Baseline heart rate was elevated in participants with diabetes compared to participants without diabetes and this was associated with reduced high frequency power or vagal tone. Baseline heart rate was highest in participants that reported IAH, who had a longer duration of disease, despite otherwise normal cardiac autonomic function testing.

Cardiac autonomic responses to hypoglycaemia were time dependent and differed according to self reported awareness of hypoglycaemia. Participants without diabetes showed a transient increase in heart rate associated with vagal withdrawal that recovered quickly. Participants with diabetes and intact awareness of hypoglycaemia showed an increase in heart rate, associated with vagal withdrawal that did not show evidence of recovery during the clamp study.

Participants with IAH showed little change in heart rate or HRV during hypoglycaemia. This could be due to either true autonomic dysfunction or a failure of autonomic responses to hypoglycaemia. We suspect that the second hypotheses is more likely and associated with both a long duration of disease and reduced autonomic responses following repeated episodes of hypoglycaemia.

Future work could explore these findings further by repeating the studies with larger patient groups. A longer period of hypoglycaemia would establish if those participants with intact awareness of hypoglycaemia would show evidence of recovery as illustrated by participants without diabetes. Studies investigating cardiac autonomic responses during spontaneous hypoglycaemia would give more

information about cardiac autonomic function when the length and depth of hypoglycaemia is not controlled.

Chapter 6: Conclusions

The three studies described in this thesis examine the relationships between IAH and DAN in T1DM. The first study compared the prevalence of DAN in participants with T1DM and IAH against participants with TIDM and intact symptomatic awareness of hypoglycaemia. The second study explored the question of whether the presence of DAN impaired the patient's ability to respond to treatment for IAH. The final study investigated cardiac autonomic responses to experimental hypoglycaemia.

The first study concluded that cardiac autonomic neuropathy was not associated with IAH. The prevalence of cardiac autonomic neuropathy in long standing T1DM was surprisingly low. The results also indicated that the severity of autonomic neuropathy increased with an increased duration of disease.

The second study concluded that the presence of DAN did not impede the ability of participants to improve their symptomatic awareness of hypoglycaemia. It also found that IAH was associated with a longer duration of disease and an increased prevalence of SH. Younger participants and those with a shorter duration of disease showed greater improvements in symptomatic awareness than older participants with a longer duration of disease.

The third study concluded that heart rate response to hypoglycaemia was time dependent and differed according to self reported awareness of hypoglycaemia. Participants with diabetes had a higher resting heart rate compared to participants without diabetes. During the hypoglycaemic clamp studies participants with IAH had reduced symptomatic and metanephrine response to hypoglycaemia than those with reported normal awareness.

In the remainder of this chapter, Sections 6.1 – 6.4 explore these key findings in more depth, and relate them to previous studies. Section 6.5 considers their clinical implications. Section 6.6 discusses the limitations of our experimental settings. Finally Section 6.7 offers final conclusions and discusses future work.

6.2 The Low Prevalence of Diabetic Autonomic Neuropathy

The prevalence of cardiac autonomic neuropathy was low in the participants enrolled in these studies with only seven of the 144 (5%) patients having definite cardiac autonomic neuropathy. This is low when compared to other studies. In the DCCT (Pop-Busui et al., 2016), patients with T1DM of duration of 1-15 years, the prevalence of DAN was 9% which rose to 31% in the EDIC (Group, 2005) studying participants with a duration of diabetes greater than 15 years. A study by Ziegler et al (Ziegler et al., 1992) in 1992 reported a prevalence of cardiac autonomic neuropathy in almost seventeen percent of participants with T1DM. A French study published in 2003 (Valensi et al., 2003) report the prevalence of moderate or severe cardiac autonomic neuropathy in T1DM, defined as an abnormality in two or more tests, as eighteen percent.

One reason for this disparity is due to differences in the way that DAN is defined. This is corroborated by a report by Vinik et al. (Vinik et al., 2003), who found that the prevalence of DAN ranges between 0 and 90% depending upon the study population and diagnostic criteria. In our studies definite cardiac autonomic neuropathy was defined as the presence of two or more abnormal cardiac reflex tests (Tesfaye et al., 2010) following the 2010 Toronto consensus statement released by the Diabetic Neuropathy Study Group of the European Association for the Study of Diabetes. O'Brien (O'Brien et al., 1986) stated in 1986 that autonomic neuropathy should not be diagnosed on the basis of one abnormal cardiac reflex test as thirteen percent of a healthy population without diabetes had an inadequate heart rate response to one of four reflex tests in a study in 1986. Less than two percent of participants had an abnormality in two or more tests.

In the DCCT and EDIC studies cardiac autonomic neuropathy was defined as an abnormality in any one of three cardiac reflex tests. Applying the same diagnostic criteria to participants in this study 24% of participants would be classified as having cardiac autonomic neuropathy. The DCCT and EDIC did not use age-related normal ranges for the cardiac reflex tests which were used in this study which may account for the reduction in prevalence of DAN in the subjects in the hypoCOMPaSS studies.

Another reason could be the fact that improved treatment has led to a reduction in the prevalence of complications. Intensive insulin therapy has led to significant improvements in glycaemic control. Other microvascular complications of diabetes, specifically nephropathy, have significantly declined over the past thirty years with a reduction from thirty two percent to eleven percent (Nordwall et al., 20014).

6.3 Cardiac Autonomic Neuropathy is not Associated with Impaired Awareness of Hypoglycaemia

The clinical syndrome of IAH was not associated with the presence or absence of DAN. Seventy one percent of participants with normal awareness of hypoglycaemia had completely normal cardiac autonomic function tests in comparison to eighty percent of the participants with IAH. Historically DAN was described as the mechanism causing IAH, but the data in the literature is conflicting (Stephenson et al., 1996, Meyer et al., 1998, Ryder et al., 1990, Hepburn et al., 1990). Our findings tally with the findings of a recent study by Olsen and colleagues (Olsen et al., 2016), who also concluded that IAH was not associated with autonomic neuropathy.

The aforementioned studies were also inconclusive on the question of whether DAN can affect the ability of participants to improve their awareness of hypoglycaemia. In our study DAN did not affect this ability. The hypoCOMPaSS trial showed that improvement of hypoglycaemia awareness with a reduction of SH was possible in longstanding T1DM (Little et al., 2014). Participants had a mean reduction in Gold score from five to four and average episodes of SH reduced from four to zero with the percentage of participants affected reducing from 92% to 6%.

When comparing the improvement in IAH, there was no difference in mean reduction of Gold score in participants with definite DAN and those with normal cardiac autonomic function tests. Both groups reduced their Gold score by 1.2. These results corroborate the findings of a recent study by Kamel et al (Kamel et al., 2015), who showed that reversal of hypoglycaemia unawareness following

islet cell/pancreatic transplant was not hampered by the presence of autonomic neuropathy.

IAH was associated with an increased incidence of episodes of SH. Participants that reported intact awareness of hypoglycaemia reported an absence of episodes of SH in the twelve months preceding the study. Participants reporting IAH described a median incidence of SH of four in the same time period. The relationship between IAH and SH is well established and was described by Gold et al (Gold et al., 1994). They reported an average annual incidence of severe hypoglycaemia of 2.8 in participants with IAH compared to 0.5 episodes per year in patients with normal symptomatic responses to hypoglycaemia.

6.4 Cardiac Autonomic Regulation during Experimental Hypoglycaemia

During the hypoglycaemic clamp studies there was a reduction in symptomatic and metanephrine response to hypoglycaemia in participants with IAH. Plasma metanephrines and reported symptoms increased in all three participant groups but autonomic symptom score and metanphrine thresholds were not reached in 63% of participants with IAH. Participants with diabetes reported normal awareness of hypoglycaemia also showed reduced symptomatic and metanephrine response to hypoglycaemia in comparison to the group without diabetes. Glucose thresholds for metanpehrine response were 2.92 mmol/l in the group with diabetes versus 3.29 mmol/l in control group. This reduction in symptom and sympathoadrenal response is established and have been reported previously (Heller et al., 1987, Amiel et al., 1988).

Resting heart rate in participants with diabetes was higher than those without diabetes indicating an element of cardiac autonomic dysfunction. Baseline cardiac autonomic function testing was normal in all participants undergoing a clamp study but baseline heart rate was higher in the participants with IAH (79bpm), in comparison to participants with intact awareness (72bpm) and those without diabetes (65bpm). This corresponded to a reduction in high frequency (HF) power or vagal tone of 1.7 in participants with IAH in comparison to 2.1 in participants with intact awareness and 2.6 in those without diabetes. Studies have

consistently reported increased baseline heart rate in participants with diabetes (Ewing et al., 1981, Vinik and Ziegler, 2007, Maser and Lenhard, 2005, Koivikko et al., 2005) and vagal neuropathy has been described as a clinical implication of cardiac autonomic neuropathy (Vinik and Ziegler, 2007, Maser and Lenhard, 2005).

Heart rate response to hypoglycaemia was time dependent and differed according to self reported awareness of hypoglycaemia. Participants without diabetes showed a transient increase in heart rate with recovery to baseline, caused by transient vagal withdrawal illustrated by a reduction in HF power. Participants with T1DM and intact awareness of hypoglycaemia showed an increase in heart rate caused by a withdrawal in vagal tone, which did not show evidence of recovery during the clamp protocol. The participants with IAH showed little changes in heart rate with minimal reductions in both vagal and sympathetic tone.

Our group has previously reported heart rate response during hypoglycaemia in participants with and without T2DM (Chow et al., 2017) and the results were similar to those reported here. Heart rate was higher in participants with diabetes (78bpm) in comparison to participants without diabetes (64bpm). Both groups showed an increase in heart rate associated with a reduction in vagal tone during hypoglycaemia. The participants with diabetes did show recovery of heart rate to baseline but this occurred later than in the participants without diabetes. We suspect that if clamp studies reported here were longer we would have also seen recovery of heart rate and HRV measures. The main difference between the participants with T1DM in our study and those in the T2DM study was duration of diabetes. The participants in these studies with T1DM had a longer duration of diabetes in comparison to those in the T2DM study (21years versus 11 years respectively).

6.5 Duration of Diabetes

Our studies have shown that duration of diabetes is a major influencing factor of the development of IAH and DAN. There was an increased prevalence of DAN and IAH in participants with a longer duration of disease. Increased disease

duration was also associated with inability of participants to improve their awareness of hypoglycaemia.

Although the prevalence of cardiac autonomic neuropathy was low in these studies there was an increase in prevalence as duration of diabetes increased. All participants with duration of diabetes of less than 15 years had normal cardiac autonomic function. Participants with duration of 15 to 30 years had a prevalence definite DAN of five percent compared to eight percent seen in those with duration of over 30 years. Data from the DCCT and EDIC also show the increase in prevalence of DAN as duration of disease increases with a prevalence of nine percent in participants with disease duration of less than fifteen years and 31% in those with a duration of greater than fifteen years (Pop-Busui et al., 2016).

IAH was associated with a longer duration of disease. Despite attempting to match study populations for duration of disease participants with IAH had a mean duration of disease of 29 years in comparison to those with intact awareness of 22 years ($p<0.001$). The association of duration of disease and IAH has been previously reported (Hepburn et al., 1990, Mokan et al., 1994). Hepburn et al described a group of patients reporting absent awareness of hypoglycaemia with only nineteen percent having duration of diabetes of less than fifteen years.

There may be structural changes occurring to glucose sensing neurones that are associated with a long duration of disease, which are irreversible, similar to the irreversible changes seen in peripheral and autonomic neuropathy. Glucose sensing neurones have been identified in the brain, intestine, hepatoportal vein and carotid body as well as pancreatic beta cells (McCrimmon, 2009). Animal studies have shown that the detection of hypoglycaemia in the brain is essential. When precipitating peripheral hypoglycaemia, whilst maintaining brain normoglycaemia, counterregulatory responses are significantly reduced (Biggers et al., 1989). Recurrent hypoglycaemia results in altered glucose sensing in the brain and mechanisms such as an increased ability to use glucose or an ability to use different fuels to maintain brain normoglycaemia have been suggested (McCrimmon, 2009).

Observation during the clamp studies showed that baseline heart rate was elevated in participants with IAH in comparison to participants with normal awareness of hypoglycaemia and this is most likely due to the differences seen in duration of disease. This sub-study reported that participants with IAH had a mean duration of disease of 31 years, in comparison to 21 years in participants with intact awareness. This increase in baseline heart rate is most likely due to subclinical cardiac autonomic neuropathy.

We hypothesise that this subclinical autonomic neuropathy is more pronounced in participants with IAH because of a longer duration of disease. The relationship between subclinical and clinical autonomic neuropathy possibly follows a similar pattern to that of diabetic peripheral neuropathy. Fifty percent of patients with diabetes have abnormal nerve conduction tests indicating the presence of peripheral neuropathy, however only a third of patients have any clinical symptoms (Tesfaye and Selvarajah, 2012).

Duration of diabetes had a negative impact on the ability of participants to improve their awareness of hypoglycaemia. Forty seven participants had duration of diabetes of less than 30 years and had a mean reduction in gold score of -1.3 and participants with a duration of greater than 30 years had a mean reduction of -0.9. Similarly Fanelli et al (Fanelli et al., 1993b) found that participants with a longer duration of disease showed improvement in hypoglycaemia awareness but the recovery of responses was inversely correlated with duration of disease.

Although both IAH and DAN were associated with a long duration of disease, the development of these clinical syndromes do not invariably affect patients who have had diabetes for a significant length of time. We have shown in these studies that the prevalence of DAN can be low even in a group of patients with a long duration of disease. And although the mean length of duration of diabetes was higher in the participants with IAH, five people in this group had a duration of diabetes of less than ten years, and thirteen participants in the normal awareness group had a duration of diabetes greater than thirty years.

6.6 Clinical Implications

The confirmation that a long duration of disease is associated with IAH rather than DAN has important clinical implications. The National Institute of Clinical Excellence (Guidance, 2015) have recognised the importance of identifying patients with IAH and recommend assessing awareness of hypoglycaemia on an annual basis. It is recognised that patients often underestimate the frequency of hypoglycaemia and under report impairment of warning symptoms (Heller et al., 1995). This increased risk in patients with a long duration of disease should be recognised and emphasised in clinical consultations.

This work also confirmed that younger participants with a shorter duration of disease were more likely to improve their symptomatic awareness of hypoglycaemia than older patients with longer duration of disease. Programmes with intensive support have been shown to improve hypoglycaemia awareness and reduce episodes of SH (Zoysa et al., 2013, Little et al., 2014). Once IAH is identified patients should be referred urgently to specialised clinics to ensure the best outcomes.

The presence of subclinical cardiac autonomic neuropathy, in participants with diabetes and a long duration of disease have potential clinical implications, because of the previous reports of the cardioprotective role of vagal activity (LaRovere et al., 1998). Although caution should be applied when extrapolating the results of experimental work to the clinical scenario of spontaneous hypoglycaemia, the risk of prolonged vagal suppression should be considered. Cardiovascular health should be optimised in all patients with long standing T1DM.

6.7 Limitations

This study is limited by the number of participants recruited, but increasing participant numbers would be unlikely to change the conclusions made. Power calculations were based on studies with a higher prevalence of DAN (Hepburn et al., 1990) and therefore the studies are underpowered. However we have shown that the difference in the prevalence of definite cardiac autonomic neuropathy between participants with impaired and normal awareness of hypoglycaemia

differs only by one percent. Therefore, increasing the number of participants is unlikely to change our conclusions that there is no association between IAH and DAN.

There will be an element of selection bias in the recruitment for these studies particularly elicited by the exclusion criteria of the clamp studies. These excluded participants with ischaemic heart disease, peripheral vascular disease and nephropathy. Microvascular complications such as nephropathy are associated with DAN as are cardiovascular risk factors such as hypertension and high risk lipid profiles (Vinik et al., 2003). The exclusion criteria were included to ensure safety during the clamp studies but will have caused a selection bias.

Spontaneous breathing was used in comparison to paced breathing during ECG recordings. Paced breathing is recommended for the interpretation of HF power (Tesfaye et al., 2010). HRV is affected by the respiratory rate and depth of breathing. As participants were being investigated by different clinical teams in different parts of the country it was felt that spontaneous breathing would limit inter-investigator differences. During the clamp studies participants were instructed to breathe spontaneously during ECG recording rather than to pace their breathing at a particular rate. This was because of the concern about cognitive impairment during hypoglycaemia which could potentially impair the ability of the participants to pace their breathing consistently. All participants had their respiratory rate measured to ensure that this was not fewer than 10 breaths per minute.

Further consideration should be given to other measures of autonomic dysfunction. This study used cardiac reflex tests to assess autonomic function and did not utilise other tests of the autonomic nervous system including tests of pupillomotor or sudomotor function. Tests for cardiac autonomic function are simple, quick, non-invasive and can be done at the bedside. Testing was limited to these tests to allow the tests to be done in each centre and then analysed in one site to improve reliability.

A specific limitation during the clamp studies was the use of two questionnaires and metanephrine results at baseline to calculate thresholds . Ideally three questionnaires and three blood samples for metanephrines would have been used to calculate thresholds for participants. It was felt that this was too cumbersome in these studies and therefore two baseline samples were used instead.

6.8 Future Work

We did not collect information about the prevalence of mild hypoglycaemia and the onset of episodes of SH in relation to symptomatic awareness and this would be an interesting focus to explore further during future work. Continuous glucose monitoring is now used frequently clinically and would be a viable tool to collect this information. The development of clinically relevant IAH is reported to be about twenty percent (Geddes et al., 2008), but the mechanism for developing IAH is still not clear. Recurrent and frequent exposure to hypoglycaemia has been suggested as possible cause (Frier et al., 2014) with the mechanism suggested as the increase in glucose threshold (a lower blood glucose is required to initiate a counterregulatory response) caused by protracted hypoglycaemia (Boyle et al., 1988). When this occurs neuroglycopenic symptoms can occur before autonomic responses are elicited (Hepburn et al., 1991).

Further large epidemiological studies are required to document an accurate prevalence of DAN using the new definitions of cardiac autonomic neuropathy (Tesfaye et al., 2010) and association with other complications of diabetes. The prevalence of DAN reported here was low potentially due to the characteristics of this particular study population that had a low prevalence of diabetic nephropathy. DAN is an important complication of diabetes because of the increased risk of death but it is one of the least studied.

The natural history of DAN should be documented particularly focusing on the potential progressive nature of the condition. There would be difficulties in recruiting participants for these studies. The study would be screening for a condition that has no treatment and a poor prognosis with no personal advantages for participants. Participants with severe cardiac autonomic neuropathy are likely

to be symptomatic and have other complications of diabetes making attendance for study visits difficult. The studies would potentially need to continue for decades to document any progression or new onset of neuropathy.

It would be interesting to repeat the hypoglycaemic clamp studies with an extended period of hypoglycaemia to observe whether there was cardiovascular recovery in patients with diabetes. Recovery of heart rate and vagal withdrawal was observed in participants without diabetes but this was not seen in participants with diabetes. However, recruiting participants for clamp studies is challenging because of the cumbersome nature of the protocols. A longer period of hypoglycaemia may prevent participants consenting to these studies.

Participants with IAH showed little change in heart rate and associated vagal and sympathetic tone during hypoglycaemia. We would hypothesise that this is due to a higher glucose threshold and if blood glucose was lowered further than this clamp study allowed then an autonomic response would eventually be seen with the corresponding cardiovascular changes seen in the control populations. This level of hypoglycaemia would potentially cause harm to participants and therefore remains a hypothesis currently. Spontaneous hypoglycaemia may be at this level for a significant amount of time in patients with IAH without their knowledge due to a lack of warning symptoms. This could be measured by blinded CGM while a continuous holter monitor is worn to capture heart rate and HRV changes.

Section 7: References

ADAMSON , U., LINS, P.-E., EFENDIC, S., HAMBERGER, B. & WAJNGOT, A. 1984. Impaired Counter Regulation of Hypoglycemia in a Group of Insulin-dependent Diabetics with Recurrent Episodes of Severe Hypoglycemia. *Acta Med Scand,* 216**,** 215-222.

ADLER, G. K., BONYHAY, I., FAILING, H., WARING, E., DOTSON, S. & FREEMAN, R. 2009. Antecedent Hypoglycemia Impairs Autonomic Cardiovascular Function. Implications for Rigorous Glycemic Control. *Diabetes,* 58**,** 360-366.

AICKIN, M. & GENSLER, H. 2011. Adjusting for multiple testing when reporting research results : the Bonferroni vs Holm methods. *AJPH,* 86**,** 726-728.

AKSELROD, S., GORDON, D., UBEL, A., SHANNON, D. C., BARGER, A. C. & COHEN, R. J. 1981. Power spectral analysis of heart rate fluctuation; a quantitative probe of beat to beat cardiovascular control. *Science,* 213**,** 220-222.

AMIEL, S. A., SHERWIN, R. S., SIMONSON, D. C. & TAMBORLANE, W. V. 1988. Effect of Intensive Insulin Therapy on Glycemic Thresholds for Counterregulatory Hormone Release. *Diabetes,* 37**,** 901-907.

AMIN, R., EDGE, J. A., ROSS, K., WARNER, J., ACERINI, C. & DUNGER, D. 2003. Hypoglycaemia Prevalence In Prepubertal Children with Type 1 Diabetes on Standard Insulin Regimen: Use of Continous Glucose Monitoring System. *Diabetes CAre,* 26**,** 662-667.

BANTING, F. G., CAMPBELL, W. R. & FLETCHER, F. G. 1923. Further Clinical Experience with Insulin (Pancreatic Extracts) in the Treatment of Diabetes Mellitus. *The British Medical Journal***,** 8-12.

BEREGSZASZI, M., TUBIANA-RUFI, N., BENALI, K., NOEL, M., BLOCH, J. & CZERNICHOW, P. 1997. Nocturnal hypoglycaemia in children and adolescents with insulin dependent diabetes mellitus: Prevalence and Risk Factors. *The Journal of Pediatrics,* 131**,** 27-33.

BERLIN, I., GRIMALDI, A., PAYAN, C., SACHON, C., BOSQUET, F., THERVET, F. & PUECH, A. 1987. Hypoglycaemic Symptoms and Decreased Beta Adrenergic Sensitivity in Insulin-Dependent Diabetic Patients. *Diabetes Care,* 10**,** 742-747.

BERNARDI, L. 2000. Clinical evaluation of arterial baroreflex activity in diabetes. *Diabetes, Nutrition and Metabolism,* 13**,** 331-340.

BERNARDI, L., SPALLONE, V., STEVENS, M., HILSTED, J., FRONTONI, S., POP-BUSUI, R., ZIEGLER, D., KEMPLER, P., FREEMAN, R., LOW, P., TESFAYE, S. & VALENSI, P. 2011. Methods of investigation for cardiac autonomic dysfunction in human research studies. *Diabetes Metab Res Rev,* 27**,** 654-664.

BERNTSON, G., BIGGER, T., ECKBERG, D., GROSSMAN, P., KAUFMAN, P., MALIK, M., NAGARAJA, H., PORGES, S., SAUL, P., STONE, P. & VANDER MOLAN, M. 1997. Heart rate variability: Origins, methods and interpretative caveats. *Psychophysiology,* 34**,** 623-648.

BIGGER, J. T., FLEISS, J. L., ROLNITZKY, L. M. & STEINMAN, R. C. 1993. The ability of several short-term measures of RR variability to predict mortality after myocardial infarction. *Circulation,* 88**,** 927-932.

BIGGER, T., FLEISS, J. L., STEINMAN, R. C., ROLNITZKY, L. M., KLEIGER, R. & ROTTMAN, J. 1992. Frequency Domain Measures of Heart Period Variability and Mortaility After Myocardial Infarction. *Circulation,* 85**,** 164-171.

BIGGERS, D. W., MYERS, S. R., NEAL, D., STINTON, R., COOPER, N. B., JASPAN, J. B., WILLIAMS, P. E., CHERRINGTON, A. D. & TYLER, F. R. 1989. Role of Brain Counterregulation of Insulin-Induced Hypoglycemia in Dogs. *Diabetes,* 38**,** 7-16.

BILCHICK, K. & BERGER, R. 2006. Heart Rate Varaibility. *Journal of Cardiovascular Electrophysiology,* 17**,** 691-694.

BILLMAN, G. 2013. The LF/HF ration does not accurately measure cardiac sympatho-vagal balance. *Frontiers in Physiology,* 4**,** 1-5.

BOLLI, G., CALABRESE, G., DE FEO, P., COMPAGNUCCI, P., ZEGA, G., ANGELETTI, G., CARTECHINI, M. G., SANTEUSANIO, F. & BRUNETTI, P. 1982. Lack of Glucagon Response in Glucose Counter-Regulation in Type 1 (Insulin-Dependent) Diabetics: Absence of Recovery After Prolonged Optimal Insulin Therapy. *Diabetologia,* 22**,** 100-105.

BONDS, D. E., MILLER, M. E., BERGENSTAL, R. M., BUSE, J. B., BYINGTON, R. P., CUTLER, J. A., DUDL, R. J., ISMAIL-BEIGI, F., KIMEL, A. R., HOOGWEF, B., HOROWITZ, K. R., SAVAGE, P. J., SEAQUIST, E. R., SIMMONS, D. L., SIVITZ, W. I., SPERIL-HILLEN, J. M. & SWEENEY, M. E. 2010. The association between symptomatic, severe hypoglycaemia and mortality in type 2 diabetes: retrospective epidemiological analysis of theACCORDstudy. *BMJ,* 340**,** b4909.

BOTTINI, P., BOSCHETTI, E., PAMPANELLI, S., CIOFETTA, M., DEL SINDACO, P., SCIONTI, L., BRUNETTI, P. & BOLLI, G. 1997. Contribution of Autonomic Neuropathy to Reduced Plasma Adrenaline Responses to Hypoglycemia in IDDM: Evidence for a Nonselective Defect *Diabetes***,** 814-823.

BOULTON, A. J. M., FREEMAN, R., VINIK, A. I., MALIK, R. A., AREZZO, J. C., MASER, R. E., BRIL, V., SOSENKO, J. M., FIELDMAN, E. L. & ZIEGLER, D. 2005. Diabetic Neuropathies. *Diabetes Care,* 28**,** 956-962.

BOYLE, P. J., KEMPERS, S. F., O'CONNOR, A. M. & NAGY, R. J. 1995. Brain Glucose Uptake and Unawareness of Hypoglycemia in Patients with Insulin -Dependent Diabetes Mellitus. *N Engl J Med,* 333**,** 1726-1731.

BOYLE, P. J., SCHWARTZ, N. S., SHAH, N. D., CLUTTER, W. E. & CRYER, P. E. 1988. Plasma Glucose Concentrations at the Onset of Hypoglycemic Symptoms in Patients with Poorly Controlled Diabetes and in Nondiabetics. *N Engl J Med,* 318**,** 1487-1492.

CARLSTEN, A., FOLKOW, B. & HAMBERGER, A. 1957. Cardiovascular Effects of Direct Vagal Stimulation in Man. *Acta. Physiol. Scandinav.,* 41**,** 68-76.

CHOUDHARY, P., LONNEN, K., EMERY, C. J., MACDONALD, I. A., MACLEOD, K. M., AMIEL, S. A. & HELLER, S. R. 2009. Comparing hormonal and symptomatic responses to experimental hypoglycaemia in insulin-and sulphonylurea-treated Type 2 diabetes. *Diabetic Medicine,* 26**,** 665-672.

CHOW, E., BERNJAK, A., WALKINSHAW, E., LUBINA-SOLOMON, A., FREEMAN, J., MACDONALD, I. A., SHERIDAN, P. & HELLER, S. R. 2017. Cardiac autonomic regulation and repolarization during acute experimental hypoglycaemia in type 2 diabetes. *Diabetes,* 66**,** 1322-1333.

CHRISTENSEN, T. F., CICHOSZ, S. L., TARNOW, L., RANDLOV, J., KRISTENSEN, L. E., STRUIKJK, J. J., ELDRUP, E. & HEJLESEN, O. K. 2014. Hypoglycaemia and QT interval prolongation in type 1 diabetes - bridging the gap between clamp studies and spontaneous episodes. *Journal of diabetes and its complications*.

CLARKE, B. F., EWING, D. J. & CAMPBELL, I. W. 1979. Diabetic Autonomic Neuropathy. *Diabetologia,* 17**,** 195-212.

CONSENSUS, P. 1988. Report and Recommendations of the San Antonio Conference on Diabetic Neuropathy. *Annals of Neurology,* 24**,** 99-104.

CRANSTON, I., LOMAS, J., AMIEL, S. A., MARAN, A. & MACDONALD, I. A. 1994. Restoration of hypoglycaemia awareness in patients with long-duration insulin-dependent diabetes. *Lancet,* 344**,** 283-287.

CRYER, P. E. 2001. Hypoglycemia-associated autonomic failure in diabetes. *Am J Physiol Endocrinol Metab,* 281**,** E1115-E1121.

CRYER, P. E. 2002. Hypoglycaemia: The limiting factor in the glycaemic management of Type 1 and Type 2 Diabetes. *Diabetologia,* 45**,** 937-948.

CRYER, P. E., DAVIS, S. N. & SHAMOON, H. 2003. Hypoglycaemia in Diabetes. *Diabetes Care,* 26**,** 1902-1912.

DAGOGO-JACK, S., RATTARASARN, C. & CRYER, P. E. 1994. Reversal of hypoglycaemia unawareness, but not defective glucose counterregulation, in IDDM. *Diabetes,* 43**,** 1426-1434.

DAGOGO-JACK, S. E., CRAFT, S. & CRYER, P. E. 1993. Hypoglycemia-associated Autonomic Failure. *J. Clin. Invest.,* 91**,** 819-828.

DEARY, I. J., HEPBURN, D. A., MACLEOD, K. M. & FRIER, B. M. 1993. Partitioning the symptoms of hypoglycaemia using multi-sample confirmatory factor analysis. *Diabetologia,* 36**,** 771-777.

DIDANGELOS, T., ARSOS, G., KARAMITSOS, D., ATHYROS, V., GEORGA, S. & KARATZAS, N. 2006. Effect of quinapril or losartan alone and in combination on left ventricular systolic and diastolic functions in asymptomatic patients with diabetic autonomic neuropathy. *Journal of diabetes and its complications,* 20**,** 1-7.

DUANE, T. D. 1972. Valsalva hemorrhagic retinopathy. *Trans Am Ophthalmol Soc.,* 70**,** 298-313.

DUCKWORTH, W., ABRAIRA, C., MORITZ, T., REDA, D., EMANUELE, N., REAVEN, P., ZIEVE, F., MARKS, J., DAVIS, S. N., HAYWOOD, R., WARREN, S., GOLDMAN, S., MCCARREN, M., VITEK, M. E., HENDERSON, W. G. & HUANG, G. 2009. Glucose Control and Vascular Complications in Veterans with Type 2 Diabetes. *N Engl J Med,* 360**,** 129-139.

DYRBERG, T., BENN, J., SANDAHL CHRISTIANSEN, J., HILSTED, J. & NERUP, J. 1981. Prevalence of Diabetic Autonomic Neuropathy Measured by Simple Bedside Tests. *Diabetologia,* 20**,** 190-194.

ELECTROPHYSIOLOGY, T. F. O. T. E. S. O. C. A. T. N. A. S. O. P. 1996. Heart Rate Variability. Standards of Measurement, Physiological Interpretation, and Clinical Use. *Circulation,* 93**,** 1043-1065.

EWING, D. J., CAMPBELL, I. W. & CLARKE, B. F. 1980. The Natural History of Diabetic Autonomic Neuropathy. *Quarterly Journal of Medicine,* 193**,** 96-108.

EWING, D. J., CAMPBELL, I. W. & CLARKE, B. F. 1981. Heart rate changes in diabetes mellitus. *The Lancet,* 317**,** 183-186.

EWING, D. J. & CLARKE, B. F. 1982. Diagnosis and management of diabetic autonomic neuropathy. *BMJ,* 285**,** 916-918.

EWING, D. J., MARTYN, C., YOUNG, R. & CLARKE, B. F. 1985. The Value of Cardiovasuclar Autonomic Function Tests: 10 Years Experience in Diabetes. *Diabetes Care,* 8**,** 491-498.

FANELLI, C., PAMPANELLI, S., LALLI, C., DEL SINDACO, P., CIOFETTA, M., LEPORE, M., PORCELLATI, F., BOTTINI, P., DI VINCENZO, A., BRUNETTI, P. & BOLLI, G. 1997. Long-Term Intensive Therapy of IDDM Patients With Clinically Overt Autonomic Neuropathy. Effects on Hypoglycemia Awareness and Counterregulation. *Diabetes* 46**,** 1172-1181.

FANELLI, C. G., EPIFANO, L., RAMBOTTI, A. M., PAMPANELLI, S., DI VINCENZO, A., MODARELLI, F., LEPORE, M., ANNIBALE, B., CIOFETTA, M. & BOTTINI, P. 1993a. Meticulous prevention of hypoglycemia normalizes the glycemic thresholds and magnitude of most of neuroendocrine responses to, symptoms of, and cognitive function during hypoglycemia in intensively treated patients with short-term IDDM. . *Diabetes,* 42**,** 1683-1689.

FANELLI, C. G., EPIFANO, L., RAMBOTTI, A. M., PAMPANELLI, S., DI VINCENZO, A., MODARELLI, F., LEPORE, M., ANNIBALE, B., CIOFETTA, M., BOTTINI, P., PORCELLATI, F., SCIONTI, L., SANTEUSANIO, F., BRUNETTI, P. & GEREMIA, B. B.

1993b. Meticulous Prevention of Hypoglycemia Normalizes the Glycemic Thresholds and Magnitude of Most of Neuroendocrine Responses to, Symptoms of, and Cognitive Function During Hypoglycemia in Intensively Treated Patients With Short-Term IDDM. *Diabetes,* 42**,** 1683-1689.

FANNELLI, C., PAMPANELLI, S., EPIFANO, L., RAMBOTTI, A. M., DI VINCENZO, A., MODARELLI, F., CIOFETTA, M., LEPORE, M., ANNIBALE, B., TORLONE, E., PERRIELLO, G., DE FEO, P., SANTEUSANIO, F., BRUNETTI, P. & BOLLI, G. B. 1994. Long-term recovery from unawareness, deficient counterregulation and lack of cognitive dysfunction during hypoglycaemia, following institution of rational , intensive insulin therapy in IDDM. *Diabetologia,* 37**,** 1265-1276.

FISHER, B. M., GILLEN, G., DARGIE, H. J., INGLIS, G. C. & FRIER, B. M. 1987. The effects of insulin-induced hypoglycaemia on cardiovascular funstion in normal man: studies using radionuclide ventriculography. *Diabetologia,* 30**,** 841-845.

FISHER, B. M., GILLEN, G., HEPBURN, D. A., DARGIE, H. J. & FRIER, B. M. 1990. Cardiac responses to acute inulin-induced hypoglycemia in humans. *American Journal of Physiology,* 258**,** 1775-1779.

FISHER, M. 2002. *Heart Disease and Diabetes*, Informa Healthcare.

FISHER, M. 2003. *Heart Disease and Diabetes*, Martin Dunitz, the Taylor Francis Group.

FRATTOLA, A., PARATI, G., GAMBA, P., PALEARI, F., MAURI, G., DI RIENZO, M., CASTIGLIONI, P. & MANCIA, G. 1997. Time and frequency domain estimates spontaneous baroreflex sensitivitry provide early detection of autonomic dysfunction in diabetes mellitus. *Diabetologia,* 40**,** 1470-1475.

FREEMAN, R. 2005. Autonomic peripheral neuropathy. *Lancet,* 365**,** 1259-70.

FRIER, B. M., HELLER, S. R. & MCCRIMMON, R. J. 2014. *Hypoglycaemia in Clinical Practice*, Wiley Blackwell.

GEDDES, J., DEARY, I. J., WRIGHT, R., FRIER, B. M. & ZAMMITT, N. N. 2007. An Evaluation of Methods of Assessing Impaired Awareness of Hypoglycemia in Type 1 Diabetes. *Diabetes Care,* 30**,** 1868-1870.

GEDDES, J., SCHOPMAN, J. E., ZAMMITT, N. N. & FRIER, B. M. 2008. Prevalence of impaired awareness of hypoglycaemia in adults with Type 1 diabetes. *Diabetic Medicine,* 25**,** 501-504.

GIBBONS, C., CHESHIRE, W. & FIFE, T. 2014. Model Coverage Policy: Autonomic Testing. *American Academy of Neurology*.

GOLD, A. E., MACLEOD, K. M. & FRIER, B. M. 1994. Impaired awareness and frequency of hypoglycemia. *Diabetes Care,* 17**,** 697-703.

GOTO, A., ONYEBUCHI, A., GOTO, M., TERAUCHI, Y. & NODA, M. 2013. Severe Hypoglycaemia and cardiovascular disease: systemic review and meta-analysis with bias analysis. *BMJ,* 347**,** 1-11.

GRAVELING, A. J. & FRIER, B. M. 2010. Impaired awareness of hypoglycaemia: a review. *Diabetes & Metabolism,* 36**,** S64-74.

GROUP, A. T. C. C. R. I. D. S. 2008. Effects of intensive glucose lowering in type 2 diabetes. *N Engl J Med,* 358**,** 2545-59.

GROUP, T. D. C. A. C. T. E. O. D. I. A. C. D. E. S. R. 2005. Intensive Diabetes Treatment and Cardiovascular Disease in Patients with Type 1 Diabetes. *N Engl J Med,* 353**,** 2643-2653.

GROUP, T. D. C. A. C. T. R. 1993. The Effect of Intensive Treatment of Diabetes on the Development and Progression of Long-Term Complications in Insulin-Dependent Diabetes Mellitus. *N Engl J Med,* 329**,** 977-986.

GROUP, T. D. R. 1991. Epidemiology of severe hypoglycemia in the diabetes control and complications trial. *Am J Med,* 90**,** 450-459.

GUIDANCE, N. 2015. *Type 1 diabetes in adults: diagnosis and management.* [Online].

GUZIK, P., PISKORSKI, J., RAUZE, T., SCHNEIDER, R., WESSELING, K. H., WYKRETOWICZ, A. & WYSOCK, H. 2007. Correlations between the Poincaré Plot and Conventional Heart Rate Variability Parameters Assessed during Paced Breathing. *J, Physiol. Sci. ,* 57**,** 63-71.

HAMLIN, R. & SMITH, R. 1968. Effects of vagal stimulation on S-A and A-V nodes. *American Journal of Physiology,* 215**,** 560-568.

HELLER, S. R., CHAPMAN, J., MCCLOUD, J. & WARD, J. 1995. Unreliability of reports of hypoglycaemia by diabetic patients. *BMJ,* 310**,** 440.

HELLER, S. R. & CRYER, P. E. 1991. Reduced Neuroendocrine and Symptomatic Responses to Subsequent Hypoglycaemia After 1 Episode of Hypoglycaemia in Nondiabetic Humans. *Diabetes* 40**,** 223-226.

HELLER, S. R., HERBERT, M., MACDONALD, I. A. & TATTERSALL, R. B. 1987. Influence of Sympathetic Nervous System on Hypoglycaemic Warning Symptoms. *The Lancet,* 330**,** 359-363.

HEPBURN, D. A., PATRICK, A. W., BRASH, H. M., THOMPSON, I. & FRIER, B. M. 1991. Hypoglycaemia Unawareness in Type 1 Diabetes: A Lower Plasma Glucose is Required to Stimulate Sympathoadrenal Activation. *Diabetic Medicine,* 8**,** 934-945.

HEPBURN, D. A., PATRICK, A. W., EADINGTON, D. J., EWING, D. J. & FRIER, B. M. 1990. Unawareness of Hypoglycaemia in Insulin-treated Diabetic Patients; Prevalence and Relationship to Autonomic Neuropathy. *Diabetic Medicine,* 7**,** 711-717.

HILLSON, R. 2008. *Diabetes Care: A Practical Manual*, OUP Oxford.

HILSTED, J., BONDE-PETERSEN, F., NORGAARD, M.-B., GRENIMAN, M., CHRISTENSEN, N. J., PARVING, H.-H. & SUZUKI, M. 1984. Haemodynamic changes in insulin-induced hypoglycaemia in normal man. *Diabetologia,* 26**,** 328-332.

HILSTED, J., MADSBAD, S., KRARUP, T., SESTOFT, N. J., CHRISTENSEN, N. J., TRONIER, B. & GALBO, H. 1981. Hormonal, metabolic and cardiovascular responses to hypoglycaemia in diabetic autonomic neuropathy. *Diabetes,* 30**,** 626-633.

HILSTED, J., RICHTER, E., MADSBAD, S., TRONIER, B., CHRISTENSEN, N. J., HILDEBRANDT, P., DAMKJAER, M. & GALBO, H. 1987. Metabolic and Cardiovascular Responses to Epinephrine in Diabetic Autonomic Neuropathy. *N Engl J Med,* 317**,** 421-426.

HOELDTKE, R. D., BODEN, G., SHUMAN, C. & OWEN, O. 1982. Reduced Epinephrine Secretion and Hypoglycemia Uawareness in Diabetic Autonomic Neuropathy. *Annals of Internal Medicine,* 96**,** 459-462.

HOWORKA, K., PUMPRLA, J., HABER, P., KOLLER-STRAMETZ, J., MONDRZYK, J. & SCHABMANN, A. 1997. Effects of physical training on heart rate variability in diabetic patients with various degrees of cardiovascular autonomic neuropathy. *Cardiovascular research,* 34**,** 206-214.

HULL, S., EVANS, A., VANOLI, E., ADAMSON, P., STRAMBA-BADIALE, M., ALBERT, D., FOREMAN, R. & SCHWARTZ, P. 1990. Heart rate variability before and after myocardial infarction in conscious dogs at high and low risk of sudden death. *JACC,* 16**,** 978-985.

KAMEL, J. T., GOODMAN, D., HOWE, K., MJ, C., GM, W. & ROBERTS, L. 2015. Assessment of the relationship between hypoglycaemia unawareness and autonomic function following isley cell/pancreas transplantation. *Diabetes Metab Res Rev,* 31**,** 646-650.

KARDOS, A., WATTERICH, G., DE MENEZES, R., CSANADY, M., CASADEI, B. & RUDAS, L. 2001. Determinants of Spontaneous Baroreflex Sensitivity in a Healthy Working Population. *Hypertension,* 37**,** 911-916.

KAUFMAN, F. R., AUSTIN, J., NEINSTEIN, A., JENG, L., HALVORSON, M., DEVOE, D. & PITUKCHEEWANONT, P. 2002. Nocturnal hypoglycemia detected with the

Continuous Glucose Monitoring System in pediatric patients with type1 diabetes. *The Journal of Pediatrics,* 141**,** 625 - 630.

KLATT, E., BEATIE, C. & NOGUCHI, T. 1988. Evaluation of Death from Hypolglycemia. *The American Journal of Forensic Medicine and Pathology,* 9**,** 122-125.

KLEIGER, R., MILLER, P., BIGGER, J. T., MOSS, A. & GROUP, M. P.-I. R. 1987. Decreased Heart Rate Variability and it's Assocation with Increased Mortality After Acute Myocardial Infarction. *Am J Cardiol.,* 59**,** 256-262.

KOIVIKKO, M. L., SALMELA, P. I., JUHANI AIRAKSINEN, K. E., TAPANAINEN, J. S., RUOKONEN, A., MA KIKALLIO, T. H. & HUIKURI, H. V. 2005. Effects of Sustained Insulin-Induced Hypoglycemia on Cardiovascular Autonomic Regulation in Type 1 Diabetes. *Diabetes,* 54**,** 744-750.

LAITINEN, T., HUOPIO, H., VAUHJONEN, I., CAMAROS, C., HARTIKAINEN, J., LAAKSO, M. & NISKANEN, L. 2003. Effects of euglycaemic and hypoglycaemic hyperinsulinaemia on sympathetic and parasympathetic regulation of haemodynamics in healthy subjects. *Clinical Science,* 105**,** 315-322.

LAROVERE, M. T., BIGGER, T., MARCUS, F., MORTARA, A. & SCHWARTZ, P. 1998. Baroreflex sensitivity and heart-rate variability in prediction of total cardiac mortality after myocardial infarction. *The Lancet,* 351**,** 478-484.

LAWRENCE, R. D. 1941. Insulin hypoglycemia: changes in nervous manifestations. *Lancet,* ii602.

LEE, G., PC, J., AB, A., D, M., P, A. & SJ, H. 2011. A comparison of plasma-free metanephrines with plasma catecholamines in the investigation of suspected pheochromocytoma. *J Hypertens,* 29**,** 2422-2428.

LEELARATHNA, L., LITTLE, S., WALKINSHAW, E., KAI TAN, H., LUBINA-SOLOMON, A., KUMARESWARAN, K., LANE, A., CHADWICK, T., MARSHALL, S., SPEIGHT, J., FLANAGAN, D., HELLER, S. R., SHAW, J. & EVANS, M. 2013. Restoration of Self-Awareness of Hypoglycemia in Adults With Long-Standing Type 1 Diabetes. *Diabetes Care,* 36**,** 4063-4070.

LEESE, G. P., MORRISON, W., WANG, J., FRIER, B. M., BROOMHALL, J., MORRIS, A. D., KELLY, P. & MARSDEN, A. 2003. Frequency os Severe Hypoglycemia Requiring Emergency Treatment in Type 1 and Type 2 Diabetes. *Diabetes Care,* 26**,** 1176-1180.

LITTLE, S., CHADWICK, T., CHOUDHARY, P., BRENNAND, C., STICKLAND, J., BARENDSE, S., OLATEJU, T., LEELARATHNA, L., WALKINSHAW, E., TAN, H., MARSHALL, S., THOMAS, R., HELLER, S. R., EVANS, M., KERR, D., FLANAGAN, D., SPEIGHT, J. & SHAW, J. 2012. Comparison of Optimised MDI versus Pumps with or without Sensors in Severe Hypoglcyaemia (the Hypo COMPaSS trial). *BMC Endocrine Disorders,* 12**,** 1-14.

LITTLE, S., LALANTHA, L., WALKINSHAW, E., TAN, H. K., OLIVIA, C., LUBINA-SOLOMON, A., CHADWICK, T., BARENDSE, S., STOCKEN, D., BRENNAND, C., MARSHALL, S., WOOD, R., SPEIGHT, J., KERR, D., FLANAGAN, D., HELLER, S. R., EVANS, M. & SHAW, J. 2014. Recovery of Hypoglycemia Awareness in Long-standing Type 1 Diabetes: A Multicenter 2x2 Factorial Randomized Controlled Trial Comparing Insulin Pump With Multiple Daily Injections and Continuous With Conventional Gluocse Self-Monitoring (HypoCOMPaSS). *Diabetes Care,* 37(8)**,** 2114-2122.

MALLIANI, A., LOMBARDI, F. & PAGANI, M. 1994. Power spectrum analysis of heart rate variability: a tool to explore neural regulatatory mechanisms. *Br Heart J,* 71**,** 1-2.

MALLIANI, A., PAGANI, M., LOMBARDI, F. & CERUTTI, S. 1991. Cardiovascular neural regulation explored in the frequency domain. *Circulation,* 84**,** 482-492.

MARQUES, J. L. B., GEORGE, E., PEACEY, S. R., HARRIS, N. D., MACDONALD, I. A., COCHRANE, T. & HELLER, S. R. 1997. Altered ventricular repolarisation during hypoglycaemia in patients with diabetes. *Diabetic Medicine,* 14**,** 648-654.

MASER, R. E. & LENHARD, J. 2005. Cardiovascular Autonomic Neuropathy Due to Diabetes Mellitus: Clinical Manifestations, Consequences and Treatment. *J Clin Endocrinol Metab,* 90**,** 5896-5903.

MCAULAY, V., DEARY, I. J. & FRIER, B. M. 2001. Symptoms of hypoglycaemia in people with diabetes. *Diabetic Medicine,* 18**,** 690-705.

MCCOY, R., SHAH, N. D., VAN HOUTEN, H. K., WERMERS, R. A., ZIEGENFUSS, J. Y. & SMITH, S. A. 2012. Increased Mortality of Patients With Diabetes Reporting Severe Hypoglycaemia. *Diabetes Care,* 35**,** 1897-1901.

MCCRIMMON, R. 2009. Glucose Sensing During Hypoglycemia: Lessons From the Lab. *Diabetes Care.,* 32**,** 1357-1363.

MEYER, C., VENEMAN, T., GROBMANN, R., GERICH, J., MITRAKOU, A., BRETZEL, R. & MAHLER, R. 1998. Effects of Autonomic Neuropathy on Counterregulation and Awareness of Hypoglycemia in Type 1 Diabetic Patients. *Diabetes Care,* 21**,** 1960-1966.

MITRAKOU, A., FANELLI, C. G., VENEMAN, T., PERRIELLO, G., CALDERONE, S., PLATANISIOTIS, D., RAMBOTTI, A. M., RAPTIS, S., BRUNETTI, P., CRYER, P. E., GERICH, J. & BOLLI, G. 1993. Reversibility of Unawareness of Hypoglycemia in Patients with Insulinomas. *N Engl J Med,* 329**,** 834-839.

MITRAKOU, A., RYAN, C., VENEMAN, V., MOKAN, T., JENSSEN, T., KISS, I., DURRANT, J., CRYER, P. E. & GERICH, J. 1991. Hierarchy of glycemic thresholds for counterregulatory hormone secretion, symptoms, and cerebral dysfunction. *AJP,* 260**,** E67-E74.

MOKAN, M., KORYTKOWSKI, M. T., MITRAKOU, A., CRYER, P. E., VENEMAN, V., GERICH, J. & RYAN, C. 1994. Hypoglycemia Unawareness in IDDM. *Diabetes Care,* 17**,** 1397-1403.

MONTANO, N., RUSCONE, T., PORTA, A., LOMBARDI, F., PAGANI, M. & MALLIANI, A. 1994. Power spectrum analsysis of heart rate variability to assess the changes in sympathovagal balance during graded orthostatic tilt. *Circulation,* 90**,** 1826-1831.

NATHAN, D. M., ZINMAN, B., CLEARY, P., BACKLUND, J.-Y. C., GENUTH, S., MILLER, R., ORCHARD, T. & GROUP, D. C. A. C. T. E. O. D. I. A. C. D. E. R. 2009. Modern-Day Clinical Course of Type 1 Diabetes Mellitus After 30 Years' Duration. *Arch Intern Med,* 169**,** 1307-1316.

NEUROLOGY, A. A. O. 1996. Assessment: Clinical Autonomic Testing. Report of the Therapeutics and Technology Assessment Subcommittee of the Amercian Academy of Neurology. *Neurology,* 46**,** 873-880.

NORDWALL, M., BOJESTIG, M., ARNQVIST, H. J. & LUDVIGSSON, J. 20014. Declining incidence of severe retinopathy and persisting decrease of nephropathy in an unselected population of Type 1 diabetes—the Linköping Diabetes Complications Study. *Diabetologia,* 47**,** 1266-1272.

O'BRIEN, I., MCFADDEN, J. & CORRALL, R. 1991. The Influence of Autonomic Neuropathy on Mortality in Insulin-dependent Diabetes. *Quarterly Journal of Medicine,* 290**,** 495-502.

O'BRIEN, I. A., O'HARE, P. & CORRALL, R. 1986. Heart rate variability in healthy subjects: effect of age and the derivation of normal ranges for tests of autonomic function. *Br Heart J,* 55**,** 348-354.

OLSEN, S. E., ASVOLD, B. O., FRIER, B. M., AUNE, S. E., HANSEN, L. I. & BJORGAAS, M. R. 2014. Hypoglycaemia symptoms and impaired awareness of hypoglycaemia in adults with type 1 diabetes: the association with diabetes duration. *Diabetic Medicine,* Accepted 9th May.**,** 1-8.

OLSEN, S. E., BJORGAAS, M. R., ASVOLD, B. O., SAND, T., STJERN, M., FRIER, B. M. & NILSEN, K. 2016. Impaired awareness of Hypoglycaemia in Adults with Type 1

Diabetes Is Not Associated With Autonomic Dysfunction or Peripheral Neuropathy. *Diabetes Care*

39**,** 426-433.

OMBONI, S., PARATI, G., FRATTOLA, A., MUTTI, E., DI RIENZO, M., CASTIGLIONI, P. & MANCIA, G. 1993. Spectral and sequence analysis of finger blood pressure variability: comparison with analysis of intra-arterial recordings. *Hypertension,* 22**,** 26-33.

PASO, G. A. R. D., LANGEWITZ, W., MULDER, L., VAN ROON, A. & DUSCHEK, S. 2013. The utility of low frequency heart rate variability as an index of

sympathetic cardiac tone: A review with emphasis on a reanalysis

of previous studies. *Psychophysiology,* 50**,** 477-487.

POLINSKY, R., KOPIN, I., EBERT, M. & WEISE, V. 1980. The Adrenal Medullary Response to Hypoglycemia in Patients with Orthostatic Hypotension. *JCEM,* 51**,** 1401-1406.

POMERANZ, B., MACAULAY, R. J., CAUDILL, M. A., KUTZ, I., ADAM, D., GORDON, D., KILBURN, K. M., BARGER, A. C., SHANNON, D. C. & COHEN, R. J. 1985. Assessment of autonomic function in humans by heart rate spectral analysis. *Am J Phyl,* 248**,** 151-3.

POP-BUSUI, R. 2010. Cardiac Autonomic Neuropathy in Diabetes. *Diabetes Care,* 33**,** 434-441.

POP-BUSUI, R., BROFFETT, B., ZINMAN, B., MARTIN, C., WHITE, N. H., HERMAN, W. H., GENUTH, S., GUBITOKI-KLUG, R. & GROUP, D. E. 2016. Cardiovascular Autonomic Neuropathy and Cardiovascular Outcomes in Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) Study. *Diabetes Care,* Online**,** 1-6.

R.E.SCHMIDT 2002. Neuropathology and pathogenesis of diabetic autonomic neuropathy. *Int Rev Neurobiol,* 50**,** 257-292.

ROBINSON, R., HARRIS, N., IRELAND, R., LEE, S., NEWMAN, C. & HELLER, S. R. 2003a. Mechanisms of Abnormal Cardiac Repolarisation During Insulin-Induced Hypoglycaemia. *Diabetes,* 52.

ROBINSON, R. T., HARRIS, N., IRELAND, R., LINDHOLM, A. & HELLER, S. R. 2003b. Comparative effect of human soluble insulin and insulin aspart upon hypoglycaemia-induced alterations in cardiac repolarization. *Br. J. Clin. Pharmacol.,* 55**,** 246-251.

ROBINSON, R. T. C. E., HARRIS, N., IRELAND, R., MACDONALD, I. A. & HELLER, S. R. 2004. Changes in cardiac repolarization during clinical episodes of nocturnal hypoglycaemia in adults with type 1 diabetes. *Diabetologia,* 47**,** 312-315.

ROSENGARD-BARLUND, M., BERNARDI, L., HOLMQVIST, J., DEBARBIERI, G., MANTYSAARI, M., BJORKESTEN, C.-G., FORSBLOM, C. & GROOP, P.-H. 2011. Deep breathing improves blunted baroreflex sensitivity even after 30 years of type 1 diabetes. *Diabetologia,* 54**,** 1862-1870.

ROVERE, M. T. L., PINNA, G. D., MAESTRI, R. & SLEIGHT, P. 2012. Clinical value of baroreflex sensitivity. *Netherlands Heart Journal,* 21**,** 61-63.

RUSSELL-JONES, D. & KHAN, R. 2006. Insulin-associated weight gain in diabetes - causes, effects and coping strategies. *Diabetes, Obesity and Metabolism.,* 9**,** 799-812.

RUSSELL, R. R., CHYUN, D., SONG, S., SHERWIN, R. S., TAMBORLANE, W. V., LEE, F. A., PFEIFER, M. A., RIFE, F., WACKERS, F. J. T. & YOUNG, L. H. 2001. Cardiac responses to insulin-induced hypoglycaemia in non-diabetic and intensively treated type 1 diabetic patients. *American Journal Physiol Endocrinol Metab,* 281**,** E1029-1036.

RYDER, R. E. J., OWENS, D. R., HAYES, T. M., GHATEI, M. A. & BLOOM, S. R. 1990. Unawareness of hypoglycaemia and inadequate hypoglycaemic counterregulation: no causal relation with diabetic autonomic neuropathy. *BMJ,* 301**,** 783-787.

SCHACHINGER, H., PORT, J., BRODY, S., LINDER, L., WILHELMS, F. H., HUBER, P., COX, D. & KELLER, U. 2004. Increased high-frequency heart rate variability during insulin-induced hypoglycaemia

in healthy humans. *Clinical Science,* 106**,** 583-588.

SCHOPMAN, J. E., GEDDES, J. & FRIER, B. M. 2011. Frequency of symptomatic and asymptomatic hypoglycaemia in Type 1 diabetes; effect of impiared awareness of hypoglycaemia. *Diabetic Medicine,* 28**,** 352-355.

SCHWARTZ, N. S., CLUTTER, W. E., SHAH, S. D. & CRYER, P. E. 1987. Glycemic Thresholds for Activation of Glucose Counterregulatory Systems are Higher than the Threshold for Symptoms. *J. Clin. Invest.,* 79**,** 777-781.

SCHWARTZ, P., BILLMAN, G. & LOWELL STONE, H. 1984. Autonomic mechanisms in ventricular fibrillation induced by myocardial ischaemia during exercise in dogs with healed myocardial infarction. *Circulation,* 69**,** 790-800.

SONG, H.-S. & LEHRER, P. M. 2003. The Effects of Specific Respiratory Rates on Heart Rate and Heart Rate Variability. *Applied Psychophysiology and Biofeedback,* 28**,** 12-23.

SONG, S. H., MCINTYRE, S. S., VELDHUIS, J. D., HAYES, P. C. & BUTLER, P. C. 2000. Direct Measrement of Pulsatile Insulin Secretion from Portal Vein in Human Subjects. *JCEM,* 85**,** 4491-4499.

STEPHENSON, J. M., KEMPLER, P., CAVALLO PERIN, P., FULLER, J. H. & GROUP, E. I. C. S. 1996. Is autonomic neuropathy a risk factor for severe hypoglycaemia? The EURODIAB IDDM Complications Study. *Diabetologia,* 39**,** 1372-1376.

STUDY, T. E. O. D. I. A. C. E. 2003. Sustained effect of intensive treatment of type 1 diabetes mellitus on and progression of diabetic nephropathy. *JAMA,* 16**,** 2159-2167.

SWENNE, C. A. 2013. Baroreflex sensitivity: mechanisms and measurement. *Netherlands Heart Journal,* 21**,** 58-60.

TAN, H. K., LITTLE, S., LEELARATHNA, L., WALKINSHAW, E., LUBINA-SOLOMON, A., HOSKING, J., SPEIGHT, J., KERR, D., HELLER, S. R., EVANS, M. L., SHAW, J. A. M. & FLANAGAN, D. 2016. Low-Blood Glucose Avoidance Training Improves Glycemic Variability in Adults with Type 1 Diabetes Complicated by Imapired Awareness of Hypoglycemia: HypoCOMPaSS Trial. *Diabetes Care,* 39**,** e56-e58.

TATTERSALL, R. B. & GILL, G. V. 1991. Unexplained Deaths of Type 1 Diabetic Patients. *Diabetic Medicine,* 8**,** 49-58.

TESFAYE, S., MALIK, R. A., BOULTON, A. J. M., SPALLONE, V., DYCK, P. J., VINIK, A. I., FREEMAN, R., BERNARDI, L., HOROWITZ, M., VALENSI, P., KEMPLER, P., GIUSEPPE, L. & GROUP, T. D. N. E. 2010. Diabetic Neuropathies: Update on Definitions, Diagnostic , Estimation of Severity and Treatments. *Diabetes Care,* 33**,** 2285-2293.

TESFAYE, S. & SELVARAJAH, D. 2012. Advances in the epidemiology, pathogenesis and management of diabetic peripheral neuropathy. *Diabetes Metab Res Rev,* 28**,** 8-14.

THOMAS, R. M., ALDIBBIAT, A., GRIFFIN, W., COX, M., LEECH, N. & SHAW, J. 2007. A randomized pilot study in Type 1 diabetes complicated by severe hypoglycaemia, comparing rigorous hypoglycaemia avoidance with insulin analogue therapy, CSII or education alone. *Diabetic Medicine,* 24**,** 778-783.

TRIPOSKIADIS, F., KARAYANNIS, G., GIAMOUZIS, G., SKOULARIGIS, J., LOURIDAS, G. & BUTLER, J. 2009. The Sympathetic Nervous System in Heart Failure. Physiology, Pathophysiology and Clinical Implications. *Journal of the American College of Cardiology,* 54**,** 1747-1762.

VALENSI, P., PARIES, J. & ATTALI, J. 2003. Cardiac autonomic neuropathy in diabetic patients: influence of diabetes duration, obesity, and microangiopathic complications—the french multicenter study. *Metabolism, clinical and experimental,* 52**,** 815-820.

VANDER, A., SHERMAN, J. & LUCIANO, D. 1998. *Human Physiology. The Mechanisms of Body Function.*, McGraw-Hill Companies.

VINIK, A. I., MITCHELL, B. D., MASER, R. E. & FREEMAN, R. 2003. Diabetic Autonomic Neuropathy. *Diabetes Care,* 26**,** 1553-1579.

VINIK, A. I. & ZIEGLER, D. 2007. Diabetic Cardiovascular Autonomic Neuropathy. *Circulation,* 115**,** 387-397.

WESSELING, K. H. 1990. Finapres, continuous noninvasive finger arterial pressure based on the method of Peñáz. *In:* MEYER-SABELLEK, W., R, G., M, A. & L, S. (eds.) *In Blood Pressure Measurements.* Steinkopff.

WHITING, M. J. & DOOGUE, M. P. 2009. Advances in Biochemical Screening for Phaeochromocytoma using Biogenic Amines. *Clin Biochem Rev.,* 30**,** 3-17.

WITTE, D. R., TESFAYE, S., CHATURVEDI, N., EATON, S. E. M., KEMPER, S. F., FULLER, J. H. & GROUP., E. P. C. S. 2005. Risk factors for cardiac autonomic neuropathy in type1 diabetes mellitus. *Diabetologia,* 48**,** 164-171.

WORKGROUP, A. 2005. Defining and reporting hypoglycaemia in diabetes: A report from the American Diabetes Association Workgroup on Hypoglycaemia. *Diabetes Care,* 28**,** 1245-1249.

YAU, J. W. Y., ROGERS, S., KAWASAKI, R., KOWALSKI, J., CHEN, S.-J. & DEKKER, J. 2012. Global Prevalence and Major Risk Factors of Diabetic Retinopathy. *Diabetes Care,* 35**,** 556-64.

ZIEGLER, D., DANNEHL, K., VOLKSW, D., MUHLEN, H., SPULER, M. & GRIES, F. 1992. Prevalence of cardiovascular autonomic dysfunction assessed by spectral analysis and standard tests of heart rate variation in newly diagnosed IDDM patients. *Diabetes Care,* 15**,** 908-911.

ZOUNGAS, S., PATEL, A., CHALMERS, J., GALAN, B. E. D., LI, Q., BILLOT, L., WOODWARD, M., NINOMIYA, T., NEAL, B., MACMAHON, S., GROBBEE, D., KENGNE, A. P., MARRE, M. & HELLER, S. R. 2010. Severe Hypoglycaemia and Risks of Vascular Events and Death. *N Engl J Med,* 363**,** 1410-1418.

ZOYSA, N. D., ROGERS, H., STADLER, M., GIANFRANCESCO, C., BEVERIDGE, S., BRITNEFF, E., CHOUDHARY, P., ELLIOTT, J., HELLER, S. R. & AMIEL, S. A. 2013. A Psychoeducational Program to Restore Hypoglycemia Awareness: The DAFNE-HART Pilot Study. *Diabetes Care,* 37**,** 863-866.

Appendix 1: ADA/WHO Definition of T1DM

1. Fasting plasma glucose ≥ 7.0 mmol/l. Fasting is defined as no caloric intake for at least 8 h.

OR

2. Symptoms of hyperglycaemia and a casual plasma glucose ≥11.1 mmol/l. Casual is defined as any time of day without regard to time since last meal. The classic symptoms of hyperglycaemia include polyuria, polydipsia, and unexplained weight loss.

OR

3. 2-h plasma glucose ≥ 11.1 mmol/l during an OGTT. The test should be performed as described by the World Health Organization, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.

In the absence of unequivocal hyperglycaemia, these criteria should be confirmed by repeat testing on a different day.

Appendix 2: Gold Score

The Gold Score

Do you know when your hypos are commencing? Please circle a number:

	Always aware						Never aware
Awareness	1	2	3	4	5	6	7

Appendix 3: Hypoglycaemic Questionnaire

Hypoglycaemic Questionnaire

Date: Time:

Initials:

ON A SCALE OF 1 (NOT AT ALL) TO 7 (VERY SEVERE), CIRCLE THE NUMBER THAT YOU THINK BEST DESCRIBES YOUR AWARENESS OF EACH SYMPTOM.

	NOT AT ALL						VERY SEVERE
ODD BEHAVIOUR	1	2	3	4	5	6	7
POUNDING HEART	1	2	3	4	5	6	7
ITCHING	1	2	3	4	5	6	7
DROWSINESS	1	2	3	4	5	6	7
DIFFICULTY SPEAKING	1	2	3	4	5	6	7
SHAKING/TREMOR	1	2	3	4	5	6	7
HEADACHE	1	2	3	4	5	6	7
HUNGER	1	2	3	4	5	6	7
CLUMSINESS/ INCOORDINATION	1	2	3	4	5	6	7
SWEATING	1	2	3	4	5	6	7
CONFUSION	1	2	3	4	5	6	7
NAUSEA	1	2	3	4	5	6	7
DIZZINESS	1	2	3	4	5	6	7
BLURRED VISION	1	2	3	4	5	6	7
ANXIETY	1	2	3	4	5	6	7
WEAKNESS	1	2	3	4	5	6	7
TINGLING	1	2	3	4	5	6	7
WARMTH	1	2	3	4	5	6	7
IRRITABILITY	1	2	3	4	5	6	7

DO YOU FEEL AS THOUGH YOUR BLOOD GLUCOSE LEVEL IS LOW? YES ___ NO ____

Please tick (√) appropriate box

What do you think your glucose level is?_____mmol/l