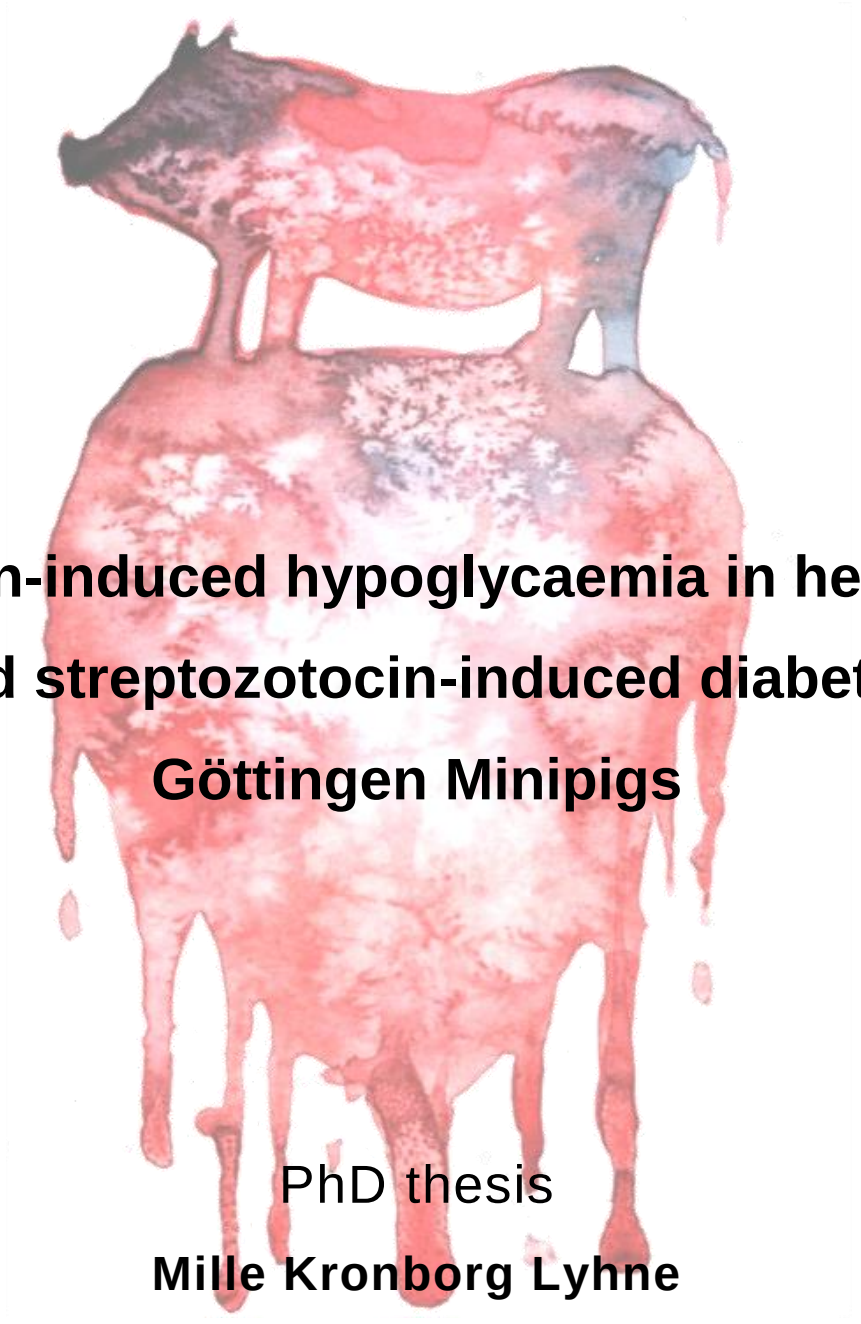
UNIVERSITY OF COPENHAGEN
FACULTY OF HEALTH AND MEDICAL SCIENCES



Insulin-induced hypoglycaemia in healthy and streptozotocin-induced diabetic Göttingen Minipigs

PhD thesis

Mille Kronborg Lyhne

This thesis was submitted to the Graduate School of Health and Medical Sciences, University of Copenhagen, on October 4th, 2022.

Insulin-induced hypoglycaemia in healthy and streptozotocin-induced diabetic Göttingen Minipigs

PhD thesis

Mille Kronborg Lyhne

University of Copenhagen

Faculty of Health and Medical Sciences

Department of Veterinary and Animal Science

Section for Experimental Animal Models

&

Large Animal Pharmacology

Novo Nordisk A/S

This thesis was submitted to the Graduate School of Health and Medical Sciences, University of Copenhagen, on October 4th, 2022.

Submitted on: October 4th 2022

Principal supervisor: **Lisbeth Høier Olsen**, Professor, DVSc, DVM
Department of Veterinary and Animal Sciences,
University of Copenhagen, Denmark

Co-supervisors: **Ulrik Pedersen-Bjergaard,** Professor, DMSc, MD
Department of Endocrinology and Nephrology
Hospital of North Zealand, Hillerød, Denmark

Jeppe Sturis, Senior Scientific Director, PhD, MSc
Andreas Vegge, Research Project Vice President, PhD, DVM
Jonas Kildegaard, Director, PhD, MSc
Rita Slaaby, Principal Scientist, PhD, MSc
Gro Klitgaard Povlsen, Research Project Vice President, PhD, MSc
Global Drug Discovery, Novo Nordisk A/S, Måløv, Denmark

Assessment committee: **Axel Kornerup Hansen**, Professor, DVSci, DVM
Department of Veterinary and Animal Sciences,
University of Copenhagen, Denmark

Aage Kristian Olsen Alstrup, Associate Professor, DVSc, PhD, DVM
Department of Clinical Medicine, Aarhus University, Aarhus, Denmark
Nuclear Medicine and PET, Aarhus University Hospital, Skejby, Denmark.

Alison Delamare McNeilly, Dr, PhD
Principal Investigator (Tenure Track)
Systems Medicine, University of Dundee, Dundee, United Kingdom

Funding: This PhD project was funded by the LIFEPHARM Centre at University of Copenhagen and Novo Nordisk A/S, Måløv, Denmark.

Conflict of interest: The author declares no personal or financial conflicts of interest.

Front page: Watercolour by the author.

Table of contents

Preface

This PhD project was funded by the LIFEPHARM Centre, a collaboration between University of Copenhagen and Novo Nordisk A/S, with enrolment in the In Vivo Pharmacology Graduate Programme at the Faculty of Health and Medical Sciences at University of Copenhagen with employment at the Department of Veterinary and Animal Sciences, Section for Experimental Animal Models. The animal studies included in this thesis were carried out at Novo Nordisk A/S Animal Unit in Ganløse, with the help of the Large Animal Pharmacology department at Novo Nordisk A/S, Måløv. The work was carried out from 2019 until 2022, with a period of maternity leave in between.

This thesis is a synopsis of the three, main studies conducted during my PhD. It contains a short summary, objectives of the studies, background, methods used and finally, a brief summary and discussion of the results with conclusions and perspectives.

Mille Kronborg Lyhne
October 4th 2022

Acknowledgements

While the initial premise of this PhD project was somewhat different to studies presented in this thesis, I am still immensely satisfied with the overall work process, learnings and results from this project.

Throughout this project, I have received help and support from many different people. I would like to thank my present and past supervisors - Lisbeth Høier Olsen, Ulrik Pedersen-Bjergaard, Andreas Vegge, Jonas Kildegaard, Jeppe Sturis, Gro Klitgaard Povlsen and Rita Slaaby, as well as my manager at Novo Nordisk, Tanja Pedersen, for their guidance and intelligent insights.

I would also like to thank the fantastic lab techs who helped me in making the practical part of this project come to fruition. Anja, Merete, Sebrina, Charlotte, Trine, Wei, Lotte and Sanne from Novo Nordisk A/S and Marianne and Carl at UCPH – without you, there would be no data.

Parts of this project were also made into three master theses. Terese Helgogaard, Kathrin Hinz and Rükiye Köse – thank you for lending a helping hand in my studies. I truly enjoyed working with you. Also, thank you to guest scientist Caroline Degel for dealing with a bit of data I did not manage to finish analysing myself.

Also, thank you to my collaborators – Steen Larsen and Caroline de Blanck at Xlab, UCPH, Jason Li and Giovanni Traverso, MIT, as well as Markus Hodal Drag and Jonathan Belanich, CBMR, UCPH.

To all my PhD colleagues, Karina, Heidi, Ditte, Louise, Laura, Victoria and more – thank you for +3 years of mutual support.

Lastly, I would like to thank my family for their support and flexibility during this project, allowing me to focus on science without neglecting what is most important in my life.

Original manuscripts

Main papers

I: Electrocardiography and heart rate variability in Göttingen Minipigs: Impact of diurnal variation, lead placement, repeatability and streptozotocin-induced diabetes.

Mille Kronborg Lyhne, Karina Poulsdóttir Debes, Terese Helgogaard, Andreas Vegge, Jonas Kildegaard, Ulrik Pedersen-Bjergaard and Lisbeth Høier Olsen

Journal of Pharmacological and Toxicological Methods, volume 118, November–December 2022, (107221), https://doi.org/10.1016/j.vascn.2022.107221

II: Hyperinsulinaemic hypoglycaemia in non-anaesthetized Göttingen Minipigs induces a counter-regulatory endocrine response and electrocardiographic changes.

Mille Kronborg Lyhne, Andreas Vegge, Gro Klitgaard Povlsen, Rita Slaaby, Jonas Kildegaard, Ulrik Pedersen-Bjergaard and Lisbeth Høier Olsen

Scientific Reports, volume 11, Article number: 5983 (2021), https://doi.org/10.1038/s41598-021-84758-w

III: Healthy and streptozotocin-induced Göttingen Minipigs as a model of counter-regulatory failure in recurrent hypoglycaemia.

Mille Kronborg Lyhne, Kathrin Hinz, Caroline Degel, Rükiye Köse, Heidi Young Nilsen, Jeppe Sturis, Andreas Vegge, Jonas Kildegaard, Ulrik Pedersen-Bjergaard and Lisbeth Høier Olsen

Manuscript in preparation.

Conference abstracts and posters

Not included in the thesis.

381-P: Recurrent Hypoglycemia Alters Endocrine Counter-regulation during Insulin-Induced Hypoglycemia in Healthy and Diabetic Göttingen Minipigs

Mille Kronborg Lyhne; Kathrin Hinz; Andreas Vegge; Jeppe Sturis; Jonas Kildegaard; Ulrik Pedersen-Bjergaard; Lisbeth Høier Olsen

American Diabetes Association Scientific Sessions 2022.

Diabetes 2022; 71 (Supplement 1): 381-P (abstract and poster)

Cardiac and endocrine responses to hyperinsulinaemic hypoglycaemia in healthy and diabetic Göttingen Minipigs.

Mille Lyhne Skøttrup, Andreas Vegge, Gro Klitgaard Povlsen, Rita Slaaby, Jonas Kildegaard, Ulrik Pedersen-Bjergaard and Lisbeth Høier Olsen

European Association for the Study of Diabetes Annual Meeting 2020.

Diabetologica 2020, P964 (abstract and poster)

Abbreviations

AV block	2nd degree atrio-ventricular block
CAN	Cardiac autonomic neuropathy
CVD	Cardiovascular disease
ECG	Electrocardiography
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
HF	High frequency power
HRV	Heart rate variability
I.V.	Intra-venous
IAH	Impaired awareness of hypoglycaemia
LF	Low frequency power
LF/HF	LF to HF ratio
PG	Plasma glucose
PLT	Platelet
QTc	R-R interval corrected QT-interval
RH	Recurrent hypoglycaemia
S.C.	Sub-cutaneous
STZ	Streptozotocin
SVE	Supraventricular ectopic complex
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
VE	Ventricular ectopic complex
VHF	Very high frequency power
VLF	Very low frequency power
QTcf	QT-interval corrected using Fridericia's formula
QTcb	QT-interval corrected using Bazett's formula

English summary

Hypoglycaemia is a common adverse effect of insulin treatment in people with diabetes, ranging from mild, self-corrected episodes to severe incidents requiring medical assistance. Hypoglycaemia in diabetes has been associated with increased risk of death and cardiovascular events, however, no direct mechanistic link between cardiovascular disease and hypoglycaemia has yet been identified. Impaired awareness of hypoglycaemia is diminished symptoms and counter-regulatory response to hypoglycaemia, often occurring in people with type 1 diabetes and is thought to be caused by recurrent hypoglycaemic episodes, resulting in an increased risk of further, more severe, hypoglycaemic episodes. While effects on the endocrine counter-regulation and myocardium of single and repeated episodes of hypoglycaemia have been investigated in rodent models, studies in large animals are sparse.

The aim of this PhD project was to investigate healthy and streptozotocin-induced diabetic Göttingen Minipigs as a translational model of hyperinsulinaemic hypoglycaemia. The studies investigated the normal minipig electrocardiogram, electrocardiographic changes and endocrine counter-regulation during hypoglycaemia as well as platelet activity.

Paper I investigated the electrocardiographic features of healthy and diabetic minipigs and described diurnal variation in both morphology and heart rate variability, lead differences in morphology and change in parameters over time, but could only detect minor changes after diabetes induction.

Paper II utilised healthy and diabetic minipigs to demonstrate a human-like endocrine counter-regulatory response to hyperinsulinaemic hypoglycaemia, with increases in plasma glucagon and epinephrine. Hyperinsulinaemic hypoglycaemic clamping also induced QTc-interval prolongation in diabetic minipigs. An increase in arrhythmic events was seen in both healthy and diabetic minipigs, changes also reported in humans experiencing hypoglycaemia. However, no changes in heart rate variability was found. Platelet aggregation response decreased during hypoglycaemia, contrary to the hypoglycaemia-induced increase reported in human studies.

Paper III investigated the effects of antecedent hypoglycaemic episodes on the counter-regulatory response and electrocardiogram in healthy and diabetic minipigs during hyperinsulinaemic hypoglycaemic clamping. The study demonstrated a diminished glucagon response in groups preconditioned with three consecutive days of hypoglycaemic clamping, with no effect of diabetes status. There was no significant difference in the epinephrine response between hypoglycaemia preconditioned groups and controls. Analysis of electrocardiographic recordings are pending.

Overall, the Göttingen Minipig model of insulin-induced hypoglycaemia demonstrated translational value in regards to counter-regulatory responses and morphological electrocardiographic changes. The model shows potential for future studies of biology, pharmacological interventions and development of devices and monitors for detection and prevention of hypoglycaemia with perspectives to better clinical management of people living with diabetes.

Resumé (Danish summary)

Hypoglykæmi er en hyppig bivirkning af insulinbehandling i mennesker med diabetes, rangerende fra milde, selv-korrigerede episoder til alvorlige hændelser som kræver lægehjælp. Hypoglykæmi i diabetes er blevet associeret med øget risiko for død og kardiovaskulære komplikationer, men der er endnu ikke fundet en direkte mekanistisk sammenhæng mellem kardiovaskulær sygdom og hypoglykæmi. Manglende opfattelse af hypoglykæmi (impaired awareness of hypoglycaemia) er associeret med mindre forekomst af symptomer og nedsat mod-regulatorisk respons på hypoglykæmi. Det er hyppigt forekommende hos mennesker med type 1 diabetes og menes at skyldes gentagende, forudgående hypoglykæmiske episoder. Dette resulterer i en øget risiko for endnu flere, og mere alvorlige, hypoglykæmi-episoder. Selvom effekterne af hypoglykæmi-episoder på det endokrine mod-regulatoriske og kardiovaskulære respons er undersøgt i gnavermodeller, findes der kun ganske få studier i større dyr.

Formålet med dette Ph.d.-projekt var at undersøge raske og streptozotocin-inducerede diabetiske Göttingen Minipigs som en translationel model for hyperinsulinæmisk hypoglykæmi. Studierne validerede længerevarende optagelser af elektrokardiografi i vågne grise samt undersøgte den endokrine mod-regulation, elektrokardiografiske ændringer samt ændringer i blodpladeaggregation som respons på enkeltstående eller gentagende hypoglykæmiske episoder.

Artikel I undersøgte raske og diabetiske minigrises elektrokardiografiske karakteristika. Der fandtes signifikant døgnvariation i både morfologi og hjerterytmevariabilitet, signifikante forskelle i morfologi mellem afledninger og en ændring i parametre over tid, men der kunne kun detekteres mindre forandringer fire måneder efter streptozotocin-induceret diabetes.

I artikel II blev raske og diabetiske minigrise brugt til at demonstrere et menneskelignende endokrint mod-regulatorisk respons til hyperinsulinæmisk hypoglykæmi, med øget plasma glukagon og adrenalin. Hyperinsulinæmisk, hypoglykæmisk clamping inducerede også QTc-interval forlængelse i diabetiske minigrise og øgede antallet af arrytmiske begivenheder i både raske og diabetiske minigrise, forandringer der også er set i mennesker. Der forekom dog ikke nedsat hjertefrekvensvariabilitet. Blodpladeaggregation responset blev nedsat under hypoglykæmi hvilket er modsat den hypoglykæmi-induceret øget blodpladeaggregation som er fundet i mennesker.

Artikel III undersøgte effekterne af gentagende hypoglykæmiske episoder på det mod-regulatoriske respons og elektrokardiogrammet i raske og diabetiske minigrise ved at bruge

hyperinsulinæmisk, hypoglykæmisk clamping. Studiet fandt et nedsat glukagonrespons i grupperne prækonditioneret med tre på hinanden følgende dage hypoglykæmisk clamping, men ingen effekt af diabetes. Der var ikke en signifikant forskel på adrenalinresponset i mellem grupper prækonditioneret med hypoglykæmi og den normoglykæmiske kontrolgruppe. De elektrokardiografiske målinger afventer analyse.

Overordnet viste Göttingen Minipig-modellen for insulin-induceret hypoglykæmi translationel værdi i forhold til de hypoglykæmi-inducerede mod-regulatoriske responser og morfologiske elektrokardiografiske forandringer som blev demonstreret i disse studier. Modellen har potentiale for fremtidige studier til at undersøge biologi, farmakologiske interventioner og udvikling af apparater og monitorering for at kunne detektere, forebygge og behandle hypoglykæmi med perspektiver til bedre klinisk behandling til mennesker der lever med diabetes.

Objectives and hypotheses

The Göttingen Minipig is widely used in cardiometabolic research, however, this large animal model is not yet commonly used as a translational model of insulin-induced hypoglycaemia in diabetes. The overall objective of this PhD project was to investigate the endocrine counter-regulatory and cardiovascular effects of insulin-induced hypoglycaemia in healthy and streptozotocin-induced (STZ) diabetic Göttingen Minipigs.

Study 1: Electrocardiography and heart rate variability in Göttingen Minipigs: Impact of diurnal variation, lead placement, repeatability and streptozotocin-induced diabetes.
This study investigated 24-hour Holter electrocardiographic (ECG) recordings in Göttingen Minipigs before and after STZ-induced diabetes. The aim was to describe a standardised method of recording and analysis of Holter ECG in Göttingen Minipigs, with focus on diurnal changes, changes over four months, differences in ECG lead and repeatability. The hypotheses were, that features of the ECG would have diurnal variations but be constant over four months. Features would vary depending on ECG lead and measurements would have low inter- and intra-observer variability. It was also hypothesised, that STZ-induced diabetes would prolong the QT-interval, increase ST-segment height and decrease HRV parameters.

Study 2: Hyperinsulinaemic hypoglycaemia in non-anaesthetized Göttingen Minipigs induces a counter-regulatory endocrine response and electrocardiographic changes.
This study aimed to develop a translational, large animal model of insulin-induced hypoglycaemia in healthy and STZ-induced diabetic Göttingen Minipigs, comparing bolus dosage and hyperinsulinaemic clamping. The hypotheses were, that hypoglycaemia would induce a counter-regulatory endocrine response similar to what have been observed in humans, ECG changes and alter platelet aggregation response.

Study 3: Healthy and streptozotocin-induced Göttingen Minipigs as a model of counter-regulatory failure in recurrent hypoglycaemia.
The aim of this study was to investigate the counter-regulatory response and ECG changes during insulin-induced hypoglycaemia after hypoglycaemic preconditioning. The hypotheses were, that preconditioning with hypoglycaemia would blunt glucagon and epinephrine release during subsequent hypoglycaemia and ECG changes caused by this would be blunted, compared to a control group. Furthermore, it was hypothesised, that STZ-induced diabetes would exaggerate these changes compared to healthy controls.

Description of international state-of-the-art research in context of the research project

Epidemiology of diabetes and hypoglycaemia

The prevalence of diabetes in the population is increasing at an alarming rate. An estimated 463 million people are living with diabetes in 2019 with an expected increase to 578 million people in 2030[1].

Type 1 diabetes mellitus (T1DM) is caused by autoimmune, inflammatory destruction of the β-cells of the pancreas, leading to a progressive loss of β-cell mass and endogenous insulin production, resulting in hyperglycaemia[2]. T1DM is thought to account for approximately 10% of all diabetes cases, but the prevalence is increasing[2]. The cause of type 2 diabetes mellitus (T2DM) is thought to multifactorial, with both genetic and life-style components, but is characterised by a marked insulin resistance and steadily progressing insulin secretory deficiency due to ongoing loss of β-cell mass[2]. Consequently, the markedly altered glucose homeostasis results in the need for exogenous insulin administration in people with T1DM and long-standing T2DM to control hyperglycaemia and the number of people in insulin treatment is rising[3]. Treatment with insulin or insulin analogues, in combination with the insulin resistance found in most people with T1DM and T2DM, creates a state of hyperinsulinaemia in which hypoglycaemia can occur[3].

Hypoglycaemia is a common adverse effect of insulin treatment[3] and is considered to be an important barrier to glycaemic control in people with diabetes[3,4]. Hypoglycaemia is most prevalent in people with T1DM, but the amount of people with insulin-treated T2DM is rising, resulting in an increasing population of people at risk for hypoglycaemia[3]. Adults with T1DM are estimated to experience around two episodes of mild hypoglycaemia a week and 30% experience at least one severe episode a year[3,5]. The frequency of severe hypoglycaemia in people with T2DM is lower than in T1DM, but is increasing with duration of insulin use, corresponding to the progression of β-cell failure in the disease[3]. Deficiency in counter-regulatory endocrine responses, resulting in impaired awareness of hypoglycaemia, also increases the risk of experiencing severe hypoglycaemia, particularly in people with T1DM[6].

The fear of hypoglycaemia has a major impact in people living with diabetes, affecting many aspects of daily life[4,7] and is a barrier to reaching treatment goals[4,7]. In a Norwegian study, death directly caused by hypoglycaemia had a prevalence of up to 8% in people with T1DM[8]. In the

ACCORD study from 2008, intensive glucose lowering therapy strategy resulted in increased mortality, resulting in a premature termination of the study[9]. The study identified a previously unknown connection between intensive treatment of diabetes and mortality. The DEVOTE study of 2018 revealed, that the risk of death of any cause increased even after a single episode of severe hypoglycaemia and this risk persisted up to one year after the event[10].

There are several effects of severe hypoglycaemia that persists after the event, including an increase in circulating inflammatory markers days after the episode and alterations in haemostasis, creating an environment that might be conductive to a thrombotic event[3]. Cardiovascular disease is common in people with diabetes and the stress put on the vasculature and heart during hypoglycaemia is speculated to exacerbate the pathology[3]. Several studies have associated cardiovascular events and hypoglycaemia, however, the mechanistic link seems to be multifactorial[11] and needs to be investigated further.

Endocrine counter-regulation during hypoglycaemia

Hypoglycaemia occurs when insulin is over-dosed according to need and the presence of excess exogenous insulin causes glucose to be removed from the circulation, resulting in low plasma glucose. The normal range of plasma glucose in healthy people is 4.0-8.0 mM[12] but already when plasma glucose drops below 4.5 mM, a series of counter-regulatory responses takes place[13].

The first responses to low plasma glucose is direct sensing of glucose levels in the pancreatic β- and α- cells, resulting in decrease of insulin secretion from pancreatic β-cells[13] and later, an increase in glucagon secretion from pancreatic α-cells[13]. Glucagon stimulates glycogenolysis and gluconeogenesis to increase circulating glucose[13]. In people with T1DM and long-standing T2DM, the glucagon response to hypoglycaemia is diminished[13,14] with epinephrine having an increased importance in the counter-regulatory response to hypoglycaemia in these populations[13].

Decreasing plasma glucose levels are detected by the glucose sensing areas of the brain, initiating a sympathetic response with effects on both the adrenal gland and pancreas[15]. At a plasma glucose level below 3.6-3.9 mM, the sympathoadrenal response causes secretion of the catecholamines epinephrine and norepinephrine from the adrenal gland and sympathetic neural activation[16]. Catecholamines, in particular epinephrine, released from the adrenal gland cause hemodynamic and cardiovascular changes during hypoglycaemia[17], as well as symptoms including sweating and palpitations, while the neural activation is responsible for hunger, tremors and anxiety[16]. Epinephrine released from the adrenal gland also works on α1-and β2-adrenoceptors in skeletal muscle to reduce glucose uptake and in the liver, together with

glucagon, stimulates gluconeogenesis to increase plasma glucose[13,17] as well as further stimulation of the pancreatic release of glucagon[13].

In people with T1DM and long-standing T2DM, the epinephrine response seems to be the primary glucose-restoring mechanism[18], as these populations have impaired glucagon response to hypoglycaemia[17]. Growth hormone and cortisol are also secreted in response to hypoglycaemia and have a more delayed response compared to epinephrine and glucagon[12], with both hormones limiting glucose utilisation and increase glucose production hours after the event[13].

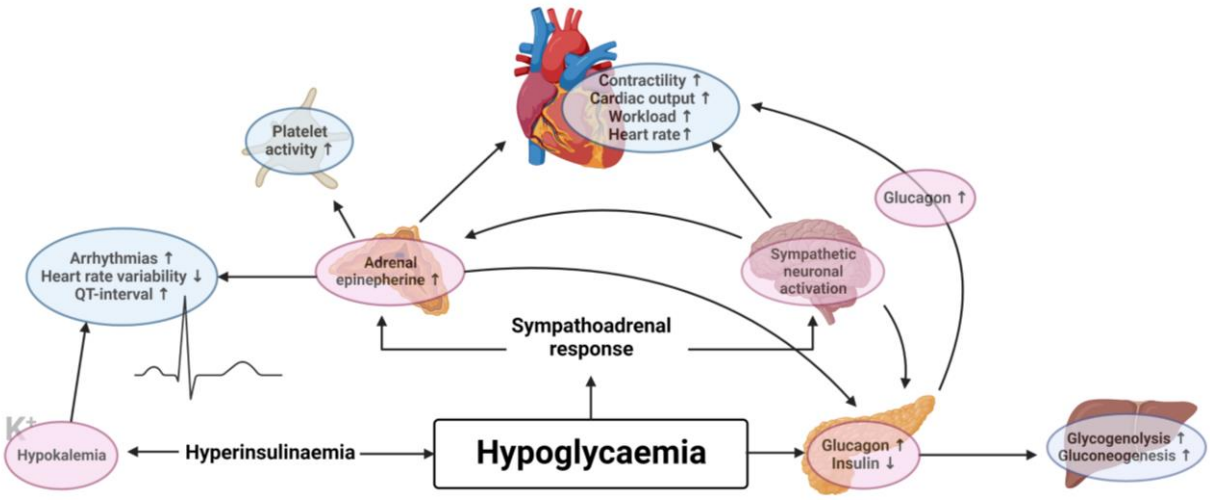


Figure 1: Simplified overview of the main effects of hyperinsulinaemic hypoglycaemia as described in the text. K^+ indicates blood potassium levels. Created with Biorender.com

Impaired awareness of hypoglycaemia

In humans, a fasting plasma glucose level above 3.9 mM is considered normal[19]. While counter-regulatory mechanisms to hypoglycaemia in people without diabetes are already initiated at a plasma glucose around 4.4 mM, the threshold for initiation of regulatory mechanisms can be altered in people with diabetes. People with diabetes are at risk of developing impaired awareness of hypoglycaemia (IAH) and a study has shown that IAH is present in 19% of people with T1DM[6]. This is mediated by counter-regulatory failure which both alters plasma glucose threshold of symptoms of hypoglycaemia but also the establishment of an appropriate endocrine counter-regulatory response[20]. As little as a single episode of hypoglycaemia alters the endocrine and symptomatic responses to hypoglycaemia, setting the premise for IAH[21].

In IAH, the threshold for hypoglycaemia awareness is lowered with the sympathoadrenal response being initiated at lower plasma glucose levels resulting in later onset of symptoms[20]. The mechanism of which recurrent episodes of hypoglycaemia induce IAH is not entirely

understood, but is thought to arise from a series of glyopenic adaptations throughout the nervous system[22] and is also theorised to stem from habituation to hypoglycaemia[23]. Glucose sensing neurons in the brain readily reacts to hypoglycaemia to induce a sympathoadrenal response, but are also subjected to the adaptive effects of recurrent episodes of hypoglycaemia, lowering the level of glucose needed to activate this response[15,22]. In an individual with normal glucose homeostasis, the adaptation might be advantageous in regards to starvation[22]. However, in people with diabetes receiving exogenous insulin and who are therefore not able to switch off the glucose-depriving effect of insulin, this results in an increased risk of hypoglycaemia[22]. In more severe episodes of hypoglycaemia, a person with IAH might not sense their low blood glucose and take measures to increase it, resulting in an increased risk of experiencing neuroglycopenia. Severe, acute neuroglycopenia can lead to brain damage, while in the long term, mild episodes can alter brain functions[22,24].

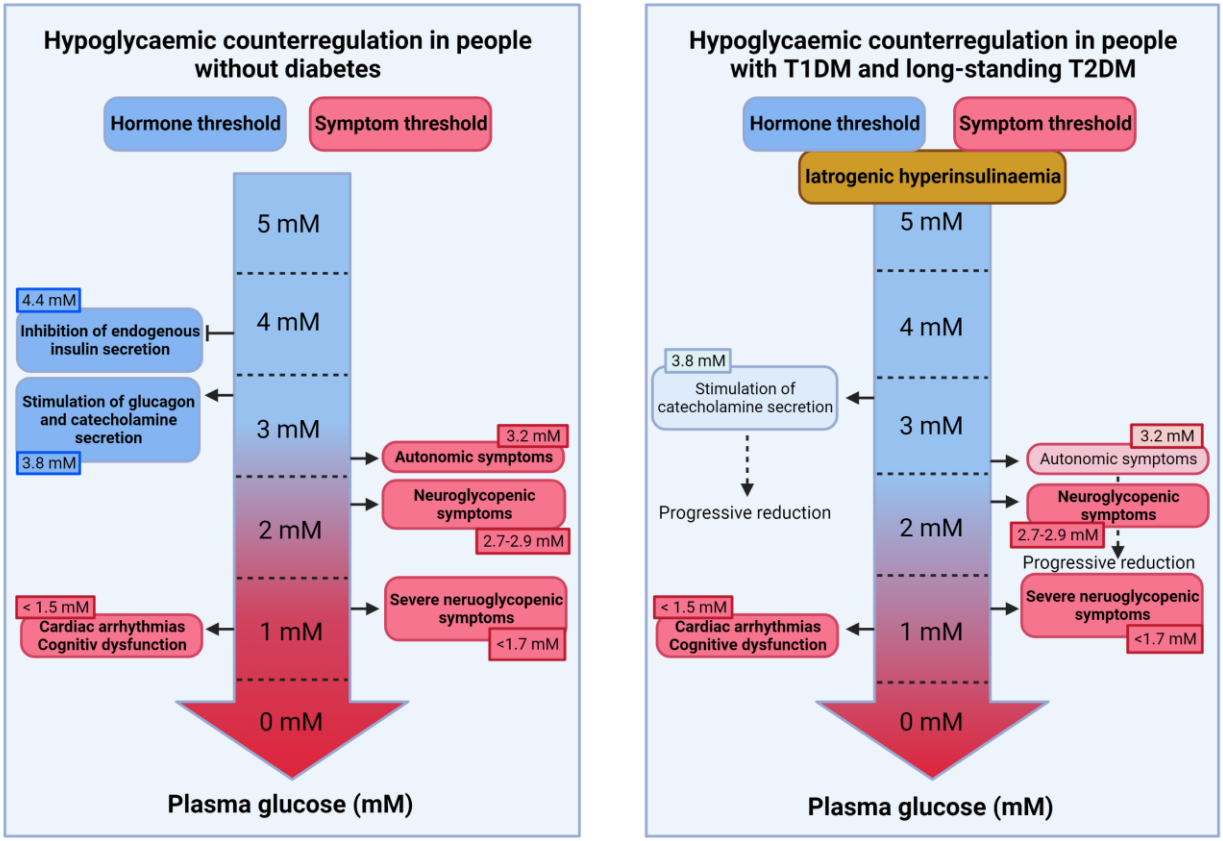


Figure 2: Schematic overview of endocrine-counter regulation in people with and without diabetes. In people with type 1 diabetes (T1DM) and long-standing type 2 diabetes (T2DM), the endocrine counter-regulation in hypoglycaemia is dominated by the sympathoadrenal response. Recurrent hypoglycaemia progressively diminishes the sympathoadrenal response, resulting in an altered threshold for catecholamine release and subsequent hypoglycaemic symptoms. Modified (figure on the right side with arrows for progressive reduction and removal of inhibition of insulin and secretion of glucagon) with author permission from Sankar *et al.* 2020[25] https://doi.org/10.1016/j.tem.2020.05.008 under Creative Commons Attribution-NonCommercial-No Derivatives License https://creativecommons.org/licenses/by-nc-nd/4.0/. Created with Biorender.com.

Cardiovascular complications of diabetes and hypoglycaemia

The incidence and risk of CVD is higher in people with diabetes compared to the general population[26,27] and is the most prevalent cause of morbidity and mortality in people with diabetes[11] with up to 80% dying from a cardiovascular event[28].

Hyperglycaemia has long been considered the major cause of microvascular complications[29], with diabetes treatment aiming to keep blood glucose within an acceptable range[19]. However, the opposite, hypoglycaemia, might be true for major adverse cardiovascular events such as stroke and ischaemic heart disease[28]. Analysis of data from the DEVOTE study of people with T2DM revealed that there was an increased risk of death of cardiovascular cause at any time after an episode of severe hypoglycaemia[10]. While several studies have confirmed the link between CVD and hypoglycaemia, the exact underlying mechanisms have not yet been found[28].

Several pathologies caused by hypoglycaemia have been suggested to increase the risk of CVD, including altered blood coagulation and inflammation[30], changes in cardiac repolarisation and arrhythmias[31,32], endothelial dysfunction[33] and myocardial ischemia[3]. Hypoglycaemia also induces a sympathoadrenal response where catecholamines increasing the workload of the heart, which can be particularly stressful for the cardiovascular system in people with existing heart disease[3]. Also, sudden death in younger people with T1DM with nocturnal hypoglycaemia is speculated to be related to fatal, hypoglycaemia-induced arrhythmias[34].

Influence of diabetes and hypoglycaemia on myocardial function

Hypoglycaemia induces a sympathoadrenal response leading to the secretion of epinephrine. While the rise in epinephrine levels is a counter measure to hypoglycaemia, as described above, the effects on the myocardium might be stressful in people with pre-existing CVD[35]. During hypoglycaemia, epinephrine increases sympathetic tone of the heart, resulting in increased heart rate and systolic blood pressure[36] mediated via β-adrenoreceptor activation[37]. The myocardial contractility and cardiac output increase[38] with a decline in peripheral vascular resistance[36] and increase in left ventricular ejection fraction[28,36].

The other main defence against hypoglycaemia is increased glucagon secretion to mobilise glucose, however, glucagon also exerts positive inotropic and chronotropic effects on the heart[39]. These effects increase the cardiac workload, further increasing the oxygen and fuel consumption of the heart[40]. Glucagon has been demonstrated to have anti-arrhythmic properties[40], which are thought to be mediated by alterations in the sinus node firing rate[41].

Studies in people with both T1DM[42] and T2DM[43] have demonstrated increased prevalence of arrhythmias during hypoglycaemia. Alterations in cardiac repolarisation, seen as prolongation of the QT-interval, have been widely documented in studies of both experimentally induced[44,45] and spontaneous hypoglycaemia[31,42,46]. QT-interval prolongation is considered pro-arrhythmic and new pharmacological therapies are required to screen for potential off-target QT-interval effects[47]. QT-interval prolongation can lead to a potential fatal arrhythmias, such as Torsade-de-pointes and ventricular fibrillation[48].

Prolongation of the QT-interval can be prevented by pharmacological β1-adrenoreceptor blockade prior to induced hypoglycaemia and has also been associated with high epinephrine levels[44,49], indicating sympathetic activation[50]. Hyperinsulinaemia during normoglycaemia also prolongs the QT-interval, most likely through epinephrine stimulation and hypokalemia[51].

Hyperinsulinaemia alone can cause hypokalemia as insulin promotes entry of potassium ions into hepatic and skeletal cells by increasing the activity of the Na+-K+-ATPase pump[52]. Hypokalemia in hypoglycaemia also seems to be exacerbated by sympathetic stimulation from epinephrine[53]. Arrhythmias such as Torsades-de-pointes, ventricular fibrillation, ventricular ectopies and polymorphic ventricular tachycardia have all been associated with hypokalemia[54]. The arrhythmias are thought to arise from altered cardiac repolarisation due to altered K^+ and Ca^{2+} currents and homeostasis, caused by the hypokalemia[54,55].

Nocturnal, severe hypoglycaemia is a particular concern in regards to arrhythmias as it is often more severe than daytime episodes, is of longer duration, and often go undetected by the person in their sleep[11,42]. Arrhythmias have been recorded during nocturnal hypoglycaemia[34] and are theorised to be the cause of ‘dead-in-bed’ syndrome in younger people with T1DM[56].

Considering the theories that hypoglycaemic episodes increase the risk of cardiovascular events[11], rat studies have demonstrated a protective effect of recurrent hypoglycaemia in regards to fatal arrhythmias, which is most likely mediated by a diminished epinephrine response[57]. Human studies have shown, that IAH in people with T1DM is associated with progressive loss of heart rate variability (HRV)[58] which is a diagnostic marker of cardiac autonomic neuropathy, a serious and common complication of diabetes that has a major impact on development of CVD[59].

Heart rate variability in diabetes and hypoglycaemia

Cardiovascular autonomic neuropathy (CAN) is a late-stage complication of diabetes thought to affect up to 60% of people with T1DM, 15 years after diagnosis[60]. Multiple risk factors have been described, such as poor glycaemic control, dyslipidaemia and hypertension, but the pathogenesis of the complication is not truly understood[60]. Several mechanisms have been proposed, the main being hyperglycaemic damage. Hyperglycaemia can increase the production of reactive oxygen species from the mitochondria, resulting in oxidative damage to the microvasculature in relation to the nerves[60-62]. To diagnose CAN, heart rate variability is used in the array of tests to conclude the diagnosis[60].

Heart rate variability (HRV) is the variation in the time interval between consecutive heart beats over time. This variation in interval is dependent on heart rate, which in turn is modulated by the autonomic nervous system. The autonomic regulation of the heart depends on direct innervation and circulating hormones, such as epinephrine. There is a dynamic balance between sympathetic and parasympathetic stimulation of the heart, resulting in an ever changing variation in heart rate. A high, but not too high, HRV is considered an indicator of good cardiac health[63] and is thought to demonstrate the ability of the heart to respond to sympathetic and parasympathetic simuli[64]. Parameters of the HRV can be described using different mathematical methods. Time-domain analysis investigates the R-R intervals of the ECG to describe the overall variation over a specified time period. Frequency domain analysis transforms the R-R interval data, often using Fast Fourier-transformation. The result is given as a specific power in predefined bands spanning high to low frequencies which have been associated with different components of autonomic regulation of the heart[63]. Frequency-domain parameters have been associated with different physiological functions and specific pathologies. Very low frequency (VLF) power is thought to arise from the parasympathetic innervation of the heart as well as the intrinsic nervous system and has been associated with all-cause mortality, with low VLF indicated in arrhythmic death[65], but also psychological disorders[63]. The low frequency (LF) power band mostly reflect baroreceptor activity during rest and is influenced by breathing[63]. The high frequency (HF) power band corresponds to respiration due to vagal modulation during inhalation and exhalation and reflects parasympathetic activity with increased band power at night[63]. The ratio of LF to HF power reflects the autonomic balance of the heart, with high values indicating sympathetic dominance and low values parasympathetic dominance[63].

During both spontaneous and induced hypoglycaemia in healthy people and people with diabetes, decreases in HRV parameters can be detected[66-70]. Alterations in HRV have been investigated as a method of early detection of hypoglycaemia by wearable Holter ECG devices[70,71].

The concurrence of decreased HRV and hypoglycaemia in the setting of heart disease, underlines the need for investigation and prevention of hypoglycaemia in regards to cardiac health. Decreased time- and frequency-domain HRV has been associated with increased risk of mortality and cardiovascular events in people with pre-existing CVD[72]. Alterations in time- and frequency-domain parameters of HRV have been shown to predict development of CVD in people with T2DM[73] and presence of impaired awareness of hypoglycaemia (IAH) is associated with progressive loss of HRV in people with T1DM[58].

Platelet function in diabetes and hypoglycaemia

Alterations of the thrombotic state is considered a factor in development of CVD[74,75]. Platelet dysfunction has been demonstrated in both T1DM and T2DM[75,76], resulting in a pro-thrombotic state in these populations. The effect of diabetes on platelets is not clear. A study demonstrated decreased platelet aggregation after insulin administration in healthy subjects and argue, that the increased platelet activity seen in diabetes could be caused by insulin deficiency[77]. Another study using platelets from people with T2DM showed that the platelets lost their responsiveness to insulin compared to platelets from healthy subjects. Furthermore, the platelets from people with T2DM showed an exaggerated response to agonists of platelet activation[78]. Increased platelet activation has also been associated with insulin resistance[79].

Increased platelet activation plays a role in thrombo-inflammatory diseases such as atherosclerosis, systemic inflammation and stroke, underlining the importance of platelets in development of these diseases and anti-platelet therapy in population groups at risk, such as people with diabetes and platelet dysfunction[80].

Studies of platelets from both healthy people and people with diabetes subjected to hypoglycaemia have shown increased platelet activation and aggregation[30,81-83]. This effect is thought to be due to the counter-regulatory rise in plasma epinephrine levels[84,85], mechanisms involving a direct effect of glucose levels[86,87] or inflammation[30,88]. The increased platelet aggregation during hypoglycaemia coupled with general platelet dysfunction with loss of responsiveness to platelet inhibitory agents in people with diabetes[75] might be a mechanistic link between hypoglycaemia and increased risk of cardiovascular events[11].

Animal models of diabetes

Animal models are important tools in basic and preclinical research to investigate pathophysiology and pathogenesis of disease, in particular on a cellular level, in experimental setups that might not be feasible in humans. Furthermore, animal models allow for preclinical testing and screening for drugs and targets. These essential functions of animal models call for thorough characterisation of disease models to assess their validity as translational models.

Both spontaneous, genetic and induced models of diabetes exist[89]. The spontaneous, genetic models are currently limited to rodent species with groups working on developing a transgenic, diabetic pig[90]. Even though dogs can develop spontaneous diabetes, this phenomenon is sporadic and rare and is therefore not a reliable model[91]. The pig is currently a popular model of cardiometabolic disease[92,93] and more commonly used than dogs[89], however, pigs do not develop diabetes spontaneously[93,94].

Diabetes can be experimentally induced by either a surgical or pharmacological intervention. Pancreatectomy will result in hyperglycaemia, but can only be done with major, invasive surgery and will also result in a defect of the exocrine pancreas, making the translational value low[93].

Pharmacological induction is most commonly used. Streptozotocin and alloxan are the two most common pharmacological agents used to destroy the β-cells of the pancreas to induce hyperglycaemia by endogenous insulin deficiency. Their mechanisms of action are different, but result in similar damage[93,95]. Streptozotocin exerts its toxic effect when it is transported into the β-cells by the GLUT2 receptor[95,96]. Here, it induces DNA damage resulting in cellular damage and death[95,96]. Depending on the dosage and protocol used, streptozotocin can induce partial or complete insulin deficiency with resulting hyperglycaemia[93]. While the streptozotocin-induced model is considered to be type 1 diabetes-“like”, it still lacks the inflammatory process of T1DM development[93].

Streptozotocin induction have been used widely in rodents and pigs to induce experimental diabetes. However, streptozotocin is a genotoxic agent working on a number of cells[96] and several other effects have been reported. In vitro studies of hepatic mitochondria[97], neurons[98] and myocytes[99] have demonstrated damaging effects of streptozotocin. In vivo, several off-target effects have been demonstrated, including renal and hepatic toxicity[100,101], introducing confounding factors and lowering the validity of studies utilizing the streptozotocin-induced model.

Animal models of insulin-induced hypoglycaemia

Preclinical studies of hyperinsulinaemic hypoglycaemia have most commonly used rodents, and in particular rats, to investigate disease mechanism and potential interventions. Rats are relatively easy and inexpensive to keep and are well characterised in regards to glucose metabolism[102]. Studies of hyperinsulinaemic hypoglycaemia using rats have shown endocrine counter-regulation at plasma glucose thresholds similar to humans[57]. In studies using protocols to induce severe hypoglycaemia, something that is not ethical to induce experimentally in humans, rats experience fatal arrhythmias that are thought to be caused by a combination of sympathoadrenal activation and hypokalemia[57,103-105]. Rats also develop an attenuation of the counter-regulatory response to hypoglycaemia after antecedent hypoglycaemic episodes[57], as seen in humans with impaired awareness of hypoglycaemia.

Though these findings in rats highlight the species as a model of human hyperinsulinaemic hypoglycaemia, there are several anatomical and physiological aspects of which rats differ from humans. Rats have a higher β-cell mass-to-body weight ratio compared to humans, resulting in a higher insulin production and turnover[106]. Rats also have a higher physiological range of their plasma glucose compared to humans as well as a higher rate of metabolism[102,107,108].

Especially when investigating the cardiovascular consequences of hypoglycaemia, the translational value of rat models is in question. With a heart rate above 300 beats per minute[108], the QTc-interval of rats is also low, at around 120 ms[105], making the comparison to humans difficult, with correction formulas often over-correcting at high heart rates[109]. In rats subjected to severe hypoglycaemia, the arrhythmias observed preluding death were atrio-ventricular blocks[105]. In humans, arrhythmias associated with hypoglycaemia have been reported to be bradycardia and supraventricular ectopies in spontaneous, nocturnal hypoglycaemia[42] and tachycardia, supraventricular and ventricular ectopies, and altered repolarisation in acute experimental hypoglycaemia[69].

The pig as a model of insulin-induced hypoglycaemia

Large animal models offer several advantages compared to rodent models. They allow for multiple blood sampling and can be kept for a long duration of time, even with reuse in other studies. Larger animals are generally domesticized and can be trained to allow experimental procedures while awake[93]. Minipigs are preferred over domestic pigs, as they have a smaller size, slower rate of growth[110] and are being increasingly characterised in preclinical research[111]. The pancreas of pigs is similar to humans in regards to macro- and microscopic anatomy[93,102,112,113]

as well as the endocrine responses to fasting, glucose and insulin kinetics and post-absorptive metabolism[93]. Shortcomings of the pig as a model of human glucose metabolism are higher glucose tolerance[93] and higher rates of hepatic gluconeogenesis in case of decreasing plasma glucose concentrations[107]. The fasting plasma glucose of domestic pigs is similar to humans[114], but should be considered in the context of the domestic pig's genetic predisposition for rapid growth. Minipigs are not breed for rapid growth and have a plasma glucose level below what is considered normal in humans, with fasting levels around 3.5-4.5 mM[115]. This lower level of physiological normal plasma glucose in the minipig could consequently result in a lower threshold for hypoglycaemic counter-regulation than seen in humans. Also, no standardised test for cognitive effects of hypoglycaemia[116] has been developed for pigs and clinical signs might be hard to perceive for the observer contrary to humans self-reporting their symptoms.

Only few studies of domestic pigs have investigated the counter-regulatory response to hypoglycaemia[114,117]. One study investigated glucose-sensitive insulin using alloxan-induced diabetic Yucatan minipigs and demonstrated an epinephrine response to hypoglycaemia but did not investigate glucagon levels[117]. Another study utilised healthy, commercial pigs and saw only a slight increase in epinephrine and glucagon during hypoglycaemia with a target of 2.0 mM plasma glucose[114]. No hypoglycaemia studies of pigs investigated cardiovascular outputs and the streptozotocin-induced diabetic Göttingen Minipig has yet to be characterised in regards to the effects of hypoglycaemia.

The porcine heart is considered to be anatomically and physiologically quite similar to humans, however, several differences exist though mostly contributed to the unguligrade stance of pigs compared to the orthograde of humans[118]. The electrophysiology of the heart is also different, where it is generally considered easy to provoke arrhythmias in pigs[119]. The cardiac repolarisation and ventricular activation is also slightly different from humans, with RR-relative QT-intervals generally being longer (QT/RR ratio, 0.425 in humans and 0.472 in Göttingen Minipigs) and the QRS complex morphology being slightly different[119]. The dissimilarities in QRS morphology in pigs compared to humans arise from the anatomically different ventricular anatomy[118], activation pathways and distribution of Purkinje fibres as well as left and right ventricular bundle anastomoses[120]. The PR-interval has also been described as shorter than human, representing a relatively fast sinoatrial conduction, attributed to the difference in atrio-ventricular anatomy[118] and short length of the penetrating atrioventricular bundle[120]. The same study also found extensive nerve fibres throughout the atrioventricular bundle and ventricular bundle branches, with human hearts described to have less nerve fibre content, suggesting the porcine heart to be more susceptible to autonomic innervation[120]. The T-wave, representing ventricular repolarisation, has also been described as different to humans, with reports of spontaneous

amplitude and polarity-shifts occurring in individual pigs during one recording and with polarity dependant on heart rate, suggesting a autonomic mechanism involved in the repolarisation pathways of the ventricles[121].

Though these dissimilarities to humans exist, the argumentation for pigs being a good preclinical model for cardiac toxicology can still be made, as adverse cardiac effects might easily be demostrated[119]. In the context of cardiovascular research, the pig is emerging as a translational model of ischaemic heart failure[110], atherosclerosis[122] and structural heart disease[123].

While the pig, and in particular the Göttingen Minipig, offers many advantages in terms of anatomy and physiology for studying the endocrine and cardiovascular effects of hypoglycaemia, only few studies investigating hypoglycaemia in pigs exist. A large knowledge gap persists in terms of characterising the counter-regulatory and myocardial effects of hypoglycaemia in pigs as a translational model of the complication in people with diabetes.

Methods

Animals

All animal studies were approved by the Danish Animal Experiment Inspectorate and carried out according to Novo Nordisk A/S internal ethical guidelines as well as rules and regulations set forth by the inspectorate.

For all studies, lean, female, adult Göttingen Minipigs (Ellegaard Göttingen Minipigs A/S, Dalmose, Denmark) were used and housed at Novo Nordisk A/S animal facility in Ganløse, Denmark. All pigs were received and initially group-housed for a three-week acclimatisation period. Some pigs had been in other pharmacological studies prior to inclusion, which has been specified in the individual papers. A two-week 'wash-out' period was allowed before inclusion in studies. All pigs were fed once daily with a standard chow (specified in papers) in either the morning or at midday, with free access to water. Pigs were kept with wood chip and straw bedding, and, when single housed, with possibility of visual and snout contact, at a 12/12 hour light cycle with windows for natural light, at 18-22°C with relative air humidity of 30-70%.

Catheters

Prior to diabetes induction and hypoglycaemia studies, pigs had permanent venous catheters implanted to enable quick and stress-free blood sampling and i.v. infusion. Pigs had at least two venous accesses, either auricular catheters with access to the jugular vein or vena cava caudalis catheters, enabling both blood sampling and infusion at the same time. Techniques for implantation of catheters are described in paper II.

Diabetes induction and maintenance

Streptozotocin is widely used for experimental diabetes induction and is a genotoxic compound that induces irreversible damage to the β-cells of the pancreas[96]. Several studies have investigated diabetes in Göttingen Minipigs using this chemically induced model. A variety of protocols exist, with doses and dosing intervals aiming at inducing a type 1 or type 2 "like" diabetes. Co-morbidities can also be introduced, such as obesity or non-alcoholic steatohepatitis to develop multifactorial disease models[122,124]. In the studies described in this thesis, lean pigs received a dosing regimen to induce type 1-"like" diabetes.

For all three studies, diabetes was induced using streptozotocin (Sigma Aldrich Denmark A/S, cat. no. S0130-5G). Pigs were dosed with 50 mg/kg i.v. for three consecutive days (protocol modified from Schumacher *et al*. 2019[124]). Pigs were then included in other experimental insulin studies until inclusion in these studies. At least two weeks prior to inclusion in the studies of this thesis, hyperglycaemia in the diabetic pigs was managed by once-daily s.c. injections of insulin glargine (Lantus, Sanofi S.A., Paris, France) in conjunction with feeding. Insulin doses were determined based on previous day's doses and fasting plasma glucose curves. Specific plasma glucose targets are specified in each paper.

Hypoglycaemia induction

Insulin was used to induce endocrine counter-regulatory responses, electrocardiographic and platelet aggregation changes to hypoglycaemia. Hypoglycaemia can be induced by either bolus injection, a common method in rodent studies or through the hyperinsulinaemic clamping technique[25], which is considered the most reliable and safe method of inducing hypoglycaemia in both preclinical and clinical studies[125].

Hypoglycaemia was either induced by i.v. bolus injection or clamp technique. Before each induction, pigs were fasted from the day before and had not received any maintenance insulin glargine. After each procedure, pigs were fed and, if diabetic, received a modified dosage of maintenance insulin glargine, which has been specified in paper II and III.

For all hyperinsulinaemic, hypoglycaemic clamps, a constant i.v. infusion of 16 pmol/kg/min human insulin (formulated in-house) was used for up to seven hours (specified in papers). Plasma glucose target was set as low as 0.8 mM (see paper I and III for specific targets), and PG levels were controlled by measuring PG every ten to fifteen minutes, then individually adjusting an i.v. glucose infusion rate (GIR).

For the bolus protocol, a single i.v. injection of human insulin was given at doses 0.4 nmol/kg for healthy and 0.6 nmol/kg for diabetic pigs. Diabetic pigs received a larger dose due to expected lower insulin sensitivity.

The insulin doses for both the clamp and bolus protocols were determined from data generated in pilot studies of healthy and diabetic Göttingen Minipigs (data not shown).

Blood sampling and analysis

To characterise the endocrine counter-regulatory response to hypoglycaemia, blood was sampled frequently through permanent venous catheters, ensuring handling- and stress-free sampling from the minipigs.

For plasma glucose determination, 1.5 mL of blood was collected and stabilised with ethylenediaminetetraacetic acid (EDTA) and directly centrifuged and analysed. Glucose in plasma was quantified directly using the YSI-2900 (Yellow Springs Inc., OH, USA).

For plasma glucagon, C-peptide and human insulin analysis, 1.5 mL of blood was stabilised with EDTA and aprotinin to prevent glucagon degradation (Trasylol 10.000 KIE/mL, Nordic Drugs AB, Limhamn, Sweden). Blood was centrifuged at 3000*x*g at 4°C for 10 minutes, and plasma was directly frozen and stored at -20°C until analysis. Levels of glucagon, C-peptide and human insulin were analysed in plasma using luminescence oxygen channelling immunoassays developed in-house. Lower limit of quantification (LLOQ) was 4 pM for glucagon, 45 pM for C-peptide and 10 pM for human insulin.

For epinephrine analysis, whole blood was added to ice-cold ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) and glutathione solution (E3889, 2.88 mg/mL and G4251 1.44 mg/dL, Merck Life Science A/S, Søborg, Denmark) and centrifuged at 3000*x*g for 10 minutes at 4°C and plasma was directly frozen and later stored before analysis at -80°C. Epinephrine in plasma was measured using liquid chromatography-mass spectrometry with a LLOQ of 0.05 ng/mL[126].

For analyses requiring serum, whole blood was collected in tubes containing gel clot activator and let coagulate for at least 30 minutes before centrifugation at 3000*x*g, 4°C for 10 minutes and then directly frozen and stored at -80°C. For specific analyses, see paper I and III.

For each study, the total amount of blood drawn from the minipigs was calculated and an appropriate recovery period was planned[127].

Light transmission platelet aggregometry

Light transmission platelet aggregometry quantifies the ability of isolated blood platelet to clump together in response to an added platelet agonist[128]. Plasma containing platelets is slightly cloudy, letting some light through shine through. When a platelet agonist is added, the platelets react with

a specific series of events, resulting in them clumping together and ultimately letting more light through the tube containing plasma. This light is measured by a sensor in the assay and is quantified as a percentage of light transmission through the plasma sample, with 0% representing the un-activated, cloudy plasma and 100% representing complete aggregation of platelets, calibrated by using platelet-poor plasma[128].

To investigate platelet aggregation during hypoglycaemia, blood samples were drawn at specified time point throughout the clamp (see paper II).

Citrate stabilised whole blood was centrifuged to obtain platelet rich plasma (PRP) and platelet poor plasma (PPP). Doses of 0-20 µM adenosine diphosphate (ADP, HB-5502-FG, Hart Biologicals, Hartlepool, UK) were used to stimulate the PRP and generate dose-response curves using the PAP-8E light transmission platelet aggregometer (Bio/Data Corporation, PA, USA). Light transmission was recorded for ten minutes and maximum aggregation response (MaxA), area under the curve (AUC) and effective concentration of ADP to generate a 50% response for both MaxA ($EC50_{MAX}$) and AUC ($EC50_{AUC}$) were calculated for each sampling time.

Electrocardiography

Holter electrocardiographic recordings

To evaluate the electrocardiac function in the minipigs, continuous Holter ECG recordings were obtained at predefined time-points. Recordings were 4-24 hours long, depending on the protocol used (see papers). The Holter ECG recorder (Lifecard CF, Spacelabs Healthcare, WA, USA) was fitted to either trained, awake minipigs or minipigs having received a dose of i.v. propofol for restraint.

Two leads were placed as previously described by Suzuki et al. 1998[129]. A positive electrode (3M Red Dot 2670, 3M A/S, Copenhagen, Denmark) was placed on the dorsal neck area (A) and a negative on the processus xiphoideus (B) to create lead I. A positive electrode on manubrium sterni (C) and a negative on processus xiphoideus (D) created lead II (see figure 3).

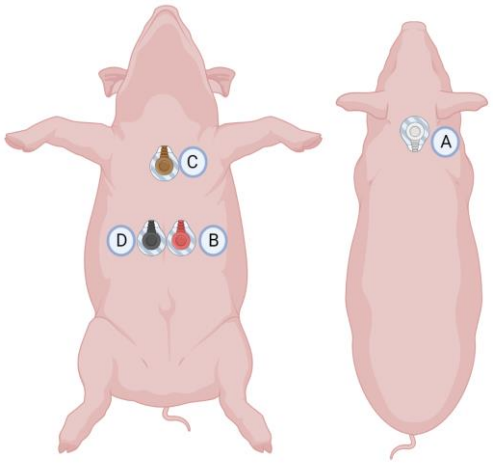


Figure 3: Electrode placement for Holter ECG recordings in Göttingen Minipigs. A) Positive electrode A in dorsal neck area. B) Negative electrode B on processus xiphoideus. C) Positive electrode C on manubrium sterni. D) Negative electrode on processus xiphoideus. A+B lead I and C+D lead II. First published by Lyhne et al. 2022[130] and reused with general permission from Journal of Pharmacological and Toxicological Methods. Created with Biorender.com

Electrocardiographic analyses

The recordings were analysed using Pathfinder SL (Spacelabs Healthcare, WA, USA). Manual editing of R-wave and arrhythmia detection was performed in the software. Recordings were screened for artefacts and quality, in case of poor skin contact, movement or electrodes falling off.

Arrhythmia analysis

To evaluate the prevalence of arrhythmic events, each recording was manually investigated. The software could automatically detect some arrhythmias (see supplemental for arrhythmia detection criteria of the software), but since it is a program developed for human use, manual screening was also conducted.

Arrhythmic events were placed into three categories (see figure 4). A second-degree atrioventricular (AV) block was defined as one or more P-waves without a corresponding QRS complex[131]. A ventricular ectopic complex (VE) was defined as a QRS complex with longer duration and different shape than normal, possibly with altered electrical axis resulting in a lower or higher amplitude of the QRS. The RR-interval of the preceding QRS could either be longer (escape complex) or shorter (premature complex) than the sinus RR-interval. There is no coupled P-wave[132]. A supraventricular ectopic complex (SVE) was defined as a QRS complex with a normal QRS duration and morphology with either a non-visible P-wave or a P'-wave. The P-P'-interval should be shorter or longer than the normal P-P interval. The RR-interval could be shorter or longer than the sinus RR-interval, indicating a premature or escape complex, respectively[132].

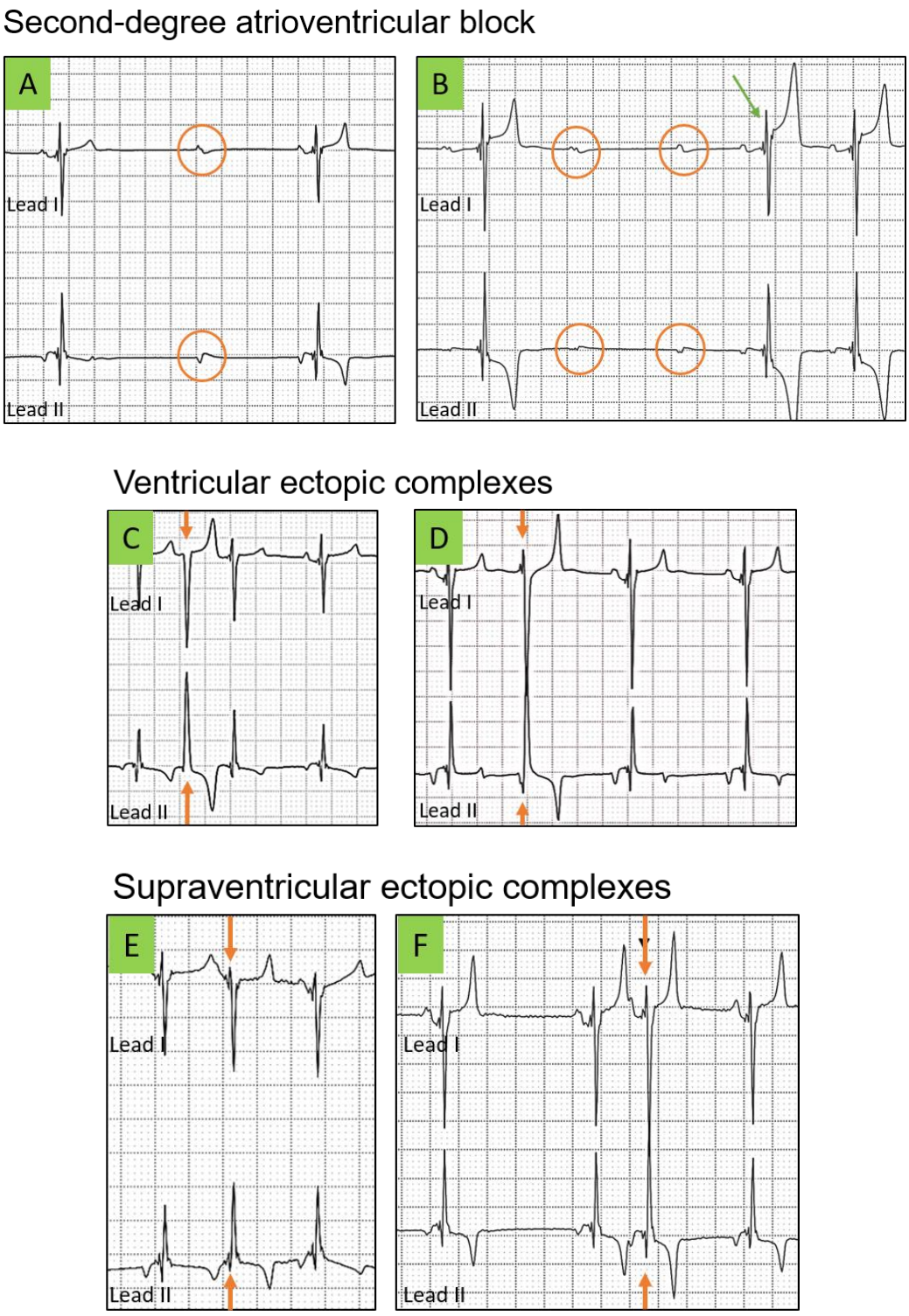


Figure 4: Examples of arrhythmic events in original electrocardiographic Holter recordings from Göttingen Minipigs recorded in lead I and II. A) Second degree 2:1 AV-block with a singular P-wave (orange circle) without a corresponding QRS complex. B) 3:1 AV block with two P-waves (orange circles) without a corresponding QRS complex. Note that the ST-segment generally shows a slight elevation, in particular after the block and in lead I (green arrow) C) and D) premature ventricular ectopic complexes (orange arrows). Note the increased duration of the QRS and altered electrical axis. E) and F) premature supraventricular ectopic complexes (orange arrows). Note the lack of corresponding P-wave.

Morphological analysis

To evaluate morphological features of the ECG, ten consecutive RR-intervals were printed from the recordings at specified time-points (see paper I-III). Durations (ms) and amplitudes (mV) were manually measured using ImageJ (National Institute of Health Sciences, Kawasaki, Japan) (see figure 5). Values were noted as an average of ten complexes.

QRS duration was measured from the first deflection of the complex until the return to the isoelectric line[133]. The duration of the PR segment was measured from the end of the P-wave until the first deflection of the QRS[134]. ST-segment elevation was measured from the J-point of the end of the QRS with the PR-segment as the isoelectric line[135]. The amplitude of the T-wave was measured from the isoelectric line after the T-wave to the top of the T-wave. The duration of the T-wave was measured from start to end of the T-wave, where it returned to the isoelectric line[136]. The amplitude of the P-wave was measured from the isoelectric line of the PR-segment until the top of the P-wave. Polarity of the T- and P-waves were noted[137].

The QT-interval was measured from the first deflection of the QRS until the return of the T-wave to the isoelectric line and was corrected with the RR-intervals using Bazett's (QTcb) and/or Fridericia's (QTcf) formulas[47,109].

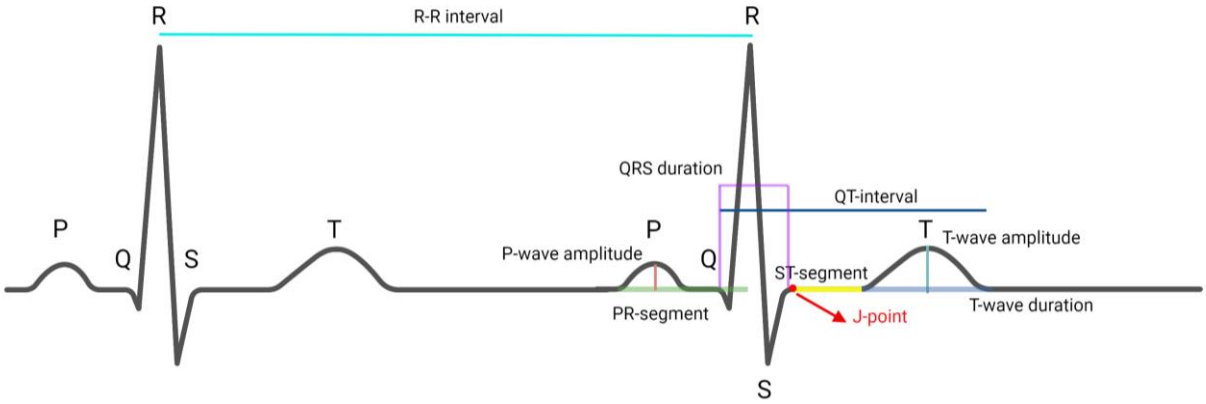


Figure 5: Morphological features extracted from the electrocardiogram. R-R interval (light blue), PR-segment (light green), P-wave amplitude (red vertical line), QRS duration (purple lines), QT-interval (dark blue), ST-segment (yellow), T-wave amplitude (green), T-wave duration (grey), J-point (red arrow). Created with Biorender.com.

Heart rate variability analysis

The principle of HRV is the variation in time intervals between heartbeats that constantly changes due to input from the autonomic nervous system and changes in breathing and body position[63]. The variability can be quantified by the variation in the beat-to-beat intervals into time-domain features. The standard deviation of normal beat-to-beat intervals (SDNN) and root mean square

of all successive R-to-R waves interval differences (RMSSD) are the two most commonly reported time-domain features and supply an overall assessment of the HRV[63].

Frequency-domain features of HRV can be calculated from ECG data transformed into frequencies bands and the power in each, predefined band can be quantified[63]. The different frequency-bands have been associated with breathing and sympathetic and parasympathetic activity[63].

In this study, the HRV of the ECG recordings was calculated using the build-in software of Pathfinder SL. Time epochs were predefined in each study. Time domain variables were SDNN and RMSSD[63]. Frequency domain variables were obtained from Fast Fourier transformed data using the build-in software. Very-low frequency (VLF) power band at 0.015-0.05Hz, normalised low frequency (LF n.u.) power band at 0.05-0.15Hz, normalised high frequency (HF n.u.) power band at 0.15-0.40Hz and very high frequency (VHF) power band at 0.35-0.5Hz and LF/HF ratio were collected[63].

Summary of the results and their relation to international state-of-the-art research

Summary of results

Paper I: Electrocardiography and heart rate variability in Göttingen Minipigs: Impact of diurnal variation, lead placement, repeatability and streptozotocin-induced diabetes.

The aim of this study was to investigate 24-hour Holter ECG recordings of healthy and STZ-induced diabetic Göttingen Minipigs. It was confirmed, that several ECG features had diurnal variations. QRS-duration, PR-segment and QT-interval were longest during the night and shortest during the day. ST-segment, T-wave and P-wave amplitude were highest during feeding of the minipigs and lowest during the night. Bazett-corrected QTc-interval were longest during feeding. Of HRV parameters, SDNN, RMSSD, n.u. LF and LF/HF ratio were highest at night.

Though it was hypothesised that there would be no changes to the ECG over the course of four months in the healthy group, QRS-duration, PR-segment and QT-interval all increased, heart rate decreased and SDNN and RMSSD were higher, in both groups.

The recordings were analysed with low variability. Average inter-observer variability were under 6% in both lead I and lead II of the ECG recording. Average intra-observer variation was below 7% in both leads.

There was confirmed significant differences between measurements from lead I and lead II, with lead I having longer QRS-duration, QT-interval, T-wave duration and higher ST-segment, T-wave and P-wave amplitude at all time-point during the day.

There was an effect of STZ-induced diabetes on features of the ECG. T-wave duration, P-wave amplitude and Bazett-corrected QTc-interval were decreased after four months, however, no change in ST-segment and HRV parameters was observed.

The findings of the study support the hypotheses, that parameters of the ECG in minipigs are not static and have diurnal variations and changes over time, as well as significant influence of choice of lead, but can be obtained with low inter- and intra-observer variability. Some effects of STZ-induced diabetes were confirmed.

Paper II: Hyperinsulinaemic hypoglycaemia in non-anaesthetized Göttingen Minipigs induces a counter-regulatory endocrine response and electrocardiographic changes.

The aim of this study was to investigate the endocrine counter-regulation, ECG changes and platelet aggregation in response to hypoglycaemia in healthy and STZ-induced diabetic Göttingen Minipigs, using hyperinsulinaemic clamping and insulin bolus dosage.

It was possible to induce hypoglycaemia at 0.8-1.0 mM plasma glucose target, in both healthy and diabetic minipigs using the clamp procedure. Using the bolus procedure, only healthy minipigs reached plasma glucose target, even though the diabetic minipigs received more insulin.

It was possible to induce a significant epinephrine response in both groups using both the clamp and bolus procedure. In the clamp, both groups had an approximate 25-fold increase in plasma epinephrine after one hour of hypoglycaemia compared to baseline. In the bolus procedure, the healthy minipigs had an approximately 15-fold increase in plasma epinephrine after 15 minutes compared to baseline levels, however the diabetic minipigs only had a significant increase one hour after bolus administration.

There was a significant glucagon response to hypoglycaemia in the clamp procedure with healthy pigs having an approximate six-fold increase in plasma glucagon compared to baseline levels. Diabetic minipigs had a three-fold increase and the response seen in the healthy minipigs were significantly higher than diabetic minipigs. Only the healthy minipigs had a significant glucagon response to the insulin bolus.

Hypoglycaemia did not influence heart rate. QTc-interval was prolonged in the diabetic minipigs after two hours of hypoglycaemia in the clamp. In the bolus procedure, QTc-interval increased significantly five minutes after insulin administration in both groups. During hypoglycaemia in the clamp, ST-segment was elevated compared to baseline in both groups and T-wave amplitude was higher in the diabetic minipigs compared to the healthy group. In both groups, the total number of arrhythmic events increased during hypoglycaemia in the clamp. In the clamp, the only HRV parameter influenced by hypoglycaemia was the LF band power, which decreased in both groups.

Platelet aggregation decreased in both groups during hypoglycaemia in the clamp but not in the bolus protocol.

Paper III (in preparation): Healthy and streptozotocin-induced Göttingen Minipigs as a model of counter-regulatory failure in recurrent hypoglycaemia

The aim of this study was to investigate the counter-regulatory response and ECG changes during hypoglycaemia in healthy and STZ-induced diabetic Göttingen Minipigs preconditioned with antecedent episodes of hypoglycaemia. The study was completed using two batches of minipigs with approximately eight months between batches, due to maternity leave.

It was not possible to completely reach the predetermined hypoglycaemic plasma glucose target of 1.0 mM using the hyperinsulinaemic, hypoglycaemic clamp procedure. However, both heathy and diabetic minipigs preconditioned with hypoglycaemia had significantly lower average plasma glucose in the final 60 minutes of the four-hour clamp compared to normoglycaemia preconditioned minipigs.

The glucagon response to hypoglycaemia was significantly diminished in the hypoglycaemia preconditioned groups, with no effect of diabetic status.

A significant epinephrine response was seen in all groups, but there was no significant effect of preconditioning or diabetic status.

Results from the ECG analysis are pending.

Discussion

Preclinical research into insulin-induced hypoglycaemia has been heavily focused on rodent models. While these models show a human-like counter-regulatory response to hypoglycaemia, the translational value of cardiovascular outputs from these models might be low due to the rodent cardiac anatomy and physiology. This underlines the need for a large animal model to investigate the cardiovascular effects of hypoglycaemia.

The studies presented in this thesis underline both the similarities and differences between minipigs and humans. During hypoglycaemia, the porcine endocrine counter-regulatory response seems similar to human with regard to glucagon and epinephrine release, however, recurrent hypoglycaemia did not induce significant blunting of the epinephrine response, as previously reported in both humans[20] and rodent models[25,57]. The minipig hypoglycaemic threshold also seems lower than what is described in humans and rodents, coupled with very low glucose infusion rates needed to maintain plasma glucose. Despite these differences in glucose homeostasis, the minipig electrocardiogram during hypoglycaemia in paper II displayed some of the same characteristic changes as have been observed in humans[44]. The minipig ECG during normal conditions also has many of the same features as humans, with only minor differences.

Translational value of the minipig electrocardiogram

Paper I investigated the ECG of healthy and diabetic minipigs to both investigate the diurnal and time effects on features of the ECG as well as determine, if STZ-induced diabetes would have an effect on the ECG over time.

Pigs are considered a good model to investigate drug-induced arrhythmias and QT-interval prolongation. Arrhythmias are easier to induce in pigs compared to dogs[119] and the normal QT-interval of pigs has been reported to be close to humans, making pigs relevant to investigate drug-induced effects on the electrophysiology of the heart[47,119]. In paper I, the Bazett- and Fridericia-corrected QTc-interval was sometimes below the lower threshold of what is considered normal in humans, with the lowest QTc-interval observed during the night in diabetic pigs after four months at a median 358 ms (Bazett) and 352 ms (Fridericia). The lower threshold of what is considered pathological in humans is 390 ms[138]. These findings are in agreement with another study of the porcine QT-interval[47], but underlines the need for standardised baseline values when evaluating the QT-interval in pigs.

In paper I, the T-wave amplitude changed during the day, with highest amplitudes during feeding, where the highest heart rates were also observed. The T-wave also changed polarities, displaying multiple morphologies in individual minipigs, particularly at night at lower heart rates. The electric

alternans of the porcine T-wave has been reported in other studies[121,139], however, an increased T-wave amplitude is considered pathological in humans[138]. This variability in the porcine repolarisation of the heart needs to be taken into account when conducting preclinical studies.

The morphology of the porcine QRS-complex is similar to many other mammal species, but different from dogs, humans and primates[119]. These dissimilarities arise from the difference in ventricular activation pathways and, combined with the smaller size of the minipig heart, results in a shorter QRS-duration with different electrical axes of the QRS-complex[119]. The QRS complex of humans is considered normal at 80-100 ms[140]. In paper I, the QRS-duration was between 83-94 ms, but other studies have demonstrated a much lower QRS-duration of minipigs at 55-59 ms[110,121,139]. This discrepancy between paper I and the other studies of minipigs might be due to choice of equipment, build-in filters applied to the raw electrical signal or simple differences in measurement techniques, but could also be caused by the higher age of the minipigs included in paper I compared to the mentioned studies, as the QRS-duration increases with age in humans[141]. Again, these findings highlight the need for baseline values in ECG studies including minipigs.

The PR-segment of the ECG represents the conduction of the electrical impulse from the atria to the ventricles. The pig atria are anatomically a bit different to humans[118], which might cause the difference in PR-segment duration seen between the two species. Studies have reported the PR-segment from pigs ranging from to 86[142] in commercial breeds to 125 ms[121] in Göttingen Minipigs, while in paper I, the median range was 105-130 ms. Another study demonstrated the longest PR-interval in Göttingen Minipigs compared to several other minipig breeds[119]. In comparison, the human PR-interval is 120-200 ms[131], suggesting the Göttingen Minipig as a more appropriate breed to investigate possible cardiac conduction disturbances.

In humans, diabetes is associated with prolongation of the QT-interval[143], low heart rate variability[144], and silent myocardial infarction[145], and these changes were not observed in paper I. The lack of human specific, diabetes related changes in paper I could be due to lack of chronicity in the diabetic minipig model, with human cardiovascular pathologies increasing with duration of diabetes, counting years, not months, as is the case in this model.

Electrocardiographic changes during hypoglycaemia

Electrocardiographic changes during hypoglycaemia is relatively well-documented in both healthy people and people with diabetes. The most common change is prolongation of the QT-interval in response to hyperinsulinaemia and hypoglycaemia. In fact, hyperinsulinaemia without hypoglycaemia can increase the QT-interval, propably mediated by insulin-induced hypokalemia and epinephrine, but seemingly independent of insulin sensitivity[51]. QT-prolongation has been

documented in people with T1DM and spontaneous, severe hypoglycaemia[46] and the QT-interval has been demonstrated to be longer in a clamp procedure with hypoglycaemic target compared to an normoglycaemic clamp at same insulin dosage[44]. QT-interval prolongation has also been documented as a predictor of mortality in both people with and without diabetes[143], hinting to a possible mechanistic link between hypoglycaemia and increased mortality.

Though no previous studies have investigated the effects of hypoglycaemia on the porcine QT-interval, the pig as a model of adverse pharmacological repolarisation effects in safety pharmacology is relatively well-documented[47]. In paper II, a prolongation of the QTc-interval was seen in diabetic pigs during hypoglycaemia and in both healthy and diabetic minipigs five minutes after insulin bolus administration. In the bolus study, this effect happened before epinephrine could be measured in plasma, and before target plasma glucose was reached, in particular in the diabetic minipigs, where a somewhat hypoglycaemic level was not reached until 60 minutes after bolus and an epinephrine response was not seen until then. This indicates an acute effect of insulin that might not depend on epinephrine levels but could certainly be, in part, caused by insulin-induced hypokalemia.

In humans, several other ECG changes occur during hypoglycaemia, including prolongation of the PR-interval[45], ST-segment depression, increased R-wave amplitude[45] and flattening of the T-wave[31,44]. In paper II, an elevation of the ST-segment was seen during hypoglycaemic clamping, but the T-waves increased in amplitude in that same period, contrary to the flattening documented in humans. There was, however, noted a slight chance in T-wave shape in some minipigs, but this change was not quantified in the paper (see figure 6). The porcine T-wave, representing repolarisation of the ventricles, seems particular dissimilar to humans, as described above, and should be interpreted with care in translational studies.

Heart rate variability (HRV) has shown potential as a non-invasive marker of cardiac autonomic activity[64] and low HRV is associated with cardiovascular outcomes in clinical studies[72]. Clinically, HRV can be used as a predictor of arrhythmic events after myocardial infarction and as a diagnostic tool in identifying diabetic cardiac neuropathy[146]. Low HRV has been suggested as an early marker of diabetic neuropathy, with subjects displaying diminished HRV prior to clinical onset of neuropathy[147]. In paper I and II, there was no effect of diabetic status on parameters of HRV. While this has been demonstrated in humans, the lack of chronicity in this minipig model might not allow sufficient time for development of cardiac neuropathy. There was also little effect of hypoglycaemia on HRV parameters, with only LF power lower after two hours of hypoglycaemia. In paper II, short term HRV around feeding demonstrated low HRV and high HRV was found during the night. Paper I found the higher HRV in long term HRV during the night

compared to daytime, both confirming the diurnal and stress-related effects found in other porcine studies[148,149].

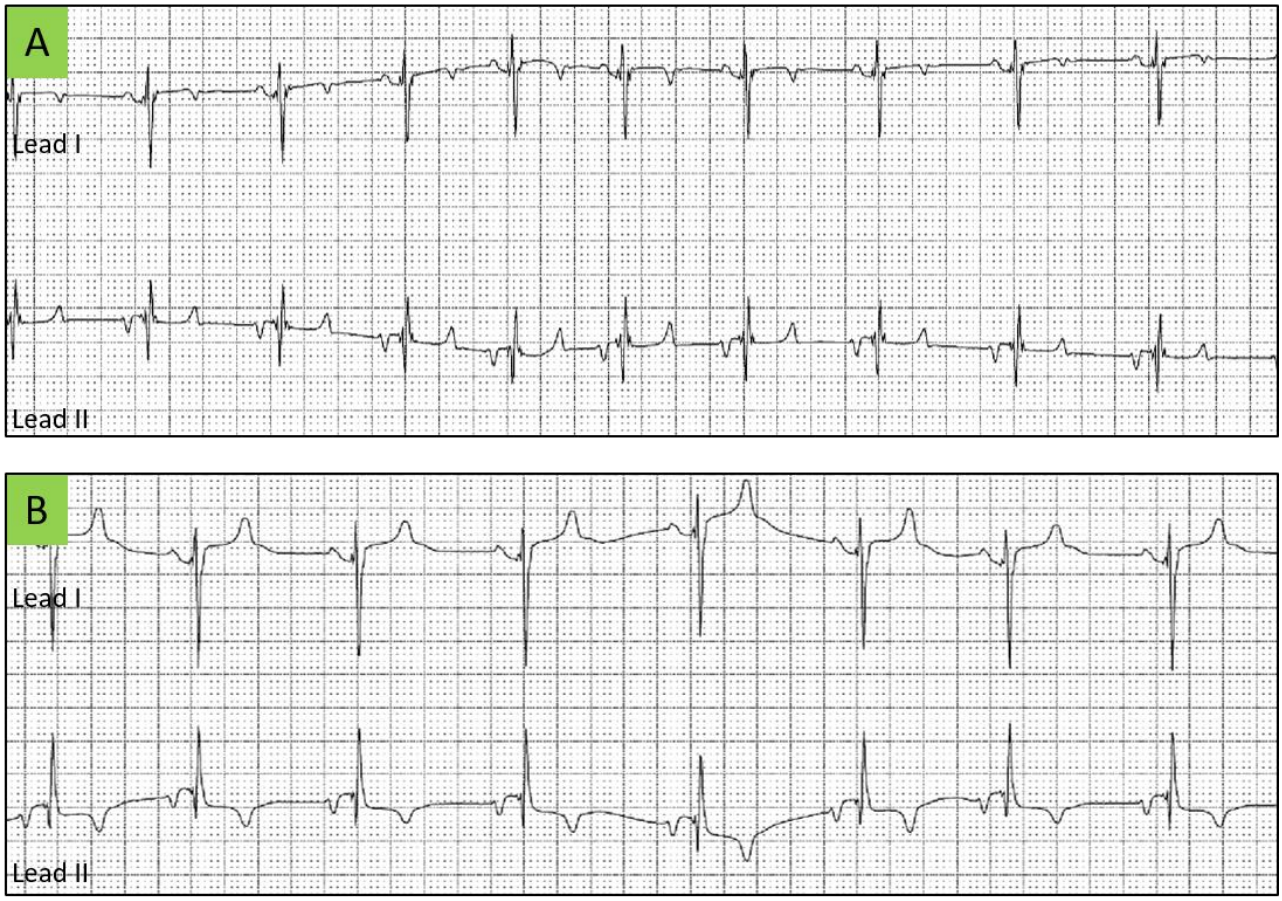


Figure 6: Example of electrocardiographic traces, recorded from lead I and II, from a diabetic Göttingen Minipig during A) hyperinsulinaemic normoglycaemia and B) after two hours of hyperinsulinaemic hypoglycaemia. In A), note the change of polarity of the T-wave from negative to biphasic in lead I. In B), note the increased R-wave amplitude, altered shape of the T-wave and slight ST-segment elevation in lead I (all complexes). Data from minipig included in paper II, recorded at 25 mm/s, 1 mV.

The porcine counter-regulation to hypoglycaemia

While the minipig offers some translational value in regards to their larger size, cardiac anatomy and physiology closely resembling that of humans, their glucose metabolism differ somewhat from humans.

In paper II, the fasting plasma glucose of female, adult, healthy Göttingen Minipigs was found to be around 3.5 mM. In growing, commercial pigs, the fasting plasma glucose is around 5.0 mM[114], which is more comparable to humans, but might also reflect the age and phenotype of rapid weight-gain seen in these breeds. Though the fasting glucose level in commercial pigs resemble that of humans, a hyperinsulinaemic, hypoglycaemic clamp plasma glucose target of 2.0 mM

demonstrated only a modest approximately 2.5-fold increase in epinephrine levels and a glucagon plasma level during hypoglycaemia comparable to that of the fasting level[114], while in paper II and III, a plasma glucose target of 1.0 mM induced approximately 25-fold increase in plasma epinephrine levels and a 15-fold increase in glucagon in Göttingen Minipigs.

The hypoglycaemic target plasma glucose level (1.0 mM) chosen in paper II and III might give rise to concern as to the severity of hypoglycaemia. In humans, moderate hypoglycaemia is defined as plasma glucose below 3.0 mM and severe hypoglycaemia is defined as an event with altered mental status and the person requiring assistance[150]. Severe hypoglycaemia can cause seizures, coma and death, if not treated in time[3]. However, in paper II, only few clinical signs of hypoglycaemia were observed, with fatigue being the most common, at a plasma glucose level that would likely cause major clinical concern in humans.

In paper III in particular, the minipigs needed very little glucose infusion to maintain the target plasma glucose during hypoglycaemia, with most minipigs requiring no glucose at all, regardless of diabetic status. This illustrates the capacity of the minipig to counter-regulate during hypoglycaemia, with high secretion of glucagon and high rate of hepatic glucose production[107]. In comparisons, humans clamped to a hypoglycaemia level need considerable glucose infusion rates to maintain plasma glucose target[151].

The Göttingen Minipig as a model of impaired hypoglycaemic counter-regulation

Currently, only rodent studies modelling impaired hypoglycaemic counter-regulation exist. Several studies have demonstrated a diminished epinephrine response in rats after different protocols of antecedent hypoglycaemia[57,105,152]. Interestingly, preconditioning rats with recurrent hypoglycaemia, and, in another experiment, β-adrenoreceptor blockade, resulted in decreased mortality during a severe, hypoglycaemic clamp, suggesting a blunted adrenergic response to be protective[105]. In paper III, three consecutive days of hyperinsulinaemic hypoglycaemic resulted in no change in epinephrine response in the hypoglycaemia preconditioned groups.

Blunting of the glucagon response after antecedent hypoglycaemia is less consistently documented in rodent studies[25], but this observation might not be of high clinical relevance, as patients with diabetes generally have a defective glucagon response to hypoglycaemia, independently of presence of impaired awareness of hypoglycaemia[20,25]. In paper III, three consecutive days of hyperinsulinaemic hypoglycaemic clamping blunted the glucagon response to a final, fourth hypoglycaemic clamp. This finding was not influenced by diabetic status, which is interesting as a diminished glucagon response was seen in paper II to a single, hypoglycaemic clamp when comparing diabetic to healthy minipigs.

Platelet effects of hypoglycaemia

Experimentally induced hypoglycaemia increases platelet aggregation[82] and pro-thrombotic markers[81], an effect thought to be caused by circulating counter-regulatory epinephrine, a known platelet agonist[85]. Platelet dysfunction and general pro-thrombosis have been documented in people with diabetes. Platelets from people with T2DM exhibit hyper-reactivity to platelet agonists [153] and lose their responsiveness to inhibitory compounds such as prostacyclin and insulin[75,78]. Even in people with well-controlled T1DM without the classic comorbidities of T2DM, such as obesity, dyslipidaemia and cardiovascular disease, factors know to affect platelet activation, platelet dysfunction is still seen[76]. This pro-thrombotic state documented in people with diabetes together with the platelet activation during hypoglycaemia, could be a possible mechanistic link between hypoglycaemia in diabetes and cardiovascular events[76].

A study using alloxan-induced diabetic commercial pigs[154] and STZ-induced diabetes in rats[155] demonstrated hyper-reactivity of platelets to thrombin, a common platelet agonist. In paper II, the platelet aggregation decreased during hypoglycaemic clamping with no effect of diabetes status, even though all minipigs had an increase in epinephrine during this period. This is contrary to what has been found in human hypoglycaemia studies[82,85], but insulin does have an inhibitory effect on platelet aggregation[77], which might be the explanation in paper II, as the clamp was hyperinsulinaemic, with super-physiological plasma levels of insulin. Paper II was also not able to reproduce the increased aggregation seen in pigs with alloxan-induced diabetes[154], however, another platelet agonist than ADP was used in that study.

Limitations

Induction and chronicity of diabetes

While STZ-dosing is a common way of inducing diabetes in both rats[101] and minipigs[93], several off-target effects of STZ has been documented in vitro and in vivo. In particular, the negative effect on isolated myocytes[99] could possible confound animal studies of the diabetic myocardium. The chemical induction of diabetes also decreases the validity of the model, as T1DM develops due to autoimmune destruction of β-cells, creating an inflammatory aspect of the disease that the STZ model does not. The outcomes of STZ-induction also varies[93], with some minipigs in paper II-III having no detectable levels of C-peptide, an equimolar marker of endogenous insulin production, while others had C-peptide levels only just lower than healthy minipigs, even though they still displayed fasting hyperglycaemia. A diagnostic cut-off for fasted C-peptide plasma levels in T1DM is 0.2 nM with a decline seen with duration of diabetes[156]. This variation in diabetes induction introduces variability to the studies in paper I-III.

Another concern for validity of the STZ diabetic minipig model is lack of chronicity. While adult[111] minipigs were used in all three papers, the duration of diabetes was below one year in all minipigs, with approximately four to eight months of diabetes duration (data not shown in paper II-III). While this is a long time in animal studies, many complications of diabetes in humans do not develop until years after disease onset[2]. While it could be possible to keep diabetic pigs for a long time, the minipig lifespan is still considerably shorter than humans, creating a need for accelerated models to investigate complications of diabetes.

Epinephrine response to hypoglycaemia

While a significant epinephrine response to hypoglycaemia was demonstrated in both paper II and III, there was no significant effect of hypoglycaemia preconditioning in paper III. The protocol, three consecutive days of hypoglycaemia preconditioning and a final, fourth episode, has been used in rat studies with a significant blunting of the epinephrine response[57,105,157]. This protocol failed to blunt the epinephrine response and might not be sufficient to induce counter-regulatory failure in minipigs. The length and number of hypoglycaemic clamps in the preconditioning period could be increased and the plasma glucose target could be lower. Variation in the epinephrine response could also cause the lack of significance in the findings, with large variations in plasma epinephrine levels in both paper II and III. The variance could be caused by the pulsatile release of epinephrine from the adrenal gland[158] and simply “missing” the high levels, as the plasma clearance is also quite rapid and best assessed in arterial blood[159]. The assay[126] to quantify the epinephrine in plasma could also be the cause of variation. As the samples were only single-determined, the variability of the assay in this particular case is unknown. To ensure less effect of the variation in plasma epinephrine, more animals could be included in future studies to increase the power.

Clamp technique

In paper II, the minipigs were clamped at a normoglycaemic level for three hours, then the glucose infusion to maintain normoglycaemia was turned completely off to induce acute hypoglycaemia. This resulted in a rapid counter-regulation and plasma glucose target of 0.8-1.0 mM was reached. In paper III, the same insulin dose (16 pmol/kg/min) was used, but a normoglycaemic period was not included in the beginning, with the goal of slowly inducing hypoglycaemia. With this protocol, it was more difficult to get the minipigs to reach the target plasma glucose of 1.0 mM, with glucose levels plateauing at around 1.5 mM in control groups and just barely reaching the target in hypoglycaemia preconditioned groups. While these results demonstrate the decrease in counter-regulatory ability to raise plasma glucose in response to hypoglycaemia, the protocol allows the minipig to respond slowly, but increasingly, to hypoglycaemia, making it more difficult to reach the plasma glucose targets. This was demonstrated by minipigs needing no, or very little, glucose

infusion during hypoglycaemia in this protocol, contrary to the protocol used in paper II. While the protocol used in paper III might better reflect the clinical situation with a progressive and steady drop in plasma glucose[11], the protocol in paper II can be used to induce more profound hypoglycaemia.

Platelet effects

In paper II, platelets from minipigs subjected to hypoglycaemia did not have increased aggregation in response to ADP. The light transmission aggregometry assay was used to evaluate the platelet aggregation response in plasma and not washed platelets, giving a more intact picture of the in vivo haemostasis. The plasma consequently contained both the pro- and anti-thrombotic factors naturally occurring in the plasma[128]. The light transmission platelet aggregometry assay is particularly sensitive to pre-analytical variables[128] and standardisation between laboratories might be very difficult. The assay is sensitive to sampling method, materials used, temperature before and during analysis, physical movement of the samples and time from sampling to analysis[128]. While the analysis conducted in paper II was standardised, the many variables affecting platelet aggregation make it difficult to compare the results to previous published literature. However, other studies have investigated the species differences in platelets, describing pig platelets as less adhesive than human platelets[160] but otherwise quite similar[161]. Another study found, that pig platelets did not respond to epinephrine[162], a well-known agonist of human platelets, which has been suggested to be the main cause of increased aggregation during hypoglycaemia in humans[85]. These fundamental differences suggest, that porcine platelets might not appropriate to investigate cardiovascular disease mechanisms involving potential platelet mediated effects.

Permanent catheters

All minipigs used had permanent peripheral or central venous catheters implanted prior to hypoglycaemia induction, to enable stress- and handling-free blood sampling. While this approach avoids stressing of the minipig and unintentionally inducing a stress or fear related epinephrine response, the catheter implantation site can introduce bacteria and chronic inflammation due to the foreign material implanted. This was evident in paper II, where the minipigs had an elevation in C-reactive protein and serum amyloid A prior to initiation of the clamp. Chronic inflammation can cause insulin resistance[163]. Furthermore, the central venous catheters used were implanted by going through the vena cava cranialis, guiding the catheter through the atrium and placing the internal tip of the catheter in the vena cava caudalis (paper II). The influence of having a catheter going through the atrium is unknown, but did not seem to cause arrhythmias in paper I, where diabetic minipigs had these kind of catheters and the healthy minipigs had auricular-to-vena jugularis catheters.

Propofol administration

Prior to fastening the Holter ECG equipment, the minipigs were dosed with propofol i.v. to induce a brief, light sedation and ensure stress-free mounting of the recorders. Propofol has been shown to lower heart rate and alter the autonomic tonus in mice, resulting in transient alterations of heart rate variability[164]. A human study found alterations of the P-wave after longer-duration propofol anaesthesia, but no other changes[165]. The anaesthetic effect of propofol is very brief with a relatively high clearance[166], but a left-over effect of the propofol dosing cannot be totally excluded in these studies. In future studies, training of the minipigs to have equipment fasted without restraint should be considered.

Conclusion

This PhD project investigated healthy and streptozotocin-induced diabetic Göttingen Minipigs as a translational model of insulin-induced hypoglycaemia in regards to endocrine counter-regulation, electrocardiographic changes and platelet effects.

While the minipig is quite similar to humans in terms of cardiac electrophysiology, electrocardiographic recordings display significant diurnal changes, lead differences and changes over time. This underlines the necessity of obtaining baseline values when conducting studies with electrocardiographic outputs. While there are many similarities between the human and porcine ECG, future studies still need to account for the pig-specific features of the ECG, such as T-wave morphology. Diabetes induction did not cause changes to morphology and heart rate variability, as otherwise seen in humans with diabetes.

The similarities to humans in the cardiovascular system indicates the Göttingen Minipig as a good model for investigation of the myocardial effects of hypoglycaemia, with electrocardiographic changes resembling what have been found in clinical studies. However, the heart rate variability of the minipig during hypoglycaemia does not display the marked lowering as observed in clinical studies.

The counter-regulatory response to hypoglycaemia in minipigs resembles that of humans, with marked increases in epinephrine and glucagon levels. However, minipigs need very little glucose infusion to maintain hypoglycaemic target plasma glucose, indicating a high capacity for glucose mobilisation during hypoglycaemia in this species. Together with the naturally low fasting plasma glucose in the breed and lower glycaemic thresholds for hypoglycaemia, this leads to concerns in terms of translation to humans.

Hyperinsulinaemic hypoglycaemia in healthy and diabetic minipigs altered platelet aggregation, but did not increase it like demonstrated in clinical studies. This indicates a different response of porcine platelets to hyperinsulinaemic hypoglycaemia that is not comparable to humans.

Antecedent hypoglycaemia diminished the glucagon response, but not the epinephrine response, to hypoglycaemia. The effects of antecedent hypoglycaemia on the electrocardiogram is yet to be analysed.

Perspectives for further research

While the Göttingen Minipig model of insulin-induced hypoglycaemia shows promising potential as a translational model, in particular with regards to cardiovascular consequences, several limitations, as demonstrated in papers I-III, need to be taken into account when planning future studies.

Further studies, including a higher number of minipigs and a more chronic hypoglycaemia protocol, could elucidate if the epinephrine response to recurrent hypoglycaemia truly is kept normal or the lack of finding is due to high variation in the response. If the minipig displays a resistance to hypoglycaemia-induced counter-regulatory failure, the mechanisms behind this resilience could also be interesting to investigate.

The minipig model could also be used to investigate the mechanisms behind the positive cardiovascular effects observed in people treated with glucagon-like peptide 1 agonists and sodium-glucose cotransporter-2 inhibitors, as the porcine cardiovascular system has a high similarity to humans. Testing of devices for detection and treatment of hypoglycaemia could also be a possibility.

Finally, tissue samples from the brain cortex, hippocampus and left ventricle of the heart from minipigs included in paper III are currently being analysed to possibly discover recurrent hypoglycaemia and diabetes-induced changes to mitochondrial respiration as a mechanism behind brain damage and cardiovascular events found in people with diabetes.

References

1 Saeedi, P. *et al.* Global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045: Results from the International Diabetes Federation Diabetes Atlas, 9(th) edition. *Diabetes Research and Clinical Practice* **157**, 107843 (2019).

2 Skyler, J. S. *et al.* Differentiation of Diabetes by Pathophysiology, Natural History, and Prognosis. *Diabetes* **66**, 241-255 (2017).

3 Frier, B. M. Hypoglycaemia in diabetes mellitus: epidemiology and clinical implications. *Nature Reviews Endocrinology* **10**, 711-722 (2014).

4 Frier, B. M. How hypoglycaemia can affect the life of a person with diabetes. *Diabetes/Metabolism Research and Reviews* **24**, 87-92 (2008).

5 listed], N. a. The Diabetes Control and Complications Trial Research Group: Hypoglycemia in the Diabetes Control and Complications Trial. *Diabetes* **46**, 271-286 (1997).

6 Geddes, J., Schopman, J. E., Zammitt, N. N. & Frier, B. M. Prevalence of impaired awareness of hypoglycaemia in adults with Type 1 diabetes. *Diabetic Medicine* **25**, 501-504 (2008).

7 Seaquist, E. R. *et al.* Hypoglycemia and diabetes: a report of a workgroup of the American Diabetes Association and the Endocrine Society. *The Journal of Clinical Endocrinology & Metabolism* **98**, 1845-1859 (2013).

8 Gagnum, V. *et al.* Causes of death in childhood-onset Type 1 diabetes: long-term follow-up. *Diabetic Medicine* **34**, 56-63 (2017).

9 Gerstein, H. C. *et al.* Effects of Intensive Glucose Lowering in Type 2 Diabetes. *New England Journal of Medicine* **358**, 2545-2559 (2008).

10 Pieber, T. R. *et al.* DEVOTE 3: temporal relationships between severe hypoglycaemia, cardiovascular outcomes and mortality. *Diabetologia* **61**, 58-65 (2018).

11 Hanefeld, M., Duetting, E. & Bramlage, P. Cardiac implications of hypoglycaemia in patients with diabetes – a systematic review. *Cardiovascular Diabetology* **12** (2013).

12 Sprague, J. E. & Arbeláez, A. M. Glucose Counterregulatory Responses to Hypoglycemia. *Pediatric Endocrinology Reviews* **9**, 463-475 (2001).

13 Cryer, P. E., Davis, S. N. & Shamoon, H. Hypoglycemia in Diabetes. *Diabetes Care* **26**, 1902-1912 (2003).

14 Siafarikas, A. *et al.* Early loss of the glucagon response to hypoglycemia in adolescents with type 1 diabetes. *Diabetes Care* **35**, 1757-1762 (2012).

15 McCrimmon, R. Glucose Sensing During Hypoglycemia: Lessons From the Lab. *Diabetes Care* **32** (2009).

16 DeRosa, M. A. & Cryer, P. E. Hypoglycemia and the sympathoadrenal system: neurogenic symptoms are largely the result of sympathetic neural, rather than adrenomedullary, activation. *American Journal of Physiology: Endocrinology and Metabolism* **287**, E32-E41 (2004).

17 Verberne, A. J., Korim, W. S., Sabetghadam, A. & Llewellyn-Smith, I. J. Adrenaline: insights into its metabolic roles in hypoglycaemia and diabetes. *British Journal of Pharmacology* **173**, 1425-1437 (2016).

18 Bolli, G. *et al.* Important Role of Adrenergic Mechanisms in Acute Glucose Counterregulation Following Insulin-induced Hypoglycemia in Type I Diabetes: Evidence for an Effect Mediated by Beta-Adrenoreceptors. *Diabetes* **31**, 641-647 (1982).

19 Association, A. D. Standards of Medical Care in Diabetes 2020. *Diabetes Care* **43** (2020).

20 Cryer, P. E. Mechanisms of Hypoglycemia-Associated Autonomic Failure and Its Component Syndromes in Diabetes. *Diabetes* **54**, 3592-3601 (2005).

21 Heller, S. R. & Cryer, P. E. Reduced Neuroendocrine and Symptomatic Responses to Subsequent Hypoglycemia After 1 Episode of Hypoglycemia in Nondiabetic Humans. *Diabetes* **40**, 223-226 (1991).

22 McCrimmon, R. J. Consequences of recurrent hypoglycaemia on brain function in diabetes. *Diabetologia* **64**, 971-977 (2021).

23 Farrell, C. M. *et al.* A randomised controlled study of high intensity exercise as a dishabituating stimulus to improve hypoglycaemia awareness in people with type 1 diabetes: a proof-of-concept study. *Diabetologia* **63**, 853-863 (2020).

24 Rooijackers, H. M., Wiegers, E. C., Tack, C. J., van der Graaf, M. & de Galan, B. E. Brain glucose metabolism during hypoglycemia in type 1 diabetes: insights from functional and metabolic neuroimaging studies. *Cellular and Molecular Life Sciences* **73**, 705-722 (2016).

25 Sankar, A., Khodai, T., McNeilly, A. D., McCrimmon, R. J. & Luckman, S. M. Experimental Models of Impaired Hypoglycaemia-Associated Counter-Regulation. *Trends in Endocrinology &Metabolism* (2020).

26 Kannel, W. B. & McGee, D. L. Diabetes and Cardiovascular Risk Factors: The Framingham Study. *Circulation* **59**, 8-13 (1979).

27 Soedamah-Muthu, S. S. *et al.* High Risk of Cardiovascular Disease in Patients With Type 1 Diabetes in the U.K. *Diabetes Care* **29**, 798-804 (2006).

28 Graveiing, A. J. & Frier, B. M. Does hypoglycaemia cause cardiovascular events? *The British Journal of Diabetes and Vascular Disease* **10**, 5-13 (2010).

29 Stehouwer, C. D. A. Microvascular Dysfunction and Hyperglycemia: A Vicious Cycle With Widespread Consequences. *Diabetes* **67**, 1729-1741 (2018).

30 Dandona, P., Chaudhuri, A. & Dhindsa, S. Proinflammatory and prothrombotic effects of hypoglycemia. *Diabetes Care* **33**, 1686-1687 (2010).

31 Chow, E. *et al.* Risk of cardiac arrhythmias during hypoglycemia in patients with type 2 diabetes and cardiovascular risk. *Diabetes* **63**, 1738-1747 (2014).

32 Robinson, R. T. C. E. *et al.* Mechanisms of Abnormal Cardiac Repolarization During Insulin-Induced Hypoglycemia. *Diabetes* **52**, 1469-1474 (2003).

33 Wright, R. J. *et al.* Effects of acute insulin-induced hypoglycemia on indices of inflammation: putative mechanism for aggravating vascular disease in diabetes. *Diabetes Care* **33**, 1591-1597 (2010).

34 Gill, G. V., Woodward, A., Casson, I. F. & Weston, P. J. Cardiac arrhythmia and nocturnal hypoglycaemia in type 1 diabetes - the 'dead in bed' syndrome revisited. *Diabetologia* **52**, 42-45 (2009).

35 Wright, R. J. & Frier, B. M. Vascular disease and diabetes: is hypoglycaemia an aggravating factor? *Diabetes/Metabolism Research and Reviews* **24**, 353-363 (2008).

36 Fisher, B. M., Gillen, G., Dargie, H. J., Inglis, G. C. & Frier, B. M. The effects of insulin-induced hypoglycaemia on cardiovascular function in normal man: studies using radionuclide ventriculography. *Diabetologia* **30**, 841-845 (1987).

37 Kerr, D., MacDonald, A., Heller, S. R. & Tattersall, R. B. Beta-adrenoceptor blockade and hypoglycaemia. A randomised, double-blind, placebo controlled comparison of metoprolol CR, atenolol and propranolol LA in normal subjects. *British Journal of clinical Pharmacology* **29**, 685-693 (1990).

38 Hilsted, J. *et al.* Haemodynamic changes in insulin-induced hypoglycaemia in normal man. *Diabetologia* **26**, 328-332 (1984).

39 Parmley, W. W., Glick, G. & Sonnenblick, E. H. Cardiovascular effects of glucagon in man. *New England Journal of Medicine* **279**, 12-17 (1968).

40 Ceriello, A., Genovese, S., Mannucci, E. & Gronda, E. Glucagon and heart in type 2 diabetes: new perspectives. *Cardiovascular Diabetology* **15**, 123 (2016).

41 Wilkerson, R. D., B.Partlow, D., K.Pruett, J. & Patterson, C. W. A possible mechanism of the antiarrhythmic action of glucagon. *European Journal of Pharmacology* **44**, 57-63 (1977).

42 Novodvorsky, P. *et al.* Diurnal Differences in Risk of Cardiac Arrhythmias During Spontaneous Hypoglycemia in Young People With Type 1 Diabetes. *Diabetes Care* **40**, 655-662 (2017).

43 Andersen, A. *et al.* Associations of hypoglycemia, glycemic variability and risk of cardiac arrhythmias in insulin-treated patients with type 2 diabetes: a prospective, observational study. *Cardiovascular Diabetology* **20** (2021).

44 Marques, J. L. B. *et al.* Altered Ventricular Repolarization During Hypoglycaemia in Patients with Diabetes. *Diabetic Medicine* **14**, 648-654 (1997).

45 Laitinen, T. *et al.* Electrocardiographic Alterations during Hyperinsulinemic Hypoglycemia in Healthy Subjects. *Annals of Noninvasive Electrocariography* **13**, 97-105 (2008).

46 Mylona, M., Liatis, S., Anastasiadis, G., Kapelios, C. & Kokkinos, A. Severe iatrogenic hypoglycaemia requiring medical assistance is associated with concurrent prolongation of the QTc interval. *Diabetes Research and Clinical Practice* **161**, 108038 (2020).

47 Kano, M. *et al.* QT PRODACT: usability of miniature pigs in safety pharmacology studies: assessment for drug-induced QT interval prolongation. *Journal of Pharmacological Sciences* **99**, 501-511 (2005).

48 Sanguinetti, M. C. & Tristani-Firouzi, M. hERG potassium channels and cardiac arrhythmia. *Nature* **440**, 463-469 (2006).

49 Lee, S. P. *et al.* Effect of atenolol on QTc interval lengthening during hypoglycaemia in type 1 diabetes. *Diabetologia* **48**, 1269-1272 (2005).

50 Magnano, A. R., Talathoti, N., Hallur, R., Bloomfield, D. M. & Garan, H. Sympathomimetic infusion and cardiac repolarization: the normative effects of epinephrine and isoproterenol in healthy subjects. *Journal of Cardiovascular Electrophysiology* **17**, 983-989 (2006).

51 Gastaldelli, A., Emdin, M., Conforti, F., Camastra, S. & Ferrannini, E. Insulin prolongs the QTc interval in humans. *The American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* **279**, R2022–R2025 (2000).

52 Liamis, G., Liberopoulos, E., Barkas, F. & Elisaf, M. Diabetes mellitus and electrolyte disorders. *World Journal of Clinical Cases* **2**, 488-496 (2014).

53 B. Miles Fisher, Thomson, I., Hepburn, D. A. & Frier, B. M. Effects of Adrenergic Blockade on Serum Potassium Changes in Response to Acute Insulin-Induced Hypoglycemia in Nondiabetic Humans. *Diabetes Care* **14**, 548-552 (1991).

54 Skogestad, J. & Aronsen, J. M. Hypokalemia-Induced Arrhythmias and Heart Failure: New Insights and Implications for Therapy. *Frontiers in Physiology* **9**, 1500 (2018).

55 Weiss, J. N., Qu, Z. & Shivkumar, K. Electrophysiology of Hypokalemia and Hyperkalemia. *Circulation: Arrhythmia and Electrophysiology* **10**, 1-10 (2017).

56 Secrest, A. M., Becker, D. J., Kelsey, S. F., Laporte, R. E. & Orchard, T. J. Characterizing sudden death and dead-in-bed syndrome in Type 1 diabetes: analysis from two childhood-onset Type 1 diabetes registries. *Diabetic Medicine* **28**, 293-300 (2011).

57 Reno, C. M. *et al.* Severe Hypoglycemia-Induced Fatal Cardiac Arrhythmias Are Augmented by Diabetes and Attenuated by Recurrent Hypoglycemia. *Diabetes* **66**, 3091-3097 (2017).

58 da Silva, T. *et al.* Impaired awareness of hypoglycemia is associated with progressive loss of heart rate variability in patients with type 1 diabetes. *Diabetology & Metabolic Syndrome* **7** (2015).

59 Karayannis, G., Giamouzis, G., Cokkinos, D. V., Skoularigis, J. & Triposkiadis, F. Diabetic cardiovascular autonomic neuropathy: clinical implications. *Expert Review Cardiovascular Therapy* **10**, 747–765 (2012).

60 Agashe, S. & Petak, S. Cardiac Autonomic Neuropathy in Diabetes Mellitus. *Methodist Debakey Cardiovascular Journal* **14** (2018).

61 Vinik, A. I. & Ziegler, D. Diabetic cardiovascular autonomic neuropathy. *Circulation* **115**, 387-397 (2007).

62 Ramasamy, R. *et al.* Advanced glycation end products and RAGE: a common thread in aging, diabetes, neurodegeneration, and inflammation. *Glycobiology* **15**, 16R-28R (2005).

63 Shaffer, F. & Ginsberg, J. P. An Overview of Heart Rate Variability Metrics and Norms. *Front Public Health* **5**, 1-17 (2017).

64 Rajendra Acharya, U., Paul Joseph, K., Kannathal, N., Lim, C. M. & Suri, J. S. Heart rate variability: a review. *Medical & Biological Engineering & Computing* **44**, 1031-1051 (2006).

65 Bigger, J. T. *et al.* Frequency Domain Measures of Heart Period Variability and Mortality After Myocardial Infarction. *Circulation* **85**, 164-171 (1922).

66 Rothberg, L. J., Lees, T., Clifton-Bligh, R. & Lal, S. Association Between Heart Rate Variability Measures and Blood Glucose Levels: Implications for Noninvasive Glucose Monitoring for Diabetes. *Diabetes Technology & Therapeutics* **18**, 366-376 (2016).

67 Limberg, J. K. *et al.* Autonomic control during acute hypoglycemia in type 1 diabetes mellitus. *Clinical Autonomic Research* **24**, 275-283 (2014).

68 Cichosz, S. L., Frystyk, J., Tarnow, L. & Fleischer, J. Are Changes in Heart Rate Variability During Hypoglycemia Confounded by the Presence of Cardiovascular Autonomic Neuropathy in Patients with Diabetes? *Diabetes Technology & Therapeutics* **19**, 91-95 (2017).

69 Chow, E. *et al.* Cardiac Autonomic Regulation and Repolarization During Acute Experimental Hypoglycemia in Type 2 Diabetes. *Diabetes* **66**, 1322-1333 (2017).

70 Olde Bekkink, M., Koeneman, M., de Galan, B. E. & Bredie, S. J. Early Detection of Hypoglycemia in Type 1 Diabetes Using Heart Rate Variability Measured by a Wearable Device. *Diabetes Care* **42**, 689-692 (2019).

71 Koeneman, M., Olde Bekkink, M., Meijel, L. V., Bredie, S. & de Galan, B. Effect of Hypoglycemia on Heart Rate Variability in People with Type 1 Diabetes and Impaired Awareness of Hypoglycemia. *Journal of Diabetes Science and Technology*, 19322968211007485 (2021).

72 Fang, S. C., Wu, Y. L. & Tsai, P. S. Heart Rate Variability and Risk of All-Cause Death and Cardiovascular Events in Patients With Cardiovascular Disease: A Meta-Analysis of Cohort Studies. *Biological Research for Nursing* **22**, 45-56 (2020).

73 Cha, S. A. *et al.* Time- and frequency-domain measures of heart rate variability predict cardiovascular outcome in patients with type 2 diabetes. *Diabetes Research and Clinical Practice* **143**, 159-169 (2018).

74 Alessi, M. C. & Juhan-Vague, I. Metabolic syndrome, haemostasis and thrombosis. *Thrombosis and Haemostasis* **99**, 995-1000 (2008).

75 Vinik, A. I., Erbas, T., Park, T. S., Nolan, R. & Pittenger, G. L. Platelet Dysfunction in Type 2 Diabetes. *Diabetes Care* **24**, 1476-1485 (2001).

76 Wisinski, J. A. & Kimple, M. E. Platelet Dysfunction in Type 1 Diabetes: Stressing the Thromboxanes. *Diabetes* **65**, 349-351 (2016).

77 Trovati, M. *et al.* Insulin Directly Reduces Platelet Sensitivity to Aggregating Agents. *Diabetes* **37**, 780-786 (1988).

78 Ferreira, I. A. *et al.* Platelet inhibition by insulin is absent in type 2 diabetes mellitus. *Arteriosclerosis, Thrombosis, and Vascular Biology* **26**, 417-422 (2006).

79 Basili, S. *et al.* Insulin Resistance as a Determinant of Platelet Activation in Obese Women. *Journal of the American College of Cardiology* **48** (2006).

80 van der Meijden, P. E. J. & Heemskerk, J. W. M. Platelet biology and functions: new concepts and clinical perspectives. *Nat Rev Cardiol* **16**, 166-179 (2019).

81 Moin, A. S. M., Al-Qaissi, A., Sathyapalan, T., Atkin, S. L. & Butler, A. E. Platelet Protein-Related Abnormalities in Response to Acute Hypoglycemia in Type 2 Diabetes. *Frontiers in Endocrinology* **12**, 651009 (2021).

82 Hutton, R. A., Mikhailidis, D., Dormandy, K. M. & Ginsburg, J. Platelet aggregation studies during transient hypoglycaemia. *Journal of Clinical Pathology* **32**, 434-438 (1979).

83 Kahal, H. *et al.* Platelet function following induced hypoglycaemia in type 2 diabetes. *Diabetes & Metabolism* **44**, 431-436 (2018).

84 Anfossi, G. & Trovati, M. Role of catecholamines in platelet function: pathophysiological and clinical significance. *European Journal of Clinical Investigation* **26**, 353-370 (1996).

85 Trovati, M. *et al.* Studies on Mechanisms Involved in Hypoglycemia-Induced Platelet Activation. *Diabetes* **35**, 818-825 (1986).

86 Sudic, D. *et al.* High glucose levels enhance platelet activation: involvement of multiple mechanisms. *British Journal of Haematology* **133**, 315-322 (2006).

87 Sakamoto, T. *et al.* Rapid Change of Platelet Aggregability in Acute Hyperglycemia: Detection by a Novel Laser-light Scattering Method. *Thrombosis and Haemostasis* **83**, 475-479 (2000).

88 Pretorius, L. *et al.* Platelet activity and hypercoagulation in type 2 diabetes. *Cardiovascular Diabetology* **17**, 141 (2018).

89 Kleinert, M. *et al.* Animal models of obesity and diabetes mellitus. *Nature Reviews Endocrinology* **14**, 140-162 (2018).

90 Zettler, S. *et al.* A decade of experience with genetically tailored pig models for diabetes and metabolic research. *Animal Reproduction* **17** (2020).

91 Fall, T., Hamlin, H. H., Hedhammar, Å., Kämpe, O. & Egenvall, A. Diabetes Mellitus in a Population of 180,000 Insured Dogs: Incidence, Survival, and Breed Distribution. *Journal of Veterinary Internal Medicine* **21**, 1209-1216 (2007).

92 Zaragoza, C. *et al.* Animal models of cardiovascular diseases. *Journal of Biomedicine and Biotechnology* **2011**, 497841 (2011).

93 Larsen, M. O. & Rolin, B. Use of the Göttingen Minipig as a Model of Diabetes, with Special Focus on Type 1 Diabetes Research. *ILAR Journal* **45** (2004).

94 Gerstein, H. C. Why don't pigs get diabetes? Explanations for variations in diabetes susceptibility in human populations living in a diabetogenic environment. *Canadian Medical Association Journal* **174**, 25-26 (2006).

95 Szkudelski, T. The Mechanism of Alloxan and Streptozotocin Action in B Cells of the Rat Pancreas. *Physiological Research* **50**, 536-546 (2001).

96 Bolzán, A. D. & Bianchi, M. S. Genotoxicity of Streptozotocin. *Mutation Research* **512**, 121-134 (2002).

97 Raza, H. & John, A. Streptozotocin-induced cytotoxicity, oxidative stress and mitochondrial dysfunction in human hepatoma HepG2 cells. *International Journal of Molecular Sciences* **13**, 5751-5767 (2012).

98 Isaev, N. K., Genrikhs, E. E., Voronkov, D. N., Kapkaeva, M. R. & Stelmashook, E. V. Streptozotocin toxicity in vitro depends on maturity of neurons. *Toxicology and Applied Pharmacology* **348**, 99-104 (2018).

99 Wold, L. E. & Ren, J. Streptozotocin directly impairs cardiac contractile function in isolated ventricular myocytes via a p38 map kinase-dependent oxidative stress mechanism. *Biochemical and Biophysical Research Communications* **318**, 1066-1071 (2004).

100 Norgaard, S. A. *et al.* Optimising streptozotocin dosing to minimise renal toxicity and impairment of stomach emptying in male 129/Sv mice. *Laboratory Animals* **54**, 341-352 (2020).

101 Graham, M. L. & Schuurman, H. J. Validity of animal models of type 1 diabetes, and strategies to enhance their utility in translational research. *European Journal of Pharmacology* **759**, 221-230 (2015).

102 Renner, S. *et al.* Comparative aspects of rodent and nonrodent animal models for mechanistic and translational diabetes research. *Theriogenology* **86**, 406-421 (2016).

103 Reno, C. M. *et al.* Severe hypoglycemia induced fatal cardiac arrhythmias are mediated by the parasympathetic nervous system in rats. *Diabetes* (2019).

104 Reno, C. M. *et al.* Severe Hypoglycemia-Induced Fatal Cardiac Arrhythmias Are Mediated by the Parasympathetic Nervous System in Rats. *Diabetes* **68**, 2107-2119 (2019).

105 Reno, C. M. *et al.* Severe Hypoglycemia–Induced Lethal Cardiac Arrhythmias Are Mediated by Sympathoadrenal Activation. *Diabetes* **62**, 3570-3581 (2013).

106 Montanya, E., Nacher, V., Biarnés, M. & Soler, J. Linear Correlation Between Beta-Cell Mass and Body Weight Throughout the Lifespan in Lewis Rats: Role of Beta-Cell Hyperplasia and Hypertrophy. *Diabetes* **49** (2000).

107 Müller, M., Paschen, U. & H. J. Seitz. Glucose production measured by tracer and balance data in conscious miniature pig. *American Journal of Physiology* **244**, E236-244 (1983).

108 Agoston, D. V. How to Translate Time? The Temporal Aspect of Human and Rodent Biology. *Frontiers in Neurology* **8**, 92 (2017).

109 Luo, S., Michler, K., Johnston, P. & Macfarlane, P. W. A comparison of commonly used QT correction formulae: the effect of heart rate on the QTc of normal ECGs. *Journal of Electrocardiology* **37 Suppl**, 81-90 (2004).

110 Schuleri, K. H. *et al.* The Adult Göttingen Minipig as a Model for Chronic Heart Failure After Myocardial Infarction: Focus on Cardiovascular Imaging and Regenerative Therapies. *Comparative Medicine* **58**, 568-579 (2008).

111 Pedersen, H. D. & Mikkelsen, L. F. in *Biomarkers in Toxicology* 75-89 (2019).

112 Murakami, T., Hitomi, S., Ohtsuka, A., Taguchi, T. & Fujita, T. Pancreatic Insulo-Acinar Portal Systems in Humans, Rats, and Some Other Mammals: Scanning Electron Microscopy of Vascular Casts. *Microscopy Research and Technique* **37**, 478-488 (1997).

113 Wieczorek, G., Pospischil, A. & Perentes, E. A comparative immunohistochemical study of pancreatic islets inlaboratory animals (rats, dogs, minipigs, nonhuman primates). *Experimental and Toxicologic Pathology* **50**, 151-172 (1998).

114 Gradel, A. K. J. *et al.* The counterregulatory response to hypoglycaemia in the pig. *Basic & Clinical Pharmacology & Toxicology* **00**, 1-9 (2020).

115 J.P.Canavan, Flecknell, P. A., J.P.New, Alberti, K. G. M. M. & P.D.Home. The effect of portal and peripheral insulin delivery on carbohydrate and lipid metabolism in a miniature pig model of human IDDM. *Diabetologia* **40**, 1125-1134 (1997).

116 Sejling, A. S. *et al.* Hypoglycemia-associated changes in the electroencephalogram in patients with type 1 diabetes and normal hypoglycemia awareness or unawareness. *Diabetes* **64**, 1760-1769 (2015).

117 Kaarsholm, N. C. *et al.* Engineering Glucose Responsiveness Into Insulin. *Diabetes* **67**, 299-308 (2018).

118 Crick, S. J., Sheppard, M. N. & Anderson, R. H. Anatomy of the pig heart: comparisons with normal human cardiac structure. *Journal of Anatomy* **193**, 105-119 (1998).

119 Stricker-Krongrad, A. *et al.* Comparative cardiovascular physiology and pathology in selected lineages of minipigs. *Toxicology Research and Application* **1**, 1-8 (2017).

120 Bharati, S. *et al.* The conduction system of the swine heart. *Chest* **100**, 207-212 (1991).

121 Stubhan, M. *et al.* Evaluation of cardiovascular and ECG parameters in the normal, freely moving Gottingen Minipig. *Journal of Pharmacological and Toxicological Methods* **57**, 202-211 (2008).

122 Ludvigsen, T. P. *et al.* Gottingen minipig model of diet-induced atherosclerosis: influence of mild streptozotocin-induced diabetes on lesion severity and markers of inflammation evaluated in obese, obese and diabetic, and lean control animals. *Journal of Translational Medicine* **13**, 312 (2015).

123 Tsang, H. G. *et al.* Large animal models of cardiovascular disease. *Cell Biochemistry and Function* **34**, 113-132 (2016).

124 Schumacher-Petersen, C. *et al.* Experimental non-alcoholic steatohepatitis in Gottingen Minipigs: consequences of high fat-fructose-cholesterol diet and diabetes. *Journal of Translational Medicine* **17**, 1-18 (2019).

125 Fabricius, T. W. *et al.* Hyperinsulinaemic-hypoglycaemic glucose clamps in human research: a systematic review of the literature. *Diabetologia* **64**, 727-736 (2021).

126 He, H., Carballo-Jane, E., Tong, X. & Cohen, L. H. Measurement of catecholamines in rat and mini-pig plasma and urine by liquid chromatography-tandem mass spectrometry coupled with solid phase extraction. *Journal of Chromatography B* **997**, 154-161 (2015).

127 Diehl, K.-H. *et al.* A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes. *Journal of Applied Toxicology* **21**, 15-23 (2001).

128 Breddin, H. K. Can platelet aggregometry be standardized? *Platelets* **16**, 151-158 (2005).

129 Suzuki, A. *et al.* Establishment of a 24-hour electrocardiogram recording system using a Holter recorder for miniature swine. *Laboratory Animals* **32**, 165-172 (1998).

130 Lyhne, M. K. *et al.* Electrocardiography and heart rate variability in Gottingen Minipigs: Impact of diurnal variation, lead placement, repeatability and streptozotocin-induced diabetes. *Journal of Pharmacological and Toxicological Methods*, 107221 (2022).

131 Kusumoto, F. M. *et al.* 2018 ACC/AHA/HRS Guideline on the Evaluation and Management of Patients With Bradycardia and Cardiac Conduction Delay: Executive Summary: A Report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines, and the Heart Rhythm Society. *Journal of the American College of Cardiology* **74**, 932-987 (2019).

132 Curtis, M. J. *et al.* The Lambeth Conventions (II): guidelines for the study of animal and human ventricular and supraventricular arrhythmias. *Pharmacology & Therapeutics* **139**, 213-248 (2013).

133 Kurl, S., Makikallio, T. H., Rautaharju, P., Kiviniemi, V. & Laukkanen, J. A. Duration of QRS complex in resting electrocardiogram is a predictor of sudden cardiac death in men. *Circulation* **125**, 2588-2594 (2012).

134 Caceres, C. A., Kelser, G. A. & Mize, W. R. Formation of the "P-R Segment". *Circulation* **20**, 229-233 (1959).

135 Macfarlane, P. W. *et al.* The Early Repolarization Pattern: A Consensus Paper. *Journal of the American College of Cardiology* **66**, 470-477 (2015).

136 Lepeschkin, E. & Surawicz, B. The Measurement of the Q-T Interval of the Electrocardiogram. *Circulation* **6**, 378-388 (1952).

137 Havmoller, R. *et al.* Age-related changes in P wave morphology in healthy subjects. *BMC Cardiovascular Disorders* **7**, 22 (2007).

138 Rautaharju, P. M. *et al.* AHA/ACCF/HRS recommendations for the standardization and interpretation of the electrocardiogram: part IV: the ST segment, T and U waves, and the QT interval: a scientific statement from the American Heart Association Electrocardiography and Arrhythmias Committee, Council on Clinical Cardiology; the American College of Cardiology Foundation; and the Heart Rhythm Society. Endorsed by the International Society for Computerized Electrocardiology. *Journal of the American College of Cardiology* **53**, 982-991 (2009).

139 Zhang, S.-B. *et al.* Normal Electrocardiogram of Bama Miniature Pigs (Sus scrofa domestica). *Journal of the American Association for Laboratory Animal Science* **55**, 150-152 (2016).

140 Al-Khatib, S. M. *et al.* 2017 AHA/ACC/HRS Guideline for Management of Patients With Ventricular Arrhythmias and the Prevention of Sudden Cardiac Death: A Report of the

American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines and the Heart Rhythm Society. *Circulation* **138**, e272-e391 (2018).

141 Dickinson, D. F. The normal ECG in childhood and adolescence. *Heart* **91**, 1626-1630 (2005).

142 Jones, R. D., Stuart, B. P., Greufe, N. P. & Landes, A. M. Electrophysiology and pathology evaluation of the Yucatan pig as a non-rodent animal model for regulatory and mechanistic toxicology studies. *Laboratory Animals* **33**, 356-365 (1999).

143 Veglio, M., Chinaglia, A. & Cavallo-Perin, P. QT interval, cardiovascular risk factors and risk of death in diabetes. *Journal of Endocrinological Investigation* **27**, 175-181 (2004).

144 Kudat, H. *et al.* Heart Rate Variability in Diabetes Patients. *The Journal of International Medical Research* **34**, 291-296 (2006).

145 Chiariello, M. & Indolfi, C. Silent Myocardial Ischemia in Patients with Diabetes Mellitus. *American Heart Journal* (1996).

146 Kautzner, J. & Camm, A. J. Clinical Relevance of Heart Rate Variability. *Clinical Cardiology* **20**, 162-168 (1997).

147 Pfeifer, M. A. *et al.* Quantitative Evaluation of Cardiac Parasympathetic Activity in Normal and Diabetic Man. *Diabetes* **37**, 339-345 (1982).

148 Kuwahara, M. *et al.* Power Spectral Analysis of Heart Rate Variability for Assessment of Diurnal Variation of Autonomic Nervous Activity in Miniature Swine. *Laboratory Animal Science* **49**, 202-208 (1999).

149 Jong, I. C. d. *et al.* Effects of social stress on heart rate and heart rate variability in growing pigs. *Canadian Journal of Animal Science* **80**, 273–280 (2000).

150 American Diabetes, A. 6. Glycemic Targets: Standards of Medical Care in Diabetes-2019. *Diabetes Care* **42**, S61-S70 (2019).

151 Nauck, M. A. *et al.* Effects of Glucagon-Like Peptide 1 on Counterregulatory Hormone Responses, Cognitive Functions, and Insulin Secretion during Hyperinsulinemic, Stepped Hypoglycemic Clamp Experiments in Healthy Volunteers. *The Journal of Clinical Endocrinology & Metabolism* **87**, 1239-1246 (2002).

152 McNeilly, A. D., Gallagher, J. R., Huang, J. T., Ashford, M. L. J. & McCrimmon, R. J. High-Intensity Exercise as a Dishabituating Stimulus Restores Counterregulatory Responses in Recurrently Hypoglycemic Rodents. *Diabetes* **66**, 1696-1702 (2017).

153 Sagel, J., Colwell, J. A., Crook, L. & Laimins, M. Increased Platelet Aggregation in Early Diabetus Mellitus. *Annals of Internal Medicine* **82**, 733-738 (1975).

154 Shukla, S. D. *et al.* Platelets from Diabetic Pigs Exhibit Hypersensitivity to Thrombin. *Comparative Medicine* **58**, 481-484 (2008).

155 A. Eldor, S. M., and H. Bar-On. The Effect Of Streptozotocin Diabetes On Platelet Function In Rats. *Thrombosis Research* **13**, 703-714 (1978).

156 Leighton, E., Sainsbury, C. A. & Jones, G. C. A Practical Review of C-Peptide Testing in Diabetes. *Diabetes Therapy* **8**, 475-487 (2017).

157 Vickneson, K. *et al.* Cold-induced dishabituation in rodents exposed to recurrent hypoglycaemia. *Diabetologia* **64**, 1436-1441 (2021).

158 Veldhuis, J. D., Keenan, D. M. & Pincus, S. M. Motivations and methods for analyzing pulsatile hormone secretion. *Endocrine Reviews* **29**, 823-864 (2008).

159 Best, J. D. & Halter, J. B. Release and Clearance Rates of Epinephrine in Man: Importance of Arterial Measurements. *Journal of Clinical Endocrinology and Metabolism* **55**, 263-268 (1982).

160 Pelagalli, A. *et al.* Adhesive Properties of Platelets from Different Animal Species. *Journal of Comparative Pathology* **128**, 127-131 (2003).

161 Goodman, S. L. Sheep, pig, and human platelet–material interactions with model cardiovascular biomaterials. *Journal of Biomedical Materials Research* **45**, 240-250 (1999).

162 Zurbano, M. J. *et al.* Characterization of adhesive and aggregating functions of pig platelets: comparative studies with human platelets and differential effects of calcium chelation. *Platelets* **9**, 329-337 (1998).

163 Rehman, K. & Akash, M. S. Mechanisms of inflammatory responses and development of insulin resistance: how are they interlinked? *Journal of Biomedical Science* **23**, 87 (2016).

164 Shintaku, T. *et al.* Effects of propofol on electrocardiogram measures in mice. *Journal of Pharmacological Sciences* **126**, 351-358 (2014).

165 Bolat, E. *et al.* Effects of balanced propofol sedation on QT, corrected QT, and P-wave dispersion on upper endoscopy. *The Anatolian Journal of Cardiology* **16**, 328-332 (2016).

166 Cockshott, I. D., Douglas, E. J., Plummer, G. F. & Simons, P. J. The pharmacokinetics of propofol in laboratory animals. *Xenobiotica* **22**, 369-375 (1992).

Supplemental

Software arrhythmia detection

Software standard setting for arrhythmia detection criteria in Pathfinder SL software (September 2022).

Event	**Detection criteria**
Atrial fibrillation detection	On/off
Ventricular events	
Premature ventricular ectopic complex	<90% of prevailing NN interval
Ventricular escape	>=300% of prevailing NN interval
R on T	<170 ms + 23% of prevailing RR interval
Pair	On/off
Bigemini	>=2 cycles
Trigemini	>=2 cycles
Idioventricular rhythm	>=3 beats at <=40 bpm
Ventricular-drive/accelerated idioventricular rhythm	>=3 beats at other frequencies
Ventricular tachycardia	>=3 beats at >=100 bpm
Dropped beat	>=180% of prevailing NN-interval
Pause	>=2.00 sec
Bradycardia	>=4 intervals at >=45 bpm
Tachycardia	>=5 intervals at >=300 bmp
Supraventricular events	
Supraventricular ectopic complexes	<50% of prevailing NN interval
Supraventricular escape complexes	>=300% of prevailing NN interval
Supraventricular pair	On/off
Supraventricular drive	>=3 beats
Supraventricular tachycardia	>=3 beats at >=150 bpm
Other	
Artefact detection	On/off
Sinus rhythm detection	On/off

Paper I

Electrocardiography and heart rate variability in Göttingen Minipigs: Impact of diurnal variation, lead placement, repeatability and streptozotocin-induced diabetes.

Mille Kronborg Lyhne, Karina Poulsdóttir Debes, Terese Helgogaard, Andreas Vegge, Jonas Kildegaard, Ulrik Pedersen-Bjergaard and Lisbeth Høier Olsen.

Journal of Pharmacological and Toxicological Methods, volume 118, November–December 2022, (107221), https://doi.org/10.1016/j.vascn.2022.107221

Journal of Pharmacological and Toxicological Methods 118 (2022) 107221

Contents lists available at ScienceDirect

Journal of Pharmacological and Toxicological Methods

journal homepage: www.elsevier.com/locate/jpharmtox

Electrocardiography and heart rate variability in Göttingen Minipigs: Impact of diurnal variation, lead placement, repeatability and streptozotocin-induced diabetes

Mille Kronborg Lyhne [a], Karina Poulsdóttir Debes [a], Terese Helgogaard [a], Andreas Vegge [b], Jonas Kildegaard [b], Ulrik Pedersen-Bjergaard [c], Lisbeth Høier Olsen [a,*]

[a] Department of Veterinary and Animal Sciences, University of Copenhagen, Ridebanevej 9, 1870 Frederiksberg, Denmark
[b] Global Drug Discovery, Novo Nordisk A/S, Novo Nordisk Park 1, 2760 Måløv, Denmark
[c] Department of Endocrinology and Nephrology, Nordsjællands Hospital Hillerød, Dyrehavevej 29, 3400 Hillerød, Denmark

ARTICLE INFO

Keywords:
Electrocardiography
Diabetes
Porcine
Translational
Methods
Heart rate variability
Arrhythmia
Göttingen Minipigs
Cardiovascular disease

ABSTRACT

Background: The Göttingen Minipig is widely used in preclinical research and safety pharmacology, but standardisation of porcine electrocardiography (ECG) is lacking. The aim of this study was to investigate diurnal effects, change over time and choice of lead on ECG morphology and heart rate variability (HRV) in healthy and streptozotocin (STZ) induced diabetic Göttingen Minipigs.

Methods: Diabetes was experimentally induced using STZ in 11 Göttingen Minipigs (DIA). Seven controls (CON) were included. 24-h ECG was recorded at baseline and four months. Morphological parameters (QRS and T wave duration, P- and T-wave amplitude, PR and QT (Bazett's (QTcb) or Fridericia (QTcf) correction) intervals and ST segment), presence of cardiac arrhythmias, heart rate (HR) and HRV (time and frequency domain) were analysed.

Results: Four months after induction, DIA had decreased P-wave amplitude ($P < 0.0001$) and T-wave duration ($P = 0.017$), compared to CON. QTcb was lower in DIA, but not in CON. Both groups had decreased HR ($P < 0.0001$) and QRS duration (lead II, $P = 0.04$) and length of PR-segment increased (lead I and II, $P < 0.01$) while selected HRV parameters also increased (all $P < 0.01$). Time of day influenced HR, QRS duration, PR segment, ST segment, T- and P-wave amplitude and some parameters of HRV. Inter- and intra-observer variability of morphological measurements was low (<6%).

Conclusion: ECG parameters were influenced by time setting, diurnal variation and lead. Some ECG and HRV changes were found in diabetic minipigs four months after STZ induction. The findings underline the need for standardisation of ECG and HRV in Göttingen Minipigs.

1. Introduction

The pig is a widely used large animal model in preclinical research. While dogs have been a commonly used large animal model of cardiovascular disease, pigs are now the dominant large animal laboratory species (Zaragoza et al., 2011). The pig offers several advantages in cardiovascular and metabolic research. Cardiovascular anatomy and physiology of pigs, including heart anatomy (Crick, Sheppard, & Anderson, 1998), cardiac conduction system (Stricker-Krongrad et al., 2017) and resting heart rates(Stubhan et al., 2008), closely resemble that of humans. The Göttingen Minipig is often used as an animal model of human diabetes (Larsen & Rolin, 2004) and in cardiovascular research (Diemar et al., 2015; Schuleri et al., 2008).

In both preclinical research and safety pharmacology (Kano et al., 2005), electrocardiography (ECG) is a required tool for assessing cardiac conduction and possible alterations in this. Features of the pig ECG have been described in several publications, spanning different breeds and recording techniques, but many of these studies are either conducted under restraint (Zhang et al., 2016), sedation or anaesthesia (Paslawska et al., 2014), or using recordings of short duration (Schuleri et al., 2008;

* Corresponding author.
E-mail addresses: mille@sund.ku.dk (M.K. Lyhne), karina.debes@sund.ku.dk (K.P. Debes), AVEG@novonordisk.com (A. Vegge), JKIP@novonordisk.com (J. Kildegaard), ulrik.pedersen-bjergaard@regionh.dk (U. Pedersen-Bjergaard), lisbeth.hoier@sund.ku.dk (L.H. Olsen).

https://doi.org/10.1016/j.vascn.2022.107221
Received 24 May 2022; Received in revised form 26 August 2022; Accepted 7 September 2022
Available online 11 September 2022

Zhang et al., 2016). Some studies record 24-h ECG from freely moving Göttingen Minipigs using surgically implanted leads (Kano et al., 2005; Stubhan et al., 2008). While some thoroughly describe the methods for obtaining the ECG recordings, such as fixation of the pigs, choice of lead and placement (Suzuki et al., 1998), duration and time of day of the recording (Kuwahara et al., 1999; Kuwahara et al., 2004) and housing (Jong et al., 2000), most do not (Schuleri et al., 2008), even though these factors have been show to influence the ECG. When manually measuring morphological features of the ECG, the possible observer effect is also often not addressed (Connelly et al., 2020; Paslawska et al., 2014).

Studies have demonstrated that diabetes is associated with morphological ECG changes (Ewing, Boland, Neilson, Cho, & Clarke, 1991; Gallego, Zayas-Arrabal, Alquiza, Apellaniz, & Casis, 2021). People with diabetes can have a prolongation of the QT-interval, an ECG change that is considered proarrhythmic and exacerbated by insulin-induced hypoglycaemia (Gallego et al., 2021; Marques et al., 1997). Also, few studies have reported elevated ST-segment in people with diabetes due to silent myocardial infarction (Chiariello & Indolfi, 1996; Karayannis, Giamouzis, Cokkinos, Skoularigis, & Triposkiadis, 2012). Cardiovascular autonomic neuropathy (CAN), a serious complication of diabetes, has been associated with hyperglycaemia, hypoglycaemia (Cryer, 2005), high glucose variability (Jun et al., 2019) and duration of diabetes (Karayannis et al., 2012). The neuropathy is suggested to involve both sympathetic and parasympathetic innervation, leading to autonomic dysfunction of the heart (Uehara, Kurata, Sugi, Mikami, & Shouda, 1999), causing lower heart rate variability (HRV) (Jaiswal et al., 2014; Uehara et al., 1999), a well-documented risk factor for CVD (Thayer, Yamamoto, & Brosschot, 2010) and predictive of CVD outcomes in people with type 2 diabetes (Cha et al., 2018).

While these changes are relatively well documented in people with diabetes, they have not been investigated in the streptozotocin-induced diabetic Göttingen Minipig. Even if studies investigated the translational value of the disease model, not many studies of the normal pig ECG exist, making the difference between normal and pathological difficult to distinguish. Only few studies have reported standardised morphological and HRV features of the minipig ECG and no studies have investigated the effect of streptozotocin-induced diabetes, even though studies on cardiovascular and metabolic preclinical research using pigs are steadily increasing.

This study aimed to investigate ECG morphology and HRV in healthy and diabetic, freely moving Göttingen Minipigs using 24-h Holter recordings. We hypothesised, that morphological features of the ECG would have diurnal variations, would not change over the course of four months, would vary depending on choice of ECG lead, and measurements would have low inter-observer variation. It was hypothesised that streptozotocin-induced diabetes prolonged QT-intervals, increased the ST-segment elevation and decreased time domain and frequency domain parameters of HRV after four months, compared to a healthy control group.

2. Materials and methods

2.1. Animals

The animal experimentation in this study has been approved by the Danish Animal Experiment Inspectorate and conducted in accordance with current rules and regulations and in compliance with ARRIVE guidelines.

Holter ECG recordings were prospectively recorded in eighteen intact, adult, healthy lean female Göttingen Minipigs (Ellegaard Göttingen Minipigs A/S, Dalmose, Denmark). Following baseline recordings, diabetes was induced using streptocotozin in eleven pigs (DIA), and seven pigs were kept as healthy controls (CON) (see Fig. 1). Final ECG recordings were obtained four months after the baseline. All pigs were naïve at study start but were used for other pharmacological studies between the two recordings until two weeks before the follow up ECG recording. The inclusion process was not randomized according to group but was based on availability. Pigs were kept at Novo Nordisk A/S Animal Unit (Ganløse, Denmark) at 18–22 °C with relative air humidity of 30–70% and a 12-h light/dark cycle with windows for natural light as well. Pigs were fed 400–500 g of feed (SDS minipig, Special Diets Service, Essex, UK) once daily in the morning, after procedures. Pigs were single housed with possibility of both visual and snout contact on straw and woodchip bedding with free access to water. Prior to study start, pigs were implanted with two central venous catheters using the Seldinger technique modified from Larsen et al., 2002 and placed as described by Lyhne et al., 2021, enabling intra venous (i.v.) anaesthesia, diabetes induction and blood sampling.

2.2. Diabetes induction and daily glycaemic control

Following baseline ECG recordings, pigs in the DIA group were made diabetic by once-daily i.v. injections of 50 mg/kg streptozotocin (S0130, Sigma Aldrich Denmark A/S, Søborg, Denmark) for three consecutive days as per protocol modified from Schumacher-Petersen et al., 2019. After diabetes induction, pigs were treated with insulins daily to manage hyperglycaemia. Two weeks prior to the follow-up ECG recording, treatment was changed to once-daily subcutaneous (s.c.) injections of insulin glargine (Lantus, Sanofi S.A., Paris, France), where the exogenous insulin was given in order to reach a fasting plasma glucose target of 5–10 mM.

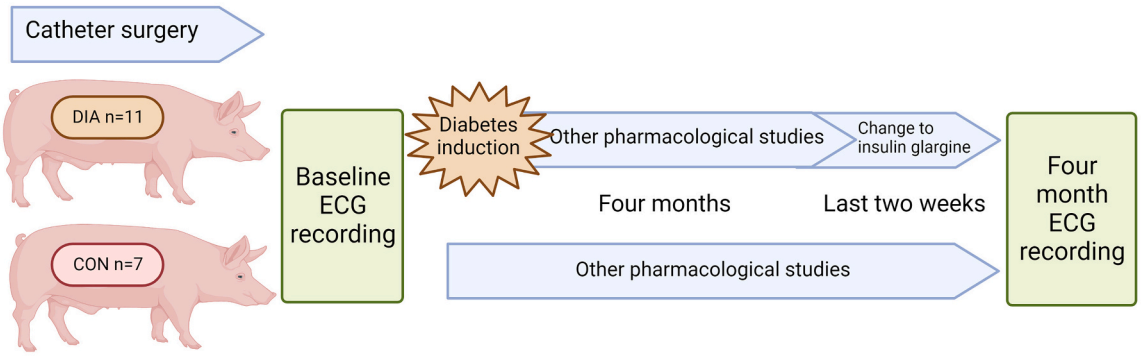


Fig. 1. Study design.
Illustration of study design. Eighteen Göttingen Minipigs were included and permanent, venous catheters were surgically implanted. All pigs had 24-h Holter electrocardiographic (ECG) recordings made. Eleven pigs were made diabetic. All pigs were included in other pharmacological studies in between recordings. Two weeks prior to the final recording, diabetic pigs were treated with insulin glargine to manage hyperlycaemia. A final recording was obtained in both groups after four months. Figure created with Biorender.com.

2.3. Continuous 24-h (Holter) electrocardiography recordings

A Lifecard CF recorder (Spacelabs Healthcare, WA, USA) with two leads were placed as previously described by Suzuki et al., 1998. Lead I was created with a positive electrode on the dorsal neck area (A) and a negative electrode on processus xiphoideus (B). Lead II was created with a positive electrode on manubrium sterni (C) and a negative electrode on processus xiphoideus (D) (see Fig. 2). Electrode attachments chosen were 3 M Red Dot 2670 electrodes patches (3 M A/S, Copenhagen, Denmark) due to the strength of the adhesive in these particular patches. Wires were secured to the pig's skin with bandaging material (Tensoplast, BSN Medical A/S, Lillerød, Denmark) and the recorder was placed in the pouch of the harness placed just cranial to the scapulae (see Fig. 2). The Holter equipment was placed in the morning before feeding and pigs were dosed with 8–10 mL propofol i.v. (Propofol "B. Braun" 10 mg/mL, B. Braun Medical A/S, Frederiksberg, Denmark) to ensure stress-free attachment of the Holter equipment. After the procedure, pigs were fed their daily allowance and DIA dosed with insulin approximately one hour after anaesthesia recovery. Equipment was removed from awake pigs 24 h later.

2.4. Electrocardiography analyses

All ECG analyses were performed by one observer. In the morphological analyses the observer was blinded for pig group (DIA or CON), time period (baseline or four month follow up) and time of day. Morphological analysis was performed on digital printed ECG traces (.pdf format) obtained in the Pathfinder SL software and manually measured using graphical software ImageJ (National Institute of Health Sciences, Kawasaki, Japan). Arrhythmia and HRV analyses were performed using the software Pathfinder SL (Spacelabs Healthcare, WA, USA).

In the Pathfinder SL software, ECG recordings were manually edited for correct R-wave detection and arrhythmia identification. Leads and ECG prints were excluded if the quality was inadequate for analysis, e.g. in the case of electrodes falling off or poor electrical signal.

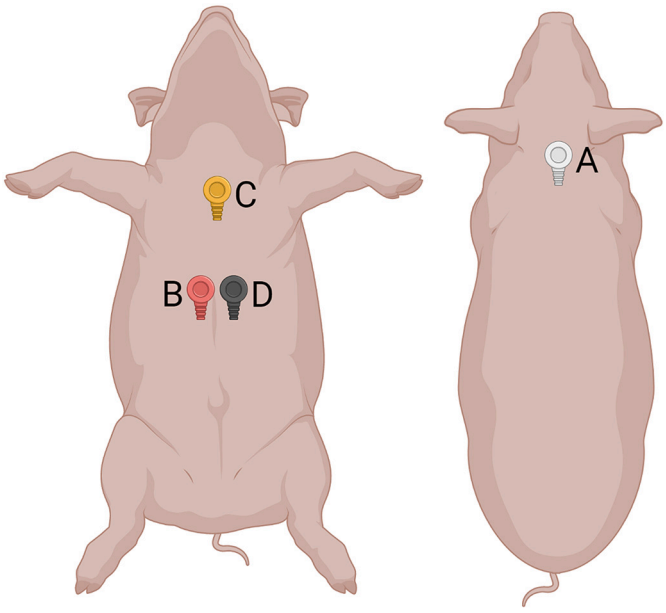


Fig. 2. Electrode placement.
Illustration of placement of electrodes. Lead I: positive electrode at dorsal neck area (A) and negative electrode on processus xiphoideus (B). Lead II: positive electrode on manubrium sterni (C) and a negative electrode on processus xiphoideus (D). Figure created with Biorender.com.

2.5. Arrhythmia analysis

Arrhythmias were evaluated in the 24 h recording and presented as a total number of arrhythmic events during 24 h. Arrhythmic events were placed into three categories: 2nd degree atrioventricular (AV) block, supraventricular ectopic complexes and ventricular ectopic complexes (Curtis et al., 2013). The 2nd degree AV blocks were defined as a singular P-wave with absent QRS complex (Kusumoto et al., 2019). Supraventricular ectopic complexes (SVE) were defined as complexes with a normal QRS morphology with either a P′ wave or non-visible P-wave and P-P′ interval shorter or longer than P—P interval (Curtis et al., 2013) and the RR interval either shorter (premature complex) or longer (escape complex) than the sinus RR interval (Curtis et al., 2013). Both premature and escape complexes were categorised as SVE. Ventricular ectopic complexes (VE) were defined as a QRS-complex with different shape with either broader duration and/or height (altered electrical axis). The RR interval in relation to the preceding QRS complex was either shorter (premature complex) or longer (escape complex) than the sinus RR interval, as well as no coupled P-wave (Curtis et al., 2013).

2.6. Electrocardiographic morphology

Prints of ten consecutive RR intervals were collected at noon (12:00, DAY), during feeding (approximately 08:00, FEED) and at midnight (00:00, NIGHT) to represent calm awake, excited and sleeping ECG traces. QRS complex duration was measured from the initial deflection until the last, regardless of polarity (Kurl, Makikallio, Rautaharju, Kiviniemi, & Laukkanen, 2012). PR segment was measured from the return of the P-wave to the isoelectric line until the very first deflection of the QRS complex (Caceres, Kelser, & Mize, 1959). ST-segment derivation was measured from the J-point, defined as the very end of the last QRS notch, using the PR segment as isoelectric line (Macfarlane et al., 2015). T-wave amplitude was measured from the isoelectric line formed right after the T-wave to ensure no ST-elevation would influence the height. T-wave duration was measured from the start to the end of the deflection created by the T-wave (Lepeschkin & Surawicz, 1952). P-wave amplitude was measured using the PR-segment as isoelectric line. Polarity of P- and T-wave was noted for each beat to be either positive, negative or diphasic (Havmoller et al., 2007). Concurrent heart rate was calculated by the Pathfinder software on the basis of the printed RR intervals and used when correcting the QT-interval (Luo, Michler, Johnston, & Macfarlane, 2004). QT-interval was measured from the beginning of the QRS complex until the end of the T wave (return of the T-wave to isoelectric line) and was corrected using both Bazett's (QTcb) and Fridericia's (QTcf) formulas (Kano et al., 2005; Luo et al., 2004). All morphological measures were reported as an average of ten RR intervals on each ECG print.

2.7. Heart rate variability

Using the build-in software of Pathfinder SL, HRV outcome was obtained for the predefined epochs. Time domain HRV variables SDNN (standard deviation of beat-to-beat intervals) and RMSSD (root mean square of successive RR differences) were obtain from 12:00 until 18:00 (daytime HRV), at 00:00 until 06:00 (night-time HRV) and for the entire 24-h recordings. Frequency domain HRV was calculated in the same time intervals as above using the build-in Fast Fourier transformation to very-low frequency (VLF) power band at 0.015–0.05 Hz, normalised low frequency (LF n.u.) power band at 0.05–0.15 Hz, normalised high frequency (HF n.u.) power band at 0.15–0.40 Hz and very high frequency (VHF) power band at 0.35–0.5 Hz as well as LF/HF ratio.

2.8. Inter- and intra-observer variability

To assess inter- and intra-observer variability, six randomly chosen ECG prints were included. For inter-observer variability, QRS complex

duration, QT-interval, PR-segment duration, ST-segment derivation, T-wave amplitude, T-wave duration, P-wave amplitude were measured as described above, by two observers. Each observer measured each recording (average of ten consecutive beats). Inter observer variability was calculated as a relative difference A-B/(A + B)*0.5. To assess intra-observer variability, one observer measured each recording six times, as described above. The standard deviation of six observations of the same print (SD) and coefficient of variation (CV) was calculated (CV=SD/mean of six observations).

2.9. Statistical analyses

Linear mixed models with individual pig as random variable were used to test if group (DIA and CON), time of recording, time of day and heart rate influenced presence of ECG morphology and HRV. Interaction between group and time of day, and in addition group and time of recording were evaluated as well. Models were reduced by backward elimination and logarithmic transformation was used as needed to obtain homogeneity and normal distribution of model residuals. Differences between groups in arrhythmia prevalence, weight and age were tested using Mann-Whitney *U* test and Wilcoxon signed rank test for weight gain, lead differences and change in arrhythmia prevalence. *P*-values were corrected using False Discovery rate (FDR) and only corrected *P*-values <0.05 were considered significant. Statistical analysis using linear mixed models was created using SAS 9.4 (SAS Institute, Cary, USA) and FDR correction, Mann-Whitney U test and Wilcoxon signed rank test was calculated using GraphPad Prism 9 (GraphPad Software Inc., San Diego, USA). Results are presented as medians and interquartile range unless specified.

3. Results

3.1. Weight, age and fasting plasma glucose

Pigs in the control group (CON) were older than pigs that were made diabetic (DIA) (at baseline: 238 days (231–245) (DIA) and 424 days (405–431) (CON); ($P = 0.0002$)). Moreover, DIA has increased body weight (BW) both at baseline (29.4 kg (27.4–30.0)) and after four months (35.2 kg (35.1–35.8)) compared to CON (25.0 kg (25.0–26.1) and 30.5 kg (30.0–32.0)), $P = 0.0006$ and 0.0002, respectively). Both groups increased in BW during the four-month period (CON ($P = 0.03$) and DIA ($P = 0.001$)).

Individual daily fasting plasma glucose concentrations were in average 8.07 mM (SD 3.43) in DIA during the four weeks prior to the last ECG recording and the mean coefficient of individual variability was 42.2% (see Fig. 3). Fasting plasma glucose was not measured in CON, however, historical data of fasting plasma glucose of lean, healthy Göttingen Minipigs at the facility is typically 3.5–4.0 mM[28].

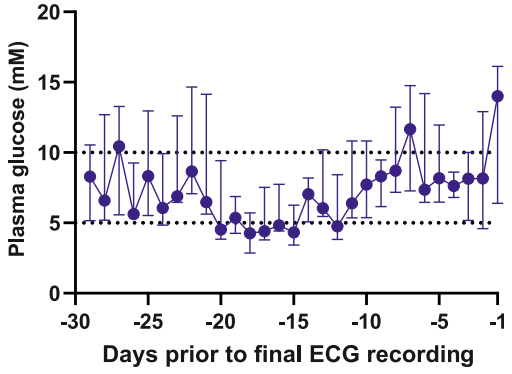


Fig. 3. Daily fasting plasma glucose of diabetic pigs.
Morning fasting plasma glucose of 11 diabetic pigs 29 days prior to the final Holter ECG recording reflecting daily glucose variations. Data is presented as median average fasted plasma glucose of the pigs. Error bars: interquartile range. Horizontal lines: plasma glucose target of 5–10 mM.

3.2. ECG morphology and lead differences

After four months, T-wave duration and P-wave amplitude were significantly decreased in DIA compared to CON (T-wave only lead I $P = 0.02$ and P-wave $P < 0.05$, both leads) (Fig. 4). In addition, Bazett-corrected QT interval (QTcb) was significantly shorter in DIA compared to CON ($P = 0.04$, lead I). In line with this, T-wave duration, P-wave amplitude (T-wave $P < 0.05$, both leads, P-wave $P < 0.0001$ both leads) and QTcb were reduced ($P < 0.01$, both leads) after four months in DIA, but CON did not differ from baseline (see Table 3).

QRS duration ($P = 0.04$, lead I), PR segment ($P < 0.01$, both leads) and non-corrected QT interval ($P < 0.0001$, both leads) all increased after four months. There was no difference between baseline and four months in Fridericia-corrected QT interval (QTcf) (see Table 3).

Several parameters had diurnal changes. QRS duration and PR segment were longer at DAY compared to FEED ($P = 0.04$ lead I, $P < 0.01$ both leads, respectively), and NIGHT was also longer than DAY ($P = 0.05$ lead I, $P < 0.0001$ lead II, respectively). ST segment was higher during DAY compared to NIGHT ($P < 0.05$, both leads) and higher at FEED compared to NIGHT ($P < 0.0001$, lead I). T-wave and P-wave amplitude were higher during FEED compared to NIGHT and DAY (all $P < 0.0001$). Uncorrected QT interval was higher during DAY and NIGHT compared to FEED (all $P < 0.05$). QTcb was higher during FEED compared to NIGHT and DAY (all $P < 0.0001$) and DAY was higher than NIGHT ($P < 0.05$ both leads) (see Table 3).

When comparing lead I and lead II at baseline measurements, lead I generally had higher QRS duration, QT interval, ST segment, T-wave duration, T-wave and P-wave amplitude with differences being found at all time points during the day (see Table 6).

3.3. Changes in heart rate (HR)

When considering heart rates recorded over one minute in conjunction with morphology analysis, HR decreased after four months ($P < 0.0001$) in both groups. HR was highest during FEED, and HR during DAY was higher than during NIGHT (all $P < 0.0001$), with no difference between groups (Table 2).

When looking at the six-hour average HR during day (from 12:00–18:00) and night (00:00–06:00) DIA was significantly lower in HR after four months compared to CON ($P = 0.03$). Both DIA and CON also decreased in average HR over the four-month period ($P < 0.0001$) (Table 4).

3.4. Heart rate variability

SDNN and RMSSD were higher after four months (both $P < 0.0001$), regardless of group. SDNN, RMSSD, normalised (n.u.) LF and LF/HF ratio were generally higher at night than at day (all $P < 0.05$).

3.5. Arrhythmia analysis

There were no significant changes in occurrence of arrhythmic ventricular or supraventricular complexes between CON and DIA and baseline and four months (see Table 1). The occurring ventricular complexes were most likely premature junctional complexes and complexes with right and left bundle branch-like morphology (Marcus, 2020) (data not shown).

3.6. Inter- and intra-observer variation of ECG morphology

Morphological measurements of six ECG prints of ten consecutive beats were performed by two observers resulting in an average relative difference of 4.5% in lead I and 5.4% in lead II. Intra-observer coefficient

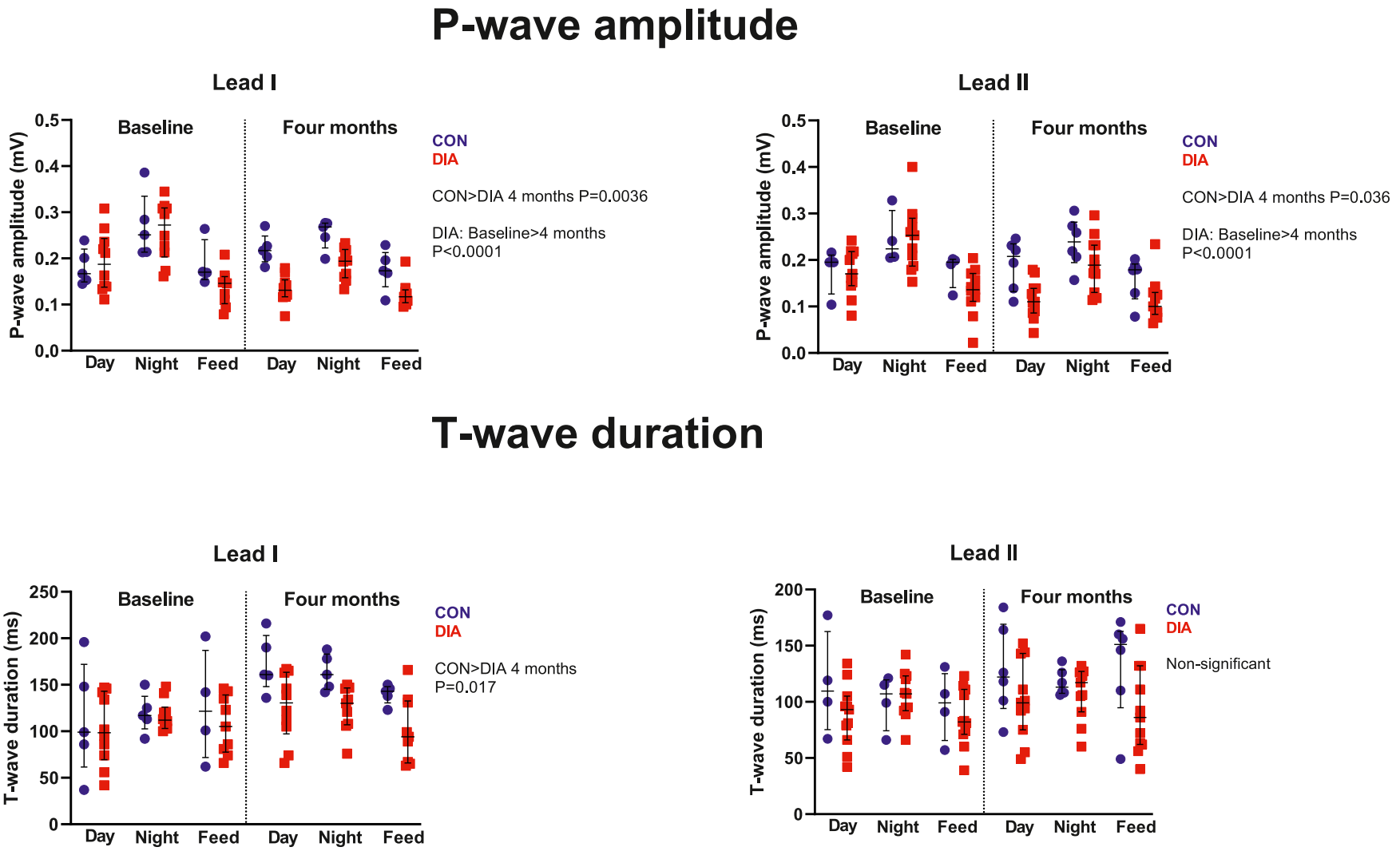


Fig. 4. P-wave amplitude and T-wave duration are decreased in DIA compared to CON after four months. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

P-wave amplitude and T-wave duration. Significant changes are written in normal black script on the right side of graphs. Blue circles: individual pigs in control group (CON). Red squares: individual pigs in diabetic group (DIA). Some values are missing due to low quality of ECG recording and can be seen as a lower number of dots/squares, with original $n = 7/11$ (CON/DIA). Black bars and lines indicate median and interquartile range. FDR-corrected P-values below 0.05 considered significant.

Table 1
Arrhythmic events in the 24-h ECG recordings.

	Control		Healthy - > diabetic	
Parameter	Baseline	4 months	Baseline	+4 months
AV-block	0 (0–1)	0 (0–0)	0 (0 13)	0 (0 11)
Supraventricular ectopic	0 (0–6)	0 (0–0)	0 (0–1)	0 (0–2)
Ventricular ectopic	0.5 (0–57)	0.5 (0–5)	0 (0 22)	0 (0–124)
Total arrhythmias	0.5 (0–63)	0.5 (0–5)	1 (0–24)	2 (0 131)

Number of arrhythmic events per whole 24-h recording. AV-block: atrioventricular block. Data presented as medians and interquartile range.

of variation (CV) was average 5.9% in lead I and 6.8% in lead II. The measurement of the QT interval had the lowest CV, with 1.8% in lead I and 1.7% in lead II of the inter-observer analysis and 1.3% in lead I and 1.6% in lead II of the intra-observer analysis. The measurement of the ST segment had the highest CV with 6.4% and 12.7% for lead I and II in the inter-observer analysis and 9.9% and 14.7% for lead I and II in the intra-observer analysis (see Table 5).

4. Discussion

There was significant influence of time of day on both ECG parameters and HRV. There was no effect of diabetes induction on arrhythmia prevalence and the number of arrhythmias did not change over the course of four months. The morphological parameters of the ECG were measured with low inter- and intra-observer variation. There was a significant difference between measurements from lead I and lead II, demonstrating the need for standardised baseline measurements and controls when using the minipig as a model of human heart disease. Few ECG and HRV parameters were changed four months after streptozotocin induced diabetes in Göttingen Minipigs. P-wave amplitude and T-wave duration were significantly decreased in DIA compared to CON and QTcb was lower in DIA compared to CON after four months. The T-wave amplitude and QTcb was lower in DIA after four months and therefore changed differently from healthy pig, over time. For HRV parameters, no effect of diabetes was seen, however, in both groups an effect of point in time were seen with HR, SDNN and RMSSD being higher after four months.

The T-wave amplitude changed over the course of the day, being highest at feeding where the highest heart rates were also observed and lowest during the night. This seemingly normal change in T-wave amplitude has also been observed in another study of minipigs (Lyhne et al., 2021) but altered axis of the T-wave is considered to be pathological in humans (Rautaharju et al., 2009). The T-wave has previously been reported to have heterogenic polarities and amplitudes between pigs, which is not influenced by electrode placement (Nahas, Baneux, & Detweiler, 2002). In the present study, T-wave also seem to change polarities, with individual pigs both having negative, positive and diphasic T-waves in both leads, mostly occurring during lower heart rates and rarely occurring during higher heart rates (Table 2). This is also reported in another study of Göttingen Minipigs using surgically implanted ECG telemetry (Stubhan et al., 2008). This is interesting because negative T waves are often considered to be pathological in human patients (Rautaharju et al., 2009). DIA had higher T-waves at baseline compared to at four months, but this difference might relate to the lower heart rate observed at four months, as in this study, T-waves were higher at higher heart rates. This variability in individual T-wave polarity, amplitude and duration need to be taking into consideration when obtaining endpoints in pig studies investigating e.g. the influence of an intervention on QT interval, as the morphology of the T-wave influence this parameter (Baumert et al., 2016).

The uncorrected QT interval was longer at four months compared to baseline, and also longer at night. Both of these changes can be attributed to the alterations in HR observed as QT interval is directly influence by heart rate (Luo et al., 2004). However, differences from baseline were also seen when correcting with Bazett's formula. These changes may be explained by an overcorrection by the Bazett's formula in regards to HR, which has been reported previously (Luo et al., 2004), as the Fridericia's correction did not find any differences between baseline and the recording after four months. It has also been suggested that Fridericia's correction is a more useful QT interval correction method in pigs (Kano et al., 2005).

Alterations in QT interval might have been expected, as some human studies have found prolongation of the QT interval in persons with diabetes (Ewing et al., 1991), which is thought to be related to autonomic dysfunction in cardiac neuropathy as the disease progresses (Karayannis et al., 2012). While these chronic comorbidities are well documented in humans, the chronicity of the minipig diabetic model is debatable, as the animals rarely are kept diabetic for many years.

P-wave amplitude decreased in DIA over the course of four months, but it was unchanged in CON. A study in landrace pigs found, that the P-wave increases with age and BW^{10}, however this was not the case in this study, even though both DIA and CON increased in BW during the four-month period. Low amplitude and short duration of the P-wave have been correlated to adverse outcomes in cardiovascular disease in humans (Alexander et al., 2017; Magnani et al., 2011; Park et al., 2016). In light of this, it is interesting that DIA had lower P amplitude after four months, however further studies are needed to elucidate the effect of streptocotozin-induced diabetes due to study limitations.

In both groups, there was a significant decrease in heart rate from baseline to the end of the study four months later. This finding could be due to conditioning of the pigs to their current environment, making them less stressed. As expected, heart rates were highest during feeding time and lowest during night time (Kuwahara et al., 1999).

The PR segment duration, as well as QRS duration (Nakagawa et al., 1998) and QT interval (Baumert et al., 2016) is dependant of heart rate in humans (Carruthers, McCall, Cordell, & Wu, 1987). In this study, heart rate decreased over the course of four months, likely influencing the QRS and QT interval duration making them longer. Schuleri et al. (2008) studied healthy Göttingen Minipigs and found the mean QRS duration was 59 ms at mean HR of 104 bpm (Schuleri et al. 2008). Stubhan et al. (2008) found a mean QRS duration of 56 ms and a PR segment at 128 ms at HR below 80 bpm, compared to QRS of 87–89 ms and PR segment of 116–129 ms at 80–88 bpm in this study. The reason for the higher QRS values obtained in this study compared to previous studies is unknown. A possible explanation might be differences in equipment used.

ST segment height should not be altered by heart rate and is reported to be below 0.1 mV in healthy humans (Macfarlane et al., 2015). Generally, the median ST segment height in this study was below 0.11 mV at all time points in both groups, however, individual values as high as 0.18 mV was seen in the healthy minipigs and there was also an effect of time of the day, where ST segments during the day and during feeding were higher than at night. ST-segment deviation has been reported in people with diabetes in relation to silent cardiac ischaemia, which is also suspected to be caused by cardiac neuropathy (Chiariello & Indolfi, 1996), but no effect of diabetes induction was seen in this study.

Diabetic cardiac neuropathy and autonomic dysfunction can be assessed by heart rate variability (Karayannis et al., 2012), and many human studies have investigated this (Jun et al., 2019; Tarvainen, Laitinen, Lipponen, Cornforth, & Jelinek, 2014). This study showed no changes in HRV in relation to diabetes induction, a result probably originating in the relative short duration of diabetes. In humans, both hyper- and hypoglycaemia contribute to neurological degradation and as the disease progresses, a general decrease in time domain heart rate variability is seen (Karayannis et al., 2012). Spectral analysis of HRV can be used for early detection of sympathetic or parasympathetic dysfunction and the LF/HF ratio is commonly assessed in this regard. While human HRV investigations are conducted with controlled breathing and posture to standardise the influence of respiration and blood pressure on the frequency components (Karayannis et al., 2012),

Table 2
Electrocardiographic morphology at baseline and after four months at different times of day.

		Control (n = 6)				Healthy - > Diabetic (n = 11)					
Parameter	Time	Baseline		4 months		Baseline		4 months		Overall P-values	
Heart rate (beats/min)	Day	88 (78–93)		80 (74–95)		103 (91–114)		83 (74–90)		Baseline>4 months***	
	Feed	148 (122–162)		132 (121–139)		146 (139–159)		132 (112–145)		Feed>Day***	
	Sleep	77 (76–85)[5]		75 (64–84)		80 (75–83)		64 (58–71)		Feed>Night***, Day>Night***	
		Lead I	Lead II	Lead I	Lead II	Lead I	Lead II	Lead I	Lead II	Lead I	Lead II
QRS duration (ms)	Day	92 (90–100)[5]	89 (33–92)[4]	89 (85–95)[5]	87 (76–91)	89 (88–91)[10]	86 (79–94)	90 (88–97)[10]	89 (79–93)	Day>Feed* Night>Feed*	4 months>Baseline*
	Feed	91 (81–95)[5]	87 (84–91)[4]	88 (84–92)[5]	92 (88–95)	89 (82–93)[10]	83 (80–88)	88 (83–90)[10]	86 (81–91)		
	Sleep	90 (78–93)[4]	82 (72–87)[4]	92 (87–99)[5]	91 (80–94)	89 (88–92)[9]	84 (76–88)	94 (89–97)[10]	88 (84–92)		
PR segment (ms)	Day	121 (109–128)[5]	116 (109–123)[4]	129 (115–134)[5]	127 (117–135)[6]	112 (101–127)[10]	113 (111 123)[11]	129 (117–126)[10]	130 (119–142)[11]	4 months>Baseline**	4 months>Baseline ***
	Feed	108 (103–119)[5]	107 (99–110)[4]	110 (104–119)[5]	108 (97–116)[6]	113 (102–126)[10]	105 (99–121)[11]	116 (108–126)[10]	115 (104–129)[11]	Day>Feed**	Day>Feed*** Night>Feed***
	Sleep	109 (104–121)[4]	125 (113–135)[4]	122 (124–143)[5]	120 (108–133)[6]	112 (106–122)[9]	120 (109–124)[11]	126 (108–141)[9]	125 (118–133)[11]		
ST segment (mV)	Day	0.08 (0.06–0.11)[5]	0.07 (0.02–0.09)[4]	0.11 (0.07–0.16)[5]	0.05 (0.04–0.07)	0.12 (0.09–0.14)[10]	0.06 (0.02–0.09)	0.10 (0.08–0.13)[10]	0.04 (0.02–0.08)	Day>Night*	Day>Night*** Feed>Night***
	Feed	0.06 (0.06–0.13)[5]	0.05 (0.02–0.64)[4]	0.10 (0.06–0.15)[5]	0.05 (0.03–0.07)	0.10 (0.06–0.16)[10]	0.06 (0.03–0.07)	0.07 (0.07–0.09)[10]	0.05 (0.04–0.06)		
	Sleep	0.07 (0.02–0.12)[4]	0.04 (0.01–0.09)[4]	0.11 (0.05–0.15)[5]	0.01 (0.00–0.06)	0.09 (0.07–0.11)[9]	0.03 (0.01–0.04)	0.10 (0.07–0.11)[9]	0.04 (0.01–0.05)		
T-wave amplitude (mV)	Day	0.15 (0.11–0.43)[5]	0.19 (0.12–0.30)[4]	0.42 (0.27–0.45)[5]	0.24 (0.15–0.41)	0.37 (0.13–0.51)[10]	0.25 (0.14–0.34)	0.16 (0.14–0.23)[10]	0.19 (0.10–0.24)	DIA: Baseline > 4 months*	DIA: Baseline > 4 months*
	Feed	0.80 (0.57–1.24)[5]	0.53 (0.21–1.07)[4]	0.88 (0.57–1.06)[5]	0.67 (0.50–0.90)	0.84 (0.59–1.18)[10]	0.70 (0.47–1.04)	0.75 (0.55–0.88)[9]	0.47 (0.41–0.68)	Feed>Day*** Feed>Night***	Feed>Day*** Feed>Night***
	Sleep	0.20 (0.13–0.26)[4]	0.12 (0.09–0.29)[4]	0.16 (0.11–0.71)[5]	0.27 (0.21–0.45)	0.18 (0.12–0.27)[9]	0.16 (0.11–0.21)	0.22 (0.11–0.25)[10]	0.13 (0.09–0.26)		
T-wave duration (ms)	Day	99 (62–172)[5]	110 (75–163)[4]	161 (145–183)[5]	122 (94–169)	99 (70–143)[10]	93 (66–105)	131 (97–164)[10]	99 (75–143)	4 months>Baseline***	CON>DIA*
	Feed	117 (103–138)[5]	107 (74–120)[4]	143 (131–148)[5]	113 (108–129)	112 (103–126)[10]	107 (92–123)	130 (107–147)[9]	117 (91–127)	CON > DIA 4 months*	
	Sleep	122 (72–187)[4]	99 (66–125)[4]	161 (148–203)[5]	151 (95–163)	105 (78–139)[9]	82 (71–111)	94 (66–133)[9]	86 (62–132)	CON: 4 months>Baseline***	
P-wave amplitude (mV)	Day	0.17 (0.15–0.22)[5]	0.20 (0.13–0.21)[4]	0.22 (0.19–0.25)[5]	0.21 (0.14–0.23)	0.19 (0.14–0.24)[10]	0.17 (0.15–0.22)	0.13 (0.12–0.15)[10]	0.11 (0.09–0.23)	Baseline>4 months** DIA: Baseline > 4 months***	Baseline>4 months* DIA: Baseline > 4 months***
	Feed	0.25 (0.21–0.34)[5]	0.22 (0.21–0-31)[4]	0.27 (0.22–0.28)[5]	0.24 (0.19–0.28)	0.27 (0.20–0.31)[10]	0.25 (0.19–0.29)	0.19 (0.16–0.22)[10]	0.19 (0.13–0.23)	CON > DIA 4 months** Feed>Day***,	CON > DIA 4 months* Feed>Day***,
	Sleep	0.17 (0.15–0.24)[4]	0.20 (0.14–0.20)[4]	0.17 (0.14–0.21) 5	0.18 (0.12–0.19)	0.15 (0.10–0.16)[9]	0.14 (0.11–0.17)	0.12 (0.10–0.13)[9]	0.10 (0.08–0.13)	Feed>Night***	Feed>Night***
P-wave polarity (+/ /diphasic)	Day	4/0/1	0/3/1	5/0/0	0/6/0	10/0/0	0/11/0	8/0/2	0/10/1	All ns	
	Feed	5/0/0	0/4/0	5/0/0	0/6/0	10/0/0	0/11/0	10/0/0	0/11/0		
	Sleep	5/0/0	0/3/1	5/0/0	0/6/0	9/0/0	0/11/0	7/0/3	0/11/0		
T-wave polarity (+/ /diphasic)	Day	3/0/2	1/2/1	5/0/0	0/4/2	8/0/2	1/6/4	5/2/3	4/5/2	All ns	
	Feed	5/0/0	0/4/0	5/0/0	0/6/0	10/0/0	0/11/0	10/0/0	1/9/1		
	Sleep	2/1/2	2/1/1	3/0/3	2/4/0	3/1/5	5/3/3	3/1/6	3/3/5		
QT interval (ms)	Day	334 (316–349)[5]	320 (312–345)[4]	343 (326–377)[5]	336 (313–353)	316 (307–338)[10]	308 (297–326)	340 (323–349)[10]	329 (324–341)	4 months>Baseline***	4 months>Baseline***
	Feed	258 (254–297)[5]	296 (265–309)[4]	302 (287–306)[5]	283 (270–305)	280 (261–304)[10]	269 (259–292)	297 (280–316)[10]	286 (263–318)	Day>Feed***	Day>Feed***

(*continued on next page*)

Table 2 (*continued*)

Parameter	Time	Control (n = 6)				Healthy - > Diabetic (*n* = 11)				Overall P-values	
		Baseline		4 months		Baseline		4 months			
	Sleep	337	335	355	338	346	328	353	352	Night>Feed***	Night>Feed***
		(330–357)[4]	(317–350)[4]	(353–379)[5]	(333–345)	(335–373)[9]	(311–347)	(343–365)[10]	(339–355)	Night>Day***	Night>Day*
Bazett	Day	392	389	412	391	420	420	397	395	DIA: Baseline > 4 months	DIA: Baseline > 4 months**
QTc (ms)		(384–400)[5]	(376–402)[4]	(407–417)[5]	(385–403)	(401–441)[10]	(381–427)	(372–409)[10]	(379–399)	***	
	Feed	424	426	435	421	434	422	434	421	CON > DIA 4 months*	Feed>Day***
		(414–442)[5]	(408–442)[4]	(420–449)[5]	(401–436)	(421–448)[10]	(414–438)	(425–440)[10]	(416–438)		Feed>Night***
	Sleep	400	387	394	380	398	381	359	358	Feed>Day***	Day>Night***
		(378–408)[4]	(377–394)[4]	(359–452)[5]	(335–406)	(382–426)[9]	(372–406)	(347–385)[10]	(350–366)	Feed>Night***	
										Day>Night*	
Fridericia	Day	377	365	382	368	379	377	375	366	All ns	All ns
QTc (ms)		(359–379)[5]	(356–375)[4]	(381–404)[5]	(365–381)	(370–405)[10]	(354–390)	(358–383)[10]	(361–376)		
	Feed	359	379	380	370	378	370	380	378		
		(352–387)[5]	(359–386)[4]	(374–394)[5]	(351–385)	(356–389)[10]	(356–383)	(373–391)[10]	(359–387)		
	Sleep	378	369	381	367	377	361	363	352		
		(361–390)[4]	(356–379)[4]	(357–426)[5]	(335–383)	(368–408)[9]	(353–382)	(354–390)[10]	(349–362)		

Superscript numbers: n, if reduced from original group size. In overall P-values, "4 months vs. Baseline" indicates a general difference between measurements at baseline compared to four months, with no effect of group. "CON vs. DIA" indicates a difference between groups with no effect of diabetes induction and time point. "DIA vs CON time point" indicates a difference between groups at the specific time point. "DIA/CON: baseline vs. 4 months" indicates an intra-group difference between baseline and four months. "Time of day vs. time of day" indicates a difference between different time points during the day, either at feeding, night or day. * $P < 0.05$, ** $P < 0.01$. *** $P < 0.001$. Ns – non-significant. P and T-wave polarity in absolute number of animals with either positive (+), negative ( ) or diphasic polarity of wave. Data is presented as median and interquartile range.

Table 3
QT interval and corrections.

Parameter	Time	Control (n = 6)				Healthy - > Diabetic (n = 11)				Overall P-values	
		Baseline		4 months		Baseline		4 months			
		Lead I	Lead II	Lead I	Lead II	Lead I	Lead II	Lead I	Lead II	Lead I	Lead II
QT interval (ms)	Day	334	320	343	336	316	308	340	329	4 months>Baseline***	4 months>Baseline***
		(316–349)[5]	(312–345)[4]	(326–377)[5]	(313–353)	(307–338)[10]	(297–326)	(323–349)[10]	(324–341)		
	Feed	258	296	302	283	280	269	297	286	Day>Feed***	Day>Feed***
		(254–297)[5]	(265–309)[4]	(287–306)[5]	(270–305)	(261–304)[10]	(259–292)	(280–316)[10]	(263–318)	Night>Feed***	Night>Feed***
	Sleep	337	335	355	338	346	328	353	352	Night>Day***	Night>Day*
		(330–357)[4]	(317–350)[4]	(353–379)[5]	(333–345)	(335–373)[9]	(311–347)	(343–365)[10]	(339–355)		
Bazett	Day	392	389	412	391	420	420	397	395	DIA: Baseline > 4 months ***	DIA: Baseline > 4 months**
QTc (ms)		(384–400)[5]	(376–402)[4]	(407–417)[5]	(385–403)	(401–441)[10]	(381–427)	(372–409)[10]	(379–399)	CON > DIA 4 months*	
	Feed	424	426	435	421	434	422	434	421	Feed>Day***	Feed>Day***
		(414–442)[5]	(408–442)[4]	(420–449)[5]	(401–436)	(421–448)[10]	(414–438)	(425–440)[10]	(416–438)	Feed>Night***	Feed>Night***
	Sleep	400	387	394	380	398	381	359	358	Day>Night*	Day>Night***
		(378–408)[4]	(377–394)[4]	(359–452)[5]	(335–406)	(382–426)[9]	(372–406)	(347–385)[10]	(350–366)		
Fridericia	Day	377	365	382	368	379	377	375	366	All ns	All ns
QTc (ms)		(359–379)[5]	(356–375)[4]	(381–404)[5]	(365–381)	(370–405)[10]	(354–390)	(358–383)[10]	(361–376)		
	Feed	359	379	380	370	378	370	380	378		
		(352–387)[5]	(359–386)[4]	(374–394)[5]	(351–385)	(356–389)[10]	(356–383)	(373–391)[10]	(359–387)		
	Sleep	378	369	381	367	377	361	363	352		
		(361–390)[4]	(356–379)[4]	(357–426)[5]	(335–383)	(368–408)[9]	(353–382)	(354–390)[10]	(349–362)		

Superscript numbers: n, if reduced from original group size. In overall P-values, "4 months vs. Baseline" indicates a general difference between measurements at baseline compared to four months, with no effect of group. "CON vs. DIA" indicates a difference between groups with no effect of diabetes induction and time point. "DIA vs CON time point" indicates a difference between groups at the specific time point. "DIA/CON: baseline vs. 4 months" indicates an intra-group difference between baseline and four months. "Time of day vs. time of day" indicates a difference between different time points during the day, either at feeding, night or day. * $P < 0.05$, ** $P < 0.01$. *** $P < 0.001$. Ns – non-significant. Data is presented as median and interquartile range.

Table 4
Long-term ffrequency and time domain heart rate variability at baseline and after four months.

		Control		Healthy - > Diabetic		
Parameter	Time	Baseline	4 months	Baseline	4 months	Overall P-value
Heart rate (beats per minute)	Day 6 h	98 (93–100)	89 (84–94)	105 (99–112)	84 (81–89)	CON: Baseline>4 months*** DIA: Baseline>4 months***
	Night 6 h	82 (80–82)[5]	73 (65–78)	80 (75–88)	64 (60–69)	CON > DIA 4 months*
	24 h	88 (83–89)[5]	78 (75–83)	87 (82–93)	74 (68–79)	Day>Night***
SDNN	Day 6 h	87 (82–138)	117 (89–136)	125 (88–134)	128 (114–138)	4 months>Baseline***
	Night 6 h	137 (84–174)[5]	155 (109–167)	113 (108–138)	164 (140–191)	Night: 4 months>Baseline***
	24 h	123 (102–154)[5]	147 (126–171)	140 (129–164)	190 (167–211)	Night>Day**
RMSSD	Day 6 h	56 (38–84)	74 (60–100)	47 (41–50)	82 (56–86)	4 months>Baseline***
	Night 6 h	128 (82–340)[5]	132 (86–153)	77 (67–94)	123 (89–147)	Night>Day***
	24 h	60 (42–71)[5]	71 (64–77)	59 (49–63)	74 (70–84)	
VLF	Day 6 h	624.2 (456.5–1079.0)	609.2 (453.8–835.3)	747.4 (635.7–1665.0)	946.1 (535.2–1124.0)	All ns
	Night 6 h	714.0 (561.1–1101.0)[5]	1169.0 (676.2–1441.0)	961.8 (694.3–1656)	1535.0 (977.7–1887.0)	
	24 h	735.9 (602.9–990.9)[5]	1047.0 (830.8–1307.0)	902.6 (790.8–1934.0)	1296.0 (912.1–1881.0)	
LF n.u.	Day 6 h	28.49 (22.30–36.48)	26.6 (11.04–44.17)	41.05 (29.80–46.50)	24.72 (21.58–34.33)	DIA > CON*
	Night 6 h	37.79 (16.46–48.42)[5]	17.02 (13.17–32.53)	38.09 (24.92–66.05)	53.51 (33.25–75.16)	Night>Day*
	24 h	36.73 (11.66–49.57)[5]	12.37 (11.05–25.10)	27.08 (22.29–33.85)	27.80 (23.97–54.63)	
HF n.u.	Day 6 h	63.79 (51.36–68.01)	59.75 (46.25–74.46)	54.71 (43.00–60.93)	70.57 (57.72–85.13)	All ns
	Night 6 h	63.38 (48.59–77.01)[5]	79.15 (65.13–83.51)	58.15 (32.31–73.41)	45.79 (24.10–64.94)	
	24 h	59.89 (46.52–84.22)[5]	81.18 (72.38–85.35)	71.01 (61.60–75.80)	70.62 (42.89–74.21)	
LF/HF	Day 6 h	0.459 (0.32–0.63)	0.45 (0.15–1.09)	0.75 (0.49–1.09)	0.34 (0.30–0.59)	DIA > CON*
	Night 6 h	0.55 (0.23–1.02)[5]	0.21 (0.16–0.50)	0.66 (0.34–2.04)	1.17 (0.51–3.10)	Night>Day*
	24 h	0.63 (0.14–1.17)[5]	0.15 (0.13–0.35)	0.38 (0.29–0.59)	0.39 (0.32–1.27)	
VHF	Day 6 h	321.6 (127.5–769.2)	389.3 (141.2–1130.0)	239.5 (129.5–535.7)	318.8 (192.3–362.5)	All ns
	Night 6 h	411.1 (159.6–519.2)[5]	413.4 (258.3–649.6)	231.8 (134.6–335.0)	205.4 (142.3–295.0)	
	24 h	477.7 (131.0–584.3)[5]	529.1 (320.7–892.7)	264.7 (159.1–549.3)	367.1 (253.2–453.5)	

Heart rate variability (HRV) of 24 h ECG recordings at baseline and four months after diabetes induction or control. In overall P-values, "4 months vs. Baseline" indicates a general difference between measurements at baseline compared to four months, with no effect of group. "CON vs. DIA" indicates a difference between groups with no effect of diabetes induction and time point. "DIA vs CON time point" indicates a difference between groups at the specific time point. "DIA/CON: baseline vs. 4 months" indicates an intra-group difference between baseline and four months. "Time of day vs. time of day" indicates a difference between different time points during the day, either at feeding, night or day. * $P < 0.05$, ** $P < 0.01$. *** $P < 0.001$. Ns – non-significant. SDNN: standard deviation of beat-to-beat intervals, RMSSD: root mean square of successive RR differences. Day: from 12:00 until 18:00, Night: from 00:00 until 06:00, 24 h: entire 24-h recordings. VLF (log): very low frequency power 0.015–0.05 Hz, LF n.u.: normalised low frequency power 0.05–0.15 Hz, HF n.u.: normalised high frequency power 0.15–0.40 Hz and VHF: very high frequency power 0.35–0.5 Hz. Superscript numbers: n, if reduced from original group size (CON n = 6, DIA n = 11). Data given as medians and interquartile range.

this is not possible in pigs and might contribute to the lack of findings in this study. Also, the non-chronicity, as well as the control of hyperglycaemia in this study, might not allow for neuropathic changes to occur, as CAN has been shown to increase with age, diabetes control and diabetes duration (Karayannis et al., 2012), making this minipig model of diabetes less appropriate for evaluation of these chronic comorbidities.

All morphological measurements made in this study were significantly different at least at one point during the day, when comparing lead I and lead II with measurements made in lead I generally being higher than lead II, most likely due to the positioning of the electrodes (Gulizia et al., 2017). Interestingly, the ST segment was consistently higher in lead I at all times of the day. This highlights the need for obtaining baseline values, for individual pigs, in the relevant lead, when doing porcine studies, where morphological parameters are an outcome. In addition, the importance of standardised lead placement and statement of this in scientific studies is clearly outlined by the results of this study, where changes seen in one lead are often not present in the other lead. Lead II is often used when nothing else is specified, however, as no official guidelines or consensus exist for porcine ECG electrode placement, it is relevant to state this in studies.

There was generally a low inter- and intra-observer CV (<10%), except for measurements of the ST segment, where CV was higher (up to 14.7%). These findings are in agreement with human ECG studies

Table 5
inter- and intra-observer variation of morphological ECG parameters.

Parameter	Inter-observer Relative difference		Intra-observer Standard deviation and coefficient of variation			
Lead	I	II	I		II	
			SD	CV	SD	CV
QRS duration ms	3.1%	3.3%	4.29	4.9%	4.35	5.2%
QT interval ms	1.8%	1.7%	4.13	1.3%	4.84	1.6%
PR interval ms	5.1%	2.4%	6.75	5.8%	5.69	5.2%
ST segment mV	6.4%	12.7%	0.01	9.9%	0.01	14.7%
T-wave amplitude mV	3.4%	9.6%	0.01	4.5%	0.01	6.2%
T-wave duration ms	7.0%	5.2%	7.82	8.0%	8.17	8.5%
P-wave amplitude mV	5.0%	2.7%	0.01	6.9%	0.01	6.3%
Average	4.5%	5.4%		5.9%		6.8%

Inter-observer and intra-observer variation in morphological ECG parameters. Inter-observer: Relative differences between observer one and observer two for each electrocardiographic print and for each parameter shown as percentage and calculated as (A-B)/((A + B)*0.5) and given in absolute percentage. SD: standard deviation. Intra-observer: Mean coefficient of variation (CV) calculated as SD divided times 100, by average of six observations made from the same ECG print. Average: average percentage relative difference or CV for each parameter in each lead.

Table 6
Differences between morphological ECG measurements in lead I and II.

Parameter/ Time	Day	Feed	Night	General
QRS duration (ms)	4 (3–8)	8 (4–11)	**5 (1−10)***	I > II
QT interval (ms)	**9 (2–16)***	**4 (0–9)***	**14 (11–18)****	I > II
PR interval (ms)	-1 (9–7)	**9 (0−12)***	-35 (13 2)	Varies
ST segment (mV)	**0.04 (0.03–0.07)****	**0.04 (0.02–0.07)****	**0.06 (0.03–0.08)****	I > II
T duration (ms)	6 (24–22)	**13 (5–24)****	19 (3–66)	I > II
T amplitude (mV)	0.11 (0.04–0.20)	**0.14 (0.09–0.22)****	0.02 (0.08–0.14)	I > II
P amplitude (mV)	**0.02 (0.00–0.03)***	**0.02 (0.00–0.04)***	0.01 (0.01–0.03)	I > II

Differences between lead I and lead II measurements for each time of day, calculated from baseline measurements. Differences calculated as lead I minus lead II for each observation. Data given as medians and interquartile range. Significance calculated with Wilcoxon signed rank test, $P < 0.05$ = *, $P < 0.01$ = **, $P < 0.001$ = ***. P-values corrected with False Discovery Rate correction.

(Hamilton, McLeod, Houston, & Macfarlane, 2005) and underline the need for proper training and consensus especially on how to measure the ST segment manually.

A limitation in this study is the relatively low number of animals included and the age- and BW differences between groups. The short duration of diabetes is also a limitation of this study, as most diabetes comorbidities develop with longer diabetes duration in human patients (Karayannis et al., 2012).

Another limitation of this study is the streptozotocin-induced diabetic Göttingen minipig, where off-target effects of streptozotocin could induce changes to non-pancreatic tissues, as shown in an in-vitro study of rat myocytes where streptozotocin directly impaired the contractility of the myocyte (Wold & Ren, 2004), suggesting an effect on myocardial tissues as well.

A third limitation is that pigs were included in other pharmacological insulin studies between the two ECG recordings (baseline and four months follow up), however all pigs were naïve at study start (before baseline recording) and both groups (CON and DIA) were included in other pharmacological studies between the two ECG recordings. Moreover, a two-week wash-out period was included before follow up ECG recording. However, it cannot be excluded that pharmacological agents received between the ECG recordings might have influenced our results. Also, in the diabetic pigs, the variability of their blood glucose over time is unknown. High glycaemic variability in humans have been discussed to predict development of diabetic complications, but with no definite conclusion (Nalysnyk, Hernandez-Medina, & Krishnarajah, 2010).

The pigs were also dosed with propofol just prior placement of the Holter equipment. While propofol has a very short duration of action, the elimination is slower (Cockshott, Douglas, Plummer, & Simons, 1992). Propofol can induce some ECG changes including P-wave and minor QT-interval changes (Bolat et al., 2016). Moreover, effects of propofol on HRV parameters have been described in mice (Shintaku et al., 2014).

In conclusion, a number of morphological ECG and HRV parameters did change during a four-month period both in diabetic and healthy Göttingen Minipigs and were influenced by time of day, indicating diurnal variations. ECG leads influenced all morphological ECG parameters, however low inter- and intra-observer variation was found. Some ECG changes were found in pigs with streptozotocin-induced diabetes compared to healthy pigs, including reduced P wave amplitude, T wave duration and QTbc in some ECG leads. Future ECG studies in Göttingen Minipigs need to account for time setting and choice of ECG lead, but due to limitations of this study, further research is needed to explore impact of streptozotocin-induced diabetes.

Data availability

The datasets generated during this study are available from the corresponding author upon request.

Authors' contributions

Mille Kronborg Lyhne: Conceptualization, Methodology, Formal Analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization, and Project administration. **Terese Helgogaard:** Formal analysis, Investigation, Writing – review & editing. **Karina Poulsdóttir Debes:** Formal analysis, Writing – review & editing. **Andreas Vegge, Jonas Kildegaard and Ulrik Pedersen-Bjergaard:** Conceptualization, Methodology, Writing – review & editing, Supervision. **Lisbeth Høier Olsen:** Conceptualization, Methodology, Formal analysis, resources, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest and funding

The authors declare the following interestes/personal relationships which may be considered as competing interests: Authors Andreas Vegge and Jonas Kildegaard are employed by Novo Nordisk A/S. Mille Kronborg Lyhne, Karina Poulsdóttir Debes, Terese Helgogaard, Ulrik Pedersen-Bjergaard and Lisbeth Høier Olsen declare no potential conflicst of interest. The study was funded by Novo Nordisk A/S and the LifePharm Centre for University of Copenhagen, Faculty of Health and Medical Sciences, Department of Veterinary and Animal Sciences.

Data availability

Data will be made available on request.

References

Alexander, B., et al. (2017). Reduced P-wave voltage in Lead I is associated with development of atrial fibrillation in patients with coronary artery disease. *Journal of Aterial Fibrillation, 10*. https://doi.org/10.4022/jafib.1657

Baumert, M., et al. (2016). QT interval variability in body surface ECG: Measurement, physiological basis, and clinical value: Position statement and consensus guidance endorsed by the European heart rhythm association jointly with the ESC working

group on cardiac cellular electrophysiology. *Europace, 18*, 925–944. https://doi.org/10.1093/europace/euv405
Bolat, E., et al. (2016). Effects of balanced propofol sedation on QT, corrected QT, and P-wave dispersion on upper endoscopy. *Anatolian Journal of Cardiology, 16*, 328–332. https://doi.org/10.5152/AnatolJCardiol.2015.6264
Caceres, C. A., Kelser, G. A., & Mize, W. R. (1959). Formation of the "P-R segment". *Circulation, 20*, 229–233. https://doi.org/10.1161/01.CIR.20.2.229
Carruthers, S. G., McCall, B., Cordell, B. A., & Wu, R. (1987). Relationships between heart rate and PR interval during physiological and pharmacological interventions. *British Journal of Clinical Pharmacology, 23*, 259–265. https://doi.org/10.1111/j.1365-2125.1987.tb03043.x
Cha, S. A., et al. (2018). Time- and frequency-domain measures of heart rate variability predict cardiovascular outcome in patients with type 2 diabetes. *Diabetes Research and Clinical Practice, 143*, 159–169. https://doi.org/10.1016/j.diabres.2018.07.001
Chiariello, M., & Indolfi, C. (1996). Silent myocardial ischemia in patients with diabetes mellitus. *American Heart Journal*. https://doi.org/10.1161/01.CIR.93.12.2089
Cockshott, I. D., Douglas, E. J., Plummer, G. F., & Simons, P. J. (1992). The pharmacokinetics of propofol in laboratory animals. *Xenobiotica, 22*, 369–375. https://doi.org/10.3109/00498259209046648
Connelly, K. A., et al. (2020). Load-independent effects of empagliflozin contribute to improved cardiac function in experimental heart failure with reduced ejection fraction. *Cardiovascular Diabetology, 19*, 13. https://doi.org/10.1186/s12933-020-0994-y
Crick, S. J., Sheppard, M. N., & Anderson, R. H. (1998). Anatomy of the pig heart: Comparisons with normal human cardiac structure. *Journal of Anatomy, 193*, 105–119. https://doi.org/10.1046/j.1469-7580.1998.19310105.x
Cryer, P. E. (2005). Mechanisms of hypoglycemia-associated autonomic failure and its component syndromes in diabetes. *Diabetes, 54*, 3592–3601. https://doi.org/10.2337/diabetes.54.12.3592
Curtis, M. J., et al. (2013). The Lambeth conventions (II): Guidelines for the study of animal and human ventricular and supraventricular arrhythmias. *Pharmacology & Therapeutics, 139*, 213–248. https://doi.org/10.1016/j.pharmthera.2013.04.008
Diemar, S. S., et al. (2015). Influence of acute glycaemic level on measures of myocardial infarction in non-diabetic pigs. *Scandinavian Cardiovascular Journal, 49*, 376–382. https://doi.org/10.3109/14017431.2015.1085079
Ewing, D. J., Boland, O., Neilson, J. M. M., Cho, C. G., & Clarke, B. E. (1991). Autonomic neuropathy, QT interval lengthening, and unexpected deaths in male diabetic patients. *Diabetologia, 34*, 182–185. https://doi.org/10.1007/BF00418273
Gallego, M., Zayas-Arrabal, J., Alquiza, A., Apellaniz, B., & Casis, O. (2021). Electrical features of the diabetic myocardium. arrhythmic and cardiovascular safety considerations in diabetes. *Frontiers in Pharmacology, 12*, Article 687256. https://doi.org/10.3389/fphar.2021.687256
Gulizia, M. M., et al. (2017). ANMCO/AIIC/SIT consensus information document: Definition, precision, and suitability of electrocardiographic signals of electrocardiographs, ergometry, Holter electrocardiogram, telemetry, and bedside monitoring systems. *European Heart Journal Supplements: Journal of the European Society of Cardiology, 19*, D190–D211. https://doi.org/10.1093/eurheartj/sux031
Hamilton, R. M., McLeod, K., Houston, A. B., & Macfarlane, P. W. (2005). Inter- and Intraobserver variability in LVH and RVH reporting in pediatric ECGs. *Annals of Noninvasive Electrocariography, 10*, 330–333. https://doi.org/10.1111/j.1542-474X.2005.00642.x
Havmoller, R., et al. (2007). Age-related changes in P wave morphology in healthy subjects. *BMC Cardiovascular Disorders, 7*, 22. https://doi.org/10.1186/1471-2261-7-22
Jaiswal, M., et al. (2014). Association between impaired cardiovascular autonomic function and hypoglycemia in patients with type 1 diabetes. *Diabetes Care, 37*, 2616–2621. https://doi.org/10.2337/dc14-0445
Jong, I. C.d., et al. (2000). Effects of social stress on heart rate and heart rate variability in growing pigs. *Canadian Journal of Animal Science, 80*, 273–280. https://doi.org/10.4141/A99-085
Jun, J. E., et al. (2019). Continuous glucose monitoring defined glucose variability is associated with cardiovascular autonomic neuropathy in type 1 diabetes. *Diabetes/Metabolism Research and Reviews, 35*, Article e3092. https://doi.org/10.1002/dmrr.3092
Kano, M., et al. (2005). QT PRODACT: Usability of miniature pigs in safety pharmacology studies: Assessment for drug-induced QT interval prolongation. *Journal of Pharmacological Sciences, 99*, 501–511. https://doi.org/10.1254/jphs.qt-c13
Karayannis, G., Giamouzis, G., Cokkinos, D. V., Skoularigis, J., & Triposkiadis, F. (2012). Diabetic cardiovascular autonomic neuropathy: clinical implications. *Expert Review of Cardiovascular Therapy, 10*, 747–765. https://doi.org/10.1586/ERC.12.53
Kurl, S., Makikallio, T. H., Rautaharju, P., Kiviniemi, V., & Laukkanen, J. A. (2012). Duration of QRS complex in resting electrocardiogram is a predictor of sudden cardiac death in men. *Circulation, 125*, 2588–2594. https://doi.org/10.1161/CIRCULATIONAHA.111.025577
Kusumoto, F. M., et al. (2019). 2018 ACC/AHA/HRS guideline on the evaluation and Management of Patients with Bradycardia and Cardiac Conduction Delay: Executive summary: A report of the American College of Cardiology/American Heart Association task force on clinical practice guidelines, and the Heart Rhythm Society. *Journal of the American College of Cardiology, 74*, 932–987. https://doi.org/10.1016/j.jacc.2018.10.043
Kuwahara, M., et al. (1999). Power spectral analysis of heart rate variability for assessment of diurnal variation of autonomic nervous activity in miniature swine. *Laboratory Animal Science, 49*, 202–208.
Kuwahara, M., et al. (2004). Effects of pair housing on diurnal rhythms of heart rate and heart rate variability in miniature swine. *Experimental Animals, 53*, 303–309.
Larsen, M. O., & Rolin, B. (2004). Use of the Göttingen Minipig as a model of diabetes, with special focus on type 1 diabetes research. *ILAR Journal, 45*. https://doi.org/10.1093/ilar.45.3.303
Larsen, M. O., et al. (2002). Mild streptozotocin diabetes in the Göttingen minipig. A novel model of moderate insulin deficiency and diabetes. *American Journal of Physiology. Endocrinology and Metabolism, 282*, E1342–E1351. https://doi.org/10.1152/ajpendo.00564.2001
Lepeschkin, E., & Surawicz, B. (1952). The measurement of the Q-T interval of the electrocardiogram. *Circulation, 6*, 378–388. https://doi.org/10.1161/01.cir.6.3.378
Luo, S., Michler, K., Johnston, P., & Macfarlane, P. W. (2004). A comparison of commonly used QT correction formulae: The effect of heart rate on the QTc of normal ECGs. *Journal of Electrocardiology*, (37 Suppl), 81–90. https://doi.org/10.1016/j.jelectrocard.2004.08.030
Lyhne, M. K., et al. (2021). Hyperinsulinaemic hypoglycaemia in non-anaesthetized Gottingen minipigs induces a counter-regulatory endocrine response and electrocardiographic changes. *Scientific Reports, 11*, 5983. https://doi.org/10.1038/s41598-021-84758-w
Macfarlane, P. W., et al. (2015). The early repolarization pattern: A consensus paper. *Journal of the American College of Cardiology, 66*, 470–477. https://doi.org/10.1016/j.jacc.2015.05.033
Magnani, J. W., et al. (2011). P wave duration is associated with cardiovascular and all-cause mortality outcomes: The National Health and nutrition examination survey. *Heart Rhythm, 8*, 93–100. https://doi.org/10.1016/j.hrthm.2010.09.020
Marcus, G. M. (2020). Evaluation and management of premature ventricular complexes. *Circulation, 141*, 1404–1418. https://doi.org/10.1161/CIRCULATIONAHA.119.042434
Marques, J. L. B., et al. (1997). Altered ventricular repolarization during Hypoglycaemia in patients with diabetes. *Diabetic Medicine, 14*, 648–654. https://doi.org/10.1002/(SICI)1096-9136(199708)14:8<648::AID-DIA418>3.0.CO;2-1
Nahas, K., Baneux, P., & Detweiler, D. (2002). Electrocardiographic monitoring in the Göttingen Minipig. *Comparative Medicine, 52*, 258–264.
Nakagawa, M., et al. (1998). Circadian rhythm of the signal averaged electrocardiogram and its relation to heart rate variability in healthy subjects. *Heart, 79*, 493–496. https://doi.org/10.1136/hrt.79.5.493
Nalysnyk, L., Hernandez-Medina, M., & Krishnarajah, G. (2010). Glycaemic variability and complications in patients with diabetes mellitus: Evidence from a systematic review of the literature. *Diabetes, Obesity & Metabolism, 12*, 288–298. https://doi.org/10.1111/j.1463-1326.2009.01160.x
Park, J. K., et al. (2016). Low P-wave amplitude (<0.1 mV) in lead I is associated with displaced inter-atrial conduction and clinical recurrence of paroxysmal atrial fibrillation after radiofrequency catheter ablation. *Europace, 18*, 384–391. https://doi.org/10.1093/europace/euv028
Paslawska, U., et al. (2014). Normal electrocardiographic and echocardiographic (M-mode and twodimensional) values in polish landrace pigs. *Acta Veterinaria Scandinavica, 56*. https://doi.org/10.1186/s13028-014-0054-2
Rautaharju, P. M., et al. (2009). AHA/ACCF/HRS recommendations for the standardization and interpretation of the electrocardiogram: Part IV: The ST segment, T and U waves, and the QT interval: A scientific statement from the American Heart Association electrocardiography and arrhythmias committee, council on clinical cardiology; the American College of Cardiology Foundation; and the Heart Rhythm Society. Endorsed by the International Society for Computerized Electrocardiology. *Journal of the American College of Cardiology, 53*, 982–991. https://doi.org/10.1016/j.jacc.2008.12.014
Schuleri, K. H., et al. (2008). The adult Göttingen Minipig as a model for chronic heart failure after myocardial infarction: Focus on cardiovascular imaging and regenerative therapies. *Comparative Medicine, 58*, 568–579.
Schumacher-Petersen, C., et al. (2019). Experimental non-alcoholic steatohepatitis in Gottingen Minipigs: Consequences of high fat-fructose-cholesterol diet and diabetes. *Journal of Translational Medicine, 17*, 1–18. https://doi.org/10.1186/s12967-019-1854-y
Shintaku, T., et al. (2014). Effects of propofol on electrocardiogram measures in mice. *Journal of Pharmacological Sciences, 126*, 351–358. https://doi.org/10.1254/jphs.14181FP
Stricker-Krongrad, A., et al. (2017). Comparative cardiovascular physiology and pathology in selected lineages of minipigs. *Toxicology Research and Application, 1*, 1–8. https://doi.org/10.1177/2397847317696367
Stubhan, M., et al. (2008). Evaluation of cardiovascular and ECG parameters in the normal, freely moving Gottingen Minipig. *Journal of Pharmacological and Toxicological Methods, 57*, 202–211. https://doi.org/10.1016/j.vascn.2008.02.001
Suzuki, A., et al. (1998). Establishment of a 24-hour electrocardiogram recording system using a Holter recorder for miniature swine. *Laboratory Animals, 32*, 165–172. https://doi.org/10.1258/002367798780599938
Tarvainen, M. P., Laitinen, T. P., Lipponen, J. A., Cornforth, D. J., & Jelinek, H. F. (2014). Cardiac autonomic dysfunction in type 2 diabetes - effect of hyperglycemia and disease duration. *Front Endocrinol (Lausanne), 5*, 130. https://doi.org/10.3389/fendo.2014.00130
Thayer, J. F., Yamamoto, S. S., & Brosschot, J. F. (2010). The relationship of autonomic imbalance, heart rate variability and cardiovascular disease risk factors. *International Journal of Cardiology, 141*, 122–131. https://doi.org/10.1016/j.ijcard.2009.09.543
Uehara, A., Kurata, C., Sugi, T., Mikami, T., & Shouda, S. (1999). Diabetic cardiac autonomic dysfunction: Parasympathetic versus sympathetic. *Annals of Nuclear Medicine, 13*. https://doi.org/10.1007/BF03164884
Wold, L. E., & Ren, J. (2004). Streptozotocin directly impairs cardiac contractile function in isolated ventricular myocytes via a p38 map kinase-dependent oxidative stress

mechanism. *Biochemical and Biophysical Research Communications, 318*, 1066–1071. https://doi.org/10.1016/j.bbrc.2004.04.138

Zaragoza, C., et al. (2011). Animal models of cardiovascular diseases. *Journal of Biomedicine & Biotechnology, 2011*, Article 497841. https://doi.org/10.1155/2011/497841

Zhang, S.-B., et al. (2016). Normal electrocardiogram of Bama miniature pigs (Sus scrofa domestica). *Journal of the American Association for Laboratory Animal Science, 55*, 150–152.

Paper II

Hyperinsulinaemic hypoglycaemia in non-anaesthetized Göttingen Minipigs induces a counter-regulatory endocrine response and electrocardiographic changes.

Mille Kronborg Lyhne, Andreas Vegge, Gro Klitgaard Povlsen, Rita Slaaby, Jonas Kildegaard, Ulrik Pedersen-Bjergaard and Lisbeth Høier Olsen.

Scientific Reports, volume 11, Article number: 5983 (2021), https://doi.org/10.1038/s41598-021-84758-w

scientific reports

Check for updates

OPEN

Hyperinsulinaemic hypoglycaemia in non-anaesthetized Göttingen minipigs induces a counter-regulatory endocrine response and electrocardiographic changes

Mille K. Lyhne[1], Andreas Vegge[2], Gro Klitgaard Povlsen[2], Rita Slaaby[2], Jonas Kildegaard[2], Ulrik Pedersen-Bjergaard[3] & Lisbeth H. Olsen[1]✉

The potentially fatal cardiovascular effects of hypoglycaemia are not well understood and large animal models of the counter-regulatory responses and cardiovascular consequences of insulin-induced hypoglycaemia are needed to understand the mechanisms in humans. The aim of this study was to develop a human-like minipig model of hypoglycaemia including healthy and diabetic pigs to investigate endocrine, electrocardiographic and platelet effects. Hypoglycaemia was induced using a hyperinsulinaemic, hypoglycaemic clamp and an insulin bolus protocol. Plasma glucose, glucagon, C-peptide, insulin, epinephrine and platelet aggregation responses were measured before, during and after hypoglycaemia. Continuous electrocardiographic recordings were obtained. Hypoglycaemia at a plasma glucose concentration of 0.8–1.0 mM in the clamp induced 25-fold increase in epinephrine and sixfold and threefold increase in glucagon for healthy and diabetic pigs, respectively. The hypoglycaemic clamp induced QTc-interval prolongation and increase in cardiac arrhythmias. In the bolus approach, the non-diabetic group reached plasma glucose target of 1.5 mM and QTc-interval was prolonged after insulin injection, but before glucose nadir. The diabetic group did not reach hypoglycaemic target, but still demonstrated QTc-interval prolongation. These results demonstrate effects of hyperinsulinaemic hypoglycaemia closely resembling human physiology, indicating the minipig as a translational animal model of counter-regulatory endocrine and myocardial effects of hypoglycaemia.

Hypoglycaemia is a common adverse effect of insulin treatment in type 1 and type 2 diabetes with serious clinical implications for the patient[1]. People with diabetes also have a higher risk of cardiovascular disease than the general population, as well as micro- and macroangiopathies, whose pathogeneses has been linked to increased platelet activation[2,3]. Large-scale clinical studies have demonstrated an increased risk of overall mortality and cardiovascular death in people with diabetes experiencing as little as a single severe hypoglycaemic episode, even days and months after the event[1,4,5], where severe hypoglycaemia was defined as blood glucose below 2.8 mM with the person needing third-party assistance to return to normal blood glucose levels[1]. The majority of data linking diabetes to cardiovascular risk have been generated in type 2 diabetes patients, potentially due to large cardiovascular outcome trials in this populations, however type 1 diabetes patients also have an increased cardiovascular risk compared to the non-diabetic population, including higher risk of cardiovascular death[6]. The mechanisms underlying this association remains poorly understood, but is thought to include an acute proarrhythmic effect of hypoglycaemia and more long-lasting effects promoting inflammation, coagulation and atherosclerosis[7]. The acute cardiovascular effects of hypoglycaemia include increased cardiac workload driven by

[1]Department of Veterinary and Animal Sciences, University of Copenhagen, Copenhagen, Denmark. [2]Global Drug Discovery, Novo Nordisk A/S, Måløv, Denmark. [3]Department of Endocrinology and Nephrology, Nordsjællands Hospital Hillerød, Hillerød, Denmark. ✉email: lisbeth.hoier@sund.ku.dk

the counter-regulatory epinephrine response and QTc-interval prolongation in healthy and diabetic humans[8–10]. Furthermore, both experimental and spontaneous hypoglycaemia in humans induces cardiac arrhythmic ventricular and supraventricular ectopic electric activity[11, 12]. The dead-in-bed syndrome in young type 1 diabetes patients has been linked to hypoglycaemia-induced, fatal cardiac arrhythmias[11, 13]. However, there are no data to definitely prove causality. In rodents, severe hypoglycaemia induces fatal arrhythmias seemingly mediated by the para- and sympathetic nervous system[14, 15], but the effects of hypoglycaemia has not been well investigated in a large animal model with more human-like anatomy and physiology. The G ttingen minipig is widely used in preclinical research[16], and their cardiac anatomy and physiology closely resembles that of humans, with cardiac conduction systems and heart size being very similar[17, 18]. In addition, porcine models appear to resemble the human cardiovascular system better than the rodent models since resting heart rate in minipigs is 85–99 beats per minute compared to the rat's mean heart rate of 350 beats per minute. Furthermore QTc-interval length in minipigs (363–383 ms)[17] is more similar to humans (360–460 ms (female))[19] than rats (127–143 ms)[14]. While the minipig is frequently used to test new pharmacological agents for diabetes treatment, only few studies have focused on modelling diabetic comorbidities and no studies have been conducted investigating the effects of insulin-induced hypoglycaemia. A translational large animal model of hyperinsulinaemic hypoglycaemia, to investigate pathological consequences of hypoglycaemia—severe hypoglycaemia in particular—and test possible interventions, has yet to be developed.

The aim of this study was to develop a human-like minipig model of hypoglycaemia in non-anaesthetized healthy and streptozotocin-induced diabetic G ttingen minipigs using two different protocols of insulin-induced hypoglycaemia, hypothesizing that hypoglycaemia induces a human-like counter-regulatory hormonal response, cardiac arrhythmias, electrocardiographic T-wave, QT interval and ST-segment changes, heart rate variability and increased platelet aggregation response, as has previously been shown in human clinical studies[9, 20–22].

Materials and methods

Animals. All animal studies have been approved by the Danish Animal Experiment Inspectorate and conducted in accordance with rules and regulations set forth by the inspectorate. Fourteen intact female, adult, lean G ttingen minipigs (Ellegaard G ttingen Minipigs A/S, Dalmose, Denmark) were included in this study. Prior to enrollment, pigs were surgically implanted with two permanent central venous catheters (Cook C-TPNS-6.5-90-REDO, William Cook Europe ApS, Bjæverskov, Denmark) and one permanent auricular vena jugularis catheter. Using the Seldinger technique, central catheters were advanced trough the vena cava cranialis, through the right atrium and into the vena cava caudalis (modified from Larsen et al. 2002[23]).

Eight pigs were kept as healthy controls (CON) and six were made hyperglycemic (DIA) three months prior to this study by once-daily i.v. injection of 50 mg/kg streptozotocin (Sigma Aldrich Denmark A/S, cat. no. S0130-5G) for three consecutive days (modified from Schumacher et al. 2019[24]). The pigs in the DIA group had been used for other pharmacological studies prior to this study, with a washout period of one month between studies. In DIA, hyperglycaemia was managed by once-daily measurement of plasma glucose and s.c. injection of insulin glargine (Lantus, Sanofi S.A., Paris, France) in conjunction to feeding, based on individual plasma glucose curves, to maintain target fasting plasma glucose of 9–12 mM. Pigs were fed 460 g of feed once-daily (SDS minipig, Special Diets Service, Essex, UK) and were single-housed with wood chip and straw bedding, possibility of snout-contact and free access to water.

Hypoglycaemia protocols. Hypoglycaemia was induced using two different protocols: hyperinsulinaemic, hypoglycaemic clamp (CLAMP) and a single bolus insulin exposure (BOLUS) with at least 14 days washout period between each protocol. Pigs were fasted for 24 h prior to study. The day prior to study DIA received a low dose of 15 U insulin glargine to control hyperglycaemia while ensuring that none of the pigs would be hypoglycaemic at the day of study.

During CLAMP, pigs were given a continuous i.v. infusion of human insulin at 16 pmol/kg/min in the auricular catheter. The dosage was determined from the linear phase of a dose–response curve of insulin infusion rate-glucose infusion rate generated in a pilot study of both healthy and diabetic minipigs (data not shown). Plasma glucose (PG) levels were controlled by individually adjusting i.v. glucose infusion rate (GIR) in one of the central vena cava catheters, based on PG measured every five to fifteen minutes during CLAMP. Glucose levels were first clamped to a minipig normoglycaemic target (3.5 mM) for three hours. To induce acute hypoglycaemia, GIR stopped, and gradually adjusted to keep PG at target 0.8–1.0 mM for two hours. The study was finalized by two hours of normoglycaemia, where after all infusions were turned off.

For the BOLUS protocol, a single bolus of human insulin was dosed i.v. and PG was monitored. Insulin dose targeted PG < 1.5 mM was determined in a pilot study in healthy pigs (data not shown). Healthy pigs received 0.4 nmol/kg human insulin and diabetic pigs 0.6 nmol/kg, due to expected lower insulin sensitivity.

For both studies, blood samples were drawn from a vena cava catheter at predetermined time-points (Fig. 1).

Clinical signs of hypoglycaemia. During both protocols, clinical signs of hypoglycaemia (ataxia, apparent nausea with or without vomiting, mild myoclonic activity or "tics", severe myoclonic activity and signs of fatigue) were scored (0 or 1).

Circulating biomarkers. Glucose was measured from EDTA plasma continuously during both studies (YSI-2900, YSI Inc. OH, USA). For plasma glucagon, human insulin and C-peptide analysis, whole blood was added to 30 µL aprotinin (Trasylol 10.000 KIE/mL, Nordic Drugs AB, Limhamn, Sweden) in a 1.5 mL EDTA coated tube to prevent glucagon degradation. Glucagon, C-peptide and human insulin was quantified using in-

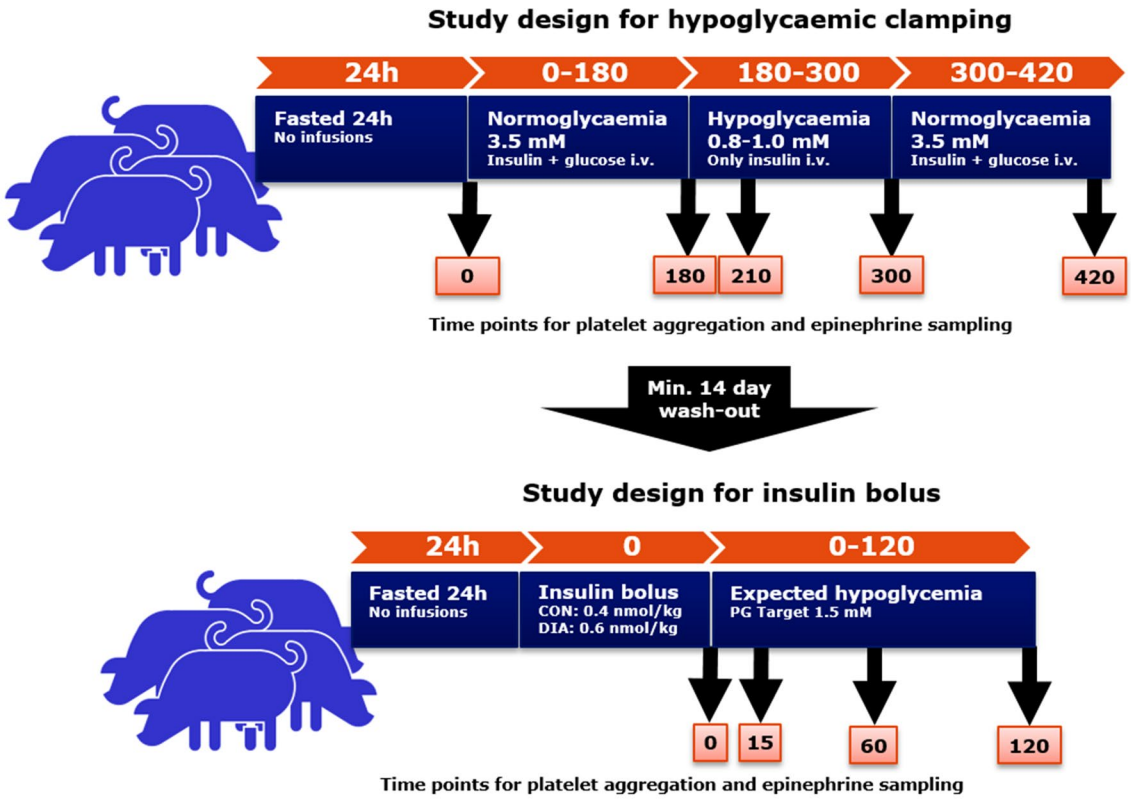


Figure 1. Study design. Hyperinsulinaemic, hypoglycaemic clamp protocol: Pigs were fasted for 24 h prior to study. Pigs received a constant infusion of human insulin (16 pmol/kg/min) throughout the study. A variable glucose infusion (GIR) was given to regulated plasma glucose (PG) to a desired level. PG was measured every five to fifteen minutes. PG target for the first 180 min of study was 3.5 mM (normoglycaemia). After 180 min, GIR was completely turned off, without turning of the insulin, to induce a rapid drop in PG. After initial drop, GIR were carefully increased on to obtain PG target of 0.8–1.0 mM (hypoglycaemia). At 300 min, GIR was increased to obtain normoglycaemia until study end at 420 min. Time-points for epinephrine and platelet aggregometry sampling is illustrated below. Insulin bolus protocol: the bolus protocol was initiated following a minimum 14 day wash-out period. Pigs were fasted for 24 h prior to study. At time 0 min, pigs received an intravenous human insulin bolus (control group (CON) 0.4 nmol/kg, diabetic (DIA) group 0.6 nmol/kg). PG was measured every five to fifteen minutes. Time-points for epinephrine and platelet aggregometry sampling is illustrated below. Software used to generate graphics: Microsoft PowerPoint 2016.

house developed luminescence oxygen channelling immunoassays. Lower limit of quantification (LLOQ) for glucagon, C-peptide and human insulin was 4, 45 and 10 pM, respectively.

For plasma epinephrine analysis, whole blood was added to ice-cold EGTA (2.88 mg/mL) and glutathione (1.44 mg/mL) solution (E3889 and G4251, Merck Life Science A/S, Søborg, Denmark) and centrifuged at 3000G for 10 min at 4 °C within 30 min of sampling. Plasma was stored at -80 °C and analysed within 6 months. Epinephrine levels was quantified using liquid chromatography-mass spectrometry with a LLOQ of 0.05 ng/mL as previously described[25].

Serum biochemistry was analysed using the Advia 1800 Chemistry System (Siemens Healthcare A/S, Ballerup, Denmark). Acute phase proteins C-reactive protein and serum amyloid A was determined as described elsewhere[26]. Haematology parameters was determined in fresh EDTA-stabilized whole blood (Sysmex XT-2000iv, Sysmex Nordic ApS, Copenhagen, Denmark).

Continuous electrocardiography (ECG). Continuous ECG was recorded with a Lifecard CF recorder (Spacelabs Healthcare, WA, USA). Electrodes were placed as previously described: "Lead I" (neck (A)—processus xiphoideus (B) and "lead II" (manubrium sterni (C)—processus xiphoideus (D))[27]. All pigs were dosed with 8–10 mL propofol i.v. (Propofol "B. Braun" 10 mg/mL, B. Braun Medical A/S, Frederiksberg, Denmark) two hours prior to study start to ensure stress-free handling when placing equipment.

ECG recordings were analysed using Pathfinder SL (Spacelabs Healthcare, WA, USA). The recording was manually edited by adjustment of R-wave indicators and arrhythmia detection, if needed. Observer was blinded to pig identification, group and time setting. Number of arrhythmic events (supraventricular ectopic beats, ventricular extra-systole and 2nd degree atrioventricular blocks[28]), ST-segment derivations, QT-interval duration and T-wave morphology were registered during normoglycaemia, hypoglycaemia and normoglycaemia after hypoglycaemia. T-wave amplitude (measured from the isoelectric line of TP segment, to highest point of

	Control		Diabetic		*P*-value	
	Clamp	Bolus	Clamp	Bolus	Clamp	Bolus
Age (months)	11.8 (11.7–12.2)		16.7 (15.3–17.5)		0.004	
Weight (kg)	25.0 (23.9–26.1)	30.0 (30–31.5)	36.6 (35.0–38.3)	36.0 (34.8–37.2)	0.004	0.004
Daily insulin glargine (IU)	–	–	26 (23–28)	25 (18.5–26.3)	–	–
CRP* (µg/mL)	51.1 (32.9–78.3)	45.1 (33.3–134.5)	33.9 (27.6–44.9)	39.4 (19.3–60.3)	ns	ns
SAA† (µg/mL)	17.4 (3.9–37.5)	3.9 (3.9–104.2)	55.6 (16.6–148.2)	12.75 (3.9–30.8)	ns	ns
Albumin (g/L)	43.85 (43.33–45.15)	45.89 (42.13–49.92)	61.67 (47.82–73.14)	53.76 (50.20–61.33)	ns	0.030
D3 hydroxybutyrate (mM)	0 (0–0.01)	0.01 (0.01–0.02)	0.03 (0.02–0.05)	0.05 (0.02–0.09)	0.042	0.033
Potassium (mM)	4.3 (4.1–4.4)	4.4 (4.1–4.4)	5.4 (4.4–6.6)	4.7 (4.4–4.8)	ns	0.028
Creatinine (µM)	73 (65–76)	74 (65–83)	94 (79–111)	80 (73–87)	ns	ns
Urea nitrogen (mM)	2.6 (2.2–3.0)	2.5 (2.3–3.2)	3.6 (2.6–4.8)	2.7 (2.4–3.6)	ns	ns
Hematocrit (%)	33.0 (28.7–33.5)	30.1 (27.9–33.5)	32.4 (30.3–36.1)	35.7 (31.2–38.1)	ns	ns
Red blood cells (10^{12}/L)	6.6 (5.9–7.0)	6.2 (5.6–6.6)	5.2 (4.6–6.2)	6.2 (5.1–6.6)	ns	ns
White blood cells (10^9/L)	13.7 (9.0–15.0)	11.9 (9.7–14.8)	7.3 (6.1–10.6)	6.9 (5.8–7.4)	0.03	0.004
Platelets (10^9/L)	488 (400–595)	512 (364–610)	358 (261–606)	398 (363–480)	ns	ns

Table 1. Basal data. Selected baseline data obtained from both groups obtained at the start of studies, including age, weight, serum biomarkers and complete blood count. Data shown as medians and interquartile range. Significant difference between groups set to $P < .05$ and tested by Mann–Whitney U-test. *CRP = C-reactive protein, †SAA = serum amyloid A.

deflection[29]), ST-segment derivation (distance from PQ segment to J-point[30]) as well as QT-interval duration (corrected for heart rate using Bazett's formula[29]) were measured as a mean of ten consecutive sinus beats using ImageJ (National Institute of Health Sciences, Kawasaki, Japan).

Frequency-domain HRV was calculated from predetermined, five-minute epochs using the build-in Fast Fourier Transformation of Pathfinder SL. Very low frequency power bands at 0.015–0.05 Hz (VLF), low frequency at 0.05–0.15 Hz (LF), high frequency at 0.15–0.40 Hz (HF) and LF to HF ratio (LF/HF) were reported[31].

Light transmission platelet aggregometry. Platelet rich (PRP) and platelet poor plasma (PPP) were prepared at previously described[32] and 250 µL of PPP and 230 µL of PRP were transferred to glass tubes containing a stirring magnet in an aggregometer (PAP-8E, Bio/Data Corporation, PA, USA). PPP represented a light signal of 100% aggregation. 20µL of ADP (HB-5502-FG, Hart Biologicals, Hartlepool, UK) diluted in modified Hepes-Tyrodes buffer was added to PRP. Each pig's PRP was stimulated with 0, 1, 2, 4, 6, 8, 10 and 20 µM ADP to generate dose–response curves. The light transmission was recorded for 10 min and was measured every 0.1 min. Maximum aggregation response (MAX) and area under the curve (AUC) were calculated for each dose of ADP. In addition, effective concentration of ADP to generate 50% response was calculated for both MAX ($EC50_{MAX}$) and AUC ($EC50_{AUC}$).

Statistical analyses. Linear mixed effect models with individual pig as random variable were used to analyse endocrine, ECG and platelet aggregation responses during hypoglycaemia using SAS 9.4 (SAS Institute, Cary, USA). Group and time were used as explanatory variables, and interaction between group and time was included as well. The models were reduced by backward elimination. Logarithmic transformation was used to enhance the fit of the models if needed. Differences between groups in baseline values of circulating biomarkers, total insulin exposures and basal characteristics (Mann–Whitney U test), and presence of clinical signs of hypoglycaemia (Fisher's Exact test) were tested. Graphical representation and calculations of EC50 were made using GraphPad Prism 8 (GraphPad Software Inc., San Diego, USA). The level of significance was set at $P < 0.05$. Data is reported as median and interquartile range.

Conference presentation. Part of this work has been presented as a poster at the 56th annual meeting of the European Association for the Study of Diabetes.

Results

The experimental procedures were generally well tolerated by the animals, however, three control pigs were excluded due to dysfunctional catheters (n = 2) or ECG abnormalities (n = 1) before study start. One diabetic pig was included in the BOLUS protocol, but was euthanized before the CLAMP due to clinical signs of systemic infection. Basal characteristics of the pigs are presented in Table 1.

Counter-regulatory endocrine response to hypoglycaemia. It was possible to induce hypoglycaemia in both CON and DIA using the CLAMP protocol to target level of 0.8–1 mM (Fig. 2a). In the CLAMP, total insulin exposure did not differ between groups (CON 891 pM (721–1319), DIA 1630 pM (1235–2110)), however, temporal differences were seen between groups (see Fig. 2c). In the BOLUS, control pigs received

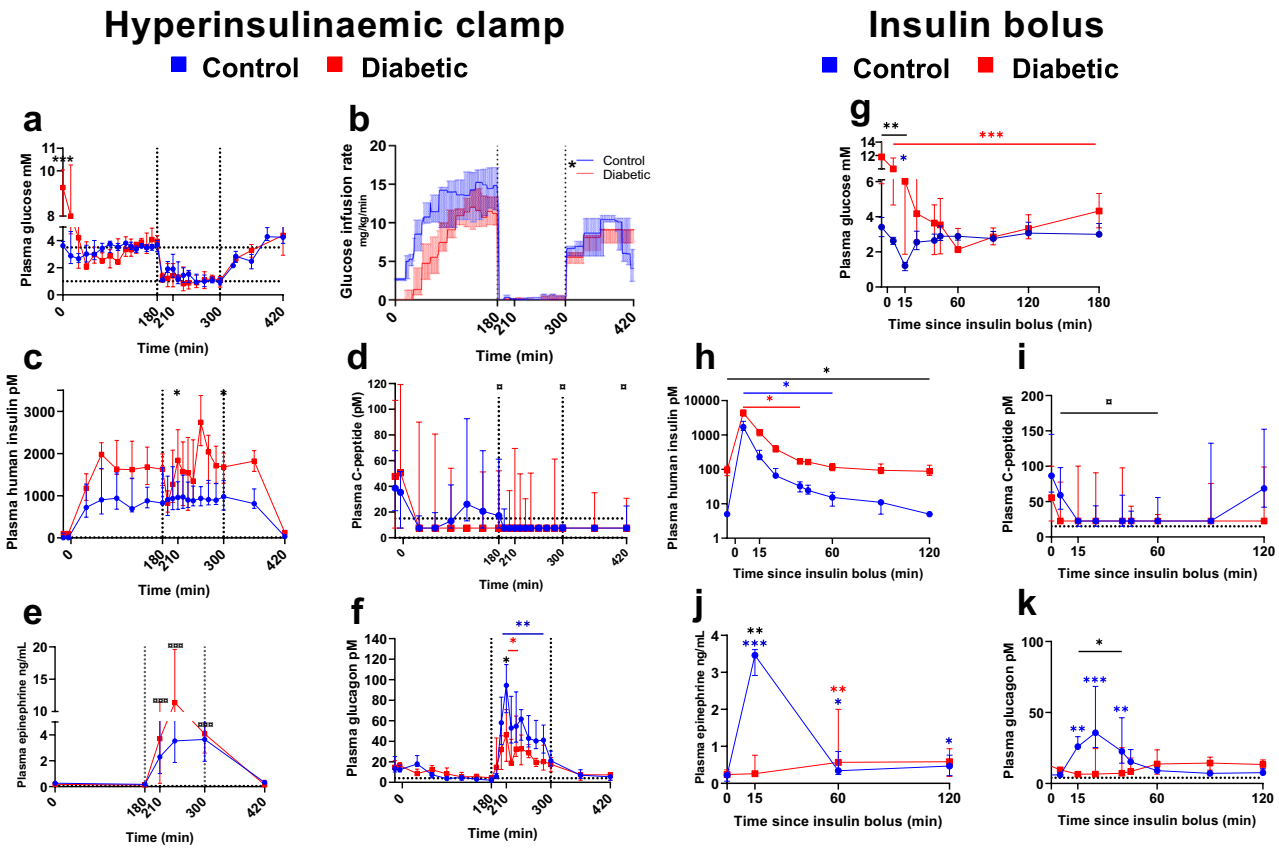


Figure 2. Endocrine response. Plasma glucose mM (**a**+**g**), human insulin pM (**c**+**h**), plasma C-peptide pM (**d**+**i**), plasma epinephrine ng/mL (**e**+**j**) and plasma glucagon pM (**f**+**k**), measured during the hyperinsulinaemic clamp and insulin bolus study. Glucose infusion rate during clamp in mg/kg/min (**b**). Clamp: blue lines: control pigs, red lines: diabetic pigs. Bolus: green lines: control pigs, orange lines: diabetic pigs. Data shown as medians ± interquartile range, except in glucose infusion rate, where each individual is illustrated. Significant difference to baseline within group is illustrated with accordingly colored stars (*), and, when both groups are considered together, as black currency sign (¤). Significance between groups as black stars (*). *$P<.05$, **$P<.001$, ***$P<.0001$, by linear mixed modelling or Mann–Whitney U-test. Horizontal dotted lines: lower limit of quantification of assay. Vertical lines: start and end of hypoglycaemia in the clamp. Software used to generate graphs: GraphPad Prism 8 (GraphPad Software Inc., San Diego, USA).

0.4 nmol/kg and diabetic pigs 0.6 nmol/kg human insulin resulting in total exposure of 12.5 nM (9.0–19.0) and 74.1 nM (65.2–88.8), respectively ($P=0.004$) (Fig. 2h). Total glucose infusion during CLAMP was higher in CON (3130 mg/kg (2508–3332)) compared to DIA (2178 mg/kg (1874–2542)) ($P=0.02$), indicating a higher insulin sensitivity in CON (Fig. 2b).

Baseline plasma C-peptide levels did not differ between groups in either the CLAMP (Fig. 2d) or BOLUS (Fig. 2i). In the CLAMP, C-peptide decreased from start of hypoglycaemia until end of study for both groups and likewise, in the BOLUS, from t15 to t60 (Fig. 2d,i), indicating suppression of endogenous insulin production.

At baseline in the CLAMP protocol, before infusions, DIA had significant higher PG levels compared to CON (Fig. 2a). No difference in median PG appeared during the first three hours of normoglycaemia (CON: 3.52 mM (3.29–3.60); DIA: 3.42 mM (2.94–4.23), and, during two hours of hypoglycaemia (CON: 1.30 mM (1.09–1.66); DIA: 1.16 mM (1.01–1.32)).

The epinephrine response to hypoglycaemia in CLAMP did not differ significantly between CON and DIA. When considering both groups together, plasma epinephrine levels increased approximately 25-fold after one hour of hypoglycaemia (5.13 ng/mL (3.19–11.60)) compared to baseline (0.2 ng/ml (0.18–0.30)) (Fig. 2e) with a return to baseline level at end of study.

In the bolus protocol, only CON reached hypoglycaemic target with PG nadir at 1.21 mM (0.89–1.46) after 15 min. PG nadir for DIA was 2.14 mM (1.87–4.02) after 60 min (Fig. 2g).

In the BOLUS, CON had approximately 15-fold increase in epinephrine at fifteen minutes compared to baseline. DIA had increased epinephrine compared to baseline only at 60 min post insulin dose (Fig. 2j).

Plasma glucagon increased approximately six-fold for CON (89.8 pM (67.7–118.5)) and three-fold for DIA (46.3 pM (20.5–70.9)) compared to baseline (CON: 14.9 pM (9.6–25.3), DIA: 14.8 (14.4–21.2)) in both groups during the CLAMP, with highest levels after 30 min (t210) of hypoglycaemia, with significantly higher levels in CON compared to DIA (see Fig. 2f). In the BOLUS, only CON had an increase in glucagon in response to insulin bolus (see Fig. 2k).

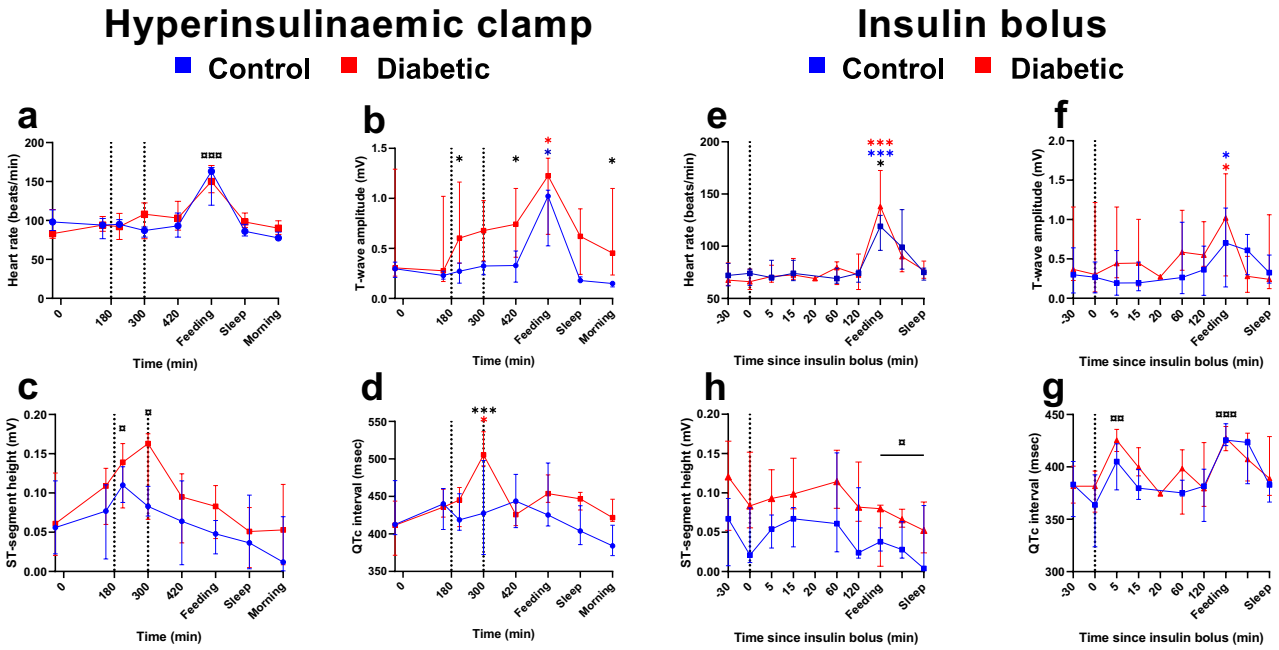


Figure 3. Electrocardiographic morphology. Measures of changing electrocardiographic morphology. Heart rate in beats-per-minute (**a**+**e**), amplitude of the T-wave in mV (**b**+**f**), height of the ST-segment in mV (**c**+**g**) and heart rate corrected duration of the QT-interval (**d**+**h**) during hyperinsulinaemic clamp and insulin bolus study. Clamp: blue lines: control pigs, red lines: diabetic pigs. Bolus: green lines: control pigs, orange lines: diabetic pigs. Data as medians ± interquartile range. Significant difference to baseline within group is illustrated with accordingly colored stars (*), and, when both groups are considered together, as black currency sign (¤). Significance between groups as black stars (*). *$P<.05$, **$P<.001$, ***$P<.0001$, by linear mixed modelling. Vertical lines: start and end of hypoglycaemia in the clamp or insulin bolus time. Software used to generate graphs: GraphPad Prism 8 (GraphPad Software Inc., San Diego, USA).

Clinical signs of hypoglycaemia. All pigs displayed clinical signs of fatigue during CLAMP ($P<0.0001$), and during BOLUS it was observed all pigs in CON and 4/6 in DIA ($P=0.0002$). During CLAMP, ataxia (1/5 CON, 3/5 DIA), mild myoclonic tics (1/5 CON, 2/5 DIA), severe myoclonic activity (1/5 DIA) and vomiting (1/5 DIA) were observed. No other signs than fatigue was seen during BOLUS.

Electrocardiographic changes. Heart rate was not influenced by hypoglycaemia (Fig. 3a,e). In the CLAMP, QTc-interval was prolonged in DIA after two hours of hypoglycaemia ($P<0.0001$), independently of heart rate, while CON that had no changes in QTc (Fig. 3d). In the BOLUS, QTc did not differ between CON and DIA, but QTc increased five minutes after insulin bolus and at feeding (Fig. 3h, $P<0.001$), with the increase during feeding most likely accounting to an over-correction due to high heart rate. During hypoglycaemia in the CLAMP, T-wave amplitude was higher in DIA compared to CON (Fig. 3b) while no changes were seen in BOLUS (Fig. 3f). The ST-segment was elevated from baseline, with no difference between groups (Fig. 3c). No increase in ST elevation was seen during hypoglycaemia in the bolus protocol, even though target PG (< 1.5 mM) was reached in CON. A decrease in ST-segment elevation compared to baseline was seen at feeding and sleep after the study, with no difference between groups (Fig. 3g).

In both CON and DIA, total number of arrhythmias increased significantly in CLAMP during hypoglycaemia to 2.25 events/hour compared to the initial normoglycaemia period of 0.04 events/hour ($P=0.03$) with no difference between groups. Number of ventricular ectopic beats changed from 0 (0–0) and 0.17 (0–0.33) during normoglycaemia to 0 (0–0.5) and 1.0 (0–1.5) per hour during hypoglycaemia in CON and DIA respectively. Number of supraventricular premature beats changed from 0 (0–26) and 0 (0–5.67) to 0.5 (0–39) and 0 (0–25) per hour for CON and DIA respectively. Second degree atrioventricular blocks changed from 0 (0–0) and 0 (0–0) to 0 (0–8.5) and 0 (0–0) per hour in CON and DIA respectively. In BOLUS, hypoglycaemia and group did not influence in total number of arrhythmic beats.

Heart rate variability. During hypoglycaemia in the CLAMP, only the LF frequency band power decreased ($P=0.004$) with no difference between groups (Fig. 4a–d). In the BOLUS, hypoglycaemia did not influence HRV (Fig. 4e–h). The VLF, LF, HF frequency band power and LF/HF ratio decreased during feeding after both protocols. After the CLAMP, the LF and HF bands increased during night and in the morning after study ($P<0.05$) while the LF/HF ratio decreased accordingly at these time points as well ($P<0.05$) (Fig. 4).

Light transmission platelet aggregometry. The platelet aggregation response decreased during hypoglycaemia in CLAMP. $EC50_{AUC}$ increased, reflecting decreased platelet aggregation response, at both 30 (7.73

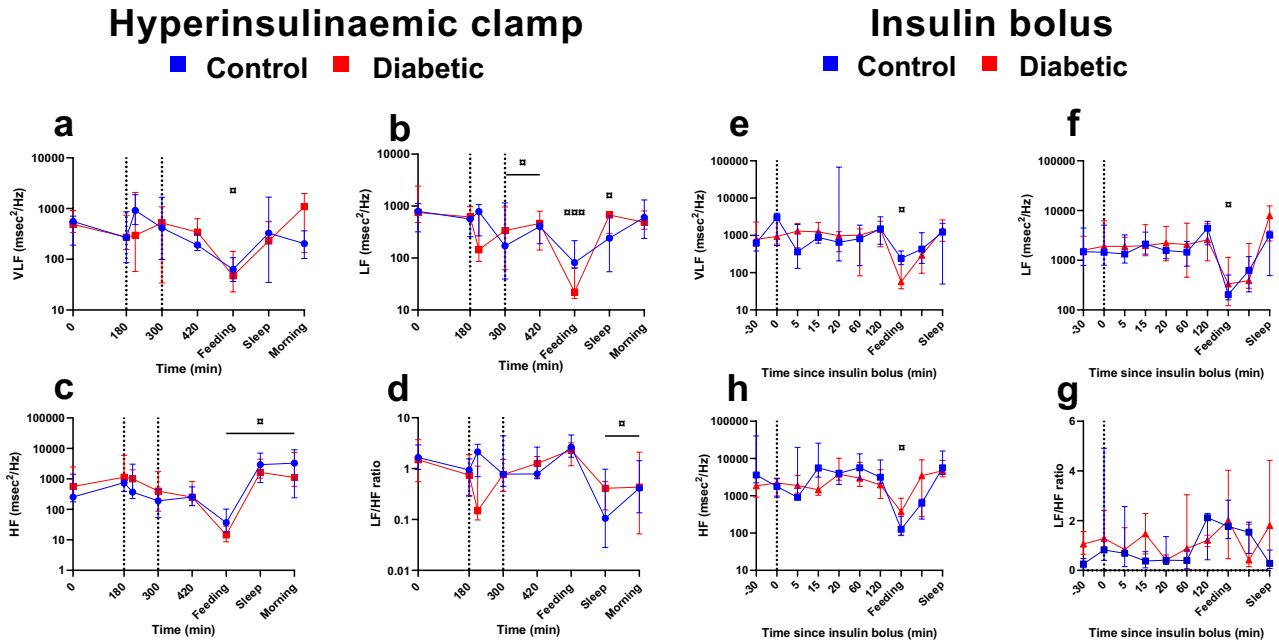


Figure 4. Frequency domain heart rate variability. Very low frequency band power (VLF) in msec^2/Hz (**a**+**e**), low frequency band power (LF) in msec^2/Hz (**b**+**f**), high frequency band power (HF) in msec^2/Hz (**c**+**g**) and ratio between LF and HF power (**d**+**h**) during hyperinsulinaemic clamp and insulin bolus study. Clamp: blue lines: control pigs, red lines: diabetic pigs. Bolus: green lines: control pigs, orange lines: diabetic pigs. Y-axis is logarithmic. Data as medians ± interquartile range. Significant difference between groups as black stars (*). Significant difference to baseline within group is illustrated with accordingly colored stars (*), and, when both groups are considered together, as black currency sign (¤). *$P<.05$, **$P<.001$, ***$P<.0001$, by linear mixed modelling. Vertical lines: start and end of hypoglycaemia in the clamp or insulin bolus time. Software used to generate graphs: GraphPad Prism 8 (GraphPad Software Inc., San Diego, USA).

(6.45–8.51), $P=0.03$) and 120 min (9.19 (7.42–9.80), $P<0.0001$) of hypoglycaemia compared to baseline (6.58 (6.18–6.98)) with no difference between groups (Fig. 5a). Furthermore, $EC50_{MAX}$ was increased after 120 min of hypoglycaemia (baseline; 3.24 (2.55–5.39), t300; 6.11 (4.32–6.86), $P<0.01$), but slightly decreased at the end of the clamp (t420 2.52 (2.17–3.11), $P<0.01$) (Fig. 5b).

Platelet aggregation response was not influenced by hypoglycaemia during the BOLUS protocol (Fig. 5c,d).

Discussion

In the present study it was possible to induce hypoglycaemia close to target of 1.0 mM plasma glucose using the clamp approach in both healthy and diabetic G ttingen minipigs. Using the bolus approach, only healthy pigs reached hypoglycaemic target while the diabetic group showed a variable response to the insulin bolus, reflecting variation in initial fasted plasma glucose and insulin sensitivity among animals in this STZ induced diabetes model. Thus, to use the bolus setup for induction of insulin-induced hypoglycaemia in STZ diabetic minipigs may require an initial insulin sensitivity determination in the individual animals. The healthy minipigs used in this study had a lower fasting blood glucose than what has been reported in studies using commercial pig breeds[33], rats[15] and in healthy humans[34], prompting a need for a quite low plasma glucose target to achieve hypoglycaemia with clinical signs. In this study, all minipigs displayed signs of fatigue during hypoglycaemia in the clamp setup, and some even signs of neuroglycopenia, such as myoclonic activity and ataxia[35], some of which has been described in rat studies[14,15] but not in a previous study of hyperinsulinaemic hypoglycaemia in awake pigs[33]. Though the clamp approach seems to be the better option to reliably induce hypoglycaemia to a specific target in both healthy and diabetic pigs, the single insulin bolus might reflect the clinical setting more closely, where patient's insulin requirements and insulin delivery might not always match[35].

The counter-regulatory autonomous response to hypoglycaemia in the clamp protocol induced an approximately 25-fold increase in plasma epinephrine, an increase similar to what has been found in human studies, where autonomous symptoms of hypoglycaemia were also recorded[8]. Release of epinephrine is a counter-regulatory mechanism to hypoglycaemia observed in humans[22] and in rodent models[14,15], but in healthy pigs, no response or only a weak response have been reported[33,36]. In the bolus setup, only the healthy group reached hypoglycaemic target to induce an epinephrine response and the lack of response in DIA is likely driven by the low insulin sensitivity, where even a 50% higher insulin dose than given the healthy group was insufficient to induce hypoglycaemia at target level.

Electrocardiography by wearable devices has been used in human studies to investigate connections between glucose fluctuations and ECG changes[37]. Cardiac arrhythmias during hypoglycaemia have been reported in both human[10,13] and rat studies[14,15], suggesting arrhythmias as potential triggers for dead-in-bed syndrome. In the

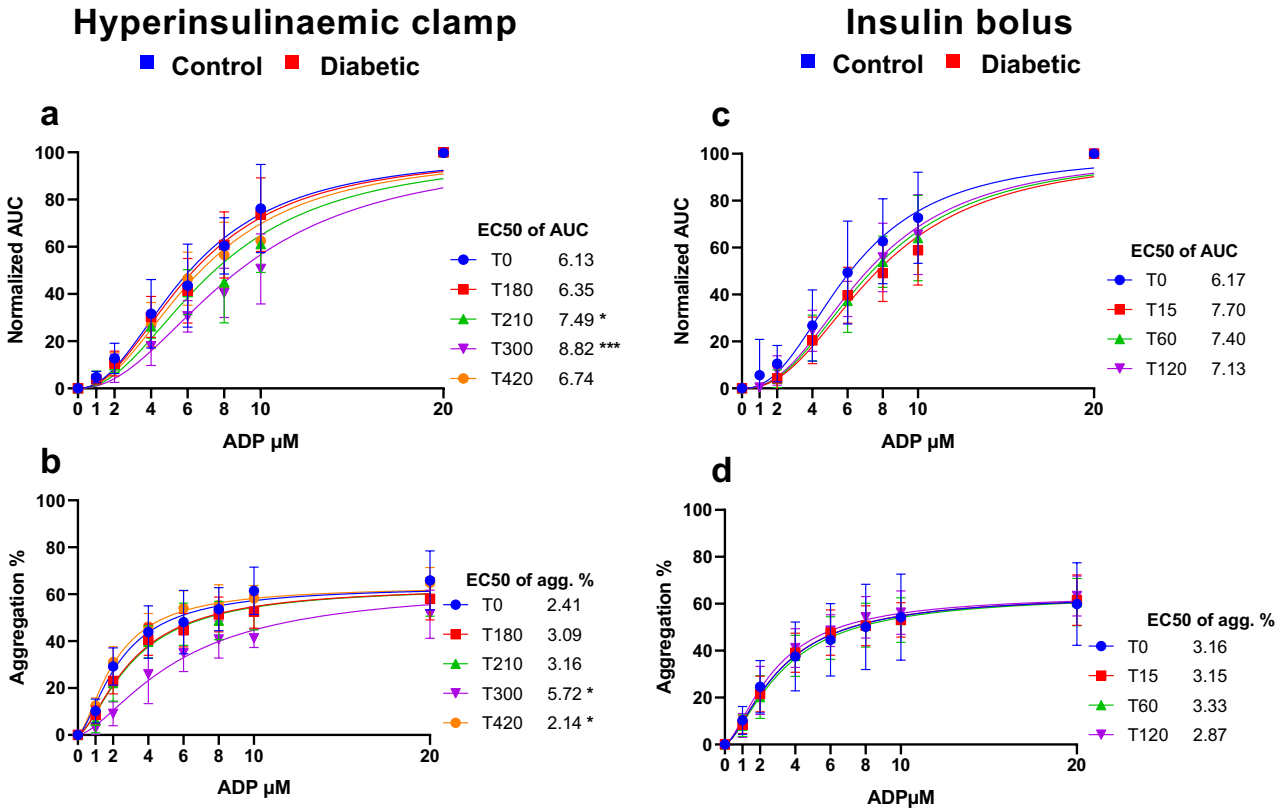


Figure 5. Platelet aggregometry. Light transmission platelet aggregometry, with no distinction between groups, at different time-points throughout the studies. Normalized (0–100%) area under the curve (AUC) at different concentrations of adenosine diphosphate (ADP) (**a** + **c**), and maximum aggregation percentage at different concentrations of ADP (**b** + **d**), both with overlays of fitted dose–response curves at the different time points. Hyperinsulinaemic clamp: blue line: time 0 representing baseline dose–response to ADP, red line: after 180 min representing two hours of normoglycaemia, green line: after 210 min representing 30 min of hypoglycaemia, purple line: after 300 min representing two hours of hypoglycaemia, orange line: 420 min representing two hours of normoglycaemia after hypoglycaemia. Data shown as medians ± interquartile range. Insulin bolus: blue line: time 0 representing baseline dose–response to ADP, red line: 15 min after insulin bolus, green line: 60 min after insulin bolus, purple line: 120 min after insulin bolus. Significant difference to baseline effective concentration for 50% reaction (EC50) labeled as stars (*) set to $*P < .05$ and $***P < .0001$ by linear mixed modelling. Software used to generate graphs: GraphPad Prism 8 (GraphPad Software Inc., San Diego, USA).

present study, the most commonly observed type of arrhythmic beats were supraventricular ectopic beats, with the overall rate of arrhythmias increasing during hypoglycaemia, possibly reflecting the proarrhythmic effect of hypoglycaemia. In a clinical study of spontaneous hypoglycaemia, supraventricular ectopic beats are the most common arrhythmia presenting[12] while another study report increase in both ventricular and supraventricular ectopic beats[11]. Hypoglycaemia also induces change in ventricular repolarization, causing QT-interval prolongation but also altered T- and U-wave morphology in humans[38]. In this study, diabetic pigs had a higher amplitude of T-waves during and after hypoglycaemia compared to healthy controls, a change contrary to what is seen in humans during hyperinsulinaemic hypoglycaemia, where hypokalemia also often is present[8,38,39]. Prolongation of the QT-interval during hypoglycaemia is thought to be an effect of sympathetic stimulation and hypokalemia[39], a pro-arrhythmogenic condition causing reduced repolarization of cardiomyocytes. The effects of hypokalemia is most often expressed in prolonged QT-interval, flattening of T-waves and can cause afterdepolarization-mediated arrhythmias[40], and the arrhythmogenic effect of hypokalemia can be somewhat reversed by potassium intravenous infusion in a rodent model of hyperinsulinaemic hypoglycaemia[15]. Importantly, in the present study, significantly increased QTc-interval without change in heart rate was seen during hypoglycaemia, which corresponds to what has previously been reported in hypoglycaemia in humans and rats[9,15]. Interestingly, in our bolus study, QTc-interval increased only five minutes after insulin dosing, and before reaching hypoglycaemia target. This suggests QTc-interval as a potential early marker of insulin-induced hypoglycaemia that need to be investigated further. A slight ST-segment elevation was also seen during hypoglycaemia, which might indicate myocardial hypoxia, a phenomenon that has been reported sporadically in people with type 1 diabetes and cardiovascular disease[20]. The finding of relative few ECG changes during hypoglycaemia in the present study can due to the hypoglycaemic level chosen in this study was not severe enough to induce the changes observed in humans, as has already been demonstrated in a rat study with hypoglycaemic target of 0.55–0.83 mM plasma glucose[15]. However, it is also possible, that the same degree of changes observed in rats and human patients cannot be induced in the pig due to species differences.

Frequency domain analysis of heart rate variability has been investigated in people with diabetes, and changes in frequency band power has been associated with altered autonomic nervous control of the heart and fluctuations in blood glucose levels[37]. In the present study, only the LF band power decreased during hypoglycaemia in groups, with no other frequency bands changing. This decrease in the LF band has been demonstrated in experimental human studies of hypoglycaemia corresponding to an increase in plasma epinephrine[41] or in hypoglycaemia detected by CGM in type 1 diabetic patients[12]. Conversely, the LF band is thought to reflect baroreceptor activity in resting states, which can be influenced primarily by parasympathetic stimulation, but also blood pressure and sympathetic stimulation[42], suggesting a counter-regulative parasympathetic cardiac modulation to elevated epinephrine during hypoglycaemia. Interestingly, parasympathetic blockade has been associated with increased survival in severe hypoglycaemia in rats[14]. The only other change seen in all frequency bands was during feeding, which is to be considered a positive control of sympathetic simulation during excitation[31].

In the present study, hypoglycaemia induced glucagon release, but diabetic pigs had an attenuated glucagon response to hypoglycaemia in the clamp compared to healthy pigs, comparable to the human counter-regulatory response in people with and without type 1 diabetes[43]. Furthermore, it should be noted that the alpha-cells can also be damaged during the STZ treatment to induce diabetes, which may explain the lower glucagon release[44]. Both groups had a decrease in C-peptide during clamp and bolus, reflecting the decrease in endogenous insulin secretion during hypoglycaemia due to suppression by the exogenous insulin. Diabetic pigs had a higher level of human insulin in plasma, even though they received the same dosage as the healthy pigs, as well as a lower glucose infusion rate to maintain target plasma glucose, further supporting the observation of a lower insulin sensitivity in this group.

Platelets are important players in the cardiovascular pathophysiology underlying the formation of atherosclerosis and coronary heart disease[3]. The most common comorbidity of diabetes is cardiovascular disease, and people with diabetes have increased platelet activation and are often resistant to anti-platelet therapy[45]. Platelet hyperreactivity has also been demonstrated in the Ossabaw pig model of metabolic syndrome[46] and in pigs with alloxan-induced diabetes[47]. However, in the present study, no difference between healthy and diabetic pigs in platelet activation was observed, indicating that the diabetic minipig model employed here does not reflect this aspect of the cardiovascular pathophysiology associated with diabetes, at least not during insulin treatment.

Healthy humans subjected to an insulin bolus to induce hypoglycaemia has been demonstrated to have increased platelet aggregation, which persists after hypoglycaemia is resolved[21]. No such effect was found in our bolus study, and in the clamp study, the opposite effect was seen, with hypoglycaemia decreasing the platelet aggregation responses. Interestingly, previous studies of healthy humans exposed to a hyperinsulinaemic euglycemic clamp demonstrated inhibition of platelet reactivity[48] similarly to what we observe in the present study, causing the speculation that the reduced platelet reactivity may a result of high insulin exposure, since insulin has been demonstrated to exert a inhibitory effect on platelet reactivity in human platelets[48].

The present study is limited by the low number of animals included and differences in age and weight between the diabetic and healthy group. The differences in age and weight between groups were due to the diabetic pigs were included in other studies prior to this study. Though the diabetic group weighted more than the control group, both groups were close to the normal weight for their age[49]. The pigs in the present study had elevated inflammatory markers compared to previous porcine studies[50], indicating some sort of subclinical inflammatory state, most likely due to the permanent central venous catheters. Diabetic pigs had slightly elevated baseline serum potassium compared to the healthy pigs, possibly creating a much steeper slope of possible hypokalemia, a common effect of hyperinsulinaemic hypoglycaemia, which can lead to ECG changes[38]. Propofol was used as a brief sedation agent to apply ECG monitors to the pigs prior to the start of study. Though propofol is known to have a short sedative duration, it cannot be excluded, that the dosing of propofol might alter the counter-regulatory response to hypoglycaemia. A limitation of this study is also the lower fasting blood glucose in the healthy G ttingen minipig compared to humans, making a direct translation of glycaemic levels more difficult. In the rat, fasting blood glucose has been shown to be 5.8–7.1 mM, which matches the human fasting level more closely[15]. Moreover, continuous glucose monitoring was not included in the diabetic group. Plasma glucose was only measured in the morning, and thereby it cannot be excluded, that the pigs had experienced hypoglycaemia prior to the study. However preceding hypoglycaemic episodes were not expected due to high fasted morning plasma glucose levels in the diabetic group.

Overall, the novel G ttingen minipig hyperinsulinaemic-hypoglycaemic clamp model appear to be a highly clinically relevant translational model. The counter-regulatory endocrine response to hypoglycaemia seems to closely match that of humans, with clinically significant glucagon and adrenalin increase, making it possible to use minipigs as models for possible intervention studies of hypoglycaemia. The electrocardiographic changes and heart rate variability fluctuations observed also match what has previously been observed in humans, which further corroborates the relevance of the model. However, the electrocardiographic changes were mainly mild and transient, prompting a need for studies conducted in more severe hypoglycaemic states to better understand the minipig as a possible model of dead-in-bed syndrome and the proarrhythmic effects of severe hypoglycaemia, which for ethical reasons cannot be studied experimentally in humans. Furthermore, the effect of recurrent hypoglycaemia needs to be examined to determine if the physiological effects are comparable to human studies[51].

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Received: 6 October 2020; Accepted: 12 February 2021
Published online: 16 March 2021

References

1. Bonds, D. E. *et al.* The association between symptomatic, severe hypoglycaemia and mortality in type 2 diabetes: retrospective epidemiological analysis of the ACCORD study. *BMJ* **340**, b4909. https://doi.org/10.1136/bmj.b4909 (2010).
2. Kannel, W. B. & McGee, D. L. Diabetes and cardiovascular risk factors: the framingham study. *Circulation* **59**, 8–13 (1979).
3. Vinik, A. I., Erbas, T., Park, T. S., Nolan, R. & Pittenger, G. L. Platelet dysfunction in type 2 diabetes. *Diabetes Care* **24**, 1476–1485 (2001).
4. Pieber, T. R. *et al.* DEVOTE 3: temporal relationships between severe hypoglycaemia, cardiovascular outcomes and mortality. *Diabetologia* **61**, 58–65. https://doi.org/10.1007/s00125-017-4422-0 (2018).
5. Gerstein, H. C. *et al.* Effects of intensive glucose lowering in type 2 diabetes. *N. Engl. J. Med.* **358**, 2545–2559 (2008).
6. Soedamah-Muthu, S. S. *et al.* High risk of cardiovascular disease in patients with type 1 diabetes in the UK. *Diabetes Care* **29**, 798–804 (2006).
7. Wright, R. J. & Frier, B. M. Vascular disease and diabetes: Is hypoglycaemia an aggravating factor?. *Diabetes Metab. Res. Rev.* **24**, 353–363. https://doi.org/10.1002/dmrr.865 (2008).
8. Laitinen, T. *et al.* Electrocardiographic alterations during hyperinsulinemic hypoglycemia in healthy subjects. *Ann. Noninvasive Electrocardiol.* **13**, 97–105 (2008).
9. Chow, E. *et al.* Cardiac autonomic regulation and repolarization during acute experimental hypoglycemia in type 2 diabetes. *Diabetes* **66**, 1322–1333. https://doi.org/10.2337/db16-1310 (2017).
10. Chow, E. *et al.* Risk of cardiac arrhythmias during hypoglycemia in patients with type 2 diabetes and cardiovascular risk. *Diabetes* **63**, 1738–1747. https://doi.org/10.2337/db13-0468 (2014).
11. Gill, G. V., Woodward, A., Casson, I. F. & Weston, P. J. Cardiac arrhythmia and nocturnal hypoglycaemia in type 1 diabetes - the "dead in bed" syndrome revisited. *Diabetologia* **52**, 42–45. https://doi.org/10.1007/s00125-008-1177-7 (2009).
12. Novodvorsky, P. *et al.* Diurnal differences in risk of cardiac arrhythmias during spontaneous hypoglycemia in young people with type 1 diabetes. *Diabetes Care* **40**, 655–662. https://doi.org/10.2337/dc16-2177 (2017).
13. Lainetti, K. R., Pimenta, J. & Vendramini, M. F. Can hypoglycemic episodes in type 1 diabetics trigger cardiac arrhythmias?. *Diabetes Res. Clin. Pract.* **158**, 107878. https://doi.org/10.1016/j.diabres.2019.107878 (2019).
14. Reno, C. M. *et al.* Severe hypoglycemia-induced fatal cardiac arrhythmias are mediated by the parasympathetic nervous system in rats. *Diabetes* **68**, 2107–2119. https://doi.org/10.2337/db19-0306 (2019).
15. Reno, C. M. *et al.* Severe hypoglycemia-induced lethal cardiac arrhythmias are mediated by sympathoadrenal activation. *Diabetes* **62**, 3570–3581. https://doi.org/10.2337/db13-0216/-/DC1 (2013).
16. Larsen, M. O. & Rolin, B. Use of the G ttingen minipig as a model of diabetes, with special focus on type 1 diabetes research. *ILAR J* **45** (2004).
17. Stricker-Krongrad, A. *et al.* Comparative cardiovascular physiology and pathology in selected lineages of minipigs. *Toxicol. Res. Appl.* **1**, 1–8. https://doi.org/10.1177/2397847317696367 (2017).
18. Crick, S. J., Sheppard, M. N. & Anderson, R. H. Anatomy of the pig heart: comparisons with normal human cardiac structure. *J. Anat.* **193**, 105–119 (1998).
19. Postema, P. G. & Wilde, A. A. M. The Measurement of the QT Interval. *Curr. Cardiol. Rev.* **10**, 287–294 (2014).
20. Desouza, C., Salazar, H., Cheong, B., Murgo, J. & Fonseca, V. Association of hypoglycemia and cardiac ischemia: a study based on continuous monitoring. *Diabetes Care* **26**, 1485–1489 (2003).
21. Hutton, R. A., Mikhailidis, D., Dormandy, K. M. & Ginsburg, J. Platelet aggregation studies during transient hypoglycaemia. *J. Clin. Pathol.* **32**, 434–438 (1979).
22. Boyle, P. J., Shah, S. D. & Cryer, P. E. Insulin, glucagon, and catecholamines in prevention of hypoglycemia during fasting. *Am. J. Physiol.* **256**, E651–E661 (1989).
23. Larsen, M. O. *et al.* Mild streptozotocin diabetes in the G ttingen minipig. A novel model of moderate insulin deficiency and diabetes. *Am. J. Physiol. Endocrinol. Metab.* **282**, E1342–E1351 (2002).
24. Schumacher-Petersen, C. *et al.* Experimental non-alcoholic steatohepatitis in Gottingen Minipigs: consequences of high fat-fructose-cholesterol diet and diabetes. *J. Transl. Med.* **17**, 1–18. https://doi.org/10.1186/s12967-019-1854-y (2019).
25. He, H., Carballo-Jane, E., Tong, X. & Cohen, L. H. Measurement of catecholamines in rat and mini-pig plasma and urine by liquid chromatography-tandem mass spectrometry coupled with solid phase extraction. *J. Chromatogr. B* **997**, 154–161. https://doi.org/10.1016/j.jchromb.2015.05.014 (2015).
26. Heegaard, P. M. H. *et al.* Optimal combinations of acute phase proteins for detecting infectious disease in pigs. *Vet. Res.* **42**, 13 (2011).
27. Suzuki, A. *et al.* Establishment of a 24-hour electrocardiogram recording system using a Holter recorder for miniature swine. *Lab. Anim.* **32**, 165–172 (1998).
28. Curtis, M. J. *et al.* The Lambeth Conventions (II): guidelines for the study of animal and human ventricular and supraventricular arrhythmias. *Pharmacol. Ther.* **139**, 213–248. https://doi.org/10.1016/j.pharmthera.2013.04.008 (2013).
29. Baumert, M. *et al.* QT interval variability in body surface ECG: measurement, physiological basis, and clinical value: position statement and consensus guidance endorsed by the European Heart Rhythm Association jointly with the ESC Working Group on Cardiac Cellular Electrophysiology. *Europace* **18**, 925–944. https://doi.org/10.1093/europace/euv405 (2016).
30. Macfarlane, P. W. *et al.* The early repolarization pattern: a consensus paper. *J. Am. Coll. Cardiol.* **66**, 470–477. https://doi.org/10.1016/j.jacc.2015.05.033 (2015).
31. Polanczyk, C. A. *et al.* Sympathetic nervous system representation in time and frequency domain indices of heart rate variability. *Eur. J. Appl. Physiol.* **79**, 69–73 (1998).
32. Olsen, L. H. *et al.* Increased platelet aggregation response in cavalier king charles spaniels with mitral valve prolapse. *J. Vet. Intern. Med.* **15**, 209–215 (2001).
33. Gradel, A. K. J. *et al.* The counterregulatory response to hypoglycaemia in the pig. *Basic Clin. Pharmacol. Toxicol.* **00**, 1–9. https://doi.org/10.1111/bcpt.13422 (2020).
34. Eckert, B. & Agardh, C.-D. Hypoglycaemia leads to an increased QT interval in normal men. *Clin. Physiol.* **18**, 570–575 (1998).
35. Abraham, M. B. *et al.* ISPAD clinical practice consensus guidelines 2018: assessment and management of hypoglycemia in children and adolescents with diabetes. *Pediatr. Diabetes* **19**, 178–192. https://doi.org/10.1111/pedi.12698 (2018).
36. Nielsen, M. F. *et al.* Impaired hepatic counterregulatory response to insulin-induced hypoglycemia in hepatic denervated pigs. *J. Clin. Transl. Endocrinol.* **2**, 131–136. https://doi.org/10.1016/j.jcte.2015.08.004 (2015).
37. Rothberg, L. J., Lees, T., Clifton-Bligh, R. & Lal, S. Association between heart rate variability measures and blood glucose levels: implications for noninvasive glucose monitoring for diabetes. *Diabetes Technol. Ther.* **18**, 366–376. https://doi.org/10.1089/dia.2016.0010 (2016).
38. Marques, J. L. B. *et al.* Altered ventricular repolarization during hypoglycaemia in patients with diabetes. *Diabet Med.* **14**, 648–654 (1997).
39. Robinson, R. T. C. E. *et al.* Mechanisms of abnormal cardiac repolarization during insulin-induced hypoglycemia. *Diabetes* **52**, 1469–1474 (2003).
40. Weiss, J. N., Qu, Z. & Shivkumar, K. Electrophysiology of Hypokalemia and Hyperkalemia. *Circ. Arrhythm. Electrophysiol.* **10**, 1–10. https://doi.org/10.1161/CIRCEP.116.004667 (2017).

41. Vlcek, M., Radikova, Z., Penesova, A., Kvetnansky, R. & Imrich, R. Heart rate variability and catecholamines during hypoglycemia and orthostasis. *Auton. Neurosci.* **143**, 53–57. https://doi.org/10.1016/j.autneu.2008.08.001 (2008).
42. Shaffer, F. & Ginsberg, J. P. An overview of heart rate variability metrics and norms. *Front. Public Health* **5**, 1–17. https://doi.org/10.3389/fpubh.2017.00258 (2017).
43. Siafarikas, A. *et al.* Early loss of the glucagon response to hypoglycemia in adolescents with type 1 diabetes. *Diabetes Care* **35**, 1757–1762. https://doi.org/10.2337/dc11-2010 (2012).
44. Mythili, M. D., Vyas, R., Akila, G. & Gunasekaran, S. Effect of streptozotocin on the ultrastructure of rat pancreatic islets. *Microsc. Res. Tech.* **63**, 274–281. https://doi.org/10.1002/jemt.20039 (2004).
45. Angiolillo, D. J. *et al.* Insulin therapy is associated with platelet dysfunction in patients with type 2 diabetes mellitus on dual oral antiplatelet treatment. *J. Am. Coll. Cardiol.* **48**, 298–304. https://doi.org/10.1016/j.jacc.2006.03.038 (2006).
46. Kreutz, R. P. *et al.* Morbid obesity and metabolic syndrome in Ossabaw miniature swine are associated with increased platelet reactivity. *Diabetes Metab. Syndr. Obes.* **4**, 99–105. https://doi.org/10.2147/DMSO.S17105 (2011).
47. Shukla, S. D. *et al.* Platelets from diabetic pigs exhibit hypersensitivity to thrombin. *Comp. Med.* **58**, 481–484 (2008).
48. Trovati, M. *et al.* Insulin directly reduces platelet sensitivity to aggregating agents. *Diabetes* **37**, 780–786 (1988).
49. Simianer, H. & Kohn, F. Genetic management of the Gottingen Minipig population. *J. Pharmacol. Toxicol. Methods* **62**, 221–226. https://doi.org/10.1016/j.vascn.2010.05.004 (2010).
50. Christoffersen, B. Ø. *et al.* Age- and sex-associated effects on acute-phase proteins in g ttingen minipigs. *Comp. Med.* **65**, 333–341 (2015).
51. Cryer, P. E. Mechanisms of hypoglycemia-associated autonomic failure and its component syndromes in diabetes. *Diabetes* **54**, 3592–3601 (2005).

Author contributions

M.K.L. executed animal experiments, data collection and analysis, and wrote the manuscript. A.V. contributed to experimental design, data analysis, discussion of results, and revision of manuscript. G.K.P. contributed to experimental design, data analysis, discussion of results, and revision of manuscript. R.S. contributed to experimental design, discussion of results, and revision of manuscript J.K. contributed to experimental design, discussion of results, and revision of manuscript. L.H.O. contributed to experimental design, data analysis, discussion of results, and revision of manuscript. U.P.B. contributed to experimental design, discussion of results and revision of manuscript.

Funding

The study was funded by Novo Nordisk A/S and the LifePharm Centre for University of Copenhagen, Faculty of Health and Medical Sciences, Department of Veterinary and Animal Sciences, Section for Experimental Animal Models.

Competing interests

AV, GKP, RS and JK are employed by Novo Nordisk A/S. MKL, UPB andLHO declare no potential conflicts of interest.

Additional information

Correspondence and requests for materials should be addressed to L.H.O.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Paper III

Healthy and streptozotocin-induced Göttingen Minipigs as a model of counter-regulatory failure in recurrent hypoglycaemia.

Mille Kronborg Lyhne, Kathrin Hinz, Caroline Degel, Rükiye Köse, Heidi Young Nilsen, Jeppe Sturis, Andreas Vegge, Jonas Kildegaard, Ulrik Pedersen-Bjergaard and Lisbeth Høier Olsen.

Manuscript in preparation.

Title page

Healthy and streptozotocin-induced Göttingen Minipigs as a model of counter-regulatory failure in recurrent hypoglycaemia.

Authors:

Mille Kronborg Lyhne[1], Kathrin Hinz[1], Caroline Degel[1], Heidi Young Nilsen[1], Andreas Vegge[2], Jeppe Sturis[2], Jonas Kildegaard[2], Ulrik Pedersen-Bjergaard[3] and Lisbeth Høier Olsen[1*]

*Corresponding author lisbeth.hoier@sund.ku.dk

1 Faculty of Health and Medical Sciences, Copenhagen University, Copenhagen, Denmark.

2 Global Drug Discovery, Novo Nordisk A/S, Måløv, Denmark.

3 Department of Endocrinology and Nephrology, Nordsjællands Hospital Hillerød, Hillerød, Denmark.

Word count: 2245 (main text)

Number of tables: 2

Number of figures: 2

Figures and tables are located at the end of the manuscript.

Abstract

Pending.

Introduction

Pending.

Aim and hypotheses

The aim of this study was to investigate the influence of antecedent hypoglycaemia on the myocardial and endocrine counter-regulatory response to hypoglycaemia in healthy and streptozotocin-induced diabetic Göttingen Minipigs. The hypotheses were that preconditioning with hypoglycaemia on three consecutive days would decrease glucagon and epinephrine responses to hypoglycaemia compared to a control group not experiencing antecedent hypoglycaemia. Furthermore, hypoglycaemia preconditioned groups will require more glucose infusion to maintain plasma glucose target and have fewer ECG changes compared to controls, due to lower counter-regulatory response.

Materials and methods

Animals

This animal study was approved by the Danish Animal Experimentation Inspectorate and conducted in accordance with current rules and regulations. Thirty-three lean, female Göttingen Minipigs (Ellegaard Göttingen Minipigs A/S, Dalmose, Denmark) were included in two batches: 1. Eight diabetic pigs and eight healthy pigs (February 2021), and 2: Twelve diabetic and ten healthy pigs (May-June 2022). For the final group sizes after exclusions, see figure 1. Pigs were housed at Novo Nordisk A/S (Ganløse, Denmark) and were acclimatized in groups for three weeks before study. During the study, pigs were kept in single pens with possibility of snout and visual contact with other pigs, at 18-22°C with relative air humidity of 30-70% and a 12-hour light/dark cycle with natural light. Pigs had straw and woodchip bedding and were feed once-daily in the afternoon with 460g of feed (Altromin Minipig diet 9069, Altromin Spezialfutter GmbH & Co. KG, Lage, Germany).

Prior to the study, pigs had venous catheters implanted by either bilateral auricular vein to jugular vein catheters (n=8, only CON) or two central venous vena cava caudalis catheters (n=28 CON and DIA) (Cook C-TPNS6.5-90-REDO, William Cook Europe ApS, Bjæverskov, Denmark) using a Seldinger technique in a protocol modified from Larsen et al. 2002[1]. The pigs were allowed convalescence for one to two weeks after implantation of catheters.

Diabetes induction and maintenance

Diabetes was induced in 20 pigs by once-daily intravenous (i.v.) injections of 50 mg/kg streptozotocin (Sigma Aldrich Denmark A/S, cat. no. S0130-5G) for three consecutive days. The protocol was modified from Schumacher et al. 2019[2]. Pigs were included in other pharmacological insulin studies, but no other pharmacological interventions, at Novo Nordisk A/S for approximately four months prior to this study, with a two-week washout period before the present study. During wash-out, and in the present

study, hyperglycaemia was managed by once-daily subcutaneous injections of insulin glargine (Lantus, Sanofi S.A., Paris, France) dosed according to previous days fasted plasma glucose curves, targeting a fasted plasma glucose of 7-10 mM. Pigs were dosed in conjunction with feeding. After the first study day, pigs were dosed with half the dose of the previous day's insulin glargine, to account for the shorter acting human insulin received during the hyperinsulinaemic clamps. The insulin glargine dose was hereafter adjusted continuously, based on morning fasted plasma glucose.

Groups

Both healthy (CON) and diabetic (DIA) pigs were randomized to receive a preconditioning treatment of either hyperinsulinaemic, normoglycaemic clamp (recurrent normoglycaemia (RN)) or hyperinsulinaemic hypoglycaemic (recurrent hypoglycaemia (RH)) clamp resulting in the following groups, after exclusions, consisting of CON-RN (n=8), CON-RH (n=8), DIA-RN (n=9) and DIA-RH (n=8) (figure 1).

Hyperinsulinaemic clamps

Studies were commenced in the morning, before feeding. On three consecutive days, pigs received either the control treatment of an normoglycaemic clamp with a target PG of 3.5 mM, which was considered to represent fasted plasma glucose of an adult, healthy minipig[3], or the test treatment of hypoglycaemic clamp with a target PG of 1.5 mM. After the clamps, pigs were feed their daily feed as described above. All pigs were then subjected to a final, hyperinsulinaemic, hypoglycaemic clamp with a lower PG target of 1.0 mM on the fourth day. The clamping procedures included continuous, constant infusion of human insulin at 16 pmol/kg/min with a variable glucose infusion rate (GIR) to keep pigs at target PG. Preconditioning clamps lasted three hours, while the final clamp, on day four,

lasted four hours. Pigs were euthanized directly after the final clamp with an i.v. injection of 3000 mg pentobarbital (approximately 100 mg/kg, Exagon vet., Salfarm Danmark A/S, Kolding, Denmark).

Continuous electrocardiographic (Holter) recordings

Pigs had continuous electrocardiography (ECG) (Holter) recorded at a baseline (one week prior study) and during the final, hypoglycaemic clamp. Pigs were briefly anaesthetised with an i.v. injection of 100 mg propofol (Propomitor Vet. 10mg/ml, Orion Pharma A/S, Copenhagen, Denmark) during fastening of the Holter equipment (Lifecard CF recorder (Spacelabs Healthcare, WA, USA) and electrodes). ECG were recorded in two leads with lead I from the dorsal neck area at the base of the skull to processus xiphoideus and lead II from processus xiphoideus to manibrium sterni[3, 4].

Four hour baseline recording was included, which was time matched with the final, four-hour hypoglycaemic clamp. All recordings were analysed using Pathfinder SL (Spacelabs Healthcare, WA, USA) as previously described[3]. Arrhythmic events were recorded as either supraventricular ectopic complexes[5] (complexes with a normal QRS morphology with either a non-visible P-wave or a P'-wave, with a P-P' interval shorter (premature) or longer (escape) than the previous P-P intervals), ventricular ectopic complexes[5] (complexes with abnormal QRS morphology, either broader or higher, with RR interval either shorter (premature) or longer (escape) than the sinus RR interval) or second-degree atrioventricular blocks (P-wave(s) without a corresponding QRS complex)[6].

Heart rate variability (HRV) parameters were obtained using the build-in software of Pathfinder SL. One-hour epochs (0-60, 60-120, 120-180, 180-240 minutes) were chosen. Time-domain variables, standard variation of the RR intervals (SDNN) and root mean square of RR intervals (RMSSD), were obtained. From Fast-Fourier transformed data, frequency domain power band variables were obtained at very low frequency at 0.015-0.05Hz (VLF), low frequency at 0.05-0.15Hz (LF), high frequency at 0.15-0.40Hz (HF) and very high frequency at 0.35-0.5Hz (VHF), as well as LF/HF ratio.

For morphological analysis of the ECG recordings, prints of ten, consecutive RR intervals were identified at time 0, 60, 120, 180 and 240 since start of final clamp and time-matched print in the baseline recordings. Variables were measured as an average of ten consecutive beats. QRS duration[7], T-wave amplitude, ST segment elevation[8] and QT interval, corrected with Fridericia's formula[9, 10] were measured as previously described[3].

Blood sample collection and analysis

During the hypoglycaemic clamps, blood samples were collected every ten minutes in tubes containing EDTA for direct PG measurements (YSI-2900, YSI Inc. OH, USA). EDTA plasma samples for porcine glucagon, porcine C-peptide and human insulin were stabilized with aprotinin (Trasylol 10.000 KIE/mL, Nordic Drugs AB, Limhamn, Sweden) to prevent glucagon degradation and analysed using in-house luminescence oxygen channeling immunoassays with a lower limit of quantification (LLOQ) at 4, 45 and 10 pM, respectively[11, 12].

The samples were collected and analysed every ten minutes, however, C-peptide and human insulin were only analysed every thirty minutes during the preconditioning clamps

Blood samples for epinephrine analysis were collected in tubes with and stabilized with EGTA (2.88 mg/mL) and glutathione (1.44 mg/mL) solution (E3889 and G4251, Merck Life Science A/S, Søborg, Denmark) every 30 minutes during the final clamp. Blood was centrifuged at 3000x*g* at 5°C for ten minutes and frozen directly. Plasma was stored at -80°C until analysis. Epinephrine levels in plasma were quantified using liquid chromatography-mass spectrometry with a LLOQ of 0.05 ng/mL, as previously described by He et al. 2015[13].

Blood for serum was sampled at baseline of the final clamp for determination of fasted β-hydroxybutyrate as an indicator of possible diabetic ketoacidosis.

Statistical analysis

To assess the effect of recurrent hypoglycaemia on outcomes in this study, linear mixed effect models were used (SAS 9.4, SAS Institute, Cary, USA). Individual pig was chosen as random variable and diabetes status, group, time, and batch as explanatory variables. Interactions between diabetes status and precondition, as well as diabetes status and batch were considered as well. To better fit data to the model, logarithmic transformation was used when needed and the models were then reduced by backward elimination. Difference between groups in single-measurement parameters (area under the curve of glucagon, C-peptide and human insulin and fasted plasma glucose as well as weight and age) were investigated with Mann-Whitney U-test and area under the curve (AUC) were calculated using GraphPad Prism 8 (GraphPad Software Inc., San Diego, USA)

Data in tables is presented as medians and interquartile (IQR) range, while data analysed with linear mixed models is estimates with standard deviation(SD), with a significance level at $P<0.05$.

Results

Animals

The age, weight, fasted plasma glucose and insulin glargine doses of the pigs are presented in table 1, with no significant differences between CON and DIA, not RH and RN groups.

Four diabetic pigs were excluded (two due to defective catheters, one due to acute colitis and one due to acute endocarditis). Three healthy pigs were excluded (two due to defective catheters and one due to acute pyometra). After reviewing data, three additional diabetic pigs were excluded in the data analysis as they had high fasted plasma glucagon (more than five times higher than what has previously been reported in fasted, diabetic pigs[3]), as well as fasted plasma glucose over 15 mM on the final study day, indicative of diabetic ketoacidosis[14, 15]. Results from analysis of β-hydroxybutyrate in serum are pending.

Preconditioning clamps

Descriptive data from preconditioning clamps is presented in table 2. It was possible to clamp both RN and RH groups, however, average PG of DIA-RH between 120-180 minutes were increasing day to day, resulting in pigs in DIA-RH not reaching PG target on day three (average PG 120-180 minutes 2.91 mM (1.35-3.50). Fasted PG also increased from day to day in both DIA groups.

GIR AUC was significantly different between RN and RH groups on all preconditioning days ($P<0.05$ for all). Plasma human insulin was not significantly different in CON and DIA groups. Fasted C-peptide was lower in DIA compared to CON groups on all preconditioning days ($P<0.05$ for all), while C-peptide levels at 120 minutes were all similar (table 2). Plasma glucagon AUC was significantly higher in CON-RH compared to CON-RN ($P<0.05$ for all), which was not observed in DIA groups.

Final hypoglycaemic clamp

On the final clamp, average PG levels in the last hour of the clamp were 1.45 mM (1.06-1.81) and 1.02 mM (0.90-1.27) in CON-RN and CON-RH and 1.48 mM (1.36-1.70) and 1.18 mM (1.04-1.52) in DIA-RN and DIA-RH. While there was no effect of diabetes status or batch, groups preconditioned with hypoglycaemia (RH) were significantly lower in PG between 180-240 minutes (*P*=0.02, see table 2).

The AUC of the GIR was 0.0 (0.0-0.0) and 0.0 (0.0-89.0) in CON-RN and CON-RH and 0.0 (0.0-1.1) and 1.0 (0.0-18.7) in DIA-RN and DIA-RH with no significant difference between groups (Mann-Whitney U-test). The AUC of plasma human insulin and plasma C-peptide at 120 minutes was similar in all groups, with no significant difference between groups (Mann-Whitney U-test, see table 2), confirming similar hyperinsulinaemia and suppression of endogenous insulin in the pigs. There was an effect of batch in regards to human insulin exposure, with batch 1 generally having higher plasma concentrations than batch 2 (data not shown).

Endocrine counter-regulation during the final, hypoglycaemic clamp

While no significant difference between groups were found when considering plasma glucagon and epinephrine AUC (see table 2), changes caused by preconditioning were seen when applying linear mixed modelling.

The glucagon response was higher in the normoglycaemia preconditioned groups (RN) compared to the hypoglycaemia preconditioned groups (RN), without an effect of diabetes status (see figure 2.E and 2.F). There was also an effect of batch, with batch 1 of CON being lower in plasma glucagon compared to batch 2 of CON (*P*=0.0002).

An epinephrine response was seen in all groups, however, there was no significant effect of diabetes status, preconditioning or batch (see figure 2.G and 2.H). The highest increase compared to baseline was seen at time 210 minutes (*P*<0.0001).

Continuous electrocardiographic Holter recordings

Data analysis of ECG recordings is pending.

Discussion

Pending.

Preliminary conclusion:

In conclusion, preconditioning with hypoglycaemia on three, consecutive days blunted the glucagon response to hypoglycaemia in both the healthy and diabetic minipigs, while no such effect was seen in the epinephrine response. There was no difference in required glucose infusion to maintain target plasma glucose. As the ECG analyses have not yet been conducted, the conclusion on the effect of hypoglycaemia preconditioning on features of the ECG is still pending.

Future studies are needed to investigate the potential blunting of the epinephrine response in this minipig model, however, the model presented in this study could still be suitable for testing interventions in recurrent hypoglycaemia to prevent blunted release of glucagon during hypoglycaemia.

Competing interests and funding

AV, JS and JK are employed by Novo Nordisk A/S. MKL, KH, CD, HYN, UPB and LHO declare no potential conflicts of interest. The study was funded by Novo Nordisk A/S and the LifePharm Centre for University of Copenhagen, Faculty of Health and Medical Sciences, Department of Veterinary and Animal Sciences.

Author contribution

MKL designed the study, carried out animal experimentation, analyzed and interpreted the data, and drafted the manuscript. KH carried out animal experimentation and contributed to data analysis and interpretation and revised the manuscript. CD carried out animal experimentation, contributed to data analysis and manuscript revision. HYN contributed to data analysis and manuscript revision. AV, JS, JK and UPB contributed to study design, data interpretation and manuscript revision. LHO contributed to study design, data analysis and interpretation and manuscript revision.

Data availability

Datasets used in this study can be made available upon request to the corresponding author.

References

1. Larsen, M.O., et al., *Mild streptozotocin diabetes in the Göttingen minipig. A novel model of moderate insulin deficiency and diabetes.* Am J Physiol Endocrinol Metab, 2002. **282**: p. E1342–E1351.
2. Schumacher-Petersen, C., et al., *Experimental non-alcoholic steatohepatitis in Gottingen Minipigs: consequences of high fat-fructose-cholesterol diet and diabetes.* Journal of Translational Medicine, 2019. **17**(110): p. 1-18.
3. Lyhne, M.K., et al., *Hyperinsulinaemic hypoglycaemia in non-anaesthetized Gottingen minipigs induces a counter-regulatory endocrine response and electrocardiographic changes.* Sci Rep, 2021. **11**(1): p. 5983.
4. Suzuki, A., et al., *Establishment of a 24-hour electrocardiogram recording system using a Holter recorder for miniature swine.* Lab Anim, 1998. **32**: p. 165-172.
5. Curtis, M.J., et al., *The Lambeth Conventions (II): guidelines for the study of animal and human ventricular and supraventricular arrhythmias.* Pharmacol Ther, 2013. **139**(2): p. 213-248.
6. Kusumoto, F.M., et al., *2018 ACC/AHA/HRS Guideline on the Evaluation and Management of Patients With Bradycardia and Cardiac Conduction Delay: Executive Summary: A Report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines, and the Heart Rhythm Society.* J Am Coll Cardiol, 2019. **74**(7): p. 932-987.
7. Kurl, S., et al., *Duration of QRS complex in resting electrocardiogram is a predictor of sudden cardiac death in men.* Circulation, 2012. **125**(21): p. 2588-94.
8. Macfarlane, P.W., et al., *The Early Repolarization Pattern: A Consensus Paper.* J Am Coll Cardiol, 2015. **66**(4): p. 470-477.
9. Luo, S., et al., *A comparison of commonly used QT correction formulae: the effect of heart rate on the QTc of normal ECGs.* J Electrocardiol, 2004. **37 Suppl**: p. 81-90.
10. Kano, M., et al., *QT PRODACT: usability of miniature pigs in safety pharmacology studies: assessment for drug-induced QT interval prolongation.* J Pharmacol Sci, 2005. **99**(5): p. 501-511.
11. Poulsen, F. and K.B. Jensen, *A luminescent oxygen channeling immunoassay for the determination of insulin in human plasma.* J Biomol Screen, 2007. **12**(2): p. 240-7.
12. Haagensen, A.M., et al., *High fat, low carbohydrate diet limit fear and aggression in Gottingen minipigs.* PLoS One, 2014. **9**(4): p. e93821.
13. He, H., et al., *Measurement of catecholamines in rat and mini-pig plasma and urine by liquid chromatography-tandem mass spectrometry coupled with solid phase extraction.* J Chromatogr B, 2015. **997**: p. 154-161.
14. Meek, T.H., et al., *Evidence That in Uncontrolled Diabetes, Hyperglucagonemia Is Required for Ketosis but Not for Increased Hepatic Glucose Production or Hyperglycemia.* Diabetes, 2015. **64**(7): p. 2376-87.
15. Müller, W.A., G.R. Faloona, and R.H. Unger, *Hyperglucagonemia in Diabetic Ketoacidosis: Its Prevalence and Significance.* The American Journal of Medicine, 1973. **54**: p. 52-57.

Figures

Figure 1: Study design

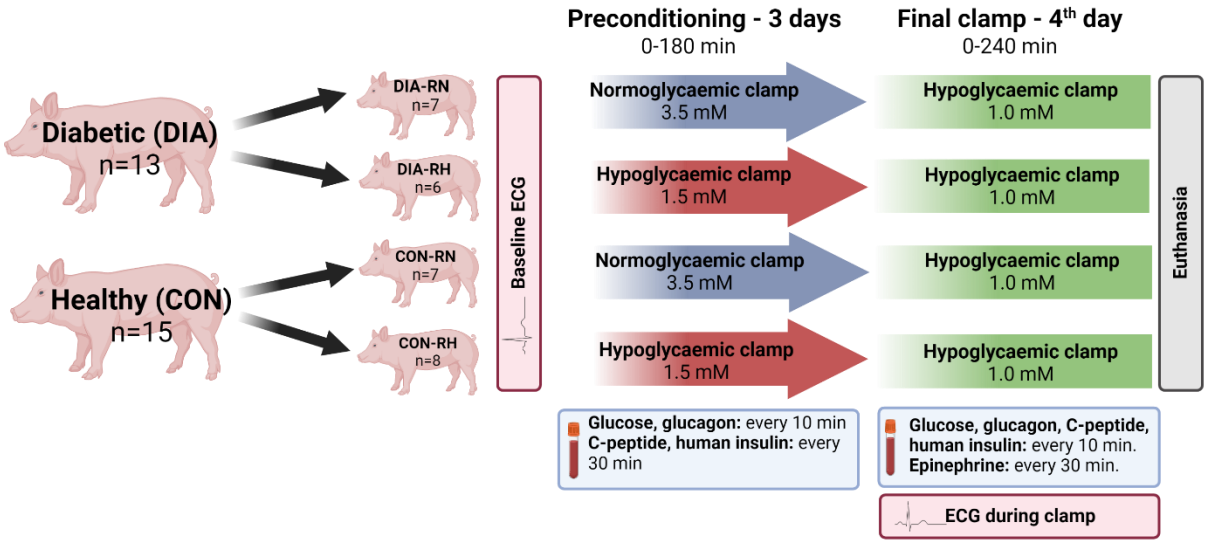
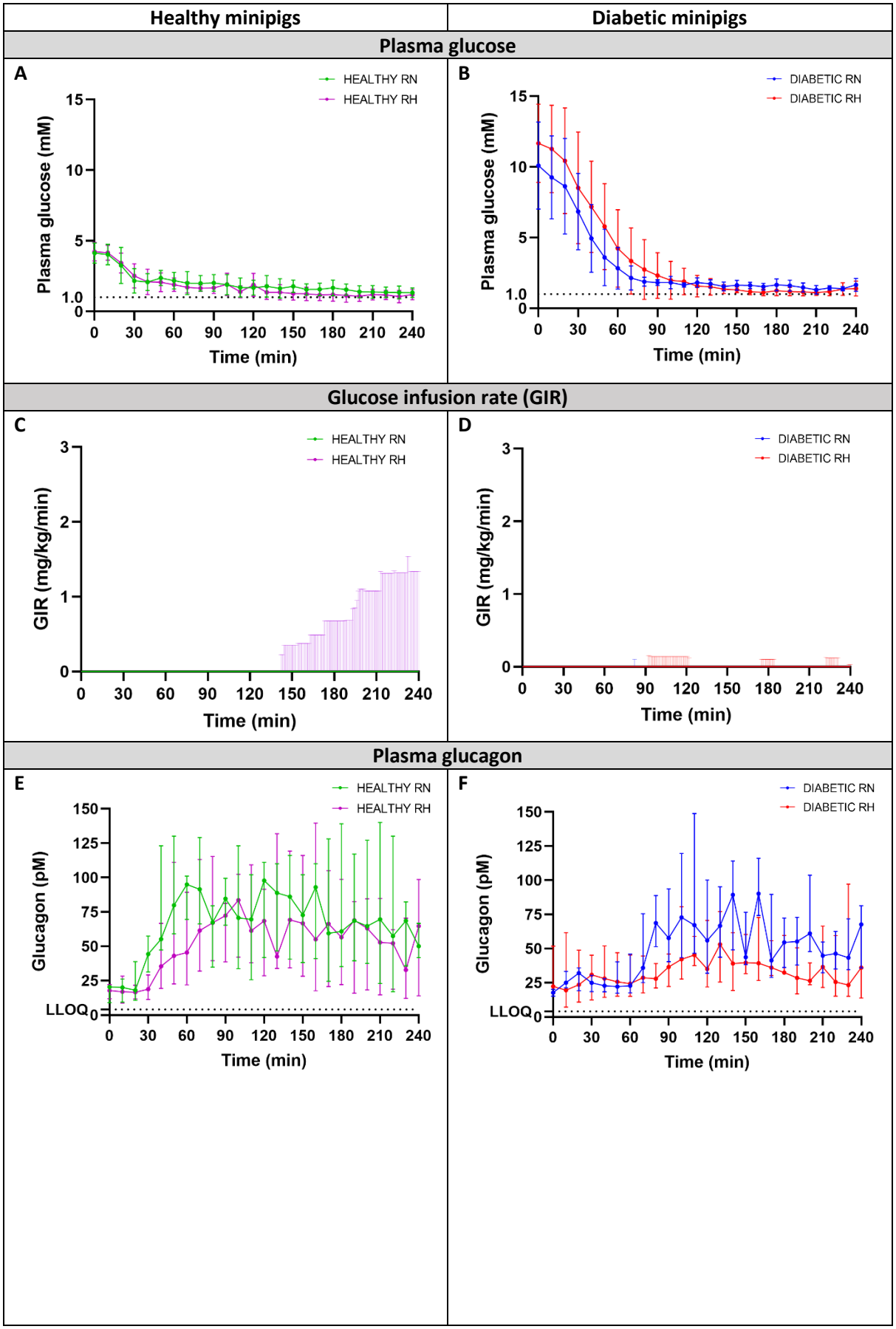


Figure 1 legend: Illustration of study design. Diabetic (DIA) and healthy (CON) pigs were split into either recurrent normoglycaemia (RN) or recurrent hypoglycaemia (RH) preconditioning groups. Pigs had baseline Holter electrocardiography (ECG) recorded. Pigs were then preconditioned with their respective treatment for three consecutive days. On the final, fourth day, all pigs underwent a hypoglycaemic clamp followed by euthanasia. Number of animals (n) reflect the final number, after exclusions. Created with Biorender.com.

Figure 2: Final hyperinsulinaemic, hypoglycaemic clamp



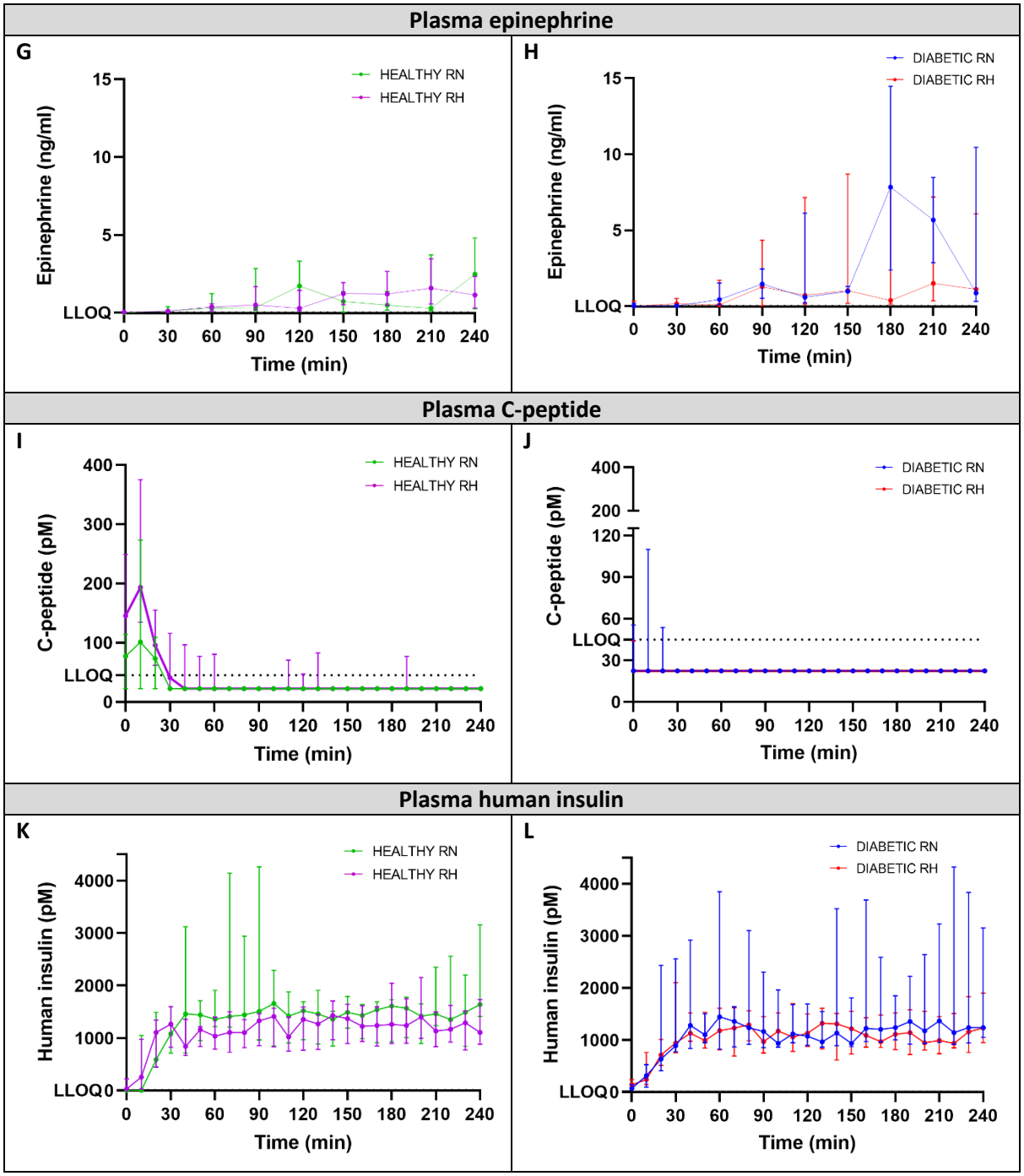


Figure 2 legend: Data from the final, hyperinsulinaemic hypoglycaemic clamp.

A+B: Plasma glucose. Horizontal dotted line indicates clamp target of 1.0 mM plasma glucose. C+D: Glucose infusion rate measured every minute. Observe, that all medians are zero. E+F: Plasma glucagon. G+H: Plasma epinephrine. I+J: Plasma C-peptide. K+L: Plasma human insulin. Graphs A+B and E-L: median and interquartile range (vertical bars). Time indicates minutes since beginning of clamp. Horizontal lines indicate lower limit of quantification of the assay (LLOQ). RN: recurrent

normoglycaemia preconditioning group. RH: recurrent hypoglycaemia preconditioning group. Data is presented as medians and vertical bars indicate interquartile range.

Tables

Table 1: animal weight, age and daily insulin and plasma glucose

	Healthy	**Diabetic**
N (RN/RH)	7/8	7/6
Weight (kg)	31.8 (29.7-35.0)	30.9 (28.8-35.6)
Age (days)	471 (469-530)	497 (487-525)
Average insulin glargine dose (IU)	-	21.3 (18.5-25.0)
Average fasted plasma glucose (mM)	-	8.9 (8.2-10.6)

Table 1 legend: Average insulin glargine dose and average fasted plasma glucose: average dose/plasma glucose in the week prior to study start. Data is presented as medians and interquartile range. RN: recurrent normoglycaemia preconditioning. RH: recurrent hypoglycaemia reconditioning. IU: international units. Comparisons made with Mann-Whitney U-test and all non-significant.

Table 2: Summary data of plasma glucose, glucose infusion rate, plasma glucagon, plasma human insulin and plasma C-peptide in preconditioning and final hyperinsulinaemic clamps

	Healthy		**P-values**	**Diabetic**		**P-values**	**P-values**
Preconditioning group	**Normoglycaemia n=7**	**Hypoglycaemia n=8**	**Healthy**	**Normoglycaemia n=7**	**Hypoglycaemia n=6**	**Diabetic**	**Healthy vs. diabetic**
Preconditioning day 1							
Fasted plasma glucose (mM)	3.66 (3.36-5.31)	3.62 (3.15-4.10)	ns	4.91 (2.37-7-35)	6.16 (3.25-9.91)	ns	RN ns RH ns
Average plasma glucose 60-120 min (mM)	3.60 (3.20-3.99)	1.66 (1.50-2.06)	**P=0.0012**	3.36 (2.91-3.98)	1.33 (1.14-1.63)	**P=0.0048**	RN ns RH ns
Average plasma glucose 120-180 min (mM)	3.43 (3.09-4.03)	1.42 (1.33-1.54)	**P=0.0012**	3.23 (3.14-3.51)	1.35 (1.28-1.47)	**P=0.0048**	RN ns RH ns
Glucose infusion rate (mg/kg/min) AUC	1246.0 (1159.0-1801.0)	0.4 (0.0-24.0)	**P=0.0012**	1295.0 (1072.0-1682.0)	151.3 (59.7-278.3)	**P=0.0024**	RN ns **RH: CON<DIA P=0.0012**
Plasma glucagon (pM) AUC	3572.0 (2660.0-5163.0)	15871.0 (6411.0-20852.0)	**P=0.0088**	3331.0 (2936.0-3668.0)	5093.0 (4589.0-9140.0)	**P=0.0023**	RN ns RH ns
Plasma human insulin (pM) AUC	210225 (174255-289545)	173010 (133853-188220)	ns	222794 (179550-292230)	224760 (140415-335955)	ns	RN ns RH ns
Fasted plasma C-peptide (pM)	189.0 (146.0-308.0)	169.5 (124.3-242.8)	ns	22.5 (22.5-70.9)	22.5 (22.5-48.3)	ns	**RN: DIA<CON P=0.0048 RH: DIA<CON P=0.0012**
Plasma C-peptide 120 min (pM)	22.5 (22.5-120.0)	22.5 (22.5-22.5)	ns	22.5 (22.5-22.5)	22.5 (22.5-22.5)	ns	RN ns RH ns
Preconditioning day 2							
Fasted plasma glucose (mM)	3.91 (3.73-4.48)	4.08 (3.86-4.68)	ns	10.25 (6.06-13.15)	12.30 (10.70-13.40)	ns	**RN: CON<DIA P=0.0048 RH: CON<DIA P=0.0012**
Average plasma glucose 60-120 min (mM)	3.52 (3.21-3.61)	1.70 (1.46-1.93)	**P=0.0012**	3.72 (3.22-4.44)	2.04 (1.54-6.42)	ns	RN ns RH ns
Average plasma glucose 120-180 min (mM)	3.49 (3.46-3.90)	1.46 (1.30-1.69)	**P=0.0008**	3.56 (3.03-3.72)	1.64 (1.34-1.93)	**P=0.0048**	RN ns RH ns
Glucose infusion rate (mg/kg/min) AUC	1366.0 (1156.0-1465.0)	0.0 (0.0-6.5)	**P=0.0012**	843.3 (259.3-1170.0)	46.1 (5.3-162.8)	**P=0.0024**	RN ns RH ns
Plasma glucagon (pM) AUC	3992.0 (1853.0-4703.0)	10021.0 (5995.0-12833.0)	**P=0.037**	3843.0 (2975.0-5009.0)	5060.0 (4587.0-6317.0))	ns	RN ns RH ns
Plasma human insulin (pM) AUC	218925 (168030-231528)	189870 (145748-215186)	ns	228577 (196807-342413)	190146 (102168-295200)	ns	RN ns RH ns
Fasted plasma C-peptide (pM)	171.0 (159.0-219.0)	221.0 (187.0-348.8)	ns	74.55 (22.5-144.0)	22.5 (22.5-60.3)	ns	RN ns **RH: DIA<CON P=0.0012**
Plasma C-peptide 120 min (pM)	22.5 (22.5-164.0)	22.5 (22.5-22.5)	ns	22.5 (22.5-22.5)	22.5 (22.5-22.5)	ns	RN ns RH ns
Preconditioning day 3							
Fasted plasma glucose (mM)	3.75	4.27	ns	12.80	12.10	ns	**RN: CON<DIA P=0.0092**

	(3.58-5.01)	(3.78-5.08)		(7.47-16.43)	(9.78-14.00)		**RH: CON<DIA P=0.0012**
Average plasma glucose 60-120 min (mM)	3.44 (2.95-3.61)	1.66 (1.46-2.25)	**P=0.0024**	3.33 (3.20-4.03)	3.15 (1.40-3.44)	ns	RN ns RH ns
Average plasma glucose 120-180 min (mM)	3.44 (3.30-3.48)	1.24 (1.16-1.71)	**P=0.0012**	3.32 (3.11-3.52)	2.75 (1.30-3.59)	ns	RN ns RH ns
Glucose infusion rate (mg/kg/min) AUC	1578.0 (1259.0-1701.0)	0.0 (0.0-8.5)	**P=0.0012**	515.1 (200.6-1081.0)	8.3 (1.8-177.2)	**P=0.016**	**RN: DIA<CON P=0.0092** RH ns
Plasma glucagon (pM) AUC	3277.0 (2129.0-4472.0)	9528.0 (5253.0-18176.0)	**P=0.0024**	3542.0 (2866.0-4914.0)	4539.0 (3714.0-6813.0)	ns	RN ns RH ns
Plasma human insulin (pM) AUC	206175 (151290-222675)	187703 (153401-323003)	ns	243422 (179526-412733)	179070 (116273-247269)	ns	RN ns RH ns
Fasted plasma C-peptide (pM)	106.0 (83.1-211.0)	260.5 (138.3-411.0)	ns	37.8 (22.5-89.5)	22.5 (22.5-58.3)	ns	RN ns **RH: DIA<CON P=0.0012**
Plasma C-peptide 120 min (pM)	71.9 (22.5-212.0)	22.5 (22.5-22.5)	ns	22.5 (22.5-30.7)	22.5 (22.5-22.5)	ns	RN ns RH ns
Final clamp							
Fasted plasma glucose (mM)	4.15 (3.40-4.75)	4.20 (3.79-4.82)	ns	10.41 (8.09-12.25)	11.80 (11.40-13.10)	ns	**RN: CON<DIA P=0.0048** **RH: CON<DIA P=0.0012**
Average plasma glucose 60-120 min (mM)	2.06 (1.22-2.34)	1.63 (1.42-2.14)	ns	1.99 (1.68-2.25)	1.85 (1.46-4.45)	ns	RN ns RH ns
Average plasma glucose 120-180 min (mM)	1.94 (1.06-2.00)	1.36 (0.98-1.57)	ns	1.67 (1.53-1.79)	1.39 (1.12-1.75)	ns	RN ns RH ns
Average plasma glucose 180-240 min (mM)	1.45 (1.06-1.81)	1.02 (0.90-1.27)	ns	1.48 (1.36-1.70)	1.18 (1.04-1.52)	ns	RN ns RH ns
Glucose infusion rate (mg/kg/min) AUC	0.0 (0.0-0.0)	0.0 (0.0-89.0)	ns	0.0 (0.0-1.1)	1.0 (0.0-18.7)	ns	RN ns RH ns
Plasma glucagon (pM) AUC	15507.0 (8115.0-25996.0)	14134.0 (6028.0-19524.0)	ns	12894.0 (10502.0-15971.0)	7641.0 (6168.0-16060.0)	ns	RN ns RH ns
Plasma human insulin (pM) AUC	365285 (236785-520825)	281821 (201974-346028)	ns	269127 (208810-697769)	272805 (182890-351255)	ns	RN ns RH ns
Fasted plasma C-peptide (pM)	77.0 (22.5-114.0)	145.5 (76.0-249.3)	ns	22.5 (22.5-86.8)	22.5 (22.5-51.1)	ns	RN ns **RH: DIA<CON P=0.0048**
Plasma C-peptide 120 min(pM)	22.5 (22.5-22.5)	22.5 (22.5-47.1)	ns	22.5 (22.5-22.5)	22.5 (22.5-22.5)	ns	RN ns RH ns
Epinephrine (ng/mL) AUC	187.1 (46.0-423.9)	489.7 (336.1-1539.0)	ns	244.4 (143.5-338.4)	262.1 (77.5-1412.0)	ns	RN ns RH ns

Table 2 legend: Summary data of preconditioning clamps. Average plasma glucose values represent the average of values between time 0-60 minutes, 60-120 minutes and 120-180 minutes to represent clamp level in one-hour intervals. AUC: area under the curve calculated with the trapezoidal method. Lower

limit (LLOQ) of quantification for plasma C-peptide is 50 pM and levels below detection rate are set to 0.5*LLOQ (22.5 pM). Plasma C-peptide at 120 minutes is included to represent level of suppression of endogenous insulin. P-values are calculated using Mann-Whitney U-test and corrected for multiple comparisons by Bonferroni correction. P-values for “healthy” are comparisons between recurrent normoglycaemia and recurrent hypoglycaemia groups in healthy pigs, while P-values for “diabetic” are comparisons between recurrent normoglycaemia and recurrent hypoglycaemia groups in diabetic pigs. P-values in “healthy vs. diabetic” are comparisons between diabetic and healthy pigs in their respective recurrent normoglycaemia or recurrent hypoglycaemia groups. P-values below 0.05 are considered significant. Data is presented as medians and interquartile range.