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Doctoral thesis

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Den bihormonale kunstige bukspyttkjertelen: Ny innsikt i farmakokinetikken og farmakodynamikken til glukagon

Kort tittel: Ny kunnskap om virkningene av glukagon i en kunstig bukspyttkjertel

En kunstig bukspyttkjertel er en elektronisk enhet som kan regulere blodsukkeret til individer med diabetes mellitus type 1 gjennom automatiske infusjoner med hormonet insulin, som senker blodsukkeret, og eventuelt også hormonet glukagon, som øker blodsukkeret. Foreløpig markedsføres bare halvautomatiske enheter som kun kan gi insulin, og som krever at brukeren informerer om måltider.

Insulin gis vanligvis i underhuden, og tas deretter langsomt opp i blodet. Forsinkelsen fra insulin gis til det tas opp i blodet og kan påvirke blodsukkeret gjør det vanskelig å utvikle en helautomatisk kunstig bukspyttkjertel som ikke krever måltidsvarsler fra brukeren. For å løse dette trengs det metoder som reduserer tiden fra insulin gis til det har effekt.

Insulin virker raskere når det gis i bukhulen, sammenliknet med i underhuden. Det første formålet med denne avhandlingen var å undersøke om dette også gjelder for glukagon, ettersom et slikt funn ville støtte videre forskning på en kunstig bukspyttkjertel som kan gi både insulin og glukagon i bukhulen. Det andre formålet var å undersøke om samtidig infusjon av glukagon og insulin på samme sted kunne forbedre opptaket av insulin fra underhuden, ettersom glukagon gir en kraftig økning av den lokale blodgjennomstrømningen.

Avhandlingen viser at glukagon tas opp og virker på blodsukkeret like raskt når det gis i bukhulen som når det gis i underhuden. Ettersom risikoen for alvorlige komplikasjoner generelt er høyere når man gir legemidler i bukhulen, konkluderer vi med at glukagon bør gis i underhuden.

Videre viser avhandlingen at samtidig infusjon av glukagon og insulin øker det totale opptaket av insulin fra underhuden. Kliniske studier er nødvendige for å undersøke om små doser med glukagon kan brukes for å forbedre opptaket av måltidsdoser med insulin fra underhuden og bedre blodsukkerkontrollen hos pasienter med diabetes mellitus type 1.

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Acknowledgements

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Finally, I thank my lovely son, Henrik Matheus. Heart beneath my heart. Thank you for all the light, happiness and love you bring into my life. You remind me every day of the beauty of existence.

Trondheim, November 2022

Ingrid Anna Teigen

Abstract

Diabetes mellitus type 1 is an autoimmune disease that causes selective destruction of the insulin-producing β-cells in the pancreatic islet of Langerhans. Insulin induces glucose storage and reduces blood glucose concentrations. As it is essential for normal glucose regulation, patients with diabetes mellitus type 1 require life-long treatment with exogenous insulin.

The treatment of diabetes mellitus type 1 has evolved significantly since insulin was discovered in 1921. Modern treatment comprises continuous self-monitoring of glucose concentrations and self-administration of appropriate insulin dosages. Most patients cover their basal and prandial insulin needs through multiple manual injections or infusions via a pump into subcutaneous tissue. Hypoglycaemic episodes are a common side-effect of insulin treatment, and can be reversed with glucagon, which mobilises glucose from the liver and increases blood glucose concentrations.

Lately, artificial pancreas technology has emerged as a promising new treatment option for patients with diabetes mellitus type 1. An artificial pancreas is a device that automatically calculates the demand for insulin based on continuous glucose feedback. Both unihormonal devices, delivering only insulin, and bihormonal devices, delivering insulin and glucagon, have been developed. However, only hybrid (partially automated), unihormonal devices that require meal announcements from the user are commercially available.

Developing a fully automated subcutaneous artificial pancreas that can achieve excellent glucose control without meal announcements is challenging. One major barrier is the slow absorption rate of insulin from the subcutaneous tissue, which makes timing and adjustments of meal boluses of insulin difficult. As such, measures that can promote insulin absorption are needed. Alternatively, a more efficient route of insulin administration could be chosen.

Intraperitoneal insulin administration mimics normal physiology and has a favourable pharmacokinetic profile compared to subcutaneous insulin administration. The first aim of this thesis was to investigate if the absorption, elimination, and utilisation of glucagon may also be improved with intraperitoneal drug delivery, as this could support developing a bihormonal intraperitoneal artificial pancreas, that would deliver both insulin and glucagon into the intraperitoneal space. The second aim was to investigate if subcutaneous insulin absorption may be accelerated by simultaneous subcutaneous glucagon administration, as glucagon is a potent vasodilator.

This thesis demonstrates that intraperitoneal delivery of glucagon results in lower systemic glucagon concentrations than subcutaneous administration, probably because of a large first-pass metabolism of glucagon in the liver. However, the effects on glucose metabolism are equal after intraperitoneal and subcutaneous glucagon administration. Because of the increased risk of complications with intraperitoneal drug delivery, we conclude that the subcutaneous route for glucagon delivery is preferable for most patients.

Simultaneous administration of glucagon did not accelerate subcutaneous insulin absorption. However, the total amount of insulin absorbed after a single bolus was significantly increased over an observation period of three hours. Clinical trials are needed to investigate if micro-doses of glucagon can be used to enhance insulin absorption and improve postprandial glucose control in subjects with diabetes mellitus type 1.

List of Papers

Paper I

Pharmacokinetics of Intraperitoneally Delivered Glucagon in Pigs: A Hypothesis of First Pass Metabolism

Ingrid Anna Teigen, Marte Kierulf Åm, Sven Magnus Carlsen, Sverre Christian Christiansen

European Journal of Drug Metabolism and Pharmacokinetics Jun 7, 2021.

doi:10.1007/s13318-021-00692-2

Paper II

Pharmacokinetics of Glucagon After Intravenous, Intraperitoneal and Subcutaneous Administration in a Pig Model

Ingrid Anna Teigen, Marte Kierulf Åm, Sven Magnus Carlsen, Sverre Christian Christiansen

Basic & Clinical Pharmacology & Toxicology 2022;130(6):623-31.

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Paper III

Vasodilatory Effects of Glucagon: A Possible New Approach to Enhanced Subcutaneous Insulin Absorption in Artificial Pancreas Devices

Ingrid Anna Teigen, Misbah Riaz, Marte Kierulf Åm, Sverre Christian Christiansen, Sven Magnus Carlsen

Frontiers in Bioengineering and Biotechnology

Sec. Biosensors and Biomolecular Electronics 2022;10.

doi: 10.3389/fbioe.2022.986858

Paper IV

Effects of Low-Dose Glucagon on Subcutaneous Insulin Absorption in Pigs

Ingrid Anna Teigen, Marte Kierulf Åm, Misbah Riaz, Sverre Christian Christiansen, Sven Magnus Carlsen

Abbreviations

APT: Artificial Pancreas Trondheim Research Group

$AUC_{0\text{-}last}$: Area under the time-plasma concentration curve from time zero to the last time point.

C_{max}: Maximum plasma concentration.

CGM: Continuous glucose monitor

DCCT: Diabetes Control and Complications Trial

DM1: Diabetes mellitus type 1.

EDIC: Epidemiology of Diabetes Interventions and Complications study

HbA1c: Glycosylated haemoglobin

IP: Intraperitoneal.

IQR: Interquartile range.

IV: Intravenous.

SC: Subcutaneous.

SD: Standard deviation.

$T_{1/2}$: Plasma elimination half-life.

TIR: Time in near-normoglycaemic range (3.9 – 10.0 mmol/L)

T_{last}: Time to last measurable concentration.

T_{max}: Time to maximum plasma concentration.

It's life that matters, nothing but life –the process of discovering, the everlasting and perpetual process, not the discovery itself, at all.

From «The Idiot» by Fyodor Dostoyevsky

1. Introduction

1.1 Pathogenesis and Prevalence of Diabetes Mellitus Type 1

Diabetes mellitus type 1 (DM1) is characterised by selective, autoimmune destruction of the β-cells in the pancreatic islet of Langerhans, which leads to extensive and usually absolute loss of endogenous insulin production (1). Insulin acts as a potent anabolic hormone and plays a crucial role in glucose regulation, general metabolism, and cell growth. Without treatment, DM1 universally leads to insulinopenia, severe hyperglycaemia, metabolic acidosis, and eventually death. As such, patients with DM1 require life-long insulin substitution and are dependent on close monitoring to keep their blood glucose concentrations within an acceptable range and avoid long-term complications.

According to the *National guideline on diabetes* from the Norwegian Directorate of Health, the diagnostic criteria for diabetes mellitus are either a glycosylated haemoglobin concentration (HbA1c) of more than 48 mmol/mol (6.5 per cent) or a fasting blood glucose concentration of more than 7.0 mmol/L or a blood glucose concentration of more than 11.1 mmol/L two hours after a 75 g oral glucose tolerance test or any blood glucose concentration higher than 11.1 mmol/L together with symptoms of hyperglycaemia (2).

Although the pathogenesis is dissimilar, differentiation between different types of diabetes mellitus can be challenging, and there are no standardised criteria separating DM1 from diabetes mellitus type 2. However, plasma C-peptide, an indirect measurement of endogenous insulin production, should be low despite high blood glucose concentrations in patients with DM1. In addition, β-cell-specific autoantibodies are commonly detected in the patient's blood (2).

The global incidence of DM1 is increasing (3, 4). In 2017, the World Health Organization reported that DM1 affected more than nine million individuals

worldwide (5). The prevalence of DM1 is higher in Scandinavia than the global average. According to the Norwegian Institute of Public Health, 23.000 persons were estimated to live with DM1 in Norway in 2021 (6). As is observed globally, the incidence rate of DM1 in Norway has been steadily increasing over the last decades, although mortality has decreased, presumably because of improved treatment (6). Currently, the underlying causes of DM1 and the factors promoting disease progression are not fully known. Although some progress has been made toward developing specific immunotherapy and β-cell replacement (7), DM1 remains a chronic condition for those affected, and no effective preventive or curative measures against the disease exist.

1.2 Morbidity and Mortality: Consequences of Diabetes Mellitus Type 1

The blood glucose concentration is normally between 4.0 and 5.4 mmol/L in healthy, fasting adults, and it seldom exceeds 7.8 mmol/L regardless of food consumption and exercise, although it may be higher the first two hours after a carbohydrate-rich meal (8, 9). Even though normal physiology is the ultimate guideline, this degree of glucose control is unrealistic for patients with DM1. As such, the target range for these patients is usually set to a near-normoglycaemic range of 3.9 to 10 mmol/L (10).

Both hyperglycaemia and hypoglycaemia cause morbidity. Although there have been major advancements in diabetes therapy, patients with DM1 continue to have reduced life expectancy. Standardised mortality rates for patients with DM1 differ globally but are estimated to be three to four times higher than the general population in European countries, mainly due to diabetes-related acute and chronic complications (11, 12).

1.2.1 Acute Complications

1.2.1.1 Acute Hyperglycaemia and Diabetic Ketoacidosis

Insulin regulates blood glucose concentrations directly and indirectly through multiple modes of reducing hepatic glucose output and increasing hepatic glucose

uptake. It also increases the uptake of glucose in peripheral tissue by stimulating glucose transporter protein type 4, GLUT4 (13). Insulin normally suppresses the secretion of the counterregulatory hormone glucagon, and when insulin is removed, a glucagon excess may develop (14, 15). Secretion of stress-hormones, mainly catecholamines and cortisol, may also be stimulated (16, 17). As a result, hepatic glycogenolysis and gluconeogenesis are paradoxically increased, and glucose utilisation in skeletal muscle and adipose tissue is diminished. All this contributes to escalating hyperglycaemia, causing glucosuria, osmotic diuresis and dehydration (18).

With severe insulinopenia and excessive secretion of glucagon and catecholamines, peripheral fat stores and muscle tissue are metabolised to provide gluconeogenic precursors. This results in increased concentrations of amino acids, glycerol, and free fatty acids in the systemic circulation. Fatty acids are converted to ketoacids, and when the buffering capacity for neutralising acids is exceeded, a high anion gap metabolic acidosis develops. This condition is known as diabetic ketoacidosis, a potentially deadly condition characterised by neurologic deterioration, dehydration, visual disturbances, abdominal pain, hyperventilation, and fruity smell (acetone) of the breath in addition to preceding general signs of acute hyperglycaemia (18, 19).

Diabetic ketoacidosis at euglycaemic glucose concentrations has been described in the literature (20). However, the plasma glucose concentration is usually strongly elevated, often above 20 mmol/L, but it can sometimes be as low as 12 to 15 mmol/L (21).

1.2.1.2 Acute Hypoglycaemia

Hypoglycaemia in patients with DM1 is essentially an iatrogenic condition. It develops when there is a relative insulin excess, causing an abnormally low blood glucose concentration. Classic symptoms include autonomic responses (primarily mediated by epinephrine), such as tremors, anxiety, sweating, hunger, and paraesthesia, and neuroglycopenic symptoms, such as reduced consciousness,

confusion, and delirium. With profound hypoglycaemia, seizures, cardiac arrhythmias, coma and death may occur (22).

The human brain relies almost exclusively on glucose for energy. However, the brain cannot store or synthesise glucose itself, and an adequate blood glucose concentration is therefore essential for maintaining normal cerebral functions and survival (23). To accommodate this, a hierarchy of hormonal defence mechanisms rapidly correct declining glucose concentrations in healthy non-diabetic individuals (24). Firstly, endogenous insulin secretion is reduced. This mechanism is activated well before the onset of any symptoms of hypoglycaemia, starting when the blood glucose concentration falls below approximately 4.5 mmol/L. Secondly, the secretion of glucagon is increased. This stimulates hepatic glucose production and release and is activated when blood glucose concentrations fall below approximately 3.9 mmol/L. Thirdly, epinephrine secretion is increased at the same glycaemic threshold as glucagon. Epinephrine inhibits glucose utilisation in several tissues and increases the delivery of gluconeogenetic substrates to the liver. Fourthly, if hypoglycaemia is sustained for several hours, cortisol and growth hormone secretion is increased. Both these hormones reduce glucose utilisation and enhance hepatic glucose production. In addition to hormonal responses, behaviour defence mechanisms (i.e., retrieval and ingestion of food) are also triggered by increasing autonomic neural activity and symptoms of hypoglycaemia (25).

The normally highly effective physiological responses to declining blood glucose concentrations are impaired in patients with DM1, which makes them susceptible to hypoglycaemia (26-28). The first defence, reduced insulin secretion, is, by definition, lost in DM1. The second defence, an increase in glucagon secretion, becomes dysfunctional in most patients with longstanding DM1. With time and repeated hypoglycaemic episodes, many patients also develop an attenuated epinephrine response and, secondary to the weakened catecholamine reaction, reduced sensibility towards early symptoms of hypoglycaemia (hypoglycaemia unawareness), causing further impairment in the counterregulatory behavioural

responses towards the condition (29).

Most episodes of hypoglycaemia in patients with DM1 occur at night during sleep (25). This is not surprising, as it represents the longest fasting period throughout the nychthemeron. However, nocturnal hypoglycaemia is particularly dangerous as awareness towards autonomic neural responses is naturally reduced, increasing the risk of prolonged duration and consequent complications (30).

The lower normal limit for fasting blood glucose concentration is usually set at 3.9 mmol/L (2). It has been suggested that blood glucose concentrations below 3.0 mmol/L indicate clinically meaningful biochemical hypoglycaemia, as this seldom occurs under physiologic conditions and should be corrected immediately to avoid harm to the individual (31). However, the glycaemic threshold for developing symptoms of hypoglycaemia varies extensively between patients. It may be higher in patients with poorly regulated DM1 and lower in patients with strict glucose control, repeated hypoglycaemic episodes, longstanding DM1 or diabetic complications. Hypoglycaemia unawareness is defined as the onset of neuroglycopenic symptoms before any autonomic warning symptoms (32). It is widespread among patients with DM1, in some studies reported to affect up to 40 per cent of the patient population (32-35).

Severe hypoglycaemia is agonising and frightening to patients. It is dreaded, not without reason, as reports suggest that up to ten per cent of deaths in patients with DM1 are related to hypoglycaemia (24). Hypoglycaemic episodes reduce the quality of life in many patients with DM1 and may constitute a definite barrier to optimising their glucose control (36, 37). As such, it is important to recognise the burden of hypoglycaemia and include effective measures against it in future diabetes treatment and technology.

1.2.2 Long-Term Complications: Chronic Hyperglycaemia

Diabetes is associated with both macrovascular disease (atherosclerosis) and

microvascular diseases, including retinopathy, nephropathy, and neuropathy. The mechanisms through which chronic hyperglycaemia leads to vascular disease are not fully known, but both systemic and organ-specific factors and genetic predisposition probably contribute (38). Notably, not all individuals suffering prolonged hyperglycaemia due to longstanding DM1, experience vascular complications (39). Both unknown protective and progressive factors probably contribute to the large interindividual variation in diabetic complications.

1.2.2.1 Microvascular Complications

Current evidence supports a strong association between chronic hyperglycaemia and the development of microvascular complications in patients with DM1, and indicates that intensive disease management with near normalisation of blood glucose concentrations and HbA1c provide substantial benefits for these patients (40). HbA1c reflects the average blood glucose concentration over the previous six to eight weeks. In the Diabetes Control and Complications Trial (DCCT), a 50 to 75 per cent reduction in the risk for development or progression of retinopathy, nephropathy and neuropathy was demonstrated over a 6.5 year follow-up period in patients receiving intensive insulin treatment compared to patients receiving conventional insulin treatment (41). The decrease in risk was attributed to improved mean blood glucose concentrations. Average HbA1c was around 53 mmol/mol (7.0 per cent) in the intervention group and 75 mmol/mol (9.0 per cent) in the conventional treatment group (42). The risk reduction for microvascular complications correlated continuously with the reduction in HbA1c concentration, indicating an approximate 40 per cent reduction in risk of microvascular disease for each 10 per cent reduction in HbA1c (40, 43).

Microvascular complications are the leading cause of blindness and renal failure in DM1, and the DCCT was terminated prematurely because of the pronounced benefit of intensive insulin treatment. In the Epidemiology of Diabetes Interventions and Complications study (EDIC), the open-label observational continuation study of the DCCT, one demonstrated that the duration of chronically elevated plasma

glucose concentration (as determined by HbA1c) was positively correlated with increased risk of microvascular complications and that the longer patients can maintain HbA1c concentrations below 53 mmol/mol (7.0 per cent), the longer the onset of these complications are delayed (44). A large Swedish study also verified these findings (45, 46).

Throughout the EDIC, the HbA1c concentrations in the two original groups converged to approximately 64 mmol/mol (8.0 per cent), owing to both the adoption of intensive treatment in the conventional group and the return of all participants to their usual diabetes care providers. However, the rate of development and progression of microvascular complications continued to be substantially lower in the original intervention group (42). This phenomenon demonstrates what is known as metabolic memory, i.e., that a sustained period of tight glycaemic control may result in a lasting benefit.

1.2.2.1 Macrovascular Complications

Patients with DM1 have an increased risk of macrovascular complications (atherosclerosis), manifesting primarily as coronary, cerebrovascular, or peripheral arterial disease (47). The factors promoting atherosclerosis in DM1 are incompletely understood, as chronic hyperglycaemia is not linked to macrovascular complications in the same way as microvascular complications. In fact, reducing HbA1c to near-normal concentrations has not been associated with noticeable reductions in cardiovascular events in large, randomised, controlled trials examining such outcomes in patients with DM1 (40).

Although the role of chronic hyperglycaemia is uncertain, the group originally randomised to intensive insulin therapy in the EDIC study had significantly fewer fatal and non-fatal cardiovascular events (41). This demonstrates that tight glycaemic control maintained over a long period may reduce cardiovascular disease in patients with DM1.

Appropriate management of hypertension and dyslipidaemia, common comorbidities in patients with DM1, substantially reduces the risk of macrovascular complications, and should therefore be a priority in diabetes care (48).

1.3 Pharmacological Treatment for Diabetes Mellitus Type 1

Insulin replacement is the cornerstone of DM1 management (49). However, appropriate treatment of DM1 and its complications may involve using several drugs. This thesis focuses on insulin and glucagon therapy. As such, only the use of these two drugs will be addressed in the following section.

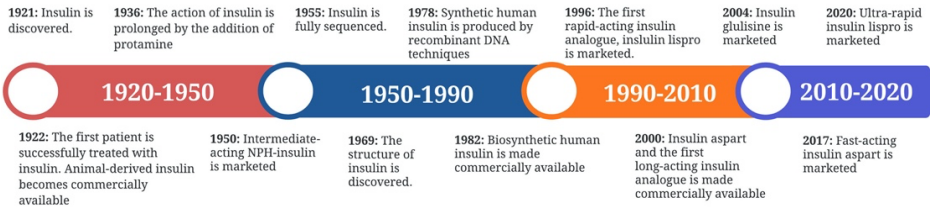
1.3.1 Insulin and Insulin Analogues

Due to the nature of DM1, all patients suffering from the disease need exogenous insulin to keep their glucose levels within the desired range (50). Pharmacological treatment for DM1 has been continuously evolving since insulin was first isolated by the team of Sir Frederick Banting and Charles Best in 1921 (51), and today several insulin analogues exist besides human insulin (52) (Figure I). Current insulin preparations may be classified as rapid-acting (insulin aspart, insulin glulisine, and insulin lispro), short-acting (insulin human), intermediate-acting (insulin human isophane), and long-acting (insulin degludec, insulin detemir, and insulin glargine) (40).

Proteolytic enzymes in the gastrointestinal tract inactivate insulin after oral consumption. No feasible insulin formulation that can resist degradation and be absorbed by the intestines has been developed yet. As such, insulin must be administered parenterally (53). Although several routes of administration are possible (54) (Table I), insulin is almost exclusively administered subcutaneously (SC) by the patients themselves outside of hospital, either as multiple daily SC insulin injections or as continuous SC insulin infusions via a pump. Because of the variation in the pharmacokinetic profile of the different insulin types and formulations, patients treated with multiple daily insulin injections usually use

rapid-acting and short-acting insulins to mitigate prandial glucose excursions and intermediate-acting and long-acting preparations to cover basal insulin needs.

Figure I: Timeline of breakthroughs in insulin discovery



Human insulin has a molecular weight of about 6000 daltons and consists of an α-chain and a β-chain of amino acids connected by disulfide linkage (40). Soluble insulin comprises oligomers, primarily monomers, dimers, and hexamers, in chemical equilibrium. When insulin is stored at high concentrations and in the presence of allosteric ligands, the oligomer equilibrium shifts towards a large fraction of hexamers. This is important to ensure sufficient shelf-life of insulin formulations, as hexamers are more resistant to chemical degradation (55). However, because of their size, insulin hexamers must dissociate before being absorbed into the systemic circulation and become biologically active as insulin monomers (56).

Presently, three rapid-acting insulin analogues are marketed: insulin lispro, insulin aspart and insulin glulisine (57). These analogues are structurally identical to human insulin except for minor changes in the amino acid sequence of the insulin β-chain. This alteration reduces the natural tendency of insulin monomers to form larger oligomers in SC tissue and increases the absorption rate after SC injection. Consequently, one achieves augmented absorption, an earlier peak concentration and more rapid insulin elimination with rapid-acting insulin analogues compared to human insulin after SC injection (52). The earlier onset of action means that insulin boluses can be administered closer to meals, at greater convenience for patients. As

such, it is unsurprising that treatment with rapid-acting insulin analogues is associated with better patient adherence and improved treatment satisfaction than treatment with regular human insulin (58, 59).

Table I: Strengths and weaknesses of administration routes for insulin used in clinical practice

Route of administration	Benefits	Weaknesses
Intravenous	No absorption delays. Rapid elimination.	Needs vascular access. Invasive with a high risk of complications. Not suitable for self-administration.
Subcutaneous	Suitable for self-administration. Relatively low risk of complications. Cost-effective.	Considerable delay in absorption and elimination. Large inter- and intraindividual variation in pharmacokinetics.
Intraperitoneal	Rapid absorption and elimination. Reduced systemic insulin concentrations.	Invasive and requires an implanted pump or IP port. Increased production of insulin antibodies in some patients. Costly.
Inhalation	Non-invasive. Rapid onset of action.	Low bioavailability. Increased risk of respiratory tract irritation. Not feasible for smokers or patients with pulmonary comorbidities.

In the last five years, two ultra-rapid-acting insulin formulations have also been commercialised: Fast-acting insulin aspart (Fiasp®), marketed in 2017, and ultra-rapid insulin lispro (Lyumjev®), marketed in 2020. Fast-acting insulin aspart contains niacinamide and L-arginine in addition to insulin aspart. Niacinamide increases the relative fraction of insulin monomers in SC tissue and causes transient local vasodilation after administration, leading to increased initial absorption of insulin aspart (60). Ultra-rapid insulin lispro contains the vasodilating drug treprostinil, a stable prostacyclin analogue, and citrate in addition to insulin lispro. These additives increase the local SC blood flow and vascular permeability, leading to accelerated insulin absorption (61).

Insulin treatment aims to match the physiologic insulin output as closely as possible, thereby preventing the negative short and long-term effects of DM1. In healthy humans, insulin is secreted from the pancreas into the branches of the portal circulation and transported directly to its main organ of action, the liver, where it undergoes a substantial first-pass metabolism (62-64). The first-pass effect entails that much of the insulin is extracted from the blood when it passes the liver, so the portion carried over to the systemic circulation is greatly reduced. Contrary, subcutaneously administrated insulin must be absorbed into the systemic circulation before it can reach the liver. Consequently, peripheral tissues in patients with DM1 are inevitably overexposed to insulin compared to healthy subjects (52). This is undesirable, as chronic hyperinsulinemia is associated with several metabolic disturbances, such as obesity, hypertension, and polycystic ovary syndrome (PCOS), and has been linked to the development of cardiovascular diseases (65-67). Notably, the prevalence of all these conditions is increased in subjects with DM1.

In the fasting and semi-fasting state, endogenous insulin is secreted in discrete pulses some minutes apart, supplemented by rapid insulin releases with meals that effectively mitigates postprandial glucose elevations. SC insulin pumps can only partially mimic this physiologic pattern, and even with rapid-acting insulin analogues, there is still a minimum delay of 30 to 60 minutes from the

administration of an insulin bolus until maximum plasma concentration is reached (40, 68-75).

The absorption of SC administered insulin is subject to extensive interindividual variation. Obesity, age, gender, smoking, and comorbidities associated with DM1 may all affect insulin pharmacokinetics. In addition, absorption may be delayed or decreased by insulin-binding antibodies, which develop in nearly all patients a few months after the start of insulin treatment (40).

At the individual level, there is a considerable variation in insulin absorption rate from different anatomical areas. Absorption is reported to be fastest from the SC tissue on the abdomen, followed by the arms, and lowest from the thighs and gluteal region (76). Absorption may also vary within the areas, and rotation between and within injection regions is likely a prominent source of intraindividual variability in insulin pharmacokinetics (56).

Another critical, alterable factor is the local perfusion at the insulin injection site. Insulin monomers and dimers are absorbed directly into SC capillaries through simple diffusion. Absorption is therefore accelerated when the capillary exchange surface in SC tissue expands with recruitment of capillaries, vasodilation, and increased blood flow (77, 78). As such, insulin absorption can be promoted through administration into areas with hyperperfusion, for instance, after direct or indirect heating or skin massage (79).

1.3.2 Glucagon

Glucagon, “the mobiliser of glucose”, was discovered as a hyperglycaemic factor of the pancreas the year after insulin, in 1922 (80). The pharmacological properties of glucagon have been less studied than insulin, but it is well established that glucagon also plays a crucial role in maintaining glucose homeostasis. It has antagonistic effects to insulin, and increases blood glucose concentrations through stimulating ketogenesis, glycogenolysis, gluconeogenesis, lipolysis and hepatic release of

glucose (81). Glucagon is secreted by the α-cells of the pancreatic islets of Langerhans and by glucagon-positive cells in the upper gastrointestinal tract (82). Like insulin, glucagon is released to the portal circulation and reaches the liver before the systemic circulation.

Unlike the β-cells, the α-cells of the pancreas are not destroyed in patients with DM1. However, their ability to secrete glucagon as a counterregulatory response to declining blood glucose levels is usually distorted (83). Regulation of glucagon secretion is complex and incompletely understood, but the dysfunctional glucagon response in DM1 is thought to be partly caused by a loss of high local insulin concentrations and paracrine communication as the β-cells of the pancreas vanishes (84).

Exogenous glucagon exerts the same effects on glucose metabolism as endogenous glucagon and can be used to reverse hypoglycaemia (85, 86). However, glucagon is only effective if hepatic glycogen is available. It has minimal value if the glycogen storages are depleted, for instance because of chronic hypoglycaemia or starvation (85). Because of this, supplemental carbohydrates should be administered as soon as possible after glucagon, to treat severe hypoglycaemia, restore hepatic glycogen and prevent secondary hypoglycaemia (87).

As with insulin, glucagon must be administered parenterally to avoid destruction in the gastrointestinal tract. Other than that, the ability of glucagon to raise plasma glucose is independent of the route of administration (88, 89). Blood glucose concentrations increase within minutes after glucagon administration, and rescue kits with supraphysiologic doses of glucagon for intravenous (IV), intramuscular, SC or intranasal use are commercially available (90).

In emergency settings outside of a hospital, the intramuscular or intranasal route is usually recommended (26, 91). However, several studies have also demonstrated

the potential benefit of using smaller doses of glucagon SC as a treatment for mild or impending hyperglycaemia in both children and adults with DM1 (92-94).

1.3.3.1 Other Effects of Glucagon

Presently, the only approved therapeutic indication for glucagon is correction of severe hypoglycaemia. However, it has several other well-documented effects. It acts as a smooth muscle relaxant and is used as a diagnostic aid to induce gastrointestinal hypotonia before some radiographic examinations. Further, it functions as a cardiac stimulant and has been successfully used as an antidote in some cases to treat severe intoxications with β-adrenergic blocking or calcium-channel blocking agents (85, 95).

Glucagon also acts as a vasodilator. Pronounced vasodilation after glucagon administration has primarily been observed on large vessels (96). However, one study on SC injection of a large glucagon dose (1 mg) demonstrated that glucagon could cause a 500 per cent increase in local SC blood flow (97). The APT research group recently discovered that SC injections of much smaller doses of glucagon (100 μg and 10 μg) could also cause a substantial increase in local SC blood flow (98). In this study, glucagon was injected SC on the abdomen of healthy individuals, and blood flow was evaluated by laser doppler technology. The median increase in SC blood flow was 250 per cent after using a dosage of 100 μg glucagon. The blood flow peaked at 2 to 4 minutes after injection before declining slowly, and the effect was still present 30 minutes after injection.

1.4 Major Technological Devices in Diabetes Treatment

1.4.1 Glucose Monitoring

SC continuous glucose monitoring systems (CGM) report glucose concentrations in the interstitial fluid in SC tissue (99). Changes in the blood glucose concentration are not immediately followed by a corresponding change in CGM measurements, as glucose must first diffuse through the capillary walls and into the interstitial space.

This process leads to a sensing delay, particularly during rapid changes in blood glucose concentrations, such as during physical activity and after meals (99, 100). Using a CGM relieves the patient from doing frequent blood glucose measurements, which usually require a fingerstick capillary sample. Satisfaction with using CGM-devices is reported to be high (101), and in 2021, 80 per cent of adult patients with DM1 in Norway possessed a CGM (102).

There have been considerable improvements in CGM-technology in recent years. Modern devices have a sensor lifetime of up to two weeks. They have decent accuracy and require seldom or no calibrations from the user (103). Further, size, weight, price, and complexity have been largely decreased, and many CGM-devices are compatible with smartphone apps for patients' convenience. However, the sensing delay is still an issue. In the literature, a delay of five to ten minutes is commonly attributed to physiological factors, such as local blood flow, tissue perfusion and permeability and convection of the interstitial fluid (100, 104). Technological factors related to the sensors also contribute to some delay within the range of a few minutes (100, 105).

1.4.2 Insulin Pumps

Modern insulin pumps provide a continuous infusion of insulin (usually a rapid-acting insulin analogue) that covers the patient's basal insulin need. The patient must also administer additional insulin boluses via the pump to manage postprandial glucose excursions (106). The basal rate commonly comprises 40 to 50 per cent of the patient's total daily insulin dose (107, 108). The required infusion rate may vary depending on diurnal and individual factors, such as concomitant infection or physical activity.

Insulin pumps contain a reservoir of insulin, which usually is administered via an infusion set into SC tissue. A pump that delivers insulin to the intraperitoneal (IP) cavity is also an option (109). The IP cavity is made up of the space between the parietal and visceral layers of the peritoneum. The parietal layer lines the abdominal

wall and accounts for approximately 30 per cent of the total surface, whilst the visceral layer covers visceral organs, and accounts for the remaining 70 per cent of the surface (110). Venous blood from the parietal peritoneum drains to the inferior vena cava, whereas venous blood from the visceral peritoneum drains to the portal circulation. As such, insulin administered IP is predominantly absorbed by vessels that empty into the portal vein (111), and mimics normal physiology.

Insulin pumps with IP insulin delivery have demonstrated superiority over SC pumps in reducing HbA1c and hypo-/hyperglycaemic episodes in humans (112). Notably, the circulating insulin concentrations are significantly reduced with IP administration, probably because of a large first-pass metabolism of insulin in the liver (113). One disadvantage of IP insulin infusion is that some patients may develop an increase in insulin-binding antibodies, increasing insulin needs over time (114). Low-medium affinity antibodies may form neutralising complexes with insulin when insulin concentrations are high, which dissociate at low insulin concentrations, causing unpredictability in insulin effect and risk of aggravated hypoglycaemia (115). Further, IP drug administration is invasive and associated with a higher risk of serious complications than SC drug delivery. As such, it is seldom preferred by patients and accounts for much less than one per cent of insulin treatment regimens used in clinical practice (113).

Most patients combine using an insulin pump with using a CGM. Sensor-augmented insulin pumps receive and use CGM data directly to inform and advise patients on their insulin needs. These pumps may also suspend insulin infusion as a response to hypoglycaemia. Other, more advanced pumps use algorithms that automatically adjust the insulin infusion rate according to the CGM values. Such inulin pumps are known as partially automated or hybrid closed-loop systems or artificial pancreata.

1.4.3 Artificial Pancreata

Diabetes technology has evolved rapidly over the last few decades (Figure II). However, managing the disease still requires a major daily effort from the individual patient. The majority of patients do not achieve the recommended treatment goals for DM1 today (116), and the risk of long-term complications due to insufficient treatment adherence constitutes a large concern for both patients and healthcare providers (117, 118). As such, developing a fully automated device that can monitor glucose concentrations and deliver insulin without any user intervention, a true artificial pancreas, has been a goal for researchers working with diabetes technology for decades (119).

Figure II: Timeline for recent major advances in diabetes technology

The term artificial pancreas was introduced as early as 1959, by the endocrinologist E. Perry McCullagh in his speech at the 41. annual meeting of The Endocrine Society (120). It translates to a system that automatically regulates insulin delivery based on continuous glucose concentration feedback. The first artificial pancreas that was tested on patients was the BioStator, in 1976 (121). However, it was a large and impractical device that based its calculations on IV glucose measurements and IV insulin delivery. As such, it was only suited for hospitalised patients.

In a more modern perspective, a SC artificial pancreas may use glucose information from a SC CGM, together with information on recent SC insulin infusions (insulin on board) and the patient's desired glucose targets to adjust the infusion rates of insulin automatically. In 2016, the Medtronic MiniMed 670G was made

commercially available, becoming the first (hybrid) artificial pancreas system fitted for outpatient care. The system adjusts basal insulin infusion rates automatically, but users must still inform the device of the carbohydrate content of intended meals for it to administer necessary meal-boluses of insulin, hence the term “hybrid”. These devices have the prospect of improving treatment outcomes, and a recent meta-analysis of 40 trials comparing treatment with hybrid artificial pancreata to any insulin-based treatment in non-pregnant patients found that the time spent in near-normoglycaemic range (TIR), defined as blood glucose concentrations between 3.9 mmol/L and 10 mmol/L, was increased by almost two and a half hours over a 24-hour period (122). The improvement of TIR was mainly due to better glucose control during the night.

Because hybrid artificial pancreata require accurate entry of meals, they cannot fully relieve patients of the daily focus and stress of managing their disease, which causes distress and reduces the quality of life among patients with DM1 (123). No completely automated artificial pancreas devices are currently commercially available. The Dutch company Inreda® Diabetic obtained European marketing approval (CE certification) for a bihormonal artificial pancreas that requires no meal announcements in 2020. However, their device is still in the testing phase and only available to selected patients in the Netherlands (124, 125).

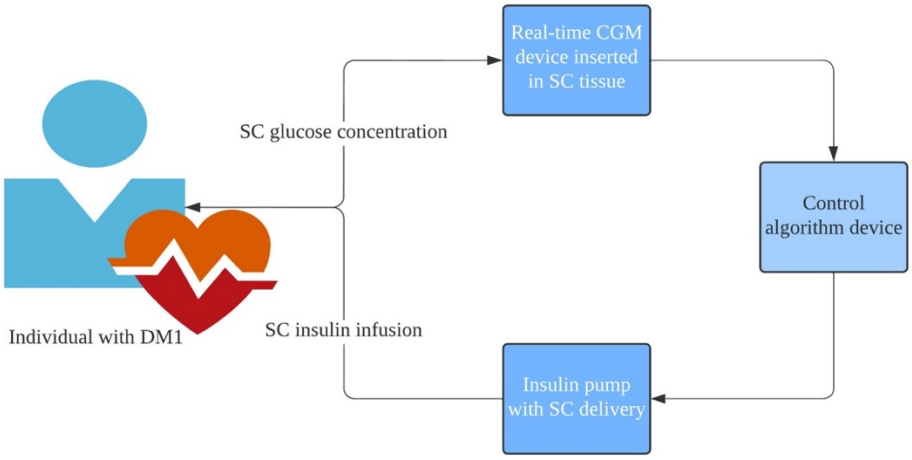
1.4.3.1 The Unihormonal Artificial Pancreas

Most research has been directed towards developing a double unihormonal SC artificial pancreas, i.e., a device that measures glucose concentrations and delivers *only* insulin SC (Figure III). This is also the approach used in all (hybrid) artificial pancreata commercially available at present (126).

Artificial pancreata generally provide better glycaemic results than conventional insulin pumps (127). However, the double unihormonal SC approach is compromised by the delay in sensing changes in blood glucose concentrations from a sensor in SC tissue and, more importantly, the delayed absorption, and hence the

glucose-lowering effect of SC delivered insulin (128, 129). These limitations are well-reflected in trials with hybrid artificial pancreata, where the TIR seldom is reported to be more than 70 to 75 per cent, even with meal announcements (130-134).

Figure III: Schematic presentation of a SC unihormonal artificial pancreas.



The system requires interaction between three components: A real-time CGM device reporting SC glucose concentrations, a control algorithm device that calculates insulin needs based on the CGM inputs, and an insulin pump that delivers the insulin directed by the control algorithm device. The control algorithm device may be its own entity (such as a computer or a smartphone) or reside in the insulin pump.

The slow absorption of insulin makes postprandial glucose excursions particularly difficult to handle for artificial pancreas devices. One possible solution to mitigate this is to use ultra-rapid-acting insulin formulations. In a four-week crossover trial comparing ultra-rapid insulin lispro to regular insulin lispro in a hybrid artificial pancreas (Medtronic MiniMed™ 670G) system in 42 patients with DM1, postprandial glycaemic control was significantly better in the ultra-rapid group. However, there were no improvements in the mean time spent in TIR, and treatment

with ultra-rapid insulin lispro resulted in more time spent in the hyperglycaemic range (blood glucose concentration >10 mmol/L) (135). Another 7-week crossover study on 40 patients with DM1 also using the Medtronic MiniMed™ 670G hybrid artificial pancreas with either fast-acting or regular insulin aspart, found that there was better postprandial glycaemic control with fast-acting insulin aspart. TIR was also statistically significantly improved (p-value =0.03), although the absolute difference was minimal with 1.8 per cent points more TIR in the fast-acting insulin aspart group (136). In another two-week pilot trial on 19 subjects comparing fast-acting insulin aspart to regular insulin aspart in established users of the Medtronic MiniMed™ 670G, there were no significant differences in glycaemic outcomes (137). In summary, the results from these studies are conflicting and provide no clear answer to whether ultra-rapid-acting insulin formulations may be advantageous in artificial pancreata. All three studies used a commercially available hybrid artificial pancreas, whose algorithm had not been customised to the ultra-rapid-acting insulin formulations. The lack of modification of the algorithm towards the altered pharmacokinetics and pharmacodynamics of the ultra-rapid-acting insulin analogues warrants a cautious interpretation of the results. Further research is needed.

1.4.3.2 The Bihormonal Artificial Pancreas (Insulin and Glucagon)

As mentioned earlier, the risk of hypoglycaemia is often the dominant limiting factor preventing tight glucose control and normalising glucose concentrations over time in patients with DM1. Because of the absorption delay of all insulins, suspension of insulin infusion may have limited effect in preventing imminent hypoglycaemia. A bihormonal artificial pancreas that utilises both insulin and glucagon may be a solution to this problem.

Unlike insulin, glucagon is rapidly absorbed from SC tissue, with an onset of effect after approximately five minutes and peak plasma concentration (T_{max}) after 10 to 20 minutes (97, 138-145). A bihormonal device could, therefore, potentially allow more aggressive insulin treatment than a unihormonal device, as glucagon could

quickly counteract insulin-induced hypoglycaemia and prevent predicted hypoglycaemia.

Several studies on bihormonal artificial pancreata have reported good glycaemic outcomes with the bihormonal approach. In short-term trials (≤ 14 days), it has been demonstrated that the TIR that can be achieved with these devices is high, up to 80 to 85 per cent (124, 125, 146-148).

Reviews comparing bihormonal and unihormonal SC artificial pancreas devices have reported that the bihormonal approach reduces the incidence and duration of daytime hypoglycaemic episodes. However, data on possible long-term improvements in TIR and HbA1c are lacking (149-151).

A bihormonal artificial pancreas would administer small doses of glucagon regularly. Historically, glucagon has been used as an emergency drug with infrequent administrations of large boluses in patients with DM1. Thus, the safety profile after long-term use with low-dose glucagon is poorly investigated. As with insulin, glucagon secreted by a functional pancreas is transported directly to the liver, its main organ of action, via the portal vein before it reaches systemic circulation. With SC administration, peripheral tissues are likely exposed to unphysiological concentrations of glucagon. As previously mentioned, glucagon exerts several hepatic and extrahepatic effects. Prolonged glucagon administration has been shown to produce a diabetic effect that may persist for several days (85), and glucagon hypersecretion is likely a cause of hyperglycaemia in patients with DM1 and diabetes mellitus type 2 (83). Patients with pronounced, chronic hyperglucagonemia because of a glucagonoma, a rare neuroendocrine tumour that produces glucagon, commonly present with hyperglycaemia, necrolytic migratory erythema, and anaemia (152). Necrolytic migratory erythema causes pruritic, erosive, and painful lesions on the skin and mucous membranes. The condition is characteristic of glucagonomas, but not specific as it can occur with other disorders and in the absence of elevated glucagon (153). Anaemia is possibly caused by a

direct effect of glucagon on erythropoiesis (154), although it could also be due to chronic illness. Whether repeated SC administration of glucagon by a bihormonal artificial pancreas may cause relative hyperglucagonemia, and which consequences this might have over time is unknown. It should be assessed in future long-term studies on users of bihormonal artificial pancreas devices.

1.4.3.3 An Intraperitoneal Artificial Pancreas

As mentioned under the section on insulin pumps, IP delivery of insulin results in faster drug absorption and provides significantly better glycaemic control than SC delivered insulin. Despite of this, research on IP artificial pancreata has been very limited. Some minor-sized clinical trials where an artificial pancreas with IP insulin infusion and either SC or IV glucose sensing have been conducted, although not with compelling results (155).

In one pilot trial comparing closed-loop control over 24 hours with SC glucose sensing and either IP or SC insulin delivery, the overall TIR for the IP approach was significantly better (mean 65.7 per cent, compared to 43.9 per cent) (156). A limitation of this study is that a rapid-acting insulin analogue (insulin lispro) was used for SC insulin delivery, whereas regular human insulin was used for the IP insulin delivery. The pharmacokinetics of these insulin formulations are very different, and IP infusion of a rapid-acting insulin analogue might have conveyed different and more overall promising results. However, the only commercially available IP port (Accu-Chek® DiaPort) is not recommended to be used with rapid-acting insulin analogues because of the increased risk of catheter obstruction (157).

IP administration of glucagon has only been studied in a few small-sized animal trials (158-162). No clinical studies on bihormonal artificial pancreata with IP glucagon delivery have been published.

1.5 Glycaemic Targets for Patients with Diabetes Mellitus Type 1

The efficiency of diabetes therapy is mainly evaluated by assessing CGM data and

HbA1c concentrations. The Norwegian Institute of Public Health recommends a treatment goal of HbA1c at or around 53 mmol/L (7.0 per cent) for most adults with DM1 (2). However, the treatment goal should be tailored to the individual patients, with a thorough risk-benefit calculation.

A low HbA1c target should only be chosen if it can be achieved without an unacceptable risk of hypoglycaemia and without greatly, negatively affecting the patient's quality of life. As such, a higher target is usually chosen for patients whose risks of stringent glucose control outweigh the potential long-term benefits, such as in elderly and fragile patients and patients with a history of severe hypoglycaemia or hypoglycaemia unawareness. Conversely, a lower target and more strict glucose monitoring are often chosen during pregnancy, as good glucose control greatly decreases the risk of maternal and foetal complications while also considering that the normoglycaemic range is physiologically lower in the pregnant state (2, 163).

2. Aims of Thesis

2.1 Overall Objectives

The project was conducted as a part of the Artificial Pancreas Trondheim (APT) research group's overall endeavour to develop a robust closed-loop glucose control system, a true artificial pancreas. The purpose of the present thesis was to gain knowledge of the pharmacokinetic and pharmacodynamic profile of glucagon, with the intention of using glucagon as a drug in a bihormonal artificial pancreas.

The first aim of this thesis was to establish if the IP route might be beneficial compared to the SC route regarding glucagon absorption, elimination, and utilisation, as this would support further investigation into developing a bihormonal IP artificial pancreas.

The second aim was to explore the vasodilatory effects of glucagon and investigate if SC insulin absorption could be enhanced by simultaneous SC glucagon administration at the site of insulin delivery.

2.2 Specific Objectives

2.2.1 Paper I: Pharmacokinetics of Intraperitoneally Delivered Glucagon in Pigs: A Hypothesis of First Pass Metabolism

The main aim of Paper I was to investigate the pharmacokinetics of glucagon after IP administration of different-sized boluses in anesthetised pigs. The study was a post hoc analysis of data collected for other reasons by APT. The results were used for hypothesis generation.

2.2.2 Paper II: Pharmacokinetics of Glucagon After Intravenous, Intraperitoneal and Subcutaneous Administration in a Pig Model

The aim of Paper II was to affirm the hypothesis presented in Paper I. It compared the pharmacokinetics of glucagon after IP, SC, and IV administration and the pharmacodynamic effects of glucagon on glucose metabolism after IP and SC

administration in a pig model.

2.2.3 Paper III: Vasodilatory effects of Glucagon: A Possible New Approach to Enhanced Subcutaneous Insulin Absorption in Artificial Pancreas Devices

The aim of Paper III was to present a hypothesis on a new possible use of glucagon in bihormonal artificial pancreata. It builds on a previous study by APT, which documented the vasodilatory effects of small doses of glucagon (98). Paper III argues for investigating if this vasodilatory effect can be used to accelerate insulin absorption and thus improve the performance of artificial pancreata.

2.2.4 Paper IV: Effects of Low-Dose Glucagon on Subcutaneous Insulin Absorption in Pigs

The aim of Paper IV was to substantiate the hypothesis presented in Paper III. It investigates the pharmacokinetics of SC delivered insulin when administered simultaneously with low-dose glucagon compared to placebo in anaesthetised pigs.

3. Research Methods

3.1 Data Collection

3.1.1 Paper I

Data for this study were retrieved from four series of pig experiments conducted by fellow researchers in the APT research group between the 12th of April 2018 and the 16th of January 2020 (162). Data from 19 non-diabetic farm pigs (*Sus scrofa domesticus*) were included in the analyses.

3.1.2 Paper II

A pig model was chosen for this study. The study had a randomised, open-label, crossover design. Data were collected between the 2nd of February 2021 and the 3rd of March 2021. Based on a power calculation for investigating the systemic bioavailability of glucagon after IP drug administration, 12 female, non-diabetic farm pigs (*Sus scrofa domesticus*) were included in the trial.

3.1.3 Paper III

Paper III builds on findings from a previous study by APT (98). It contains a theoretical discussion and presents a new hypothesis. No data were collected for this paper.

3.1.4 Paper IV

A pig model was chosen for this study. The study had a randomised, open-label design. Data were collected between the 11th of August 2022 and the 26th of August 2022. Based on a power calculation for investigating a potential difference in time to maximum insulin concentration (T_{max}), 12 non-diabetic farm pigs (*Sus scrofa domesticus*) were included in the trial.

3.2 Animal Experiments (Paper I, Paper II and Paper IV)

3.2.1 Animal Handling

All the pigs that were used in the presented trials were regular farm pigs acquired from the same local supplier at approximately 12 weeks of age.

To reduce the stress experienced by the pigs, they were brought to the research facility around one week before the experiments to become familiar with the surroundings. To accommodate the pigs' social needs, they were kept in pen groups of two or three whenever possible. Toys, wood chips, and nesting material were available to them. The pigs were fed commercial compound feed twice daily and provided with water without restriction. Food was removed on the evening before the experiments.

3.2.2 Anaesthetics and Surgery

All animal experiments reported in this thesis were performed under general anaesthesia. The pigs were sedated while still in their pen and carried manually into the operating theatre, where they were intubated after induction of general anaesthesia. The pigs were euthanised under general anaesthesia at the end of the study day, with an IV infusion of 100 mg/kg phenobarbital.

IV fluids and drugs were administered through a venous catheter placed in the left internal jugular vein. Blood samples were drawn from an arterial catheter placed in the left carotid artery. Both catheters were inserted via the same surgical incision.

IP drug infusions were administered via a tube placed into the peritoneal cavity in the upper left quadrant of the abdomen.

SC drug infusions were delivered into the SC adipose tissue behind the left ear.

At the beginning of all experiments, a bladder catheter was inserted for urine collection. The catheter was inserted directly into the bladder after exposing it surgically with a small, low laparotomy.

Vital parameters were monitored continuously during all experiments, and anaesthesia was adjusted if necessary. All surgical procedures, drug administrations (other than premedication/light sedation) and blood samplings were performed under general anaesthesia.

3.2.3 Suppression of Endogenous Glucagon and Insulin Secretion

We attempted to suppress endogenous insulin and glucagon secretion with somatostatin analogues during all the pig experiments included in this thesis.

In the study presented in Paper I, the pigs received either an IV infusion of 0.4 mg octreotide (Sandostatin®, Novartis Europharm Limited, United Kingdom) every hour or a continuous IV infusion of 150 µg octreotide per hour. The first seven pigs also received a subcutaneous injection of 0.3 mg pasireotide (Signifor®, Novartis Europharm Limited, United Kingdom) at the start of the experiment (162). Due to a temporary supply shortage for pasireotide in Norway, and a lack of support in the literature for additional benefit when pasireotide is used together with octreotide, the protocol was later changed to include only octreotide.

In the studies presented in Paper II and Paper IV, the pigs received an initial IV bolus of 5 µg/kg octreotide, followed by continuous IV infusion of 5 µg/kg/hour octreotide throughout the experiments. Treatment with octreotide was initiated one hour before the study intervention in both trials.

3.2.4 Sample Handling and Analysis

Arterial blood glucose concentrations were analysed on-site immediately after collection on heparinised syringes with a Radiometer ABL 800 FLEX blood gas analyser.

Arterial blood samples for determination of plasma glucagon, insulin and porcine insulin concentrations were collected on EDTA vacutainers and stored on ice for 10 minutes before centrifugation. After centrifugation, plasma was transferred to Eppendorf tubes that were stored at -18 ºC for the duration of the experiment day. From the end of the study day and until analysis, the tubes were stored at -80 ºC.

Glucagon concentrations were measured using Glucagon ELISA kits (Mercodia, Uppsala, Sweden), insulin concentrations were measured using Iso-Insulin ELISA kits (Mercodia, Uppsala, Sweden), and porcine insulin concentrations were measured using Porcine insulin ELISA kits (Mercodia, Uppsala, Sweden). The samples were run in singles in the studies presented in Paper I and Paper II, and in duplicate in the study presented in Paper IV.

3.3 Pharmacokinetic Analyses (Paper I, Paper II and Paper IV)

Maximum plasma concentration (C_{max}), T_{max}, and last measurable plasma concentration (T_{last}) were obtained directly from the measured drug concentrations in plasma. Plasma elimination half-life ($T_{1/2}$) and area under the time-plasma concentration curve from time zero to time to last measurable/measured concentration ($AUC_{0\text{-}last}$) were estimated using Simbiology in MATLAB version R2020B (164). The terminal rate constant, describing the decrease of the log-concentration of either glucagon or insulin, was calculated by applying a best-fit linear regression to the terminal portion of the curve. $T_{1/2}$ was calculated as $\frac{\ln 2}{\text{terminal rate constant}}$. $AUC_{0\text{-}last}$ was calculated by using the linear trapezoidal method.

3.4 Statistical Analyses

3.4.1 Paper I

Statistical analyses were performed using GraphPad Prism version 9 (165).

Medians and interquartile ranges (IQR) and means and standard deviation (SD) of

the assessed pharmacokinetic variables were calculated.

Spearman's ranks order correlation test was used to determine a possible correlation between dose size and pharmacokinetic outcomes. Correlation analyses were performed on non-transformed data. Because of lognormal distribution, a logarithmic transformation was performed before linear regression analysis in order to make equations for C_{max} and $AUC_{0\text{-}last}$.

3.4.2 Paper II

Statistical analysis was performed using GraphPad Prism version 9 (165). Non-parametric tests were predominantly chosen because of the small sample size and skewed distribution of some variables.

Medians and IQR plus means and SD of the assessed pharmacokinetic variables were calculated.

The bioavailability[1] of glucagon was assessed by comparing $AUC_{0\text{-}last}$ after IV administration with $AUC_{0\text{-}last}$ after IP and SC delivery. Possible differences in bioavailability after IP and SC administration were examined using the Wilcoxon matched-pairs signed-rank test.

Possible differences in T_{max}, C_{max}, $AUC_{0\text{-}last}$, $T_{1/2}$ and T_{last} of glucagon in relation to administration routes were examined using the Friedman test. Correction for multiple analyses was performed using Dunn's multiple comparison test.

A mixed linear model was used to compare differences in glucose concentrations over time after IP and SC administration. The model had time and administration route as fixed effects and the subject number as a random effect. Correction for

[1] In this context, bioavailability refers to the fraction of the drug that becomes available to systemic circulation.

multiple analyses was performed using Šídák's multiple comparison test.

3.4.3 Paper IV

Statistical analysis was performed using GraphPad Prism version 9 (165). Non-parametric tests were predominantly chosen because of the small sample size and skewed distribution of some variables.

Medians and IQR plus means and SD of the assessed pharmacokinetic variables were calculated.

Possible differences in T_{max}, C_{max}, $AUC_{0\text{-}last}$, and $T_{1/2}$ of insulin between the two groups were examined using the Mann-Whitney U-test.

A mixed linear model was used to compare differences in glucose concentrations over time. The model had time and adjuvant substance (glucagon/placebo) as fixed effects and the subject number as a random effect. Correction for multiple analyses was performed using Šídák's multiple comparison test.

4. Summary of Papers

4.1 Paper I

Pharmacokinetics of Intraperitoneally Delivered Glucagon in Pigs: A Hypothesis of First Pass Metabolism

Ingrid Anna Teigen, Marte Kierulf Åm, Sven Magnus Carlsen, Sverre Christian Christiansen

European Journal of Drug Matabolism and Pharmacokinetics Jun 7, 2021. doi:10.1007/s13318-021-00692-2

An IP artificial pancreas administering small glucagon boluses in addition to insulin could possibly improve glycaemic control in patients with DM1. However, the pharmacokinetic properties of glucagon after IP administration are not well studied. The aim of the study was to evaluate the dose-dependency of selected pharmacokinetic variables in a pig model.

We found that C_{max} and $AUC_{0\text{-}last}$ of glucagon in plasma correlated positively with the administered glucagon dose. However, the relationship was not linear, as administration of larger glucagon dosages resulted in a relatively larger increase in C_{max} and $AUC_{0\text{-}last}$. T_{max} and $T_{1/2}$ of glucagon in plasma seemed independent of the drug dose.

Based on the findings, we hypothesised that the liver has a satiable first-pass metabolism of glucagon.

4.2 Paper II

Pharmacokinetics of Glucagon After Intravenous, Intraperitoneal and Subcutaneous Administration in a Pig Model

Ingrid Anna Teigen, Marte Kierulf Åm, Sven Magnus Carlsen, Sverre Christian Christiansen

Basic & Clinical Pharmacology & Toxicology 2022;130(6):623-31.

doi:10.1111/bcpt.13731

This study was conducted to investigate the pharmacokinetics of glucagon. The study compared glucagon pharmacokinetics after IP, SC and IV administration of 1.5 µg/kg glucagon and investigated the pharmacodynamic effects of glucagon on glucose metabolism after IP and SC administration in a pig model.

We found that the systemic bioavailability of glucagon was median (IQR) 3 (2-5) per cent after IP administration. This was significantly lower than after SC administration. However, the effect of glucagon on glucose metabolism was equal after IP and SC administration.

We concluded that IP glucagon administration results in lower systemic glucagon exposure than SC administration without loss of efficiency. We interpreted this finding as evidence of a major first-pass metabolism of glucagon in the liver, affirming the hypothesis from Paper I.

4.3 Paper III

Vasodilatory effects of Glucagon: A Possible New Approach to Enhanced Subcutaneous Insulin Absorption in Artificial Pancreas Devices

Ingrid Anna Teigen, Misbah Riaz, Marte Kierulf Åm, Sverre Christian Christiansen, Sven Magnus Carlsen
Frontiers in Bioengineering and Biotechnology
Sec. Biosensors and Biomolecular Electronics 2022;10.
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The slow absorption rate of insulin from SC tissue is a critical element preventing the development of a fully automated artificial pancreas relying on SC insulin delivery. SC insulin absorption is influenced by several factors, among which local SC blood flow is one of the most important. This paper discusses how the local vasodilative effects of micro-doses of glucagon might be utilised to improve the

performance of SC bihormonal artificial pancreas devices. We present our hypothesis as a disruptive novel approach, where we propose using glucagon as a vasodilator to accelerate the absorption of meal boluses of insulin, besides using it conventionally to treat hypoglycaemia.

4.4 Paper IV

Effects of Low-Dose Glucagon on Subcutaneous Insulin Absorption in Pigs

Ingrid Anna Teigen, Marte Kierulf Åm, Misbah Riaz, Sverre Christian Christiansen, Sven Magnus Carlsen

This study was conducted to investigate the hypothesis presented in Paper III. It compares the pharmacokinetics of insulin aspart after SC administration with 100 µg glucagon or the equivalent volume of placebo. Insulin absorption was rapid in both groups, with median (IQR) T_{max} after 30 (30-50) minutes in the glucagon group and median (IQR) T_{max} after 30 (10-45) minutes in the placebo group. $AUC_{0\text{-last}}$ was significantly larger in the glucagon group over the observation period (three hours), indicating better overall drug absorption. The improved absorption was probably mediated by local SC vasodilation caused by glucagon. The pigs in the glucagon group had significantly higher blood glucose concentrations the first 30 minutes after insulin infusion. This was likely caused by a hyperglycaemic effect of the glucagon dosage.

5. Discussion

5.1 Ethical Considerations

5.1.1 Use of Animals for Research Purposes

Using animals in medical research is controversial. Findings from preclinical studies may have limited transferability to clinical medicine, and animal trials are known for being prone to reproducibility issues (166, 167). However, animal models remain essential to discover and understand the causes of diseases and investigate new courses of treatment to prevent and treat suffering and illness in both humans and animals. Although using animals for scientific purposes is considered an ethical dilemma, extensive surveys performed in Great Britain demonstrate that the majority of the public condones using animals in medical research, as long as suffering is minimised and other alternatives have been fully considered (168).

The three Rs (replace, reduce, refine) are core principles that protect animals that are used in research, and they are incorporated in Norwegian legislation regulating the use of animals in medical trials. The three Rs obligate researchers to *replace* animals and obtain data from methods that do not require animals when this is possible. When animals must be used, the number of animals used per study should be *reduced* to a minimum, although consistent with the scientific aims. Further, animal experiments should be *refined*, so that they provide the best possible data, whilst the pain and harm that the animals might experience is minimised.

Animals are sentient creatures with an independent worth and the capacity to feel fear and pain. Therefore, minimising harm is an obvious moral duty, as well as a legal obligation. It is also methodically beneficial, as pain and distress may alter normal physiological responses and introduce variation and confounding in experimental results (169).

All animal experiments included in this thesis were conducted following the

«Norwegian Regulation on the Use of Animals in Research» and the 2010/63 EU directive on the «Protection of Animals Used for Scientific Purposes». The trials were all preapproved by the Norwegian Food Safety Authority.

For the study presented in Paper I, we used data that had already been collected and restrained from using more animals. For the studies presented in Paper II and Paper IV, the number of animals included was the minimum number deemed necessary based on a power analysis of the primary outcome. Efficient anaesthetic and analgetic drugs were used to minimise the stress, fear, and pain the animals would feel during the experiments.

We chose to perform the studies presented in Paper II and Paper IV on anaesthetised pigs as we regarded it unethical to induce large glucose oscillations, perform multiple parenteral drug administrations and draw the required number of blood samples from conscious animals. The pigs were euthanised with a large dose of phenobarbital at the end of the experiments whilst still under general anaesthesia. The decision to euthanise the pigs after each experiment, and hence not perform multiple experiments on each animal, was based on an assessment of the risk of postoperative complications and to avoid the pain and distress that would be expected during the recovery period.

5.1.2 Ethical Considerations Regarding Implementing Automated Systems in Medicine

APT aims to develop a true artificial pancreas that functions automatically without daily user interventions. Expanding the use of artificial intelligence and robotics in medicine is controversial, and it is important that researchers working within this field explore ethical issues related to the potential “end-product” of their work. However, a comprehensive review of this large and complex subject would fall outside the scope of this thesis. As such, the topic will only be discussed very briefly in view of the four prima facie principles of clinical ethics: respect for autonomy, justice, beneficence and non-maleficence (170).

5.1.2.1 Autonomy and Informed Consent

Autonomy is the ability to make deliberate decisions, which defines us as moral agents (171). Even if an artificial pancreas allows discontinuation and manual adjustments of the treatment it has prescribed, the knowledge required to comprehend and operate the system may be beyond what could be expected from the average patient. By transferring treatment decisions to electronic devices, patients may lose ownership of their treatment, and their ability to manage their disease autonomously may be reduced. This will constitute a grave risk of harm if the technological system fails. From a societal perspective, increased use of automated medical devices could change how we perceive and value our ability to make autonomous choices as humans without computational decision support.

5.1.2.2 Justice

Our legal practices are based on humans being accountable towards other humans. As such, problems inevitably emerge if the law is applied to non-human systems (172). A physician or nurse responsible for treatment decisions would be subject to repercussions in the case of maltreatment. Conversely, it is difficult to place responsibility and culpability on an electronic device, as it has no freedom, free will or conscience.

Another critical issue is the potential for unauthorised reprogramming (hacking) of an artificial pancreas (173). Without robust prevention features against manipulation, patients' security and confidentiality could be jeopardised. This could potentially lower the trust citizens are willing to place in medical innovations.

Artificial pancreas technology is costly and will likely be available only to a highly privileged part of the patient population. One could argue that research into new treatment options that will benefit primarily wealthy patients in more economically developed countries should be discouraged. However, the lack of global equitability

is a harsh reality in health care. If advances in medical knowledge are to be made and new treatment options discovered, this must perhaps be accepted.

5.1.2.3 Non-maleficence and Beneficence

The principle of non-maleficence conveys the universal duty not to cause harm or evil. The risk of harm to patients would probably be reduced with an artificial pancreas, as it would monitor treatment effects continuously. However, this degree of vigilance may also result in overtreatment and perhaps narrow our understanding of normal inter- and intraindividual variation and change our collective attitude towards health (174).

Advanced technology provides exciting opportunities for safer and more effective medical care, and how we perceive beneficence will inevitably change as new treatment solutions emerge. It is morally controversial to withhold existing treatments that can remove or relieve diseases. One could argue that restraining from researching new technology that will potentially improve future treatment is equally controversial, even if the societal effects are unknown.

5.2 Methodological Considerations

5.2.1 Post Hoc Analyses

The first study in this thesis (Paper I) contains a post hoc analysis of data collected by fellow researchers in APT for other reasons. Because these experiments were not designed to investigate pharmacokinetic endpoints there is limited consistency in dosages, timing of blood samples and choice of comedication, causing large variation between the experiments.

A more robust design where multiple pigs could have been given a variation of standardised glucagon dosages would have been more useful and increased the quality of the results. However, this study was only conducted for hypothesis generation and to provide a preliminary overview of the pharmacokinetics of IP delivered glucagon. It inspired the second study (Paper II), that was dedicated into

investigating glucagon pharmacokinetics. This second study confirmed the hypothesis of a large hepatic first-pass metabolism of glucagon.

5.2.2 Animal Models

As previously mentioned, the transferability from animal trials to humans is limited. However, because of obvious safety issues and ethical concerns, it is custom to investigate drug pharmacology in animals before advancing to human trials. Pig models were chosen for the trials in this thesis due to the anatomical, physiological, and genetic similarities between pigs and humans. The porcine genome is three times closer to the human genome than that of rodents, and pigs may thus provide better models for human health and diseases in general (175). Further, the size of the animals made the experiments easier to perform and ensured that the necessary number of blood samples could be drawn without causing unacceptable harm to the animals.

There is one major anatomical difference between pigs and humans that might have reduced transferability in the studies on IP glucagon. Pigs have no greater omentum, which covers the anterior surface of the intestines in humans. IP administered glucagon would therefore to a larger degree be absorbed over the peritoneal lining of the intestines in pigs, rather than the peritoneal lining of the greater omentum in humans. However, venous blood from both the intestines and the greater omentum drains to the portal vein, so the consequence of this difference is unclear.

5.2.3 Suppression of Endogenous Insulin and Glucagon Secretion

Endogenous insulin and glucagon secretion was supressed in the trials presented in Paper I, Paper II and Paper IV, mainly by treatment with the somatostatin analogue octreotide. Octreotide is a well-documented, potent inhibitor of insulin and glucagon release (176). The concentration of porcine insulin was measured every hour after initiation of treatment in the pig trials presented in Paper II and Paper IV to monitor the efficiency of suppression. In the study presented in Paper II, we deem the suppression to have been efficient, as the concentration of porcine insulin

was supressed below the detection cut-off limit of 2.3 mU/L at baseline in 11 out of 12 pigs, and remained below 2.3 mU/L in 88 per cent of the samples. In the study presented in Paper IV, the suppression of endogenous insulin secretion was less satisfactory. At baseline, porcine insulin was below the detection cut-off limit of 2.3 mU/L in only four of 12 pigs. For the remaining eight pigs, mean (SD) porcine insulin concentration at baseline was 7.5 (2.8) mU/L. Suppression was least efficient during pronounced hyperglycaemia, i.e., in the first hour of the experiment. It was improved in most pigs throughout the experiment, and in total porcine insulin was supressed below the detection cut-off limit of 2.3 mU/L in 71 per cent of the samples.

5.3 Discussion of Main Findings

5.3.1 First-pass Metabolism of Glucagon (Paper I and Paper II)

The equations presented in Paper I suggest that C_{max} and $AUC_{0\text{-last}}$ are dose-dependent and indicate that larger doses provide a relatively greater increase in these parameters than smaller doses. This observation could be compatible with a satiable hepatic first-pass metabolism of glucagon, as was documented for insulin in a previous study by APT (62).

To investigate the hypothesis of first-pass metabolism of glucagon further, we conducted the study presented in Paper II. This study demonstrated that the systemic bioavailability of glucagon was only three per cent after IP administration of 1.5 µg/kg glucagon. Despite very low transition to systemic circulation, IP administered glucagon exerted comparable effects on glucose metabolism as SC administered glucagon. This finding is compatible with the presence of a major first-pass metabolism of glucagon in the liver. The first-pass effect entails that a large fraction of the administered glucagon is removed from the blood when it passes through the liver and that the portion that reaches systemic circulation is reduced. The liver is the main target organ for glucagon. As such, it is not surprising that the effect on glucose metabolism is preserved even though the glucagon concentration found in plasma is minimal.

As only one bolus size was used in the study presented in Paper II, it cannot provide any evidence on whether the observed first-pass effect is satiable or not. As such, the hypothesis proclaimed in Paper I remains partly unverified.

5.3.2 Potential Benefits of Intraperitoneal Glucagon Delivery in an Artificial Pancreas

The study presented in Paper II found no improvements in glucagon pharmacodynamics with IP administration compared to SC administration, neither in terms of faster nor larger glucose response. Therefore, the potential benefit gained from using the IP route seems limited to the possible advantage of reduced systemic exposure to glucagon.

Large doses and high systemic concentrations of glucagon are associated with more adverse reactions than smaller doses (177). As such, IP administration of glucagon could theoretically lead to less drug-related side-effects than SC administration. However, IP drug delivery is invasive and associated with a high risk of serious complications, which would likely outweigh this potential benefit.

Studies that have compared the efficiency of continuous IP insulin infusion to continuous SC insulin infusion have demonstrated significantly lower HbA1c concentrations and fewer episodes of severe hyperglycaemia and hypoglycaemia with the IP approach in patients with DM1. The improvement in glycaemic control is most prominent among patients with poorly regulated diabetes (113). It is likely that a bihormonal IP artificial pancreas would only be a feasible treatment option for the subgroup of patients who would profit greatly from IP insulin administration.

5.3.3 Rapid Absorption and Effect of Glucagon Regardless of Administration Route (Paper II)

A rather surprising finding from Paper II is the apparent equal speed of absorption

of glucagon after IP and SC administration. The study demonstrates that absorption of glucagon from SC tissue is fast, with median T_{max} after 10 minutes. Effects on glucose metabolism is also fast, and comparable with the effects observed after IP administration, as described above. As glucagon is a known vasodilator at high dosages (97), this observation led us to hypothesise that glucagon enhances its own SC absorption through local vasodilation.

5.3.4 Vasodilatory Effects of Glucagon (Paper III)

The vasodilatory effects of low doses of glucagon were investigated in a trial by other researchers in APT, which was published in 2022. In this study, injection of 10 μg and 100 μg of glucagon caused a significant increase in local blood flow on the abdomen, as evaluated by laser doppler technology. The median increase in SC blood flow was 250 per cent after injection of 100 μg glucagon (98) and 145 per cent after injection of 10 μg glucagon (unpublished data) (178).

The slow absorption rate of insulin from SC tissue is probably the most important factor that prevents the development of a fully automated artificial pancreas. The rate of SC insulin absorption is positively correlated to SC blood flow (77, 78), and vasodilatory substances are already used as additives in some insulin formulations to promote absorption (60, 61). In Paper III we suggest that micro-doses of glucagon could be administered together with meal-boluses of insulin, as the tubes delivering the two drugs may lie in immediate vicinity. We hypothesise that insulin absorption may be accelerated by glucagon, thus shortening the time until onset of insulin effect in the postprandial state.

5.3.5 Effects of Glucagon on Insulin Absorption (Paper IV)

The hypothesis presented in Paper III was tested in the study presented in Paper IV. In this study 100 μg glucagon or the equivalent volume of placebo (isotonic saline solution) was administered concomitantly with 10 international units of insulin aspart in anaesthetised, hyperglycaemic pigs. The insulin and glucagon/placebo

were delivered via two separate infusion sets, using a guide that secured deposition at the same site.

Absorption of insulin was rapid, and T_{max} of insulin was reached after median 30 minutes in both the intervention group and the control group. As such, the hypothesis from Paper III could not be verified. However, total insulin absorption, as evaluated by $AUC_{0\text{-last}}$, was significantly larger in the glucagon group over the total observation period, which was three hours long.

In healthy subjects, the blood glucose returns to preprandial concentrations within two to three hours after a meal (179). Better overall absorption of insulin, and hence more available insulin in the postprandial period may possibly translate to improved glucose control. Clinical studies that investigate the effects of low-dose glucagon on postprandial insulin and glucose concentrations in subjects with DM1 are needed to investigate this further.

5.3.6 Concerns Related to Using Glucagon to Promote Insulin Absorption (Paper III and Paper IV)

A logical objection to using glucagon to enhance insulin absorption is that glucagon exerts antagonistic effects to insulin on glucose metabolism and may increase blood glucose concentrations, which is highly undesirable in the postprandial state. The hyperglycaemic potential of glucagon is demonstrated clearly in the study presented in Paper IV, where blood glucose concentrations were significantly higher in the glucagon treated group compared to the placebo group the first 30 minutes following the insulin infusion.

In artificial pancreas devices that use glucagon to prevent hypoglycaemia, glucagon seems to be most effective in situations with low systemic insulin concentrations (180-182). In the study in Paper IV, the pigs had low levels of circulating insulin at the time of glucagon infusion, as their endogenous insulin secretion had been supressed with octreotide. It is therefore not surprising that glucagon had such a

pronounced, albeit temporary hyperglycaemic effect.

There is evidence supporting meal-induced glucagon release from the intestine in subjects with DM1 (82, 83, 183-185). This glucagon release appears within minutes of oral glucose or food ingestion. Glucagon originating from the intestines is drained directly via the portal circulation to the liver, where it might increase glucose output. A fully automated artificial pancreas would have to identify a meal through increasing SC glucose concentrations before initiating a meal-insulin bolus. If there is a saturable first-pass metabolism of glucagon in the liver, as is hypothesised in Paper I, micro-doses of glucagon administered with meal insulin are less likely to cause independent glucose excursions, as the liver could already be saturated with glucagon from the intestine.

As previously mentioned, the glucagon dosage used in Paper IV (100 μg) caused a significant increase in blood glucose concentrations. We believe the hyperglycaemic effect probably would have been lower in a situation with more circulating insulin and after oral, rather than IV glucose intake. However, using a smaller dosage of glucagon, with less potential for hyperglycaemic side-effects, would still be highly preferable. Systematic studies into whether the vasodilatory effects of glucagon are dose-dependent and at which threshold they occur should be performed before investigating glucagon as a possible method for accelerating insulin absorption in humans.

Glucagon is associated with several non-glycaemic side-effects. In short-term trials with bihormonal artificial pancreata, no serious adverse reactions have been reported. However, mild and moderate side-effects such as nausea, GI-discomfort, skin irritation and headache are common (124, 125, 146-148, 186). If micro-doses of glucagon are sufficient to cause vasodilation, they would probably cause less adverse reactions than therapeutic doses of glucagon. Safety and the occurrence and severity of potential side-effects should be assessed in future human trials.

5.3.7 Interindividual Variation in Glucagon Response

In the study presented in Paper IV we observed marked variation in response to the glucagon-infusion. Two subjects absorbed much more of the administered insulin than the other pigs in the glucagon group, presumably because of better glucagon effect in these individuals. Large variation in glucagon response was also observed in the human study by Åm et al. In this study, injection of glucagon caused increased SC blood flow in most of the study subjects. However, the effect was not observed in seven of the 22 included participants, which were classified as non-responders (98).

The mechanisms causing interindividual variation in glucagon response are currently unknown and should be elucidated in future trials. In the study presented in Paper IV the two pigs displaying the best insulin absorption were both male. This could likely be coincidental. However, findings from animal trials have suggested that oestrogen may induce changes in glucagon secretion and alter sensitivity to glucagon's hyperglycaemic effects (187). Whether oestrogen may also interact with the vasodilative effects of glucagon, and what implications this may have for using glucagon in artificial pancreas devices, for instance in relation to hormonal changes during pregnancy, menstruation, and menopause, should be investigated in future trials. Notably, sex was not identified as a factor causing difference in glucagon response in the trial by Åm at al (98).

5.3.8 Instability of Glucagon and Applicability in an Artificial Pancreas

In Europe, injectable glucagon is currently only available as human glucagon (produced through recombinant DNA-technology), that is stored as a dry powder and reconstituted before use (85). Human glucagon is relatively unstable and may aggregate and form amyloid fibrils rapidly, depending on the solution's temperature and pH-value. According to the manufacturer, reconstituted glucagon solutions should therefore be used immediately. However, there is some evidence indicating that reconstituted human glucagon could be chemically stable for up to seven days

(188, 189). In the studies presented in Paper I, II and IV, the glucagon that was used had been reconstituted on the same day as the infusion. The infusion sets were checked before each drug administration and no blockage or reduced flow was observed.

Having to change glucagon cartridges frequently and risking tube obstruction because of glucagon aggregates, reduces the feasibility of glucagon in artificial pancreas devices. This issue may possibly be overcome by using stable liquid glucagon (Gvoke®) or the glucagon analogue dasiglucagon (Zegalogue®), which were approved for clinical use in USA in 2019 and in 2021, respectively (190, 191). Both stable liquid glucagon and dasiglucagon have been tested in short-term studies with bihormonal artificial pancreata, demonstrating that they can be used to treat and prevent hypoglycaemia by such devices (192, 193).

Questions regarding the vasodilative properties of different glucagon formulations, dosages of glucagon or glucagon analogues to be used, chemical compatibility with different insulin preparations and technical details of the glucagon delivery in an artificial pancreas device are subjects for future animal trials and clinical research in patients with DM1.

5.4 Limitations

5.4.1 Single Analysis of Samples

A considerable limitation of the studies presented in Paper I and Paper II is that the glucagon and porcine insulin ELISA analyses were run in singles, which reduces the precision. In the study presented in Paper II, we regarded this as acceptable, because the short time interval between samples would have made it possible to detect any marked outliers that needed to be reanalysed. No strongly deviating outliers were detected and reanalysed. For the study presented in paper IV we chose to run all samples in duplicate to increase precision.

Notably, the inter-assay variation was much higher in the trial presented in Paper I

than in the trials presented in Paper II and Paper IV. This was likely because multiple engineers had contributed to the ELISA-analyses in the trial presented in Paper I, whereas all analyses were performed by the same engineer in the two last trials.

5.4.2 Factors Affecting Absorption from the IP Cavity

Local degradation or formation of drug-containing loculaments in the IP cavity may have reduced or delayed the absorption of glucagon. Direct drug infusion into the portal vein would have given a more precise estimate of the first-pass metabolism of glucagon, which was the aim of Paper II. However, since intraportal administration would be dangerous and not a viable treatment option in humans, we relied on IP administration to gain information on other relevant aspects for the possible use of glucagon in a bihormonal IP artificial pancreas.

5.4.3 General Anaesthesia

Prolonged anaesthesia is highly unphysiological and may induce changes in circulation and metabolism (194). Pigs undergoing prolonged anaesthesia may accumulate IP fluid, which possibly could influence drug absorption from the IP cavity (195). This phenomenon was encountered as a problem in an early pig trial by APT, and the protocol was changed to include larger (older) animals and reduce the amount of IV fluid administered to the pigs in later trials (196). These adjustments seem to have resolved the issue, and we did not observe pronounced accumulation of IP fluid in the pig experiments presented in this thesis. However, we regard it as likely that the amount of fluid could have been mildly increased.

Narcosis, prolonged supine positioning, and the trauma related to surgical procedures could potentially have affected the pharmacokinetics and pharmacodynamics of both insulin and glucagon during the trials. In addition, four of the drugs that was used in the pig trials (azaperone, thiopental, ketamine, and isoflurane) are known to affect glucose metabolism (197-202).

5.4.4 Repeated Glucagon Administrations

In the study in presented in Paper II, the pigs received three glucagon boluses in one study day. Reduced effectiveness of consecutive doses of glucagon has been observed in healthy volunteers, possibly because of glycogen depletion (203). However, other studies on human subjects with DM1, and a previous pig study conducted by APT, detected no association between glucose response and bolus number (138, 162, 204). In the study presented in Paper II, the pigs received continuous infusions of insulin aspart and glucose solution to promote hepatic glycogenesis and reduce the risk of glycogen depletion.

5.4.5 Small Sample Size

The studies included in this thesis were planned as pilot studies that should be followed by larger, more robustly designed, and preferably human, trials.

In line with the reduction-principle, we chose to use as few animals as possible in the trials based on power-calculations. Paper I, Paper II and Paper IV are based on data from 19, 12 and 12 pigs respectively. Some subjects had to be excluded from the analyses, which reduced the number even further. In summary, all studies included in this thesis have a relatively small sample size, which reduces the confidence level of the results.

6. Concluding Remarks

6.1 Benefits of the Research

The papers included in this thesis are based on animal experiments. Consequently, the findings are not directly transferable to clinical practice. However, in a medium- and long-term perspective, these preclinical studies may contribute to further development of artificial pancreas technology.

The endeavour to develop a bihormonal IP artificial pancreas is controversial because of the inherent invasiveness of the approach. An increase in risk would only be acceptable if it resulted in pronounced benefits for patients in terms of optimised glucose control. Whether the reduction in systemic glucagon exposure that can be achieved through the IP approach provides any benefit for patients is uncertain. One can hypothesise that lower systemic insulin concentrations over time may reduce the risk of cardiovascular diseases. However, extensive studies conducted over many years in a substantial number of patients would be needed to verify this. At present, it seems that only a highly selected subgroup of patients, who cannot achieve reasonable glucose control with a SC artificial pancreas, are likely to be considered candidates for an IP artificial pancreas.

If glucagon can be used as a safe vasodilator that efficiently enhances SC insulin absorption in the postprandial period, it would be a disruptive new indication for the drug. This could potentially be a step towards radically improving the dynamics in SC artificial pancreata, making it possible to “close the loop” and liberate patients from the daily burden of diabetes management. Many aspects regarding the vasodilative properties of glucagon and the possible utilisation of micro-doses of glucagon in SC artificial pancreata remain to be explored. NTNU has filed a patent application related to this research, and some of the senior members of APT are among the inventors. As such, the studies conducted as part of this thesis could spur further clinical trials and ultimately result in a novel artificial pancreas device developed by the APT research group and NTNU.

6.2 Past and Future Perspectives

The discovery of insulin changed DM1 from a deadly diagnosis to a manageable, chronic condition. With insulin, life could literally be injected back into critically ill patients, most of whom were children. It remains as one of the greatest triumphs of modern medicine.

Artificial pancreas technology can potentially improve the quality of life for patients with DM1. However, new innovations often require a high degree of vigilance and devotion from the user to function. The adverse effects of using such devices must be weighed against the possible improvements in glucose control and reduced risk of diabetes-related complications. Reaching a glycaemic target is not synonymous with having a fulfilling and pleasant life. Ultimately, patients may refuse impractical and life-constraining treatments, even if they are medically beneficial. As such, the goal of diabetes research must always be to develop technology and treatment options that empower patients, increase their freedom, and improve their quality of life. With that in mind, I end this thesis with the inspiring words of E. Perry McCullagh, the endocrinologist who first introduced the term artificial pancreas in 1959:

When all the molecules of all the hormones are dissected and all the portions of all their atoms can be placed and replaced at will, what then? There will still be human beings with their proteins, their loves, their fears, their ambitions, their anxieties and their deaths […], let us never forget that we are working for the happiness of human beings (120).

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Appendix: Publications

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ORIGINAL RESEARCH ARTICLE

Pharmacokinetics of Intraperitoneally Delivered Glucagon in Pigs: A Hypothesis of First Pass Metabolism

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Abstract

Background and Objective Artificial pancreases administering low-dose glucagon in addition to insulin have the scope to improve glucose control in patients with diabetes mellitus type 1. If such a device were to deliver both hormones intraperitoneally, it would mimic normal physiology, which may be beneficial. However, the pharmacokinetic properties of glucagon after intraperitoneal administration are not well known. Hence, the current study aims to evaluate the relationship between the amount of intraperitoneally delivered glucagon and pharmacokinetic variables in a pig model.

Methods Pharmacokinetic data was retrieved from experiments on 19 anaesthetised pigs and analysed post hoc. The animals received a single intraperitoneal bolus of glucagon ranging from 0.30 to 4.46 µg/kg. Plasma glucagon was measured every 2–10 min for 50 min.

Results Peak plasma concentration and area under the time–plasma concentration curve of glucagon correlated positively with the administered dose, and larger boluses provided a relatively greater increase. The mean (standard deviation) time to maximum glucagon concentration in plasma was 11 (5) min, and the mean elimination half-life of glucagon in plasma was 19 (7) min.

Conclusions Maximum plasma concentration and area under the time–plasma concentration curve of glucagon increase nonlinearly in relation to the intraperitoneally administered glucagon dose. We hypothesise that the results are compatible with a satiable first-pass metabolism in the liver. Time to maximum glucagon concentration in plasma and the elimination half-life of glucagon in plasma seem independent of the drug dose.

Key Points

After intraperitoneal delivery, the time to peak plasma concentration and the elimination half-life of glucagon are dose-independent.

Peak plasma concentration and area under the time–plasma concentration curve of glucagon increase with increasing intraperitoneal doses of glucagon. The relationship is nonlinear, which could indicate a satiable first-pass metabolism of glucagon in the liver.

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1 Introduction

Glucagon is secreted by the alpha cells of the pancreatic islets of Langerhans and glucagon-positive cells in the gastrointestinal tract [1]. Its main physiological effect is to stimulate hepatic glucose output in order to maintain euglycaemia [2, 3]. In individuals with diabetes mellitus type 1 (DM1), the alpha cells' responsiveness to declining blood glucose concentrations is usually diminished. Consequently, they are susceptible to hypoglycaemia [4–6], which constitutes an important factor hampering optimal glycaemic control in insulin-treated patients with DM1 [5, 7].

Exogenous glucagon exerts the same pharmacodynamic effects as endogenous glucagon [8, 9], and rescue kits with high doses of glucagon, usually 1 mg, for intravenous, intramuscular or subcutaneous injection are commercially available and have been used for decades [10]. Lately, studies have demonstrated the potential benefit of delivering smaller doses of glucagon subcutaneously (SC) to counteract mild or impending hypoglycaemia in patients with DM1 [11–13].

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Likewise, there is some evidence to support that the addition of glucagon leads to improved glucose control when utilised in SC bihormonal artificial pancreases, i.e. devices that both continuously monitor glucose levels and deliver insulin and glucagon subcutaneously [14–17].

Both insulin and glucagon are normally drained through the portal vein system after secretion from the pancreas. Thus, they reach the liver, a major site of action for both, before systemic circulation. An artificial pancreas delivering these hormones intraperitoneally (IP) could mimic normal physiology, as drugs administered IP are predominantly absorbed by the mesenteric vessels that empty in the portal vein [7, 18]. Insulin pumps with IP delivery have been tested on humans with some success [19, 20]. In contrast, the effects of IP-administered glucagon have only been studied in a few animal trials [21–24]. In a previous study on rats, our research group observed accelerated onset of action of glucagon after IP compared to subcutaneous (SC) administration [22]. In another study on pigs, we demonstrated superior glucose response after IP delivery compared to SC administration [21]. However, both of these studies focussed on the pharmacodynamics of IP glucagon. The pharmacokinetics of glucagon were not properly investigated.

A thorough knowledge of the pharmacokinetics of IP-delivered glucagon is essential for tailoring bolus sizes and administration intervals, and for designing control algorithms predicting the effects of glucagon on glucose levels in an IP bihormonal artificial pancreas. This knowledge is currently missing. Hence, the present study aims to determine the possible relationship between the size of a single IP-administered glucagon bolus and the following variables:

I. The maximum glucagon concentration achieved in plasma (C_{max}).
II. The total drug exposure, as measured by area under the plasma glucagon time-concentration curve from 0 to the last measured time point ($AUC_{0–last}$)
III. The time it takes to reach maximum plasma concentration of glucagon (T_{max}).
IV. The elimination half-life of glucagon in plasma ($T_{½}$) after peak plasma concentration is reached.

2 Methods

2.1 Data Collection

Data for this study were retrieved from four series of experiments conducted by our research group (Artificial Pancreas Trondheim, APT) between 12 April 2018 and 16 January 2020 [21]. In some experiments, the doses were fixed at 12, 50, 75 or 150 µg rather than based on weight. Thus, the total amount of glucagon delivered per kg bodyweight varied considerably. Pharmacokinetic parameters were not included in the original protocols, and, as such, were analysed post hoc. Some of the included pigs had received small doses of insulin prior to their glucagon bolus. Insulin, in contrast to glucagon, lowers blood glucose, and, as such, pharmacodynamic aspects could not be reliably assessed [9]. We have not found evidence of any pharmacokinetic drug–drug interactions between insulin and glucagon.

2.2 Animals and Animal Handling

In total, 19 pigs (8 males, 11 females) were included. Mean (SD) weight was 42.7 (5.3) kg. All the pigs were acquired from the same local supplier at around 12 weeks of age and brought to the research facility approximately 1 week in advance of the experiment to be acclimatised to the surroundings. They were fed commercial compound feed twice a day and provided with water without restriction. Food was removed in the evening approximately 10 h before the start of the experiments.

2.3 Experiment Protocols

2.3.1 Premedication and Anaesthesia

The pigs were premedicated before intubation with an intramuscular injection of either diazepam (Stesolid®; Actavis Group, Hafnarfjörður, Iceland) + azaperone (Stresnil®; Eli Lilly Regional Operations, Austria) + ketamine (Ketalar®; Pfizer, Norway), xylazine (Xysol®; CP-Pharma Handelsges, Germany) + ketamine or midazolam (Accord Healthcare, Harrow, UK) + ketamine. Anaesthesia was induced by an intravenous (IV) infusion of fentanyl (Actavis Group) + thiopental (VUAB Pharma, Roztoky, Czech Republic) and maintained by continuous IV infusion of midazolam + fentanyl and inhalation of isoflurane (Baxter, Oslo, Norway). Intubation was eased by an IV infusion of atropine (Takeda, Asker, Norway). The pigs received an IV infusion of the antibiotic cephalothin (Villerton Invest, Luxembourg) after anaesthesia was established. They were euthanised while still under anaesthesia at the end of the study day with an IV infusion of phenobarbital (NAF; Apotek, Lørenskog, Norway).

2.3.2 Suppression of Endogenous Glucagon Secretion

The effectiveness of somatostatin analogue treatment (SAT) has been evaluated in a previously published study by our research group [21]. The pigs were given SAT through either an IV infusion of 0.4 mg octreotide (Sandostatin®; Novartis Europharm, UK) every hour or a continuous IV infusion of 150 µg octreotide per hour to suppress their endogenous glucagon secretion. In addition, the first seven pigs received

a subcutaneous injection of 0.3 mg pasireotide (Signifor®; Novartis Europharm) every third hour. The protocol was changed for later experiments due to a lack of support for an additional benefit of pasireotide.

The effectiveness of SAT in the present study was assessed by comparing the mean plasma glucagon concentration in two consecutive samples drawn before initiation of SAT with the mean of the last two quantifiable glucagon concentrations measured after SAT and before administration of exogenous glucagon. For one pig (pig 14), only one sample had been drawn before initiation of SAT, and, in this case, the single, known value was used. The overall mean (SD) plasma glucagon concentration before SAT was 6.3 (3.3) pmol/L, whereas the mean (SD) before glucagon administration was 4.3 (4.3).

2.3.3 Glucagon Delivery and Blood Sampling

Freshly constituted glucagon (Novo Nordisk, Denmark) was stored at room temperature and delivered via a pump to the peritoneal cavity. The administered IP dose varied between 0.30 and 4.46 µg/kg and was followed by arterial blood sampling every 2–10 min for 50 min. However, for one pig (pig 8), no sample was drawn at 50 min. The last sample used in the analyses for this pig was drawn at 46 min. The samples were stored in ice water for 10 min before centrifugation. Plasma was then transferred to Eppendorf tubes and stored at – 18 °C until the end of the experiment day. Afterwards, the tubes were kept at – 80 °C until analysis. Glucagon was analysed in singles with Glucagon ELISA kits (10-1281-01; Mercodia, Uppsala, Sweden). The inter-assay coefficient of variation was 9–27%.

2.4 Data Analysis

2.4.1 Pharmacokinetic Analysis

The reported concentrations are not baseline corrected, as the baseline concentration of glucagon was missing or not quantifiable in six pigs. C_{max} and T_{max} were obtained directly from the measured values. $T_{½}$ and $AUC_{0–last}$ were estimated using the programme package Simbiology in MATLAB v.R2020B [25]. To calculate $AUC_{0–last}$, the linear trapezoid method was used. For the six pigs where the baseline concentration was unobtainable, the average baseline value from the other 13 pigs was used at time zero in the AUC calculations (mean 4.3 pmol/L, SD 3.9). The terminal rate constant (λ_z), describing the decrease of the log-concentration of glucagon, was calculated by applying a best-fit linear regression to the terminal portion of the curve. The elimination half-life was calculated as $\ln 2/\lambda_z$. In three pigs (nos. 3, 4 and 6), no apparent elimination of glucagon after C_{max} was observed, causing a negative value for $T_{½}$. These pigs were therefore excluded from the half-life analysis.

2.4.2 Statistical Analysis

Statistical analysis was performed using GraphPad Prism v.9 [26]. Spearman's rank-order correlation test was used to determine a possible correlation between dose size and pharmacokinetic outcomes. A 95% confidence interval (95% CI) not containing zero was considered significant. Means and standard deviations (SD) and medians and interquartile ranges (IQR) of T_{max} and $T_{½}$ were calculated. Possible outliers were identified through the ROUT method [27], using a q value of 0.01. Normality and log-normality were tested using the D'Agostino and Pearson test [28]. Correlation analyses were performed on non-transformed data. Because of the log-normal distribution, a logarithmic transformation was performed before linear regression analysis in order to make equations for C_{max} and $AUC_{0–last}$.

3 Results

Most of the pigs displayed a prominent rise and subsequent fall in glucagon concentrations after IP drug administration (Fig. 1), although some deviated from this pattern (Fig. 2).

Without correction, the mean (SD) T_{max} was 13 (8) min, and the median value (IQR) of T_{max} was 12 (8–15) min. After removal of two identified outliers [pig 5 (35 min) and pig 15 (32 min)], the mean (SD) was 11 (5) min, whereas the median (IQR) was 10 (8–15) min.

Simple linear regression after logarithmic transformation provided the following equation (Eq. 1) for C_{max} (Fig. 3):

$$C_{max} = e^{3.01+(0.26 \cdot \text{glucagon dosage})} \quad (1)$$

The 95% CIs of the intercept and slope were 2.54–3.47 and 0.06–0.46, respectively.

Simple linear regression after logarithmic transformation provided the following equation (Eq. 2) for $AUC_{0–last}$ (Fig. 4):

$$AUC_{0-last} = e^{6.40+(0.27 \cdot \text{glucagon dosage})} \quad (2)$$

The 95% CIs of the intercept and slope were 5.94–6.86 and 0.08–0.47, respectively.

Without correction, the mean (SD) $T_{½}$ was 23 (16) min, and the median value (IQR) of $T_{½}$ was 19 (14–22) min. After removal of one identified outlier [pig 8 (77 min)], the mean (SD) was 19 (7) min, whereas the median (IQR) was 19 (13–22) min.

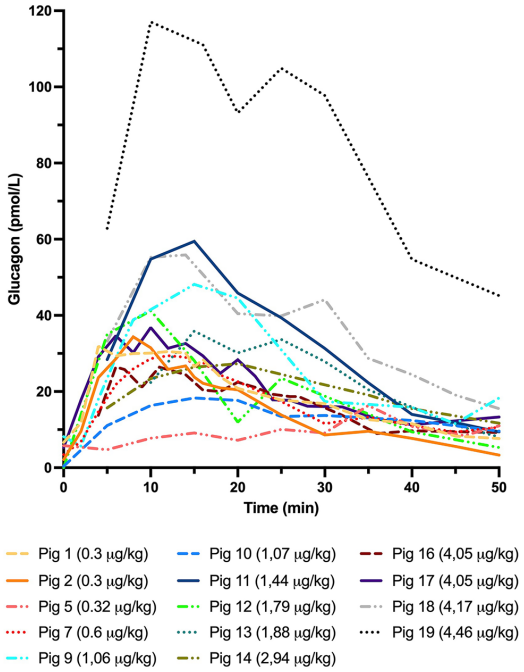

Fig. 1 A total of 14 of the 19 pigs demonstrated a rise and fall in glucagon concentration after intraperitoneal drug administration

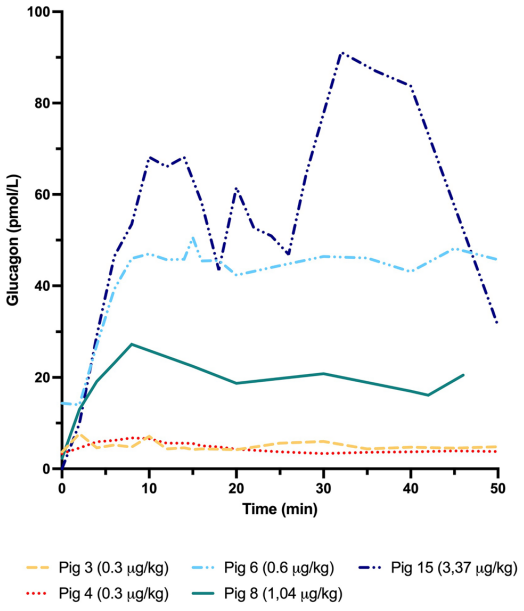

Fig. 2 Five of the 19 pigs displayed a deviating pattern without a consistent rise and fall in glucagon after intraperitoneal drug administration

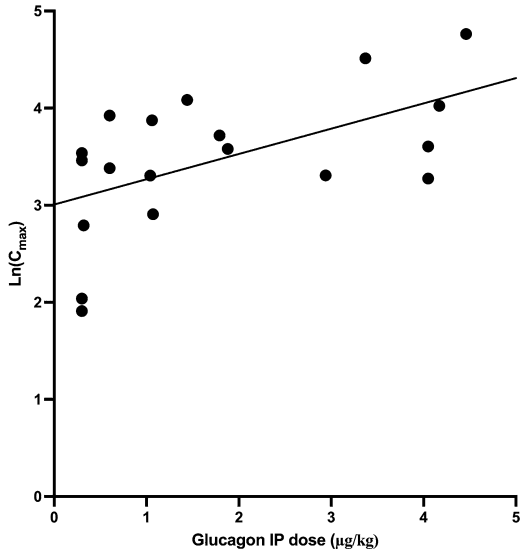

Fig. 3 Logarithmic values of the maximum plasma concentration (C_{max}) of glucagon in all pigs. The *regression line* represents our model

T_{max} did not correlate with the glucagon dose, with a Spearman's rank-order correlation coefficient (ρ) (95% CI) of 0.37 (− 0.14 to 0.73). Maximum plasma concentration

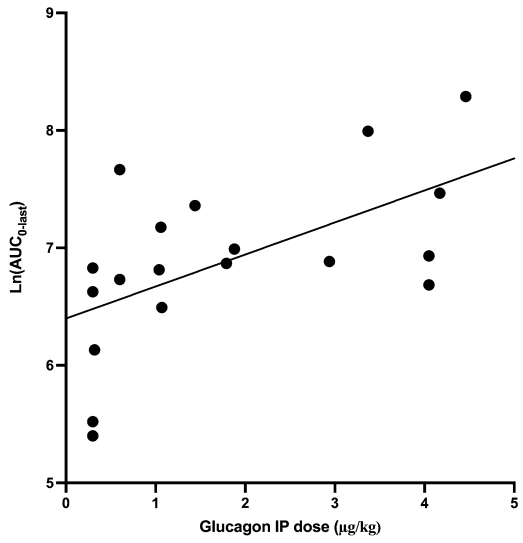

Fig. 4 Logarithmic values of the area under the time–plasma concentration curve from 0 to the last measured time point in min ($AUC_{0\text{-last}}$) for all pigs. The *regression line* represents our model

was significantly correlated with dose size, with a ρ of 0.56 (0.13–0.81). AUC_{0-last} was significantly correlated to dose size, with a ρ of 0.65 (0.26–0.86). The elimination half-life of plasma glucagon did not correlate with dose size, with a ρ of − 0.07 (− 0.57 to 0.47).

4 Discussion

The present analysis supports that there is a relationship between IP glucagon dosage and C_{max} (Eq. 1) and AUC_{0-last} (Eq. 2). Both equations are logarithmic, which suggests a linear curve with a breakpoint, where larger doses provide a relatively greater increase than smaller doses. This is compatible with a first-pass effect of glucagon in the liver that larger doses could saturate, as is observed with insulin [29, 30]. Because only a few or just one pig received the same dose, we cannot exclude the possibility that the findings are due to inter-individual variation. However, if a considerable presystemic hepatic metabolism of glucagon truly exists, it could support an IP versus a SC bihormonal artificial pancreas approach, as IP delivery may then lower the amount of glucagon entering the systemic circulation and hence possibly reduce the risk of adverse effects. This adds to our previous observation that, compared to SC glucagon delivery, half the dose given IP will achieve the same glucose-increasing effect [21]. More extensive, controlled studies with standardised doses are needed to investigate this further.

A majority of the pigs had a fairly consistent rise and fall in plasma glucagon concentration within 50 min after IP administration of the drug (Fig. 1). However, five pigs deviated from this pattern (Fig. 2). Pigs 3 and 4 (dosage 0.3 µg/kg) only achieved a minor increase in plasma glucagon concentrations compared to their baseline values, and clear elimination could not be observed after C_{max}. The curves of pigs 6 (dosage 0.6 µg/kg) and 8 (dosage 1.04 µg/kg) also flattened after their peak, strongly affecting the observed half-life. Pig 15 (dosage 3.37 µg/kg) had a second, more prominent peak after the fall following the first peak. There are several possible explanations for this inconsistency, other than a genuine inter-individual variation in absorption and metabolism of IP-delivered glucagon, among which we consider the following most plausible:

I. The IP tubes delivering glucagon could have been blocked.
II. The suppression of endogenous glucagon could have been insufficient at some point during the experiments.
III. IP fluid may have accumulated during anaesthesia, possibly causing dilution and altered absorption of the delivered drug [31, 32].
IV. Loculaments of glucagon-containing fluid could have been formed within the intraperitoneal cavity making them less available for absorption. They may later have dissolved, explaining a second peak.

T_{max} and $T_{½}$ did not seem to differ in relation to glucagon dosage. However, because of the short observation time, the values for $T_{½}$ should be interpreted with caution. Other human studies have conveyed comparable or slightly larger values for T_{max} after SC administration of considerably larger doses [33–41]. Although the values are not directly transferable, this may indicate non-superiority of the IP route compared to the SC route in regard to absorption rate to the systemic circulation.

This study is limited by several factors, among which small sample size, unknown baseline values for a considerable number of pigs, large dose variation, single analysis of samples, inconsistency in anaesthetic protocol and relatively short observation times are the most prominent. Larger studies with a more robust design should be conducted to obtain more reliable results. Furthermore, extended trials where the animals may be administered multiple doses of glucagon are needed to increase the knowledge of possible intra-individual variation.

5 Conclusions

The present study indicates a relationship between the IP glucagon bolus size and the maximum plasma concentration and total drug exposure. In contrast, the time to maximum plasma concentration and the elimination half-life of glucagon seem to be independent of the dose. The results could be compatible with a satiable hepatic first-pass effect of IP-delivered glucagon. While further research is needed, if confirmed, this would support the endeavour to develop an IP bihormonal artificial pancreas, as it would reduce the systemic drug load and hence the expected adverse effects of treatment compared to a SC approach.

Declarations

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Author contribution All authors contributed to the conception and design of the study. Marte K. Åm was responsible for the conduction of animal experiments and data collection. Ingrid Anna Teigen analysed and interpreted the data and wrote the first draft of the manu-

script. All authors reviewed and commented on previous versions of the manuscript. All authors have read and approved the final version of the manuscript.

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Conflict of Interests The authors declare no conflict of interests.

Ethics Approval The Norwegian Food Safety Authority pre-approved all the trials from which the analysed data were gathered (FOTS number 12948). The experiments were conducted in accordance with the "Norwegian Regulation on the Use of Animals in Research" and the 2010/63 EU directive on the "Protection of Animals Used for Scientific Purposes".

Data Availability Statement Data supporting the findings are available from the corresponding author upon request.

Code Availability Not applicable.

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ORIGINAL ARTICLE

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Pharmacokinetics of glucagon after intravenous, intraperitoneal and subcutaneous administration in a pig model

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Abstract

Introduction: There is increasing scientific evidence to substantiate using low-dose glucagon as a supplement to insulin therapy in artificial pancreata for diabetes mellitus type 1. The delivery of both these hormones intraperitoneally would mimic normal physiology. However, our knowledge of the pharmacological properties of glucagon after intraperitoneal administration is limited. This study compared the pharmacokinetics of glucagon after intraperitoneal, subcutaneous and intravenous administration and the pharmacodynamic effects of glucagon on glucose metabolism after intraperitoneal and subcutaneous administration in a pig model.

Materials and methods: Twelve pigs were included. Glucagon was administered intraperitoneally, subcutaneously and intravenously in a randomised order. Arterial samples were collected every 2–10 min for 150 min to determine plasma glucagon and blood glucose concentrations.

Results: The bioavailability of glucagon was significantly lower after intraperitoneal compared with subcutaneous administration with a median difference (95% confidence interval) of 13% (4–22). The effect of glucagon on glucose metabolism was equal after intraperitoneal and subcutaneous administration.

Conclusions: Intraperitoneal glucagon administration resulted in lower systemic glucagon exposure than subcutaneous administration without loss of efficiency. We interpret this as evidence of a major first-pass metabolism of glucagon in the liver.

KEYWORDS
artificial pancreas, diabetes mellitus type 1, glucagon, intraperitoneal infusion, pharmacokinetics

List of abbreviations: 95% CI, 95% confidence interval; $AUC_{0\text{-last}}$, area under the time-plasma concentration curve from time zero to time to last measurable concentration; C_{max}, maximum plasma concentration; DM1, diabetes mellitus type 1; HbA1c, glycated haemoglobin; IP, intraperitoneal; IQR, interquartile range; IV, intravenous; SC, subcutaneous; SD, standard deviation; $T_{1/2}$, plasma elimination half-life; T_{last}, time to last measurable concentration; T_{max}, time to maximum plasma concentration.

1 | INTRODUCTION

Glucagon increases hepatic glucose output in response to declining blood glucose concentrations in healthy individuals.[1] Most patients with diabetes mellitus type 1 (DM1) have a malfunctioning glucagon regulation, making them susceptible to hypoglycaemia.[2] Exogenous glucagon exerts the same pharmacodynamic effects on glucose metabolism as endogenous glucagon, and utilisation of low-dose glucagon to prevent and reverse mild hypoglycaemia has been investigated with some success, both as single subcutaneous (SC) injections and as part of SC artificial pancreata.[3–6]

After being secreted by the pancreas, insulin and glucagon are drained via the portal vein through the liver, the main action site for both hormones, before entering the systemic circulation. An artificial pancreas with intraperitoneal (IP) drug delivery could mimic this pathway, as drugs administered IP are absorbed predominantly by the splenic and mesenteric vessels that empty into the portal vein.[7] Unihormonal pumps administering solely insulin IP have demonstrated superiority over SC pumps in reducing glycated haemoglobin (HbA1c) and hypoglycaemic/hyperglycaemic episodes in humans.[8] In contrast, the pharmacological properties of IP delivered glucagon have only been studied in a few small-sized animal trials.[9–13]

To our knowledge, no comparative pharmacokinetic studies of intravenously (IV), SC, and IP delivered glucagon have been published. Thus, we investigated possible differences in glucagon pharmacokinetics based on the route of administration using a pig model. The primary aim of this study was to investigate the bioavailability of glucagon after IP administration, potentially providing evidence for our previously published hypothesis of extensive first-pass metabolism of glucagon in the liver.[12] Other relevant pharmacokinetic parameters, such as maximum plasma concentration (C_{max}), time to C_{max} (T_{max}), time to last measurable concentration (T_{last}), plasma elimination half-life ($T_{1/2}$) and area under time-plasma concentration curve from time zero to T_{last} ($AUC_{0\text{-}last}$), after IV, IP and SC glucagon administration were evaluated as secondary outcomes. The pharmacodynamic effects of glucagon on glucose metabolism after IP and SC administration were also analysed.

2 | METHODS

2.1 | Study design

The study was preapproved by the Norwegian Food Safety Authorities (FOTS number 12948), and it complied with the "Norwegian Regulation on the Use of Animals in Research" and the 2010/63 EU directive on the "Protection of Animals Used for Scientific Purposes". The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies.[14]

We conducted a randomised, open-label, cross-over trial. To detect at least a 60% reduction in bioavailability (after IP compared with IV administration) with a power of 80% and an alpha value of 0.05, we included 12 female, non-diabetic farm pigs (*Sus scrofa domesticus*).

Each pig received three separate boluses of 1.5 μg/kg glucagon over a single study day: One bolus was administered in the left internal jugular vein, one bolus was delivered via a pump to the IP space in the upper left quadrant of the abdomen, and one bolus was administered via a pump to the SC adipose tissue behind the left ear. Both the IP and the SC boluses were delivered with an infusion speed of 100 μg/min. A 1.5 μg/kg glucagon dosage was chosen to correspond to a realistic dosage in artificial pancreata for humans, that is, 75–150 μg for individuals weighing 50–100 kg. The order of the bolus administrations was decided through a simple block randomisation procedure, with two pigs randomly allocated to each possible order.

2.2 | Animals and animal handling

All pigs were acquired from the same local supplier at approximately 12 weeks of age. Mean (standard deviation [SD]) weight was 45.4 (6.5) kg.

The animals were monitored continuously during the experiments. All surgical procedures, drug administrations and blood samplings were performed under general anaesthesia.

2.3 | Study procedure

2.3.1 | Premedication and anaesthesia

The pigs were premedicated before intubation with an intramuscular injection of 10 mg/kg azaperone (Separon vet.l®, Richter Pharma AG, Austria) and 10 mg/kg ketamine (Ketalar®, Pfizer AS, Norway) and an IV infusion of 1 mg atropine (Takeda AS, Asker, Norway). Anaesthesia was induced by an IV infusion of 150–250 μg fentanyl (Actavis Group, Hafnarfjörður, Iceland), 75–125 mg thiopental (VUAB Pharma AS, Roztoky, Czech Republic) and 150–250 mg ketamine. Anaesthesia was maintained by continuous IV infusion of 0.5 mg/kg/h midazolam (Accord Healthcare Limited, Middlesex, UK) and 7.5 μg/kg/h

fentanyl, together with continuous inhalation of 0.5%–2% isoflurane (Baxter AS, Oslo, Norway). An IV infusion of 2 g cephalothin (Villerton Invest SA, Luxembourg) was given as antibiotic prophylaxis immediately after establishing anaesthesia and repeated every third hour. The pigs were euthanised while still under general anaesthesia at the end of the study day with an IV infusion of 100 mg/kg phenobarbital (NAF, Apotek, Lørenskog, Norway).

2.3.2 | Suppression of endogenous glucagon and insulin secretion

To suppress endogenous glucagon and insulin secretion, the pigs received an initial IV bolus of 5 μg/kg octreotide (Sandostatin®, Novartis Europharm Limited, United Kingdom), followed by continuous IV infusion of 5 μg/kg/hour octreotide throughout the study day. Octreotide treatment was initiated 1 h before the first glucagon bolus. The concentration of porcine insulin was measured before and after every glucagon bolus to monitor the efficiency of suppression.

2.3.3 | Insulin and glucose infusions

The pigs received separate continuous IV infusions of 0.05 IU/kg/hour insulin aspart (NovoRapid®, Novo Nordisk AS, Denmark) and 20% glucose solution (Glucos B. Braun®, Braun, Germany) throughout the study day to prevent glycogen depletion. Before each glucagon bolus, blood glucose concentration was titrated to a normoglycaemic target concentration of 4–5 mmol/L by adjusting the glucose infusion rate. According to the study protocol, the blood glucose concentration had to be stable (no more than 0.2 mmol/L variations) before every glucagon administration, without any glucose infusion rate adjustments for 20 min. The glucose infusion rate was also kept stable for the first 60 min after glucagon administration to monitor the effects on glucose metabolism.

2.3.4 | Blood sampling

Arterial blood samples were drawn immediately before each glucagon bolus, and subsequently every 2 min for the first 40 min, every 5 min for the next 60 min, and every 10 min for the remaining 50 min. We set the observation time to 150 min to ensure a wash-out period of more than five estimated $T_{1/2}$ between each bolus.[12,15]

2.3.5 | Sample handling and analysis

Arterial blood glucose concentrations were analysed immediately after collection with a Radiometer ABL 800 FLEX blood gas analyser. The inter-assay coefficient of variation was below 5%.

After centrifugation, arterial plasma samples for hormone analyses were stored at −18°C for the duration of the experiment, and later at −80°C until analysis. Glucagon concentrations were measured using Glucagon ELISA kits (Mercodia, Uppsala, Sweden), and porcine insulin concentrations were measured using Porcine insulin ELISA kits (Mercodia, Uppsala, Sweden). Both glucagon and porcine insulin samples were run in singles. All glucagon and porcine insulin ELISA kits were from the same batches. The analyses were performed in the same setup by the same engineer. Both intra-assay and inter-assay variation were below 10% for glucagon and below 5% for porcine insulin.

2.4 | Data analysis

2.4.1 | Pharmacokinetic analysis

C_{max}, T_{max} and T_{last} were obtained directly from the measured glucagon concentrations in plasma after correcting for baseline concentrations. $T_{1/2}$ and $AUC_{0\text{-last}}$ were estimated using Simbiology in MATLAB version R2020B.[16] The terminal rate constant, describing the decrease of the log-concentration of glucagon, was calculated by applying a best-fit linear regression to the terminal portion of the curve. $T_{1/2}$ was calculated as $\frac{\ln 2}{\text{terminal rate constant}}$. $AUC_{0\text{-last}}$ was calculated by using the linear trapezoidal method.

2.4.2 | Pharmacodynamic analysis

The glucose concentrations were baseline-corrected. Only IP and SC administrations were included in the pharmacodynamic analyses, as this was done primarily to verify results from a previous, smaller pig study.[13] Furthermore, the IV route is currently only used in emergency and in-hospital settings and is not feasible for artificial pancreata.

2.4.3 | Statistical analysis

Statistical analysis was performed using GraphPad Prism version 9.[17] Non-parametric tests were chosen because of

the small sample size and skewed distribution of some variables.

Medians and interquartile ranges (IQR) plus means and SD of T_{max}, C_{max}, $AUC_{0\text{-last}}$, $T_{1/2}$ and T_{last} were calculated. Possible differences in T_{max}, C_{max}, $AUC_{0\text{-last}}$, $T_{1/2}$ and T_{last} in relation to administration routes were examined using the Friedman test. Correction for multiple analyses was performed using Dunn's multiple comparison test.

Bioavailability was assessed by comparing $AUC_{0\text{-last}}$ after IV administration with $AUC_{0\text{-last}}$ after IP and SC delivery. Median and mean bioavailability for the IP and SC route was calculated. Possible differences in bioavailability after IP and SC administration were examined using the Wilcoxon matched-pairs signed-rank test.

Differences in blood glucose concentrations over time after IP and SC administration was analysed using a mixed-effects analysis with time and administration route as fixed effects and the subject number as a random effect. Correction for multiple analyses was performed using Šídák's multiple comparison test.

3 | RESULTS

3.1 | Exclusions

The following pigs were excluded from the analyses to maintain pairing:

Pig 1 was excluded from all analyses because we suspected that the SC catheter had not penetrated the skin properly upon removing it. No increase in blood glucose concentration was observed after this bolus, and only a minimal increase in plasma glucagon concentration was noted between 38 and 45 min after drug administration, which could be due to percutaneous absorption.

Pig 11 was excluded from only the pharmacodynamic analyses because the glucose infusion set was blocked shortly before SC glucagon administration, which led to a marked drop in the blood glucose concentration at baseline. It was kept in the pharmacokinetic analyses as we regard it unlikely that this temporary drop in glucose concentration, which was corrected within the following 2 min, would have affected glucagon metabolism.

Pig 12 was excluded from all analyses as it had abdominal adhesions and gross vascular anomalies compatible with previously advanced peritonitis, which would likely affect IP drug absorption. A veterinary surgeon confirmed the diagnosis on site.

3.2 | Suppression of endogenous insulin and glucagon secretion

The plasma glucagon concentration was below the quantification cut-off limit of 1.95 pmol/L before the first glucagon bolus in all included pigs and porcine insulin was below the quantification cut-off of 2.3 mU/L in all included pigs, except one (Pig 5, 3.6 mU/L). The porcine insulin concentrations remained low throughout the experiments and were below the quantification cut-off limit in 53 out of 60 samples in the included pigs. The mean (SD) porcine insulin concentration was 3.8 (0.8) mU/L in the remaining seven samples.

3.3 | Pharmacokinetic parameters

Ten pigs were included in the pharmacokinetic analyses. Pharmacokinetic findings are summarised in Table 1. Mean glucagon concentration with their respective 95% confidence intervals (95% CI) at every time point for each administration route is presented in Figures 1 and 2. Plasma glucagon concentration over time after IP and SC administration for each pig is presented in Figure 3. The concentrations were baseline-corrected. Plasma glucagon concentration was below the quantification cut-off limit of 1.95 pmol/L at baseline in 24 of 30 included boluses. For these boluses, the baseline concentration was set at 0 pmol/L. The mean baseline (SD) concentration was 4.0 (1.7) pmol/L in the six remaining boluses.

Glucagon concentrations increased rapidly in plasma regardless of administration route. Median (IQR) T_{max} was 2 (2–2), 13 (10–18) and 10 (8–12) min after IV, IP and SC administration, respectively, and mean (SD) T_{max} was 2 (0), 15 (8) and 10 (4) min. T_{max} was significantly lower with IV administration as compared with both IP and SC administration (p value < 0.001 and <0.01, respectively). There was no significant difference in T_{max} between IP and SC administration (p value > 0.99).

Median (IQR) C_{max} was 1735 (393–1965), 7 (5–10) and 31 (14–59) pmol/L after IV, IP and SC administration, respectively, and mean (SD) C_{max} was 1372 (790), 8 (6) and 37 (24) pmol/L. C_{max} was significantly higher with IV administration than with IP administration (p value < 0.0001). No significant difference in C_{max} was observed when we compared IV and SC or IP and SC administration (p value 0.08 for both comparisons).

Median (IQR) $AUC_{0\text{-last}}$ was 7670 (2389–8600), 163 (87–235) and 991 (426–1359) pmol/L/min after IV, IP and SC administration, respectively, and mean (SD) $AUC_{0\text{-last}}$ was 6476 (3383), 161 (79) and 961 (540) pmol/L/min. $AUC_{0\text{-last}}$ was significantly larger with IV administration than with IP administration

TABLE 1 Pharmacokinetic parameters

Pharmacokinetic Parameter	Intravenous Administration	Intraperitoneal Administration	Subcutaneous Administration
Time to maximum plasma concentration (minutes)	2 (2–2)	13 (10–18)	10 (8–12)
Maximum plasma concentration (pmol/L)	1735 (393–1965)	7 (5–10)	31 (14–59)
Area under the time-plasma concentration curve from time zero to time to last measurable concentration (pmol/L/min)	7670 (2389–8600)	163 (87–235)	991 (426–1359)
Plasma elimination half-life (minutes)[a]	6 (3–8)	10 (8–12)	15 (9–18)
Time to last measurable concentration (minutes)	48 (44–61)	41 (32–59)	68 (50–81)
Bioavailability (percentage)[b]	—	3 (2–5)	16 (9–22)

Note: Data are reported as medians (interquartile range) when not stated otherwise.
[a]7 animals included.
[b]Values are stated as median (95% confidence interval).

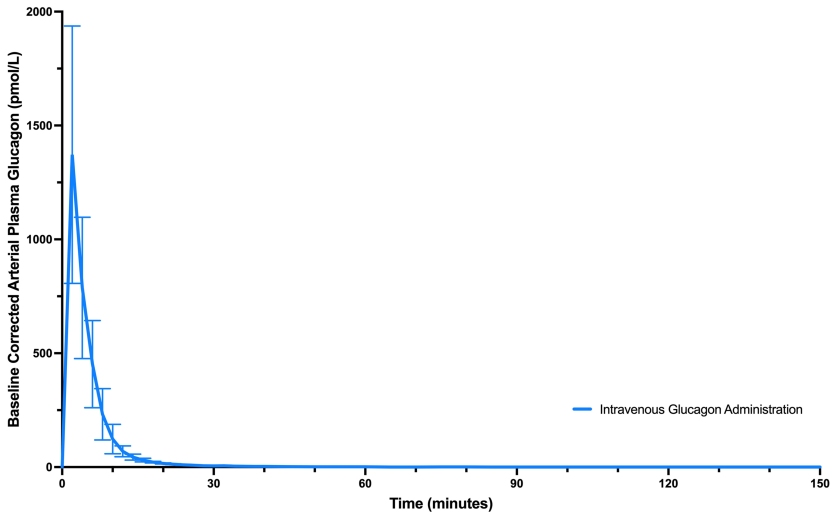

FIGURE 1 Mean glucagon concentrations in arterial plasma (with 95% confidence interval [CI] error bars) over time after intravenous administration

(p value < 0.0001). No significant difference in $AUC_{0\text{-last}}$ was observed when we compared IV and SC or IP and SC administration (p value 0.08 for both comparisons).

Irregular elimination patterns after IP administration of glucagon in Pigs 2 and 3 and after SC administration of glucagon in Pig 5 prevented reliable calculation of $T_{1/2}$ from these. Therefore, these three pigs were excluded from the $T_{1/2}$ analysis. Median (IQR) $T_{1/2}$ after IV, IP and SC administration was 6 (3–8), 10 (8–12) and 15 (9–18) min, respectively, whereas the mean (SD) was 9 (10), 13 (8) and 13 (5) min. No significant differences between administration routes were observed (p value > 0.10 for all comparisons).

Plasma glucagon concentrations returned to baseline within 150 min after all boluses except after IV and SC administration in Pig 5. Median (IQR) T_{last} was 48 (44–61), 41 (32–59) and 68 (50–81) min, respectively, whereas mean (SD) T_{last} was 59 (34), 45 (20) and 74 (30) min. No significant differences between administration routes were observed (p value > 0.10 for all comparisons).

Median (IQR) bioavailability was 3% (2–5) and 16% (9–22) with IP and SC administration, respectively, and the mean (SD) bioavailability was 3% (2) and 22% (26). The bioavailability of glucagon was significantly lower after IP compared with SC administration (p value 0.002), with a median difference (95% CI) of 13% (4–22).

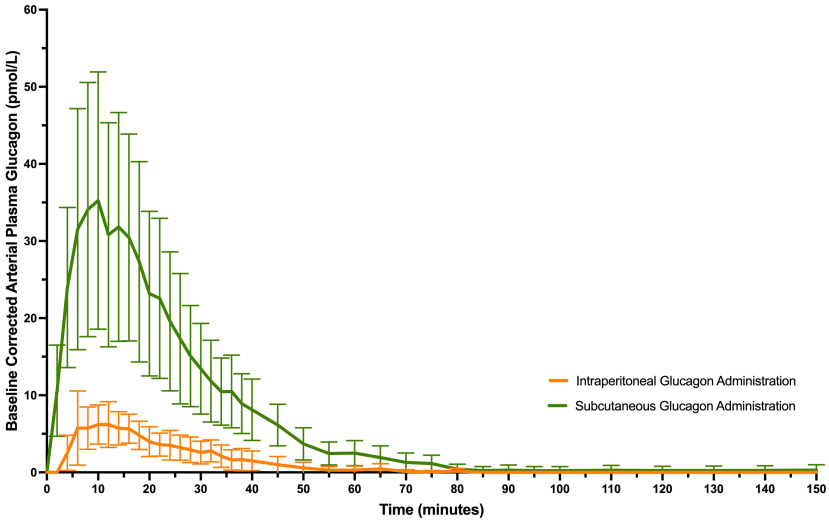

FIGURE 2 Mean glucagon concentrations in arterial plasma (with 95% confidence interval [CI] error bars) over time after intraperitoneal and subcutaneous administration

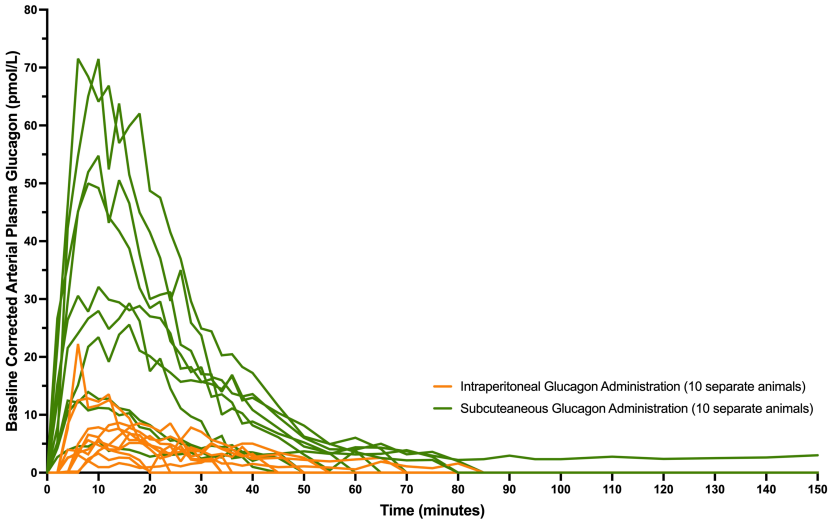

FIGURE 3 Glucagon concentrations in arterial plasma over time for all included pigs after intraperitoneal and subcutaneous administration

The bioavailability remained significantly different (p value 0.004) also after excluding Pig 11, which was excluded from the pharmacodynamic analysis, with a median difference (95% CI) of 13% (4–22).

3.4 | Pharmacodynamic parameters

Nine pigs were included in the pharmacodynamic analyses. Mean blood glucose concentrations with their respective 95% CI between 0 and 60 min after IP and SC glucagon administration are presented in Figure 4. The concentrations were baseline corrected. The mean (SD) blood glucose concentration at baseline was 4.6 (0.3) mmol/L for the IP boluses and 4.3 (0.4) mmol/L for the SC boluses.

There was no significant difference (95% CI including 0 and p value > 0.90) in mean blood glucose concentration after IP and SC administration at any point in time (Figure 3).

4 | DISCUSSION

Despite having significantly lower bioavailability, IP administered glucagon exerted comparable effects on

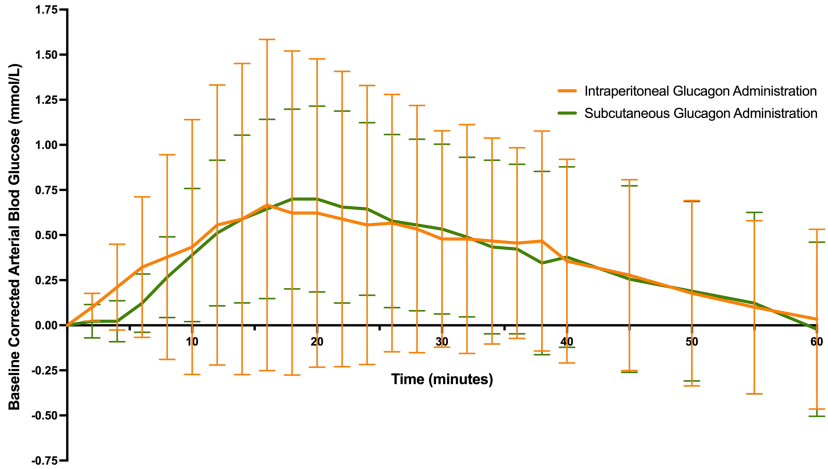

FIGURE 4 Mean glucose concentrations in arterial blood (with 95% confidence interval [CI] error bars) over time after intraperitoneal and subcutaneous administration

glucose metabolism as SC administered glucagon in the present study. A major first-pass metabolism of glucagon in the liver is the most likely explanation for this observation. In theory, this could be advantageous because high systemic concentrations of glucagon are associated with more pronounced adverse reactions.[18]

The observed equivalent effect on glucose metabolism after IP and SC glucagon administration contradicts the findings of two animal studies previously performed by our research group, Artificial Pancreas Trondheim. In those studies, IP administration was associated with either a faster[9] or a more pronounced[13] glucose response. In the previous studies, we used smaller glucagon doses, and it is possible that we saturated the liver's ability to increase glucose output regardless of administration route by administering a larger dose in the present study. However, the former pig study, which demonstrated superior glucose elevating effect through IP administration, only detected this difference upon excluding four of 10 originally included pigs. Moreover, there was no difference in total glucose elevation over time, as measured by AUC.[13] This indicates that there might not be a genuine difference in response between the two administration routes. A study examining the effects of glucagon at different doses is needed to investigate this further.

In the same pig study mentioned above, blood glucose concentrations increased more than in the present study, despite using a smaller glucagon dosage (0.6 μg/kg).[13] We believe this could be because the pigs in the previous study received no insulin during the study day, as insulin and glucagon exert antagonistic effects on glucose metabolism in the liver. Although an artificial pancreas would likely reduce and ultimately discontinue insulin infusion as a response to hypoglycaemia, a situation where there is no circulating insulin is highly unrealistic. As such, the results from our present study could be more translatable to a real-life scenario.

As expected, the initial peak in plasma glucagon concentration occurs later after SC and IP administration compared with IV administration. The first sample was drawn 2 min after the drug was administered. Therefore, the exact values of C_{max} and T_{max} after IV infusion are probably unknown. Following IP and SC administration, absorption is rapid, and T_{max} is reached at a similar pace.

There was no significant difference in C_{max} and $AUC_{0\text{-last}}$ between IP and SC administration. However, there was a tendency towards a difference, with a p value of 0.08 for both parameters after correcting for multiple comparisons. It is possible that the study became underpowered to detect any difference, as we had to exclude two pigs from the analyses, and that a larger study would have conveyed a different result.

There were no significant differences in $T_{1/2}$ between the different routes of administration. However, this result should be interpreted with caution because a stable elimination of glucagon from plasma was not observed after three administrations, which prevented a reasonable calculation of $T_{1/2}$ from these boluses. This could possibly be due to prolonged absorption of glucagon from the administration site, temporary failure of suppression of endogenous glucagon secretion or interindividual variation in metabolism.

Reduced systemic drug exposure after IP administration compared with SC administration could make the IP route preferable in a bihormonal artificial pancreas. However, IP drug delivery is invasive and associated with a high risk of serious complications. As such, it would only be an acceptable route of administration if it were to prove greatly advantageous in terms of glucose control.

Continuous IP insulin infusion is associated with lower HbA1c and fewer episodes of hyperglycaemia and hypoglycaemia than continuous SC insulin infusion in patients with DM1. Notably, the circulating insulin concentrations are also significantly reduced after IP administration, probably because of the large first-pass metabolism of insulin in the liver.[19] As hyperinsulinemia has been linked to the pathogenesis of cardiovascular diseases, reducing insulin in systemic circulation could hypothetically result in improved long-term health outcomes for patients with DM1.

4.1 | Limitations

Local degradation or formation of drug-containing loculaments in the IP cavity may have reduced the absorption of glucagon. Direct drug infusion into the portal vein would likely give a more precise estimate of first-pass metabolism. However, because this is not a viable treatment option in humans, we relied on IP administration to gain information on other relevant aspects for the possible use of glucagon in an IP artificial pancreas.

Pigs undergoing prolonged anaesthesia may accumulate IP fluid, which possibly could influence drug absorption from the IP cavity.[20] Theoretically, anaesthetic drugs and general anaesthesia may also lead to alterations in the pharmacokinetics and pharmacodynamics of glucagon. Our decision to perform these experiments under general anaesthesia was based on considerations for animal welfare.

The pigs received three glucagon boluses in one study day. Reduced effectiveness of consecutive doses of glucagon has been observed in healthy volunteers, possibly because of glycogen depletion.[21] However, other studies on human subjects with DM1, and a previous pig study conducted by our group, detected no association between glucose response and bolus number.[13,22,23] In the present study, the pigs received continuous infusions of insulin aspart and glucose solution to promote hepatic glycogenesis and reduce the risk of glycogen depletion.

A considerable limitation of the study is that both glucagon and porcine insulin samples were run in singles, which reduces the precision. However, we regarded this as acceptable, because the short time interval between samples made it possible to detect any marked outliers that needed to be reanalysed.

This study had a small sample size, with only 12 pigs. Some pigs had to be excluded from the analyses, which reduced the number even further. However, the main results regarding the bioavailability of IP glucagon are consistent and probably unaffected by this limitation.

5 | CONCLUSIONS

This study demonstrates that IP administered glucagon has lower bioavailability than SC administered glucagon, although the effect on glucose metabolism is equivalent. These results are compatible with a major first-pass metabolism of glucagon in the liver. Lower systemic drug concentrations could, in theory, lead to fewer adverse reactions. However, the SC route carries a much lower risk of complications and would doubtlessly be preferred for most patients with DM1. Nonetheless, a bihormonal IP artificial pancreas could be a possible treatment option for a subgroup of patients who may benefit from IP insulin administration.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data supporting the findings are available from the corresponding author upon reasonable request.

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Vasodilatory effects of glucagon: A possible new approach to enhanced subcutaneous insulin absorption in artificial pancreas devices

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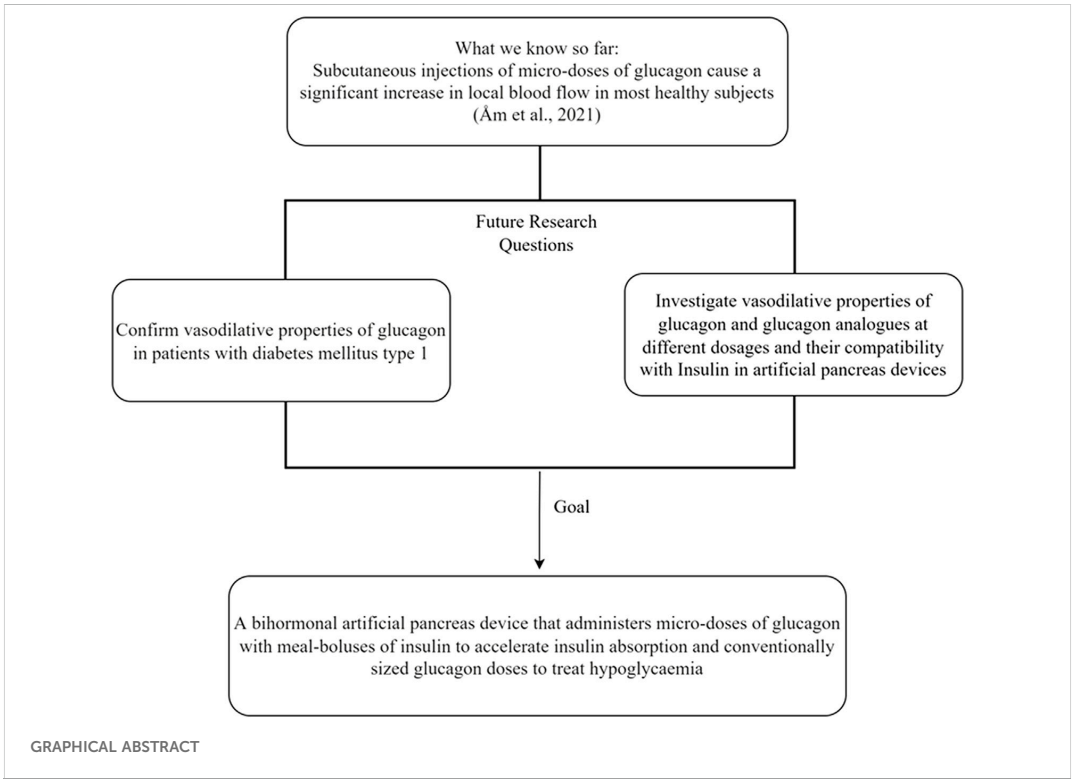
Patients with diabetes mellitus type 1 depend on exogenous insulin to keep their blood glucose concentrations within the desired range. Subcutaneous bihormonal artificial pancreas devices that can measure glucose concentrations continuously and autonomously calculate and deliver insulin and glucagon infusions is a promising new treatment option for these patients. The slow absorption rate of insulin from subcutaneous tissue is perhaps the most important factor preventing the development of a fully automated artificial pancreas using subcutaneous insulin delivery. Subcutaneous insulin absorption is influenced by several factors, among which local subcutaneous blood flow is one of the most prominent. We have discovered that micro-doses of glucagon may cause a substantial increase in local subcutaneous blood flow. This paper discusses how the local vasodilative effects of micro-doses of glucagon might be utilised to improve the performance of subcutaneous bihormonal artificial pancreas devices. We map out the early stages of our hypothesis as a disruptive novel approach, where we propose to use glucagon as a vasodilator to accelerate the absorption of meal boluses of insulin, besides using it conventionally to treat hypoglycaemia.

KEYWORDS

glucagon, pharmacokinctics, subcutaneous infusion, artificial pancreas, diabetes mellitus type 1

1 Introduction

Patients with diabetes mellitus type 1 (DM1) have lost their ability to produce and secrete insulin due to the selective destruction of beta cells in the pancreatic islets of Langerhans. Insulin is necessary for a wide range of physiological processes and is mandatory for keeping blood glucose concentrations within a narrow range. Thus, patients with DM1 depend entirely on exogenous insulin, and intensive insulin treatment and tight glucose control are recommended to reduce the risk of microvascular complications (Reichard, 1992; Nathan et al., 1993; Reichard et al.,



GRAPHICAL ABSTRACT

1996). Most patients with DM1 use multiple daily subcutaneous (SC) insulin injections or continuous SC insulin infusions administered via a pump. Recently, double SC artificial pancreas devices have also been introduced as a treatment option, i.e., devices that measure SC glucose concentrations continuously and calculate and deliver suitable insulin doses automatically (Peyser et al., 2014). Currently, only hybrid artificial pancreas devices that require meal annunciations from the user are commercially available (Knebel and Neumiller, 2019). As they require frequent user intervention, patients using these hybrid devices are not fully relieved of the daily focus and stress of managing their disease, which is known to be a factor that causes distress and reduced mental well-being among patients with DM1 (Pallayova and Taheri, 2014).

Administering appropriate meal-time insulin doses is a major daily challenge for patients with DM1, and delayed or miscalculated insulin dosages often results in postprandial glucose excursions and poor glucose control (Robinson et al., 2021). Timing and adjusting insulin dosages is difficult because all insulins, even the most rapid-acting insulin analogues, are absorbed relatively slowly from the SC tissue, with an onset of effect after 10–30 min and a maximum glucose-lowering effect one to 3 hours after administration (AHFS Drug Information, Insulins General Statement, 2022a). Whereas in healthy individuals, postprandial blood glucose concentrations have returned to baseline within the same period (Hiyoshi et al., 2017).

Patients with DM1 are advised to inject meal-time insulin 10–20 min before they start eating to accommodate the insulin absorption delay. However, despite this precaution, glucose concentrations are often strongly elevated after meals (Birkeland, 2006). If fully automated artificial pancreas devices that require no user intervention are to become a reality, this issue must be resolved, as such devices evidently must detect ingestion of a meal before increasing insulin infusion.

2 Factors delaying onset of insulin action in SC artificial pancreas devices

A fully automated SC artificial pancreas device may only upscale the insulin infusion rate in relation to a meal once a meal is detected by a rise in the SC glucose concentration or through another sensing modality. As such, two major barriers exist that

prevent the development of such a device: The delay in sensing changes in blood glucose concentrations from a sensor in SC tissue and, more importantly, the delayed absorption, and hence the glucose-lowering effect of SC delivered insulin (Christiansen et al., 2017; Gingras et al., 2018).

2.1 Glucose sensing in SC tissue

SC continuous glucose monitoring systems (CGM) report glucose concentrations in the interstitial fluid in SC tissue (Funtanilla et al., 2019). Changes in blood glucose concentration are not immediately followed by a corresponding change in CGM measurements, as glucose must first diffuse through the capillary walls and into the interstitial space. This process leads to sensing delay, particularly during rapid changes in blood glucose concentrations, such as during physical activity and after meals (Schmelzeisen-Redeker et al., 2015; Funtanilla et al., 2019).

Although there have been considerable improvements in CGM-technology in recent years, the sensing delay is still an issue that needs to be resolved. In the literature, the delay is commonly attributed to both physiological and technological factors and is estimated to be in the range of 5–10 min (Schmelzeisen-Redeker et al., 2015; Siegmund et al., 2017).

2.2 Insulin absorption from SC tissue

When administered SC compared to intravenously, the half-life of human insulin increases from approximately 6 min to 3 h (Binder, 1969; Skjaervold et al., 2012), predominantly because of the significant delay in absorption from SC tissue (Lindholm and Jacobsen, 2001). SC absorption of insulin is influenced by several factors, contributing to considerable interindividual and intraindividual variation. Of particular importance are physical-chemical factors related to the drug type, physiological factors on the administration site, such as skin temperature and SC blood flow, and differences in absorption kinetics in the anatomical regions suitable for SC injections (Gradel et al., 2018; Pitt et al., 2020).

2.2.1 Drug type

Soluble human insulin comprises oligomers, primarily monomers, dimers, and hexamers, in chemical equilibrium. When insulin is stored at high concentrations and in the presence of allosteric ligands, the oligomer equilibrium shifts towards a very large fraction of hexamers. This process ensures long shelf-life of insulin formulations, as hexamers are more resistant to chemical degradation (Gast et al., 2017). However, insulin hexamers are too large to be absorbed by the SC capillaries and must dissociate before getting absorbed into the systemic circulation and to become biologically active as insulin monomers (Gradel et al., 2018).

Rapid-acting insulin analogues, which are used in artificial pancreas devices, follow a similar pattern. Both insulin aspart, insulin glulisine, and insulin lispro are structurally identical to human insulin except for minor alterations in the amino-acid sequences that exist to reduce the tendency of insulin monomers to form larger oligomers in SC tissue (AHFS Drug Information, Insulin aspart, Insulin glulisine, Insulin lispro, 2022b). Thus, the rapid-acting insulin analogues are more easily absorbed, resulting in a faster onset and shorter duration of action (Gradel et al., 2018).

2.2.2 Insulin concentration and volume

Highly concentrated insulin formulations are usually absorbed more slowly from SC tissue than diluted preparations. However, smaller injection volumes cause less pain to the patient (Zijlstra et al., 2018) and additionally have a larger surface-to-volume ratio, which promotes absorption (Søeborg et al., 2009; Mader et al., 2013; Gradel et al., 2018).

2.2.3 Anatomical site and injection technique

There is a considerable variation in insulin absorption rate from different anatomical areas. Absorption is reported to be fastest from the SC tissue on the abdomen, followed by the arms, and lowest from the thighs and gluteal region (Bantle et al., 1993). Absorption may also vary within the areas, and rotation between and within injection regions is a likely source for intraindividual variability in insulin pharmacokinetics (Gradel et al., 2018).

2.2.4 SC blood flow

Variations in skin blood circulation are essential for thermoregulatory control and can differ substantially depending on the presence of vasoactive substances such as noradrenaline and nitric oxide (Charkoudian, 2003).

Insulin monomers and dimers are absorbed directly into SC capillaries through simple diffusion. When the capillary exchange surface in SC tissue expands with the recruitment of capillaries, vasodilation and increased blood flow, insulin absorption is accelerated. As such, insulin absorption is positively correlated to the blood flow at the injection site (Binder, 1969; Vora et al., 1992) and can be promoted through injection into areas with hyperperfusion, for instance, after direct or indirect heating or skin massage (El-Laboudi and Oliver, 2015).

The ultra-rapid insulin lispro formulation, Lyumjev® (Eli Lilly) and the fast-acting insulin aspart formulation Fiasp® (Novo Nordisk) contain vasoactive additives that increase blood flow and accelerate the absorption of the insulin analogue. Lyumjev® contains the vasodilating drug treprostinil, a stable prostacyclin analogue, and citrate in addition to insulin lispro (The Norwegian Medicines Agency Medicine Database,

Lyumjev, 2022). This combination leads to significantly faster SC absorption, elimination, and earlier onset of action of insulin lispro (Leohr et al., 2021). Fiasp® contains niacinamide and L-arginine together with insulin aspart. Niacinamide increases the relative fraction of insulin monomers in SC tissue and causes transient local vasodilation after administration, leading to increased initial absorption of insulin aspart (Kildegaard et al., 2019).

2.2.5 Individual factors

Obesity, age, gender, smoking, and comorbidities associated with DM1 all contribute to extensive interindividual variation. In addition, absorption may also be delayed or decreased by the presence of insulin-binding antibodies, which develop in nearly all patients a few months after the start of insulin treatment (AHFS Drug Information: American Society of Health-System Pharmacists, 2022e).

3 The fear of hypoglycaemia and the need for a bihormonal approach

Although intensive insulin treatment is recommended to reduce long-term microvascular complications in patients with DM1, it increases the risk of severe hypoglycaemia, an acute and dramatic condition that can be life-threatening if left untreated (Nathan et al., 1993). The fear of hypoglycaemia significantly reduces the quality of life for patients with DM1 and prevents tight glucose control (Chatwin et al., 2021).

In healthy individuals, the alpha cells in the pancreatic islets of Langerhans secrete glucagon as a response to declining blood glucose concentrations to stimulate hepatic glucose output and prevent hypoglycaemia. In patients with DM1, this pancreatic response is reduced or even absent, which makes them susceptible to hypoglycaemic episodes (Gerich et al., 1973).

SC bihormonal artificial pancreas devices that utilise both insulin and glucagon can potentially allow more aggressive insulin treatment than unihormonal devices using only insulin, as glucagon could counteract insulin-induced hypoglycaemia.

Studies comparing bihormonal and unihormonal SC artificial pancreas devices have reported that the bihormonal approach reduces the incidence and duration of daytime hypoglycaemic episodes. However, evidence of long-term improvements in the time spent in the normoglycaemic range and effects on HbA1c is lacking (Bakhtiani et al., 2013; Weisman et al., 2017; Haidar, 2019).

4 Vasodilatory effects of SC micro-doses of glucagon

For decades, it has been known that large doses of glucagon have physiologic effects beyond increasing glucose output. For instance, it may reduce gastrointestinal motility, increase heart contractility and cause vasodilation (Farah, 1983).

Glucagon has previously been shown to cause up to a 500 per cent increase in SC blood flow after SC injection of a large dose (1 mg) (Simmons and Williams, 1992). Our research group recently discovered that SC injections of much smaller doses of glucagon (0.1 and 0.01 mg) could also cause a substantial increase in local SC blood flow (Åm et al., 2022). In this study, glucagon was injected SC on the abdomen in healthy individuals, and blood flow was evaluated by laser doppler technology. The median increase in SC blood flow was 250 per cent after using a dosage of 0.1 mg glucagon. The blood flow peaked at 2–4 min after injection before declining slowly, and the effect was still present 30 min after injection. Although injection of glucagon caused increased SC blood flow in most of the study subjects, the effect was not observed in seven of the 22 included participants, which were classified as non-responders (Åm et al., 2022).

The effect of glucagon on SC blood flow appears to be local and is not present 2 cm lateral to the glucagon injection site as evaluated by both laser doppler technology and a thermal camera (unpublished data). This observation is perhaps unsurprising, as SC blood supply is organised vertical to the skin's surface, with arterioles situated 1.5–7 mm apart depending on the area of the body (Braverman et al., 1992; Raju et al., 2012).

Unlike insulin, glucagon is absorbed rapidly after SC administration, reaching maximum plasma concentrations in humans at approximately 10–20 min (Simmons and Williams, 1992; Graf et al., 1999; El-Khatib et al., 2010; El Youssef et al., 2014; Blauw et al., 2016; Castle et al., 2016; Ranjan et al., 2016; Hövelmann et al., 2018; Hövelmann et al., 2019). Previous animal trials conducted by our research group demonstrated no significant difference in the absorption speed or glucose elevating effect of glucagon after SC compared to intraperitoneal administration (Dirnena-Fusini et al., 2018; Åm et al., 2020; Teigen et al., 2022). This observation contrasts with the major delay in insulin absorption and effect after SC administration compared to intraperitoneal administration (Christiansen et al., 2017). Therefore, we hypothesise that the local vasodilatory effect of glucagon promotes its absorption from the SC space, as this would explain why there is no significant difference in absorption or the time to onset and maximum effect of SC versus intraperitoneally delivered glucagon (Teigen et al., 2022).

5 Discussion: A new approach for bihormonal artificial pancreas devices

As previously addressed, it is probably impossible to achieve excellent glucose control by a fully automated SC artificial pancreas device without any feature that can enhance insulin absorption.

If it is confirmed in future trials that micro-doses of glucagon increase local SC blood flow in patients with DM1, this effect can be utilised in SC bihormonal artificial pancreas devices where tubes that deliver insulin and glucagon could lie in immediate proximity (i.e., the hormones are delivered via a dual-lumen line). Micro-doses of glucagon could be given at the same time as meal boluses of insulin to promote postprandial insulin absorption and larger doses of glucagon may be administered when indicated to treat and prevent hypoglycaemia.

The glucagon dosages that have been used previously in SC bihormonal artificial pancreas devices to counteract hypoglycaemia resemble those we have used to demonstrate increased SC blood flow in healthy subjects (Haidar, 2019). An obvious objection to using glucagon to enhance insulin absorption is that glucagon exerts antagonistic pharmacodynamic effects on glucose metabolism to insulin and may increase blood glucose concentrations, which is highly undesirable in the postprandial state. However, in studies investigating the potential benefit of adding glucagon to artificial pancreas devices, glucagon has been administered to prevent hypoglycaemia and not with insulin infusions or in relation to meals. Glucagon's ability to increase blood glucose concentrations seems to be influenced by the concentration of circulating insulin, and it is most effective in situations with impending hypoglycaemia and low systemic insulin concentrations (Castle et al., 2010; Bakhtiani et al., 2015). This is the opposite of the situation in which we suggest using micro-doses of glucagon to increase blood flow, where insulin concentrations are increasing.

Further, there is evidence supporting a meal-induced glucagon release from the intestine in subjects with DM1 (Cryer, 2012; Knop, 2018; Yosten, 2018; Bengtsen and Moller, 2021; Ito et al., 2021). This glucagon release appears within minutes of oral glucose or food ingestion. Glucagon originating from the intestines is drained directly via the portal system to the liver, where it might increase glucose output. Results from a previous study by our research group support a saturable first-pass metabolism of glucagon in the liver (Teigen et al., 2021). Therefore, we hypothesise that micro-doses of glucagon administered with meal-time insulin would probably have no additional effects on postprandial glucose excursions as the liver will already be saturated with glucagon from the intestine.

In the study by Åm et al., there was a substantial proportion of non-responders to glucagon (Åm et al., 2022). The extent of interindividual variation and the mechanisms causing non-response are not yet known and should be elucidated in future studies. Individual response to glucagon will need to be confirmed before using it as part of a patient's artificial pancreas algorithm.

6 Future perspectives and research

We hypothesise that bihormonal SC artificial pancreas devices may use micro-doses of glucagon to induce local vasodilation and accelerate SC insulin absorption after meals. We suggest investigating the effects of micro-dosages of glucagon that are administered together with insulin boluses via a dual-lumen delivery line by the artificial pancreas when it detects a meal. The glucagon dose should be the minimum dose required to achieve adequate vasodilation while the insulin dose should be adjusted according to the effect on glucose levels. The same glucagon delivery line may also be used to deliver conventionally sized doses of glucagon during hypoglycaemic events when the insulin infusion rate is low or discontinued.

Human glucagon is relatively unstable and may aggregate and form amyloid fibrils quite rapidly, depending on the solution's temperature and pH-value. Therefore, human glucagon is recommended to be used within 24 h after reconstitution in artificial pancreas devices, although there is some evidence indicating that reconstituted human glucagon could be chemically stable for up to 7 days (El-Khatib et al., 2007; Taleb et al., 2017). The stable glucagon analogue dasiglucagon was approved for clinical use by the FDA in 2021, but it has not yet been granted marketing authorisation in Europe (AHFS Drug Information, Dasiglucagon Hydrochloride, 2022d). Questions regarding the vasodilative properties of different glucagon formulations, dosages of glucagon or glucagon analogues to be used, chemical compatibility with different insulin preparations and technical details of the glucagon delivery in an artificial pancreas device are obvious subjects for future animal trials and clinical research in patients with DM1.

7 Conclusion

The recent discovery of a pronounced local vasodilative effect of SC administered micro-doses of glucagon should be studied further in patients with DM1. If confirmed, a disruptive change in the use of glucagon in patients with DM1 may soon emerge as micro-doses of glucagon could be used to accelerate the absorption of meal boluses of insulin from SC tissue. This could potentially improve the performance of SC bihormonal

artificial pancreas devices. Thus, a fully automated bihormonal artificial pancreas that can provide excellent glucose control without daily input from the patient may be achievable.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

All authors contributed to the conception of the manuscript. IAT wrote the first draft of the manuscript. All authors reviewed and commented on previous versions of the manuscript. All authors have read and approved the final version of the manuscript.

Conflict of interest

The authors SMC and SCC are among the inventors of a pending patent application on the use of micro-doses of glucagon to enhance SC CGM performance and SC insulin absorption.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Effects of Low-Dose Glucagon on Subcutaneous Insulin Absorption in Pigs

Running title: Effects of Glucagon on Subcutaneous Insulin Absorption

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