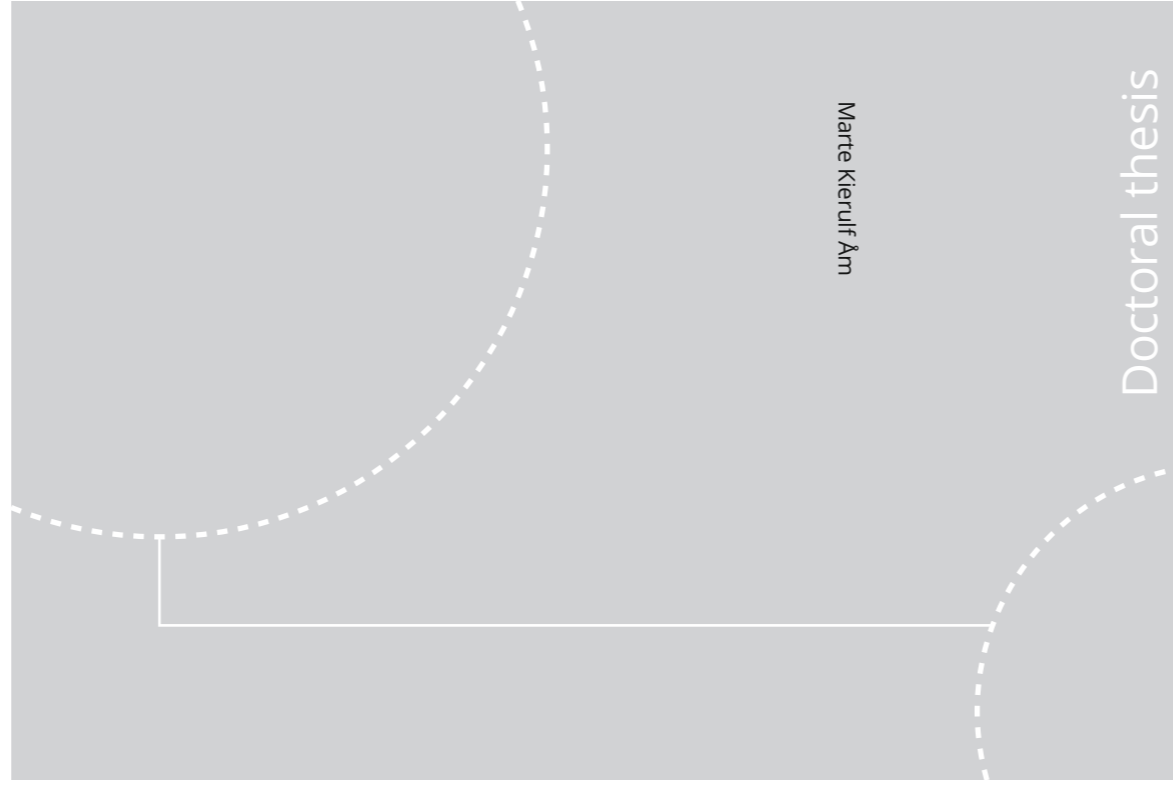


ISBN 978-82-326-4882-5 (printed ver.)  
ISBN 978-82-326-4883-2 (electronic ver.)  
ISSN 1503-8181



Doctoral theses at NTNU, 2020:267

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**NTNU**  
Norwegian University of Science and Technology  
Thesis for the Degree of  
Philosophiae Doctor  
Faculty of Medicine and Health Sciences  
Department of Clinical and Molecular Medicine

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Trondheim, October 2020

Norwegian University of Science and Technology  
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Doctoral theses at NTNU, 2020:267

Printed by NTNU Grafisk senter

## Summary

Diabetes is a heterogeneous disease affecting millions of people worldwide. Diabetes mellitus type 1 is characterised with no, or very little, production of insulin and was a fatal disease until 1922, when the first successful treatment with insulin was conducted. Although the treatment of diabetes mellitus type 1 has had a revolutionary development in the last century, and many patients invest a lot of time and effort into the management of their disease, side effects due to suboptimal glucose control are still common. The artificial pancreas is an automated glucose regulatory device to keep the blood glucose close to normal levels and thus avoid the side effects of the disease. The artificial pancreas system should also relieve the user of the constant management of the disease. The first commercially available AP system, approved by the United States Food and Drug Administration in 2016, is an insulin-only double subcutaneous system, i.e., subcutaneous continuous glucose monitoring, and subcutaneous continuous insulin infusion. The system automatically adjusts the basal insulin infusion but still relies on user intervention to control post-prandial glucose elevations.

The artificial pancreas system is a closed-loop system comprised of a glucose sensor, a hormone pump (or pumps for dual-hormone artificial pancreas) and a controller. The controller decides on the appropriate action of hormone delivery based on the detected glucose levels, and the success of the system relies on minimal delay in every part of the loop. There are physiological delays in both glucose sensing and hormone effect, and the delay in insulin effect is currently the biggest challenge to any double subcutaneous artificial pancreas system. The intraperitoneal space is, therefore, being investigated as an alternative site for an artificial pancreas system because of the fast dynamics of hormone effect and glucose sensing.

The main aim of this thesis was to investigate the intraperitoneal space as a site for glucose sensing and glucagon delivery as a part of an intraperitoneal artificial pancreas system. The first paper investigated potential spatial differences in glucose dynamics within the peritoneal space of anaesthetized pigs. There does not seem to be any clinically significant differences in glucose dynamics between the four quadrants of the intraperitoneal space. The second paper in the dissertation discusses the importance of measuring glucose as close to the peritoneal lining as possible in order to detect changes in blood glucose as fast as possible.

Investigating the effect on the blood glucose level after intraperitoneal delivery of glucagon showed that the glucose response was faster after intraperitoneal delivery in rats and gave a higher glucose response in anaesthetized pigs compared to subcutaneous delivery. It seems also possible that sufficient glucose elevations might be achieved with smaller glucagon doses by IP delivery compared with subcutaneous delivery, possible avoiding side effects of glucagon treatment.

This thesis demonstrates how the intraperitoneal space is a promising site for glucose sensing and glucagon delivery in an artificial pancreas system, however, development of appropriate sensor technology and further animal experiments are needed to fully evaluate the performance of an intraperitoneal artificial pancreas.

## Norsk sammendrag

Diabetes er en gruppe sykdommer som globalt rammer millioner av mennesker. Denne avhandlingen fokuserer på diabetes mellitus type 1 som kjennetegnes av ingen, eller veldig liten, produksjon av insulin. Diabetes mellitus type 1 var en dødelig sykdom frem til 1922, da den første pasienten mottok behandling med eksternt tilført insulin. Selv om behandlingen av DM1 har gjennomgått en revolusjonære utvikling det siste århundret, opplever mange pasienter alvorlige bivirkninger på grunn av suboptimal glukoseregulering til tross for at de investerer mye tid og krefter i sin håndtering av sykdommen. En kunstig bukspyttkjertel er en teknisk innretning som automatiserer tilførselen av insulin og på den måte holder blodglukosenivået nær normale nivåer og på den åten unngå bivirkningene av sykdommen. Det kunstige bukspyttkjertelen bør også avlaste brukeren fra den kontinuerlige oppmerksomheten personer med diabetes mellitus type 1 må ha til sin sykdom. Det første kunstige bukspyttkjertelsystemet ble godkjent av the United States Food and Drug Administration og gjort tilgjengelige for pasienter i 2016. Systemet er et såkalt dobbelt-subkutant, det vil si at både glukosemålinger og tilførsel av insulin skjer i underhuden. Systemet er ikke fullt automatisert system, da det kun justerer den basale insulininfusjonen, og er ikke i stand til å unngå den typisk store økningen i blodglukose etter et måltid. Denne hybrid-løsningen er derfor avhengig av at brukerne informerer systemet om alle kommende inntak av karbohydrater slik at insulin kan doseres i forkant av måltider.

En kunstig bukspyttkjertel er et lukket sløyfesystem som består av en glukosesensor, en hormonpumpe (eller flere pumper for systemer med flere hormoner) og en kontroller. Kontrolleren bestemmer hvilket og hvor mye hormon som skal administreres basert på målinger fra glukosesensoren. Systemet er avhengig av minimale forsinkelser i alle deler av sløyfen for å fungere kunne oppfylle intensjonen om å være et full-automatisert system. Fysiologiske forsinkelser observeres for både glukosemålinger og hormoneffekt, og forsinkelsen i insulinets effekt på glukosestoffskiftet er den største utfordringen for de systemene som benytter underhuden både til glukosemåling og insulin tilførsel. Bukhulen blir av den grunn undersøkt som et alternativt sted for en kunstig bukspyttkjertel fordi tidligere studier har vist rask dynamikk både for hormoneffekt og glukosemålinger.

Hovedmålet med denne avhandlingen var å undersøke glukosemåling og administrering av glukagon i bukshulen som en del av en kunstig bukspyttkjertel. Den første artikkelen undersøkte potensielle forskjeller i glukosedynamikk avhengig av lokalisasjon i bukshulen

hos anesteserte griser. Det ser ikke ut til å være noen klinisk signifikante forskjeller i glukosedynamikk mellom de fire kvadrantene av bukhulen. Den andre artikkelen i avhandlingen diskuterer betydningen av å måle glukose så nært peritonealhinnen som mulig for å oppnå tilstrekkelig raske målinger av forandringer i blodglukosenivået.

De to siste artiklene undersøkte effekten på blodglukosenivåene etter administrering av glukagon i bukhulen og sammenlignet dette med administrering av glukagon i underhuden. Resultatene viste at glukoseresponsen var raskere etter administrering i bukhulen på rotter og ga en høyere glukoserespons hos anesteserte griser sammenlignet med administrering i underhuden. Resultatene viser også at tilstrekkelige økninger i blodglukose kan oppnås ved bruk av mindre doser ved intraperitoneal sammenlignet med subkutan administrering. Dette kan medføre at bivirkninger av glukagonbehandlingen kan unngås ved intraperitoneal administrering av glukagon.

Denne avhandlingen viser at bukhulen er et lovende sted for måling av glukose og administrering av glukagon som en del av en kunstig bukspyttkjertel. Imidlertid må optimal sensorteknologi utvikles og ytterligere dyreforsøk utføres før man kan avgjøre om en dobbelt intraperitoneal kunstig bukspyttkjertel er en mulig framtidig behandlingsløsning for personer med diabetes mellitus type 1.

## Acknowledgements

The work presented in this thesis was conducted at the Department of Clinical and Molecular Medicine at Faculty of Medicine and Health Sciences, Norwegian University of Science and Technology (NTNU) with collaborative contributions from the Department of Engineering Cybernetics and Department of Electronic Systems, Faculty of Information Technology and Electrical Engineering (NTNU). The work was funded by The Liaison Committee for Education, Research and Innovation in Central Norway (grant number 2014/23166) and partly by the Research Council of Norway through the Centre for Digital Life Norway (grant number 248872) and the Norwegian Medical Association Johan Selmer Kvanes Endowment. The animal experiments were conducted at the Comparative medicine Core Facility (CoMed), NTNU. CoMed is funded by the Faculty of Medicine at NTNU and Central Norway Regional Health Authority.

First and foremost, I wish to express my greatest gratitude to my supervisors, Professor Sven M. Carlsen, Dr. Sverre C. Christiansen and Associate Professor Anders L. Fougner for guiding me through my time as a PhD student. I want to thank my co-authors for their contributions in writing the papers included in this thesis. I would also like to thank present and former members of Artificial Pancreas Trondheim (APT) for the many interesting discussions we have had across a wide array of topics. Working in a group with knowledge from so many different disciplines has been very valuable both professionally and personally.

I want to thank Ilze Dirnena-Fusini for being my social lifebuoy at Øya, and for all the delicious cakes. I want to thank Konstanze Kölle for our collaboration on paper I, and for the refreshing jokes shared during hard times. I want to thank Patrick Bösch, for his never-failing good mood and enthusiasm and the endless flow of ideas to solve any practical problems coming our way.

I would like to thank the staff at CoMed, and especially Oddveig Lyng for her invaluable contributions to our animal experiments and for our shared love of pigs.

I want to express my deepest gratitude to my parents for the all the time, love, and effort they have invested in my siblings and me; I'm forever grateful.

Finally, I thank my husband, Per Helge, and our children, Eirik, Arve and Kristin, for reminding me daily of what truly matters in life.





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## Abbreviations

AP	artificial pancreas
APT	Artificial Pancreas Trondheim
BG	blood glucose
BGL	blood glucose level
CGM	continuous glucose monitor (ing)
CIPII	continuous intraperitoneal insulin infusion
CNS	central nervous system
CSII	continuous subcutaneous insulin infusion
Da	Daltons
DM1	diabetes mellitus type 1
DM2	diabetes mellitus type 2
DMSO	dimethyl sulfoxide
FDA	United States Food and Drug Administration
GIP	glucose- dependent insulinotropic peptide
GLP-1	glucagon-like peptide 1
GLUT	glucose transporter
GOx	glucose oxidase
HbA1c	glycosylated haemoglobin A1
IAH	impaired awareness of hypoglycaemia
IP	intraperitoneal/ intraperitoneally
ISF	interstitial fluid
IV	intravenous
MDI	multiple daily injections
nm	nanometres
SC	subcutaneous
U	unit
Å	Ångström



## List of papers

### Paper I

#### **Effect of sensor location on continuous intraperitoneal glucose sensing in an animal model**

Marte Kierulf Åm, Konstanze Kölle, Anders Lyngvi Fougner, Ilze Dirnena-Fusini, Patrick Christian Bösch, Reinold Ellingsen, Dag Roar Hjelme, Øyvind Stavadahl, Sven Magnus Carlsen, Sverre Christian Christiansen

PLoS ONE. Public Library of Science; 2018;13(10):e0205447.

doi:10.1371/journal.pone.0205447

### Paper II

#### **Why intraperitoneal glucose sensing is sometimes surprisingly rapid and sometimes slow: A hypothesis**

Marte Kierulf Åm, Anders Lyngvi Fougner, Reinold Ellingsen, Dag Roar Hjelme, Patrick Christian Bösch, Øyvind Stavadahl, Sven Magnus Carlsen, Sverre Christian Christiansen

Medical Hypotheses 2019;109318.

doi:10.1016/j.mehy.2019.109318

### Paper III

#### **Intraperitoneal, subcutaneous and intravenous glucagon delivery and subsequent glucose response in rats: A randomized controlled crossover trial**

Ilze Dirnena-Fusini\*, Marte Kierulf Åm\*, Anders Lyngvi Fougner, Sven Magnus Carlsen, Sverre Christian Christiansen

(\*shared first authorship)

BMJ Open Diabetes Research and Care 2018;6:e000560.

doi:10.1136/bmjdr-2018-000560

### Paper IV

#### **Intraperitoneal and subcutaneous glucagon delivery in anaesthetized pigs: Effects on circulating glucagon and glucose levels**

Marte Kierulf Åm, Ilze Dirnena-Fusini, Anders Lyngvi Fougner, Sven Magnus Carlsen, Sverre Christian Christiansen

Manuscript\*

(\*Edited and published version, doi:10.1038/s41598-020-70813-5)





# 1 Introduction

## 1.1. Motivation

Diabetes is a group of metabolic diseases characterised by dysfunctional glucose regulation and categorised into four main subgroups; diabetes mellitus type 1 (DM1), diabetes mellitus type 2 (DM2), gestational diabetes and diabetes due to other causes (1). The result of untreated diabetes, however, is the same regardless of classification; persistent hyperglycaemia.

Patients with DM1 loses the ability to regulate their blood glucose levels (BGL) due to an autoimmune destruction of  $\beta$ -cells and consequently compromised insulin secretion. Restoration of the  $\beta$ -cell function is currently not possible, and the treatment of diabetes is directed towards stabilizing the BGLs rather than curing the cause of the disease. Patients are required to lower their BGLs towards the physiologic range by self-administration of insulin. Self-regulation of BGLs can be painful, bothersome, time-consuming, and difficult. The patients need to know their BGLs to plan for the right dose of insulin at the right time while coping with other factors that affect their BGLs, such as exercise, illness, stress and other environmental factors. Some patients, with unstable (“brittle”) diabetes, struggle to keep their BGLs within the desired range despite tremendous effort. Pancreas or pancreatic islet transplantation might be an option for patients with hypoglycaemia unawareness, severe hypoglycaemic episodes, and glycaemic lability, but this is an invasive procedure and requires life-long immunosuppressive therapy, which carries its own long-term adverse effects (2). Technological equipment, such as glucose sensors and insulin pumps, have been developed and proved beneficial for blood glucose (BG) control. However, improvements in glucose control and quality of life are still called for. The artificial pancreas (AP) holds the promise of automated control of glucose levels, and several groups have contributed with reports from their ongoing research in several AP systems in the last thirty years.

An AP system is a closed-loop system that calculates and automatically delivers insulin (and glucagon in a dual-hormone AP) based on glucose values obtained from a glucose sensor. For an AP to function adequately the delays in both glucose sensing and hormone effect(s) must be minimal. It might be regarded as too optimistic to assume that an AP system can regulate BGL as efficiently as glucose regulation in healthy individuals. However, the success of an AP will not be determined by how closely it

resembles the physiological glucose metabolism, but by defined outcome measures, primarily reduced glucose variability, time in range, and quality of life. Normal physiology is, however, the ultimate regulatory mechanism that guides us in the development of an AP system, and the more closely the AP system resembles normal physiology the more successful it will be.

Most of the current research on the AP focuses on the double subcutaneous (SC) approach, i.e. where both glucose sensing and hormone delivery occurs in the subcutaneous tissue. This site holds several advantages; it is easily accessible, allowing the patient to insert glucose sensors and insulin tubes themselves, and the risk of serious complications due to infections is low. The drawback of using the SC pathway is the slow, and sometimes unpredictable, dynamics of both glucose sensing and insulin delivery, besides pain and tissue changes (scars, pain, lipohypertrophy). The FDA has approved a double SC hybrid AP system by Medtronic (MiniMed 670G) (3), but this system does not represent a fully automated AP system. Patients are still required to calculate and enter the carbohydrate content of up-coming meals so that the system can deliver pre-meal boluses of insulin. Besides, the hybrid AP system is vulnerable to conditions it cannot manage, in which case the system switches to patient-steered open loop-control. To overcome the slow dynamics of glucose sensing and hormone delivery, there is a rising interest in exploring the intraperitoneal (IP) space as a possible site for glucose sensing and hormone delivery by an AP system.

Continuous IP insulin infusion (CIPII) has been studied since the late 1970s, and is currently an available treatment strategy for patients not tolerating SC insulin delivery, have severe SC insulin-resistance, experience severe hypoglycaemias, severe glucose variability, lipodystrophia and skin disorders or where other therapies failed to stabilize glucose levels (4). Several insulin IP-studies report lower levels of peripheral insulin, in addition to less glucose variability, as compared with continuous SC insulin infusion (CSII) (5–10). Continuous IP insulin delivery provides more physiologically correct insulin dynamics as much of the hormone is absorbed through the mesenteric capillaries and transported directly to the liver via the portal vein. This leads to faster absorption (11), larger first-pass proportion to the liver and consequently a more rapid effect on BGLs compared with SC insulin delivery. Continuous IP insulin delivery requires access to the IP space. This is achieved with a manufactured port through the abdominal wall and provides the opportunity of introducing also glucose sensing and glucagon delivery into the IP space. If the combined dynamics of hormone delivery and glucose sensing is faster

---

and/or more effective in the IP space as compared with the SC tissue, the goal of developing a fully automated AP system could prove possible.

### 1.2. Prevalence of diabetes

The World Health Organization estimated that 422 million adults were living with diabetes (both DM1 and DM2) in 2014 compared to 108 million in 1980 (12). The majority of adult patients with diabetes are diagnosed with DM2 (13,14). DM1 is the most common type of diabetes among children and adolescents, although the prevalence of DM2 also is increasing for this patient group (15). Estimates of the future prevalence of diabetes varies, but calculations show a continuous increase. By 2030 the prevalence in the adult population is suggested to be between 4.4% (366 million) (16) and 7.7% (439 million) (17), and by 2045 a prevalence of 9.9% (693 million) is expected (18). The prevalence of DM1 is increasing in European countries, including Norway (19), but the most rapid increase in prevalence of diabetes is experienced by developing countries mainly because of the increasing influence by western lifestyles and consequently increasing numbers of DM2 patients (18). Besides being a relatively common disease, diabetes is also a serious condition and was the 7th most common cause of death in the USA in 2016 (13).

### 1.3. Glucose metabolism

Glucose is one of the body's key sources of energy, and the primary fuel for certain cell types, such as nervous tissue (20) and red blood cells (21). The BGL is determined by the rate of glucose entering and being removed from the circulation. The regulatory mechanisms behind this fine-tuned system are highly complex and controlled by a sum of multiple neural, hormonal, and metabolic stimuli (22). The BGL in healthy fasting individuals stays between 3.5 and 5.5 mmol/L (23) with postprandial glucose elevation rarely increasing beyond 7.8 mmol/L and returns to pre-prandial levels within 2–3 hours (24,25). The liver holds a key function in glucose regulation, as it can switch from glucose storage to glucose production and release depending on glucose excess or demand (26).

Glucose molecules enter cells by special transmembrane glucose proteins called glucose transporters. Two main types of transport proteins are known: facilitated diffusion glucose transporters (GLUTs) and sodium-glucose linked transporters (SGLTs). The latter type of glucose transporter is primarily found in intestinal cells and in renal tubules where they facilitate an active absorption of glucose from intestinal content and

glomerular filtrate, respectively (27,28). Twelve different GLUTs have been identified, all with a different distribution, regulation, and glucose affinity (27). The transporters have been given numbers from 1 to 12, but the first four GLUT-receptors (class I) have been studied the most. GLUT 1 is present in all cells but is especially important in neurons and red blood cells, where they provide a constant glucose uptake. GLUT 2 is found in pancreatic  $\beta$ -cells, hepatocytes, in the kidneys, and the intestines. GLUT 3 is present in nervous tissue, and GLUT 4 is found mainly in skeletal and heart muscle cells and adipose tissue. GLUT 4 is the only GLUT receptor, of the first four, in which glucose transport is regulated by insulin, while GLUT 1–3 receptors facilitate basal glucose uptake and are, in that sense, insulin-independent (27–29).

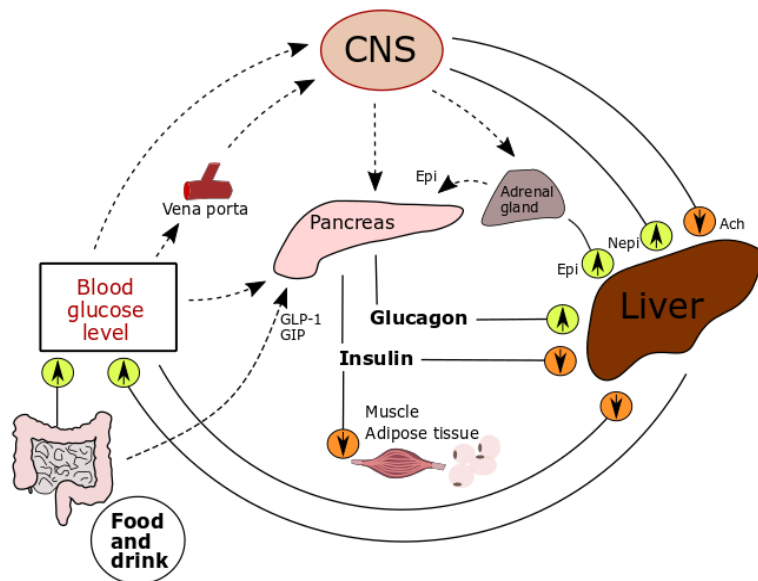
#### 1.3.1. Postprandial glucose metabolism

Eating carbohydrates leads to an increase in BGL, and most of the nutrients are absorbed in the intestines leading. However, glucose is detected even as we chew the food by specific sensors in the taste-buds on the tongue, which detect glucose and send signals to the brain preparing the body to handle the upcoming glucose load (30,31). Small amounts of glucose can also be absorbed through the mucous membrane in the mouth (32). Nervous signals are also sent to the central nervous system (CNS) when glucose is absorbed from the intestines, both from the intestinal cells themselves, but also from glucose sensors in the portal vein which is the large vein draining the intestines and mesentery. At the same time, enteroendocrine cells (L-cells and K-cells) release the incretin hormones glucagon-like-peptid-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP), respectively, as a response to the uptake of glucose from the intestines (33). GLP-1 stimulate insulin secretion, inhibits gastric emptying and glucagon secretion and slows the rate of endogenous glucose production (34).

Increasing BGLs are detected by  $\beta$ -cells in the pancreas (GLUT-2) (35), and by specific sensor cells in the CNS, mainly by cells in the brain stem and the hypothalamus (31). Insulin is released by the pancreatic  $\beta$ -cells both because they are stimulated directly by high BGLs and by the parasympathetic nervous system via the vagus nerve.  $\beta$ -cells are further stimulated by, the already mentioned, incretins (GLP-1 and GIP). In healthy subjects, the incretin effect accounts for around 50% of the secreted insulin after an oral glucose intake (34). Postprandial insulin is released in a pulsive pattern first by a rapid secretion of preformed insulin, followed by an increase in insulin synthesis (36). Insulin is released into the pancreatic vein, which drains into the splenic vein and ultimately to the liver via the portal vein (see chapter on IP anatomy) before it enters the systemic

circulation. Insulin, in combination with signals from the parasympathetic nervous system, stimulates the transportation of glucose into the hepatocytes, and the conversion of glucose to glycogen for storage (37). Insulin also binds to insulin receptors in adipose tissue and muscle cells and thereby activates translocation of GLUT 4 from intracellular vesicles to the cell membrane and allowing increased cellular uptake of glucose (29). In non-diabetic, non-obese, healthy subjects both insulin levels and BGL will be restored to their pre-meal levels within 2–3 hours (24,25).

Insulin is the major glucose-lowering hormone and directly regulates glucose removal by the liver and by stimulation of the GLUT 4, as already described. However, insulin also regulates the BGL by indirect pathways. It has a paracrine and inhibitory effect on the pancreatic  $\alpha$ -cells and thus glucagon secretion (38). Insulin also suppresses hepatic gluconeogenesis by suppressing lipolysis and thus inhibiting the production of substrates for gluconeogenesis (39). Amylin, a hormone also secreted by the  $\beta$ -cell in response to postprandial BG elevations, further suppresses glucagon secretion. Also, amylin slows the rate of gastric emptying and gives a feeling of saturation, and therefore, indirectly, lowers the BGL (40,41).



**Figure 1:** Simplified overview of normal glucose regulation. Full lines represent effects causing direct blood glucose changes, dotted lines represent stimulus causing indirect glucose changes.

Abbreviations: Epi=epinephrine, Nepi=norepinephrine, Ach=acetylcholine

### 1.3.2. Fasting glucose metabolism:

Avoiding low BGLs is important for the function of many cells, and essential for the CNS. Glucose uptake by the cells in the CNS is saturated at physiological BGLs via insulin-independent GLUTs, and the glucose consumption of the CNS accounts for 50–80% of basal glucose uptake after a night of fasting (42). Since the CNS is highly dependent on a constant supply of glucose, decreasing BGLs triggers several physiologic responses to prevent hypoglycaemia. Cells in the brain stem and hypothalamus react and activate the short term specific regulatory pathways: the sympathetic nervous system, the parasympathetic nervous system and the release of epinephrine from the adrenal glands (43,44). These pathways stimulate both glucagon release by the pancreatic  $\alpha$ -cells and glucose production by the liver (44–46).

Glucagon is considered the main glucose elevating hormone. As already mentioned, the release of glucagon is directly stimulated by signals from the CNS and adrenal glands, but the regulation of glucagon is also indirectly regulated by insulin, believed to be caused by the actual decrease of insulin concentration or by the disappearance of the inhibitory effect of insulin (43). Other glucagon suppressive factors, such as amylin and incretins, are also present in small amounts when the BGL is low, lifting their suppressive effect on glucagon release (47,48). Although glucagon secretion is considered the main glucose-elevating response to falling BGLs, there are still questions unanswered as to how glucagon secretion is regulated (38,43,49). Debated issues are whether or not low BG values directly stimulates the  $\alpha$ -cells to secrete glucagon and the role of nervous innervation of pancreatic islets (43,50).

Glucagon is released by the  $\alpha$ -cells into the pancreatic vein, in the same way as insulin, and transported directly to the liver. Glucagon receptors have been identified in several tissues but the majority are located in the liver and to a smaller degree in the kidneys (51). Glucagon binds to glucagon receptors in a time- and temperature-dependent, saturated and reversible manner (52). The hormone triggers the hepatocytes to produce glucose by glycogenolysis and gluconeogenesis. The liver produces 75–80% of the endogenous glucose in the fasting state, while the kidneys contribute with around 20–25% (53). Short-term glucose needs are provided mainly by hepatic glycogenolysis, stimulated by glucagon and sympathetic nervous system through catecholamine release (54). Glucagon is mainly eliminated by the kidneys (55).

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As the storage of glycogen diminishes after prolonged fasting, glucose is produced to a larger extent by gluconeogenesis (28). Gluconeogenesis accounts for approximately 50% of the hepatic glucose production after an overnight fast (39). The effect of glucagon on gluconeogenesis is slow and the physiological effects are not observed until hours after glucagon delivery compared to minutes after the effect of glucagon on glycogenolysis (56). In addition to glucagon, gluconeogenesis is also stimulated by glucocorticoids. Glucocorticoids are a class of steroid hormones released by the adrenal glands, with a wide range of effects in all tissues. Glucocorticoids elevate the BGL by inhibiting glucose uptake in skeletal muscle cells and white adipose tissue and cause insulin resistance (57). They also stimulate hepatic gluconeogenesis both directly and indirectly by promoting the production of gluconeogenic precursors: gluconeogenic amino acids from skeletal muscle and glycerol from adipose tissue (58). Proteins can also be utilized for glucose production, but because they serve specific functions within the body, they are the last resort for energy production (28). Growth hormone (GH) is an anabolic hormone, which is secreted from the pituitary gland and affects many tissues and processes in the body, including the BGL. The hormone stimulates lipolysis and formation and oxidation of free fatty acids in situations of fasting and/or stress. This decreases insulin sensitivity, reducing glucose uptake by the liver and promotes gluconeogenesis, all contributing to the elevation of BGLs (59). Growth hormone is secreted in pulses and higher levels are secreted during night-time (60). It is believed that the elevated levels of GH at night in combination with low levels of insulin in the early hours of the morning contribute to the “dawn-phenomenon”, an abnormal early-morning increase in BG experienced by many people with DM1.

#### 1.4. Diabetes mellitus type 1

DM1 is a progressive disease and usually a consequence of autoimmune destruction of the insulin-producing  $\beta$ -cells of the pancreas (61). DM1 is considered a multifactorial disease and has, in most cases, a strong and complex genetic association. The hereditary factors are, however, not fully understood (62). Environmental factors, such as infections, may also play an important role in the development of the disease (63).

The destruction of  $\beta$ -cells happens over some time, and there are also individual differences in the timing of the first symptoms relative to the amount of  $\beta$ -cell destruction (64).



The early stages of DM1 can be classification based on the effect of  $\beta$ -cell loss (65,66);

**Stage 1:** Development of two or more types of DM1-associated islet autoantibodies, but euglycaemia.

**Stage 2:** Development of two or more types of DM1-associated islet autoantibodies, and with a dysfunctional glucose regulation due to loss of  $\beta$ -cells, however still no manifestations of diabetic symptoms.

**Stage 3:** Manifestations of the typical symptoms of DM1, prolonged hyperglycaemia, polydipsia and polyuria, weight loss and sometimes polyphagia and blurred vision, and ultimately diabetic ketoacidosis.

The rate of  $\beta$ -cell destruction varies but often occurs more rapidly in children and adolescents compared with adults (61). In contrast, latent autoimmune diabetes in adults (LADA) develops slowly, and patients can be mistaken for DM2 patients at the initial stage of their disease (67). Detection of islet antibodies enables prediction and early diagnosis of the disease, but patients are normally diagnosed after the onset of symptoms of prolonged hyperglycaemia. Screening individuals at risk of developing DM1 might prove more relevant as the pathophysiology of DM1 is further described and interventions to stop  $\beta$ -cell destruction and improve functional residual  $\beta$ -cell mass are more successful (68).

The key "property" of DM1 is the destruction of pancreatic  $\beta$ -cells and compromised insulin (and amylin) secretion. Insulin is required for the transport of glucose molecules into adipose and muscle cells facilitated by GLUT-4 transporters. Without insulin, these cells cannot utilize and store glucose, and the BGL will rise to hyperglycaemic levels. Insulin is the main glucose-lowering hormone, but the diminishing amylin secretion also contributes to the dysfunctional glucose regulation, by reducing the normal postprandial inhibition of gastric emptying which normally reduces the postprandial glucose elevations (40). The  $\alpha$ -cells, however, are not destroyed by the autoimmune reaction and can secrete glucagon in the early stages of the disease (69). Glucagon levels are higher than expected when considering the hyperglycaemia associated with DM1 and the  $\alpha$ -cells do not respond normally to neither rising nor falling BGLs (70).

The human pancreatic islets are comprised of randomly distributed  $\alpha$ -cells,  $\beta$ -cells, and  $\delta$ -cells. This is in contrast to rodents where the  $\beta$ -cells constitutes the core of the islets surrounded by the other cell-types (71). Removal of the paracrine effect of insulin on the  $\alpha$ -cells interrupts the secretion of glucagon and the ability to correct hypoglycaemias

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(50,70). The loss of amylin and its inhibitory effect on  $\alpha$ -cells also contribute to disturbed glucagon secretion (40). Incretins are normally secreted by the enteroendocrine cells as a response to a meal, but a disturbance in the cleavage of proglucagon can result in an abnormal postprandial production of glucagon from the enteroendocrine cells (72). This contradictory glucagon secretion by the intestines are believed to contribute to the hyperglycaemia in individuals with diabetes, as increased postprandial glucagon concentrations have been observed after an oral glucose load (73,74), but not after an intravenous (IV) glucose load (73).

Diminishing insulin secretion does not only affect glucose metabolism. Insulin also holds important functions in fat metabolism, and alterations in lipid profiles and changes in lipoprotein composition are observed in youths after a relatively short duration of DM1s despite insulin treatment (75).

### 1.5. Diabetic complications

**Diabetic ketoacidosis** is a hyperglycaemic crisis and the result of uncontrolled diabetes. It is most often seen in individuals with DM1, but can also affect some people with DM2 (76). As the net effect of insulin is reduced or missing, counter-regulatory pathways, such as glucagon, epinephrine, norepinephrine, cortisol, and growth hormone are activated. This leads to an increase in endogenous glucose production via both glycogenolysis and gluconeogenesis (77). Adipose and muscle tissue are metabolized to provide gluconeogenic precursors, leading to increased concentrations of amino acids (glutamine and alanine) from protein catabolism and fatty acids from increased lipolysis of adipose tissue (77,78). The result of this dysfunctional metabolism is a potentially deadly combination of uncontrolled hyperglycaemia, metabolic acidosis and markedly increased ketone concentration due to an overload of free fatty acids (79). The fatty acids are converted to ketones, and when the kidney threshold for ketone clearance is trespassed, hyperketonemia occurs. Many factors can contribute to the development of ketoacidosis in insulin-treated DM1 individuals; such as infections, certain drugs, and concurrent somatic diseases, but omission or inadequate dosing of insulin is considered the main reason (77). The symptoms of ketoacidosis are typically varying degrees of polyuria, polydipsia, weight loss, vomiting, dehydration, weakness, Kussmaul respiration, tachycardia, hypotension, and potentially profound lethargy or coma (79). Hospitalization and critical care management are required to avoid death and to regain regulatory control (80).

**Hypoglycaemic crisis** is a potentially deadly consequence of hyperinsulinemia and compromised counter-regulation mechanisms in response to the falling BGLs (81). Hypoglycaemia can be defined as "all episodes of an abnormally low plasma glucose concentration that expose the individual to potential harm" (82). The glucose threshold for developing a hypoglycaemic crisis is individual and will also vary for each person with DM1 at different times and in different situations (83). Hypoglycaemia might lead to symptoms such as anxiety, palpitations, tremor, sweating, hunger, paraesthesia in the fingers and hands, neurological impairments, including behavioural changes, cognitive dysfunction, seizures, and coma (81). As the CNS cannot store or synthesize glucose, prolonged hypoglycaemia might damage CNS neurons leading to cerebral damage or death (84,85).

The ability to recognize hypoglycaemic symptoms differ between individuals and many individuals develop a reduced ability to perceive the onset of hypoglycaemia, i.e. impaired awareness of hypoglycaemia (IAH) (86,87). The mechanisms behind the development of this syndrome are not fully explained, and multiple factors probably influence the development. The condition can be temporarily reversible for some people as the initial treatment is avoid hypoglycaemias and to accept a higher level of glucose for some weeks, during which period the patient often will regain his/her usual hypoglycaemia symptoms. Repeated episodes of hypoglycaemia are believed to contribute to the development of IAH, and current recommendations encourage patients to avoid hypoglycaemic episodes and to temporarily raise their glycaemic targets so they can retain their ability to recognize hypoglycaemia and to avoid future episodes (88,89). "Dead in bed" syndrome is also a feared complication of diabetes. The pathogenesis is not fully understood, but a combination of nocturnal hypoglycaemia and autonomic neuropathy is believed to be the cause (90,91). The fear of hypoglycaemia is perhaps the most important reason why many people with DM1 fail to achieve the desired glucose control (92). They might either inject too small doses of insulin, eat more carbohydrates than necessary or only inject fast-acting insulin after reassuring themselves that glucose is rising during the meal, which again might lead to poor glycaemic control and long-term complications (92). For some individuals, their fear of hypoglycaemia controls their every-day life to such a degree that it has a strong negative impact on their quality of life.

**Long term complications** of diabetes are caused by prolonged hyperglycaemia, resulting in damage to both small and large blood vessels. Retinopathy, nephropathy, and neuropathy are the most common microvascular complications, caused by damage to

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endothelial cells in the retina, mesangial cells in the kidneys and neurons and Schwann cells in peripheral nerves, respectively (93,94). All cells that are incapable of modulating their glucose transport are susceptible to damage by prolonged hyperglycaemia. These cells will under hyperglycaemic conditions have an intracellular concentration of glucose close to the intercellular glucose levels, resulting in disturbed cellular energy metabolism (95–98). Hence, symptoms caused by microvascular dysfunction are observed from many organs and tissues in addition to those mentioned above, for example from the CNS, skin, and myocardium (99). The Diabetes Control and Complications Trial (DCCT) showed that intensive insulin therapy, keeping the BGL close to the normal range, delayed the onset and slowed the progression of microvascular complications (100). A four-year follow-up study showed persisting reduced risk of progressive retinopathy and nephropathy although the glycosylated haemoglobin A1c (HbA1c) levels of the two groups were not significantly different after the initial study (101). However, not all individuals develop serious microvascular complications despite prolonged hyperglycaemia, which might imply that hyperglycaemia by itself does not necessarily lead to microvascular dysfunctions, and that unknown protective factors might prevent the development of these complications in some people (102). Macrovascular complications, such as atherosclerosis and cardiomyopathy, are also common long-term complications of diabetes (96,103–106).

Diabetes is associated with reduced life expectancy (107,108) and quality of life (109). However, since the first insulin replacement treatment in 1922 this disease is no longer inevitably fatal. Further improvements in treatment and care for DM1 individuals have led to improved overall glucose control and significantly better long-term clinical outcomes and improved quality of life for patients. Although BG control can be improved further, there is an ongoing debate on how much the society can spend on people with diabetes without neglecting other patient categories and health care needs, which becomes more imminent every time new advances in insulin therapy and glucose measurement are introduced to the market.

## 1.6. Treatment of diabetes

Treatment of DM1 is not directed at the cause of the disease but towards the consequence of the disease, hyperglycaemia. Most DM1 individuals will also need to deal with the consequence of the treatment, hypoglycaemia. People with diabetes strive to keep their BGL close to normal levels but achieving a tight glucose control like non-diabetic people is unrealistic with the current treatment options. The desired target

range for DM1 individuals is therefore suggested to be between 3.9 and 10 mmol/L (110), but even keeping within this range can be difficult for many people with DM1.

**Insulin replacement:** As most DM1 individuals are not producing insulin, the hormone is usually replaced by multiple daily SC injections of insulin (MDI) or by CSII with additional boluses in relation to meals. MDI is often a combination of one or two daily injections of long-acting insulin to provide basal insulin levels, and pre-prandial injections of fast-acting insulin to counteract the typically large postprandial glucose excursion. Insulin pumps deliver a pre-programmed subcutaneous continuous infusion of fast-acting insulin to provide basal insulin levels with supplemented pre-prandial user triggered insulin boluses. Both pump users and MDI users are advised to deliver the meal bolus before eating. The "state of the art" in insulin treatment is sensor-augmented pumps with automated insulin suspension at predefined glycaemic thresholds. With such a system, the users can decide and regulate the basal infusion and bolus delivery themselves, but the system will automatically discontinue the insulin infusion for a period in situations where low glucose sensor values are present or imminent (111).

**Glucose monitoring:** To decide when and how much insulin to administer, the current BGL must be known. The most common way of monitoring BGL is by obtaining a drop of capillary blood from a finger prick and analyse it with a glucose meter. This provides a real-time glucose value, but only shows a "snapshot" of the BGL unless it is followed-up by a second measurements within minutes. To discover trends in BGLs, frequent blood samples are needed. Finger pricks, however, cause pain, discomfort and for some people embarrassment and are often avoided. Continuous glucose monitoring (CGM) by a glucose sensor implanted into the SC tissue provides the opportunity for observing historical data and trends in BGLs. This enables users to learn and make better decisions regarding both the timing and magnitude of insulin doses. Robust and reliable CGM is an absolute requirement for an AP system.

**Glucagon** is currently available as an emergency treatment against hypoglycaemia in situations when patients are not able or willing to consume carbohydrates. Glucagon is not stable when mixed with saline and the drug is preserved as a freeze-dried powder to be mixed with saline just before injection. This procedure can be challenging in a stressful situation and studies have shown that many caregivers are not able to successfully inject the drug when needed (112,113). Promising results have been published from stable premixed glucagon formulations and nasal sprays under development for easier application (114–121). The first glucagon formulation for

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application in the nasal cavity was approved by the FDA in July 2019, and the first stable liquid glucagon formulation for emergency use was approved by the FDA in September 2019. Stable glucagon formulations are also under development for inclusion in dual-hormone AP systems (see section 1.7.3).

Medications used to improve glycaemic control in individuals with DM2 may also be beneficial for DM1 individuals.

An **amylin** analogue, pramlintide, is approved by the FDA (Food and Drug Association) as a supporting treatment of both DM1 and DM2. Amylin analogues must be injected. Amylin analogues are currently under investigation as a part of an AP system (122,123).

**GLP-1** analogues are under investigation as a supportive treatment for DM1 as they have an inhibitory effect on glucagon secretion and slows gastric emptying, both reducing postprandial glucose elevations (122).

**Metformin** is an oral BG lowering drug, used as the first-line treatment of DM2. Metformin affects many different cells and cellular functions, not all related to glucose metabolism (124), but it affects the BGL by accumulating in hepatocytes, inhibiting gluconeogenesis, and enhancing insulin sensitivity (125). It also shifts the metabolism of glucose in the intestinal cells to anaerobic metabolism, resulting in reduced net uptake of glucose from the gut. The use of metformin to improve glycaemic control in DM1 individuals has shown a small but positive effect on outcome measures such as mean BG concentrations and insulin requirements, and can have a positive effect in DM1 patients who are particularly insulin resistant (126).

**Sodium-glucose cotransporter-2 (SGLT-2)** inhibitors lower the BGL by inhibiting the reabsorption of glucose from the renal filtrate in the proximal tubules of the kidneys, leading to increased excretion of glucose in the urine (127). There are several reports regarding an increased risk of diabetic ketoacidosis when SGLT2-inhibitors are used in individuals with DM1, and the FDA recently declined approval of the use of SGLT2-inhibitors as supplementary treatment of DM1 (128).

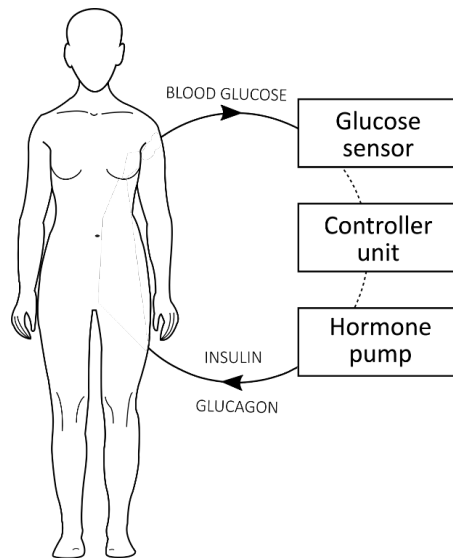
**Pancreas transplant** is an alternative treatment for some patients, especially patients suffering from end-stage diabetic kidney disease. Successful transplantation can offer patients relief from their diabetes, but human pancreases are a limited source and the procedure comes with serious side effects due to the immunosuppressive medications required. In Norway, around 25 transplants have been performed annually since 2011 (129). The survival rate of transplanted pancreases is between 90 and 77% the first year dropping to around 50% after ten years post-transplant (130,131). Graft survival was improved when the pancreas was transplanted simultaneously with kidneys compared with pancreas alone (131). Implantation of islet cells is an approved treatment

procedure in Norway with 91 treated patients between 2001 and 2017 (132). In the US it is still considered an experimental treatment and only used in clinical studies approved by the FDA (133,134).

The success of DM1 treatment is generally evaluated by observing the HbA1c level. This test gives an indication of the glucose levels over the last eight weeks, with emphasis on the last month before the test. The goal of treatment is to keep the HbA1c below 48 mmol/L (6.5%), resembling values observed in non-diabetic people (27–38 mmol/L, 4.6–5.6%). However, the HbA1c informs nothing about the fluctuations of the BGLs or the frequency or severity of hypoglycaemic incidents. Glucose variability is considered a better way of evaluating diabetic management. The recently recommended outcome measures for diabetic treatment include hypoglycaemia, hyperglycaemia, time in range, diabetic ketoacidosis (DKA), and patient-reported outcomes (PROs) (110). Time in range, and time in hyper- and hypoglycaemia are obtainable outcome measures only after the introduction of CGM.

### 1.7. The artificial pancreas

The dream of an automated insulin delivery system is not new. The term "artificial pancreas" was already used in 1959 when the idea was announced by E. Perry McCullagh in a speech to The Endocrine Society (135). The first AP system, the BioStator, was tested on patients in 1976 (136). This was a large, bedside device measuring glucose and delivering insulin intravenously and consequently only applicable to hospitalized patients (137). A mobile version was also developed but deemed impractical for daily use due to its size (comparable to a 30 L backpack). The aim of an AP is the same now as it was when the idea was first introduced; automated control of the patients BGL, keeping it as close to normal values as possible without the risk of developing hypoglycaemia and without patients needing to intervene. The target range for most published closed-loop trials has been set to 3.9–10 mmol/L, and by keeping the BGL within this range, the aim is to avoid both short-term and long-term complications of diabetes, avoid hypoglycaemia and provide the users the same quality of life and life expectancy as people without DM1. The simplest version of an AP system consists of a glucose sensor, a controller unit and a pump for insulin delivery (Figure 2). The AP system can be made more complex by adding other input data in addition to glucose values. Prediction of upcoming meals and physical activity might be especially useful (138,139). Other hormones can also be included in an AP system. Glucagon is the most commonly added hormone, but the addition of amylin is also under investigation (123).



**Figure 2.** Schematic presentation of the AP system and its components.

The AP system is highly awaited by the diabetes community. A quick search using the phrase #wearenotwaiting on Twitter and other social media shows thousands of posts and comments from patients and caregivers eager for the development of the AP system to move forward. Some patients and caregivers have taken the work into their own hands, and recipes and ways of hacking commercially available medical devices are shared online (140).

A hybrid AP system, the MiniMed 670G from Medtronic, was approved by the FDA in 2016 (3). However, the system only automatically controls the basal insulin infusion and patients are still required to enter the carbohydrate content of every up-coming meal for insulin bolusing.

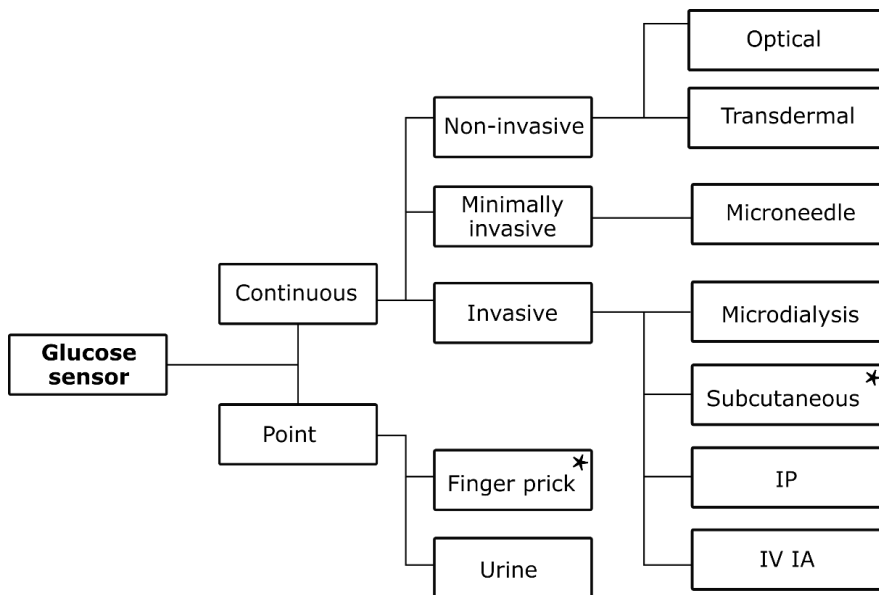
### **1.7.1. Glucose sensing**

There are several ways to measure the BGL, some methods are invasive, and some are non-invasive, some intermittent and some continuous. Figure 3 shows an overview of sensor technology currently commercially available or under development.



Optical sensor technology offers the possibility of measuring glucose without penetrating the skin. Non-invasive glucose sensing has been studied, in saliva, tears, sweat, breath, and transdermal measurements (141–143). The latter approach has been most extensively studied, but it has been shown difficult to overcome several major challenges, such as the variation in skin tones and types between patients, but also variation in skin appearance for the same patient due to sun exposure, scarring, fever, sweating, etc. Friction between the sensor and skin also imposes difficulties in gaining trustworthy results. A way of preventing such movement is keeping the sensor firmly attached to the skin, but skin irritation is a likely complication. A few non-invasive glucose sensing devices did make it to commercialization (144,145) but disappeared from the market after a short time.

Only technology related to the work of this thesis and technologies available to patients will be mentioned explicitly in this chapter, but the development of other sensor technologies might hold the future solution for glucose sensing in an IP AP.



**Figure 3.** Flowchart of current glucose sensing technology in diabetic treatment and research. Sensor technology used in clinical practice are marked with stars. The invasive subcutaneous glucose sensing box in this figure includes both electrochemical and

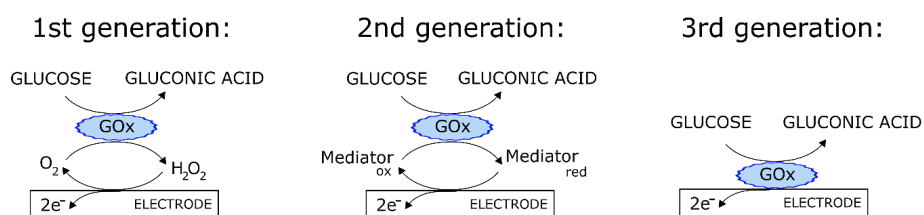
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*optical technology. Urine samples are used in the screening of diabetes, but no longer in glucose monitoring. Figure adapted from Oliver et al. (146).*

### **Electrochemical sensors**

Most commercially available CGM sensors today are electrochemical sensors utilizing the properties of glucose oxidase (GOx), an enzyme that binds selectively to glucose. The principle of this type of sensor is the oxidation of glucose to gluconic acid and the transfer of electrons to a working electrode, creating an electrical current proportional to the local glucose concentration (147).

Electrochemical glucose sensors are divided into three generations based on how the electrons are transferred to the working electrode of the sensor. The first generation of GOx-based glucose sensor converts glucose and oxygen to gluconic acid and hydrogen peroxide (148). As hydrogen peroxide is converted to oxygen and water, free electrons are transferred to the working electrode. The first-generation glucose sensor is dependent on the availability of oxygen, and since the normal physiological level of oxygen is lower than the normal physiological levels of glucose, oxygen is a potential limiting factor for these glucose sensors (148). Sensors from Medtronic and Dexcom are first generations sensors and are dependent on oxygen for glucose measurements (149). The second-generation sensors are not dependent on oxygen, as they have incorporated artificial mediator molecules together with the GOx. It is this mediator which is reduced by the enzyme and then reoxidized at the working electrode, creating the current within the sensor (150). The FreeStyle Libre sensor utilises mediator molecules and are not depending on oxygen for glucose measurements (151). The third-generation glucose sensor is currently under development (152). Electrons are transferred directly from the oxidation of glucose by GOx to the working electrode of the sensor. There is no need for either oxygen or a mediator, and this eliminates the problem of availability of oxygen and potential cross-reactions with other molecules resulting in a more stable and accurate sensor (152). Figure 4 shows the principles of the different generations of electrochemical glucose sensors.



**Figure 4.** Overview of the three generations of electrochemical sensors utilizing glucose oxidase.

The measured current for all amperometric electrochemical sensors is translated to glucose values by calibration procedures. Most sensors require calibration against BG values obtained from finger prick measurements, both at set up and throughout the sensor lifetime. FreeStyle Libre and Dexcom 6G are both pre-calibrated, but the Dexcom 6G offers the possibility of calibration against BGL, whereas the FreeStyle Libre does not. The details of this factory-calibration method are not publicly available.

Glucose (and oxygen in the 1. generation sensors) must diffuse through a semi-permeable membrane to reach the sensing element. The purpose of the membrane is to prevent unwanted molecules from interfering with the chemical reactions by discriminating molecules based on size and/or net charge (153). The diffusion of glucose through the membrane creates a time lag, which adds to the sensor dynamics. The manufacturers of the different glucose sensors are reluctant to publish the specifications of their sensors, and thus their information of the sensor dynamics is not publicly available (154). This is a potential problem when investigating glucose dynamics using CGM devices, as the sensor dynamics and limitations might lead to erroneous conclusions regarding physiological glucose dynamics (155).

Three manufacturers dominate the electrochemical CGM market: Medtronic, Dexcom, and Abbott. They all manufacture CGM devices, but Abbott also manufacture a flash glucose monitoring system, FreeStyle Libre, which is the sensor used in the animal trials described in Paper I. The FreeStyle Libre is defined as a flash glucose monitor because it only provides glucose values when scanned with a hand-held reader device. The system will also display the trend in BGLs and the historical data for the last 8 hours when scanned.

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CGM sensors have a limited lifetime. The electrochemical sensors are approved for between 7 and 14 days of use. Degradation, or consumption, of the GOx enzyme, might be a limiting factor for the lifetime of a sensor but an excess of the enzyme is incorporated into the sensors to eliminate this as a limiting factor for a use up to 14 days. Degradation of the enzyme, in combination with the immune reaction around the implanted sensing element, will, however, cause a drift in the electrical current, but most sensors account for this by requiring two or more daily calibrations by finger prick measurements of BG values (147).

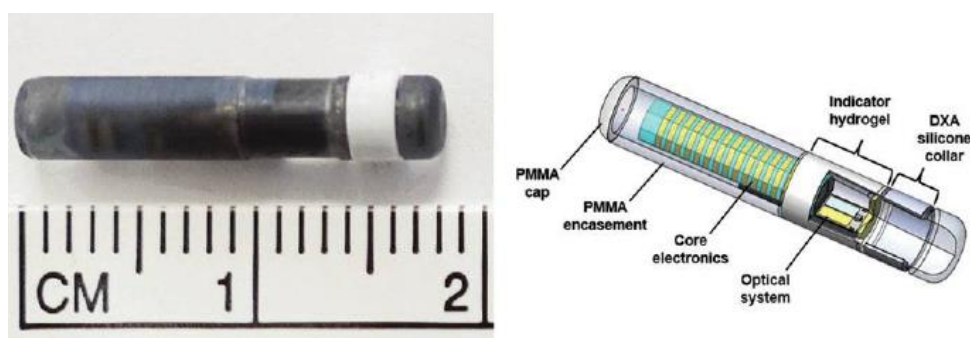
Several substances and common drugs are known to interfere with the sensors and cause erroneous measurements. A study of several substances on Medtronic Guardian Sof-Sensor and Dexcom G4 Platinum showed that ethanol, acetaminophen, albuterol, lisinopril, atenolol, and atorvastatin interfered with sensor performance (156–158). However, sensors and particular sensor membranes are refined specifically to deal with these issues and Dexcom claims to have developed an advanced membrane for their G6 and thus eliminated the unwanted cross-reaction to acetaminophen (159). However, no producer-independent confirmation of this statement is available.



**Figure 5.** Photos of glucose sensors. Left photo shows the FreeStyle Libre sensor and reader from Abbott. Middle photo shows Dexcom G6 with applicator, receiver and a smart phone from Dexcom. Right photo shows the MiniMed 670G system with sensor, BG meter, and insulin pump from Medtronic. (Images downloaded from [www.freestylelibre.no](http://www.freestylelibre.no), [www.dexcom.com](http://www.dexcom.com), and [www.medtronicdiabetes.com](http://www.medtronicdiabetes.com), October 2019).

### Optic sensors

Eversense, manufactured by Senseonics, is currently the only CGM device on the market that employs optical technology. The sensor is fully implanted in the SC tissue of the upper arm, and the external transmitter is attached to the skin over the sensor. It is approved for 180 days of use in Europe (160) and 90 days in the US (161). The sensor is composed of a cylindrical case containing a light-emitting diode (LED). The case is partially covered by a fluorescent polymer (a hydrogel) (Figure 6). Glucose will bind reversibly to this gel, which then emits fluorescent light when excited by the LED inside the case. The fluorescent light is detected by two photodiodes, and the signal is transferred to the external transmitter, which calculates glucose values and sends the data to a smartphone (162). Both implantation and extraction of the sensor are done by health professionals. In vitro tests show that other molecules can interfere with the glucose measurements, such as mannitol and tetracyclines. According to the manufacturer acetaminophen does not interfere with the performance of the Eversense (163).

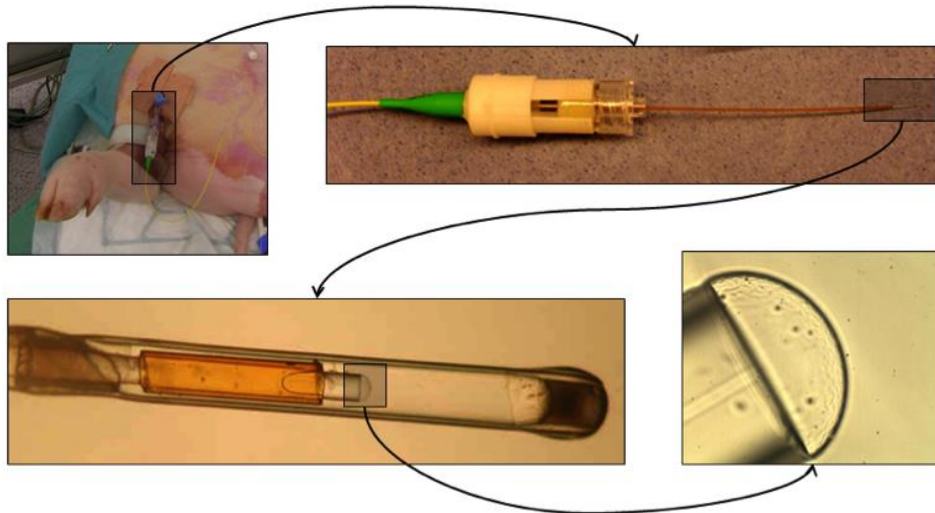


**Figure 6.** The Eversense sensor. Photo from Christiansen et al. (164).

Optical sensor technology for intravascular use is also under development. The GlucoSet sensor, which forms the base for Paper II, consists of a half-spherical biosensitive hydrogel bound to the end of an optical fibre (Figure 7). Glucose binds reversibly to this glucose specific hydrogel, which adopts an equilibrium swelling volume depending on the amount of glucose available (165). The changes in volume of the hydrogel are detected by an interferometric technique and converted into glucose values (166). The latency caused by this sensor is minor, if any, and the sensor can detect small changes in BG values (167). Intravascular glucose sensing is not a realistic approach for patients

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in free-living conditions but might be an important tool in monitoring critically ill patients in a hospital setting.



**Figure 7.** The GlucoSet sensor. The photo on the top left shows the positioning of the sensor in the femoral artery of a pig. The other photos show the sensor at increasing magnifications, and a close-up of the biosensitive gel-dome is displayed in the photo on the bottom right. Photo from Skjaervold et al. (167).

### 1.7.2. Controller

The function of the controller in an AP system is to calculate the appropriate hormone delivery based on glucose values from the glucose sensor. Different algorithms are pursued in the development of an AP system, and at present the three most common algorithms are:

**MPC** - model predictive control is a family of algorithms, which utilize a BG dynamic model to predict the future behaviour of the BG response. The algorithm solves an optimization problem at every new input to the system and estimates the optimal action response (within predefined constraints) to bring the system back to the predefined target range or value. MPC is used in many industrial applications.

**PID** - proportional integral derivative control is a family of more simplistic feedback control algorithms than the MPC. The inputs to the system are the deviation between

the measured glucose level from a defined target glucose level (the proportional component), the area under the curve between the measured and target glucose levels (the integral component) and the rate of glucose change (the derivative component) (168). The MiniMed 670 G (Medtronic) utilizes a modified PID controller (169). The details of this controller are not publicly available, and the system is only capable of adjusting the basal rate of insulin infusion, requiring the patients to plan for and execute insulin boluses related to meals.

**Fuzzy logic** - is a rule-based control system in which the input variables are translated into non-numeric values and scaled between the two extreme values of true and false. The same applies to the output variable (hormone delivery). The controller does not rely on a mathematical model of the system, and the controller can handle a great number of input and output variables. Fuzzy logic is used in computer-aided detection (CADe) as a help in the interpretation of medical images, such as X-ray, MRI, and ultrasound.

### *1.7.3. Hormone delivery*

The pump(s) of the AP system continuously, or by frequent mini-boluses, delivers the amount of hormone calculated by the controller. The insulin formulations used in pumps and AP systems are fast-acting insulins approved for this purpose. However, there is no glucagon formulation approved for use in a pump. Glucagon is highly unstable in an aqueous solution as it easily forms  $\beta$ -pleated sheets of amyloid-like fibrils after mixing (170). The amyloid-like fibrils can then turn into a firm gel (119,171) and clog the infusion set. Glucagon also degrades through oxidation and deamidation in aqueous solutions and loses its effect (172,173).

Recent interest in both improved glucagon emergency kits and glucagon as a part of an AP has initiated the development of stable liquid forms of glucagon (118,121,171,174). At least two different stable glucagon formulations are currently under investigation: BioChaperone and Dasiglucagon, and one formulation by Xeris was approved by the FDA in September 2019. Currently, there are no glucagon formulations approved for pump use.

**BioChaperone® Human Glucagon** is unchanged recombinant glucagon in an aqueous solution where the peptide is protected from degradation and aggregation by protective molecules (chaperones). It is marketed by the pharmaceutical company Adocia. Preliminary results show that two different formulations of BioChaperone glucagon

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successfully treated insulin-induced hypoglycaemia in people with DM1. The dynamics, however, were slower than commercially available glucagon (175).

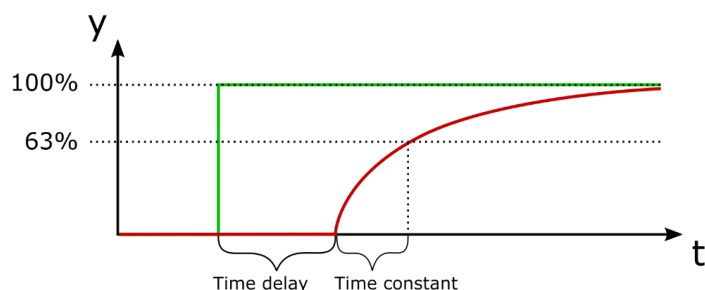
**Dasiglucagon** is a glucagon analogue under development by Zealand Pharma. Seven of the 29 amino acids of the glucagon molecule have been changed. This has altered the properties of the glucagon molecule, making it resistant to degradation and formation of fibrils when mixed in an aqueous solution. Trials on DM1 patients show that dasiglucagon restores BGL after insulin-induced hypoglycaemia, but the effect seems to be more prolonged compared with conventional glucagon (179). It has also been shown efficient in small doses for use in an AP system (117). The formulation is currently investigated for long time use in an animal model and as a part of an AP system in cooperation with Beta Bionics. They plan on conducting a phase 3 study in 2020.

**XeriSol™** is a non-aqueous, injectable suspension developed by the pharmaceutical company Xeris. The solvent in this suspension is dimethyl sulfoxide (DMSO), which dissolves both non-polar and polar molecules. DMSO is used in a wide range of products, including several medications. It also promotes the penetration of molecules through tissue (177). Because this is a non-aqueous solution the formation of glucagon fibrils is avoided. This glucagon formulation showed similar glucose dynamics as conventional glucagon formulation after SC injection in pigs, with maximal glucose rising effect at 35-40 minutes (118). The glucagon formulation has also been tested on humans and proved efficient in elevating BGL after insulin-induced hypoglycaemia in adults and children (178,179). An emergency pen and a prefilled syringe of glucagon (Gvoke, Xeris) were approved by the FDA in September 2019.

#### *1.7.4. Challenges to the AP system - latency*

Regardless of the type of glucose sensor or controller used in an AP system, there are several challenges to overcome to achieve optimal regulation of the BGL. The performance and robustness of an AP system are highly dependent on the dynamics of the different parts of the control-loop. In a perfect situation, there would be no delays in the control loop, and the controller would easily handle any disturbance to the system (i.e. meals, exercise). However, latency in the AP system seems inevitable. The latency can be described as a combination of both **time delay**, the time passed before a change in the system is detectable, and the **time constant**, the time passed before 63% of equilibrium is re-established after a sudden change to the system (Figure 8) (180).





**Figure 8.** Illustration explaining time delay and time constant.  $y$  represents the quantity to be measured (for the green line) and the sensor signal (for the red line).  $t$  represents time. The green line shows the system stimulus. The red line represents the output from the system, i.e. the detection of the change in the system. Figure adapted from Christiansen et al. (181).

Part of the latency is physiological and depends on the characteristics of the environment for the different parts of the control loop. For glucose sensing, latency is determined by how fast glucose molecules diffuse from blood to the sensor where they are detected. This latency in glucose sensing implies that the controller decides appropriate actions based on historical data. This might not be a noteworthy problem for systems with only minor disturbances to the system, but the large and fast glucose changes observed in relation to meals and exercise in persons with DM1 will represent major challenges for an AP system. For the double SC approach, a prediction of these large glucose deviations is needed. The MiniMed 670G requires the patients not only to notify the controller of an upcoming meal but also inform the system of the carbohydrate content of this meal to deliver the insulin bolus before the BGL elevation.

The latency in insulin absorption and effect will be determined by how fast the hormones are absorbed and transported to the cells they bind to and how rapid they cause an effect on the BGL. The latency after SC delivery of insulin is higher than the latency in glucose sensing and represents a greater challenge to the success of an AP system. The pumps themselves contribute very little to the total latency of the system and the latency in insulin effect is primarily caused by physiological factors. Some pumps offer the possibility to adjust bolus delivery rates. Medtronic 640G pump and Animas Vibe pump delivers insulin meal boluses at a rate of 1U per 40 seconds and 2.5 seconds, at the slowest rate, respectively. However, other technical parts will contribute to the

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overall latency of an AP system. When reporting results from AP studies, such as the MiniMed 670G, the latency is not differentiated and the results are a combination of the physiological latency, potential latency in the sensor itself and the averaging of glucose values over every 5 minutes (182).

Minimizing the latency of glucose sensing and insulin absorption and effect will improve the overall AP system and possibly omit the need for user intervention. The development of faster-acting insulins and other ways of increasing the absorption rate of insulin from the SC tissue are warranted and currently under investigation. Moving the glucose sensing and hormone delivery to an alternative site might minimize both latencies in glucose sensing and effect of delivered hormone and investigating the IP space for this purpose forms the bases of this thesis.

### 1.8. Possible sites for an artificial pancreas

The site for both glucose sensing and hormone delivery in an AP should contribute as little as possible to the delays of the system, and at the same time be as comfortable and safe as possible, i.e. it is a trade-off between efficiency and robustness on one hand and functionality on the other. When only considering the dynamics of glucose sensing and hormone delivery, the optimal positioning of an AP would be in the portal vein, closely resembling the normal physiology. This is not a realistic approach due to the invasiveness and high risk of serious complications. A larger peripheral artery or vein would also provide fast dynamics of both glucose sensing (166) and hormone delivery and effect compared with SC delivery (183). However, placing a sensor and catheter in a peripheral vessel for a prolonged period comes with a high risk of infections and thromboembolic complications. The intravascular site is also a hostile environment for foreign objects resulting in short sensor lifetime. An intravascular AP is under development as an alternative for patients under intensive care and for limited periods (167,184).

The next two sections will discuss the two most relevant sites for an AP system: the SC tissue and the IP space.

### **1.8.1. The subcutaneous tissue**

The SC tissue is an easily accessible site and both sensors and cannulas for pumps can be inserted by the patients themselves. Although the skin is penetrated, the risk of serious complications is low, and infections can be easily supervised and addressed. The double SC approach is the most studied solution for an AP system and the site for the only currently available hybrid AP system.

#### **1.8.1.1. Anatomy of skin**

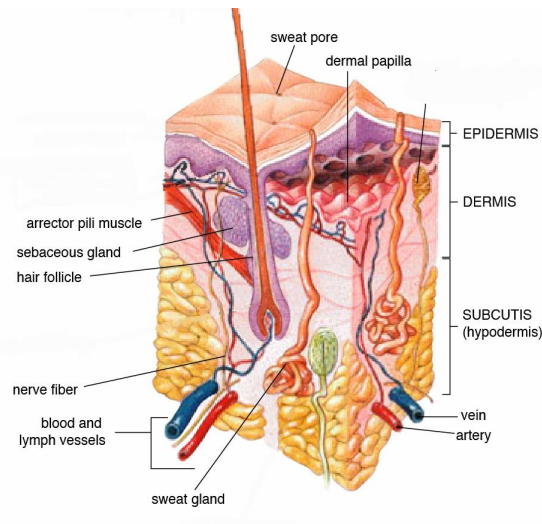
The skin is the outer barrier of the body. It serves as a protective layer, shielding the body from pathogens and mechanical and chemical influences. The skin regulates the body's temperature by both evaporation of sweat and by regulation of the blood flow in the skin. The skin also retains water and is an important sensory organ.

Skin thickness is associated with age, being thickest in young adults compared with both children and older people (185). It is divided into three distinct layers: epidermis, dermis, and hypodermis. Hypodermis is also named SC tissue or subcutis.

The **epidermis** is the outer layer and consists of several cell types, but keratocytes make up most of the cell population. The epidermis forms the eccrine and apocrine sweat glands, hair follicles and the sebaceous glands, which all protrude down into the dermis (186). The epidermis does not contain blood vessels or nerves.

The **dermis** provides the skin with its elasticity, flexibility and tensile strength. It holds water, protects against mechanical injury and contains nerve endings and receptors important in thermoregulation and pain recognition. The dermis is a complex of fibrous and amorphous connective tissue, nerve cells, vascular networks, macrophages and mast cells (186). Blood derived cells, including lymphocytes, leukocytes and plasma cells, can infiltrate the dermis in response to inflammation or other stimuli.

The **hypodermis/SC tissue** is the site for both CGM and insulin injections/infusions. The SC tissue contains large amounts of fat cells, which act as shock absorbers to blood vessels and nerve endings (187). The thickness of the SC adipose tissue varies between individuals and different body parts. Plexuses of blood vessels are formed between the SC tissue and dermis from which capillary loops enter the outer parts of the skin.



**Figure 9.** Schematic overview of skin histology. The illustration is public domain and has been modified.

[http://training.seer.cancer.gov/ss\\_module14\\_melanoma/images/illu\\_skin01.jpg](http://training.seer.cancer.gov/ss_module14_melanoma/images/illu_skin01.jpg)

Downloaded October 2019.

The blood supply of the skin is organized in interconnected blood vessel plexuses at different levels (188,189). Regulation of skin blood flow serves an important function in the body's thermoregulation, and the blood flow can go from almost zero to more than half the cardiac output in situations of heat stress (190). Local blood flow and endothelial permeability are also increased by inflammation mediators.

Interstitial fluid (ISF) surrounds all individual cells and forms, together with the extracellular matrix, the interstitium. All molecules, such as nutrients and waste-products going between the individual cells and the capillaries, different signal molecules between cells and antigens and cytokines to the local lymph nodes must all diffuse through the ISF (191). The amount of ISF varies between different organs and constitutes approximately half of the wet tissue weight in the skin (191).

### 1.8.1.2. Subcutaneous glucose sensing

The sensing element of a CGM device is inserted in the outer layer of the SC tissue. This site is easily accessible and offers a low-risk opportunity for continuous glucose monitoring. The Eversense® sensor is the only fully implanted sensor and needs to be implanted and removed by health professionals. The risk associated with this sensor is still low, and it has a significantly longer lifetime compared to the electrochemical sensors.

The glucose concentration in the ISF of the SC tissue reflects the glucose concentration in blood at a steady-state, but a time latency is observed during changes in the BGLs, for both increasing and decreasing values (192). The delay between blood glucose values and ISF glucose values is also suggested to be more complicated than a "pure" delayed response, but more that the ISF glucose represents a "distorted mirror" of BGL (193). The physiological time latency is, in an experimental setting, shown to be 5–6 minutes in healthy individuals (194), and approximately 7 minutes in patients with diabetes (195). However, the physiological time latency varies greatly from patient to patient (196,197). Clinical studies investigating the performance of CGM report time latencies between blood and sensor output of between 4 and 20 minutes (196,198–202). Unfortunately, sensor dynamics are not differentiated from the physiological time latency in these studies, which is a problem as the time latency in the sensor systems might potentially hide or mask the physiological time latency (155,203). The great variability between sensors is also a problem when comparing the results between studies (198,200). CGM devices probably compensate for the time latency in some way (196,204), but the algorithms for the different commercially available sensors are not publicly available.

CGM performance is also highly dependent on other circumstances such as foreign body reaction towards the inserted sensor (205–208), mechanical pressure to the sensor or the insertion area (207,209,210), microhemorrhages at the insertion site (211) and fluctuations in tissue perfusion (192,212). The insertion of the sensor itself, penetrating intact skin, triggers the immune system. Also, the continuous presence of a sensor will prevent normal wound healing and cause a foreign body reaction. The area around the sensor will first go through a period of acute inflammation and proteins and immune cells will adhere to the sensor membrane. This acute inflammatory reaction leads to a suboptimal sensor performance the first days after insertion (213), and advice shared among CGM users in the online diabetes-communities is to insert the new CGM sensor

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the day before removing the old. After the acute phase, the inserted sensor element will be surrounded by granulation tissue, which will start degrading the sensor membrane, and finally, a fibrous capsule will cover the foreign body, affecting the diffusion of glucose and oxygen through the membrane of the sensing element (214). This encapsulation of the sensor leads to a drift in the sensor signal, and most of the CGM devices on the market must be calibrated against BG values daily by the user to account for this drift. The pre-calibrated sensors, such as the FreeStyle Libre (Paper I), are calibrated by the manufacturer and do not require calibrations by the user. The manufacturer claims that this is achieved by optimisation of the sensor chemistry as well as the sensor biocompatibility and reducing variability in sensitivity between sensors, without providing further details (215).

Other circumstances, which influence blood perfusion in the SC tissue, such as fever, exercise, sepsis and external temperature (216), might also influence SC glucose sensing dynamics. However, no data have been published on how different skin temperatures or physiologic variations in blood flow of other causes affect the performance of CGM devices.

#### 1.8.1.3. Subcutaneous hormone delivery

**Insulin** is delivered in the SC tissue either by multiple daily injections or continuously by a pump. Both the kinetics and dynamics of SC delivered insulin are slow, regardless of the method of delivery even for fast-acting insulin formulations. The maximum concentration in blood is reached 45 minutes after injection, and the blood concentration is still markedly increased after 3 hours (217). The maximal glucose-lowering effect is observed around 90–120 minutes, and the effect is still present more than five hours after the bolus (217). This is in contrast to normal physiology where small but, significant amounts of insulin are secreted into the portal vein even at the anticipation of a meal (sight, smell and artificial sweet taste) (218) and the insulin secretion is finely tuned to the changes in BGL and restores normal BGLs within 2–3 hours. The slow effect of SC insulin delivery implies that meal boluses should be delivered before eating to match the time of maximum glucose-lowering effect with the postprandial glucose elevation. Optimal timing and choice of the correct dose can be challenging for several reasons, but especially the substantial variation in insulin absorption and effect makes insulin blousing difficult. There are several factors affecting insulin absorption and effect, causing both intra-individual and inter-individual variation. Examples of such factors are changes in SC blood perfusion caused by changes

in temperatures or physical activity, body position and lipohypertrophy (219). Inserting the infusion set in the SC tissue causes an acute inflammatory reaction and subsequent fibrous encapsulation of the inserted tube (220), probably also affecting insulin kinetics. Pump users are advised to change their infusion sets every two-three days to avoid local skin reactions to the infused insulin formulations. A small study showed that use for longer than the recommended 2–3 days caused skin irritation and discomfort at the insertion site and a significantly higher mean glucose at day seven compared to the first day (221).

**Glucagon** is currently used as an emergency treatment of severe insulin-induced hypoglycaemia when the patient is not able or willing to consume carbohydrates. In an emergency, it is not important if glucagon is injected in the SC tissue or the muscle tissue, as the glucose rising ability is not significantly different between the two routes (222). BG starts to rise shortly after administration, and the maximal glucose response after an SC injection of 1 mg is reported to be around 25 and 30 minutes (222–225). Glucagon has a short half-life in plasma of approximately 6–7 minutes after IV delivery (225–227). The half-life is reported to be longer after SC and IM delivery, 27 and 23 minutes, respectively and larger doses seem to increase the half-life (225).

The normal dose for treating severe hypoglycaemia is 1 mg for adults, and 0.5 mg for children <25 kg (223,224). The use of smaller doses of glucagon was first investigated for treating milder hypoglycaemia in children unable or unwilling to consume carbohydrates (228). Small doses of glucagon have shown good results in correcting mild hypoglycaemia in children (228,229), and adults (120,230–232) and preventing post-exercise hypoglycaemia (233). Promising results have been shown for the new development of stable glucagon formulations and glucagon analogues (117,118,175,179). Unfortunately, there is no consensus regarding the optimal glucose response after mini-dose glucagon boluses, and doses and study protocols vary between the published papers making comparisons between available data challenging.

Although mini-doses of glucagon are successful in restoring normal glucose levels in controlled settings, glucagon boluses in the AP setting have not prevented hypoglycaemic episodes, and correction by carbohydrate intake was still sometimes required (138,234–242). Several factors might influence the ability of glucagon to elevate BGLs. In the setting of an AP, glucagon would ideally be used to prevent imminent hypoglycaemia, i.e. it would be given when the BGLs are in the lower euglycemic range and not in the hypoglycaemic range. A study in dogs showed that

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glucagon induced a higher glucose response when infused in the hypoglycaemic range compared with the euglycaemic range (243). However, contrasting results have been found when studying DM1 patients where there was no apparent difference in glucose elevating ability of both small and large doses of glucagon at different BGLs (231,244). Although different BGLs might not influence glucagon efficiency, high levels of insulin-on-board seem to have a negative effect on the small glucagon doses' ability to raise euglycaemic BGLs (245,246), and should be considered when including glucagon in an AP system.

The rapid glucose elevating effect of glucagon is caused by the stimulation of glycogenolysis in the liver (see section 1.3). The amount of glycogen available in the liver might, therefore, influence the glucose response. A study on non-diabetic persons receiving two IV injections of 0.5 mg glucagon 7 hours apart showed a markedly lower glucose elevation after the second dose (247). However, the first dose was given in the morning and a fasting state while the second dose was given in the afternoon and in a postprandial state, which might have influenced the glucose response. The reduced effect of a second glucagon bolus has not been reproduced in DM1 patients. Castle et al. conducted a study on 11 DM1 patients who received eight SC glucagon boluses of 0.2 microgram/kg either in the fasting or fed state (248). The authors could not identify any difference in glucose response whether patients were fed or fasting. Neither did they observe an effect on the estimated amount of glycogen in the liver before and after the repeated glucagon boluses in either group. A study by Blauw et al. showed similar glucose results after three repeated doses of glucagon in fasting DM1 patients (231). However, a one-week study investigating how low-carbohydrate diet in DM1 patients can influence glucagon efficiency showed that the glucose response to glucagon was smaller in the low-carbohydrate diet group compared with the group eating the high-carbohydrate diet (249). The pharmacokinetic parameters of glucagon and insulin were similar in the two groups, indicating that different amounts of stored liver glycogen might explain this difference in glucose response.

#### 1.8.1.4. Clinical outcome of the double SC AP system

Meta-analysis (including both dual and single hormone) show that the AP system is superior to conventional insulin treatment when evaluating time spent in near euglycaemia, time spent in hyperglycaemic and hypoglycaemic range and mean glucose values for the wider diabetic population (250–252) and the non-adult population (253). However, these improvements are mostly observed during night-time when there are



few disturbances to the system (such as meals and physical activity) and the AP system only regulates the basal hormone delivery (181,252). Meals and exercise represent major disturbances to the AP system, and most studies on insulin-only AP systems have therefore included carbohydrate counting and meal announcement in their study protocol.

Several AP systems are under development, and the FDA approved the first AP system in 2016 (3). This a system marketed by Medtronic under the name MiniMed® 670G System, and is a hybrid closed-loop system, as the users still need to count the amounts of carbohydrates of any upcoming meal and notify the controller. Users are also advised to check their BGL with a finger-prick sample when waking up, before every meal, at bedtime and occasionally mid-sleep (254). The target level is set at 6.7 mmol/L (120 mg/dL) and can be temporarily adjusted to 8.3 mmol/L (150 mg/dL) if the user would wish to. The MiniMed 670G system will exit its “Auto mode” and enter a “Safe basal mode” if it detects a sensor fault or if insulin delivery exceeded a predetermined maximum over a predefined period (255). In the “Safe basal mode”, the system delivers insulin at a constant rate and does not adjust this delivery rate based on the CGM signals. The system can return to “Auto mode” or request the user to enter a BG value. If a BG value for calibration is not provided, the system will enter “Manual mode” (256). A three-month study on adolescents and young adults using the MiniMed 670G system showed decreasing time in “Auto mode” throughout the study period, starting at 89% in the first week and ending with 71.8% in the last week of the study (256). The great majority of these “kick-outs” from “Auto mode” was initiated by the AP system, and most often because the system detected an irregularity (sensor or pump related) or because of hyperglycaemia.

Most AP systems under development are insulin-only systems, which regulate the BGL by increasing, decreasing or discontinuing insulin delivery. By including glucagon, the AP system can actively increase BGLs in case of hypoglycaemia or imminent hypoglycaemia. Reviews on dual-hormone AP vs. single hormone AP suggests that dual-hormone systems are beneficial in terms of less time spent in hypoglycaemia (251,257) and reduced total carbohydrate intake for corrective proposes (257). However, the need for rescue carbohydrate consumption is not completely eliminated (234,235,237,240,241,258). The dual-hormone AP systems also seemed to enable better glycaemic control after announced or detected exercise (138,259,260). Prevention of overnight hypoglycaemia, glycaemic control in the post-prandial period and reduction of mean glucose levels are areas where the dual-hormone AP systems

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have not shown conclusive superior results compared with single hormone systems (257). Dual-hormone AP systems have been studied without meal announcement (240,241), with standardized meal announcements (234,237,258) and with carbohydrate counting (238), showing time spent in target range (day and night) of 78–85%, 78–80%, and 79%, respectively. These results are comparable to the results from single-hormone studies with meal announcement (252).

### *1.8.2. The peritoneal space*

The peritoneal space is the space within the abdominal cavity. It is covered by a serous lining and contains peritoneal fluid. The peritoneal fluid lubricates the lining and reduces friction and facilitates movements of the abdominal organs, such as the intestines and female reproductive organs.

#### **1.8.2.1. Anatomy**

##### **Gross anatomy**

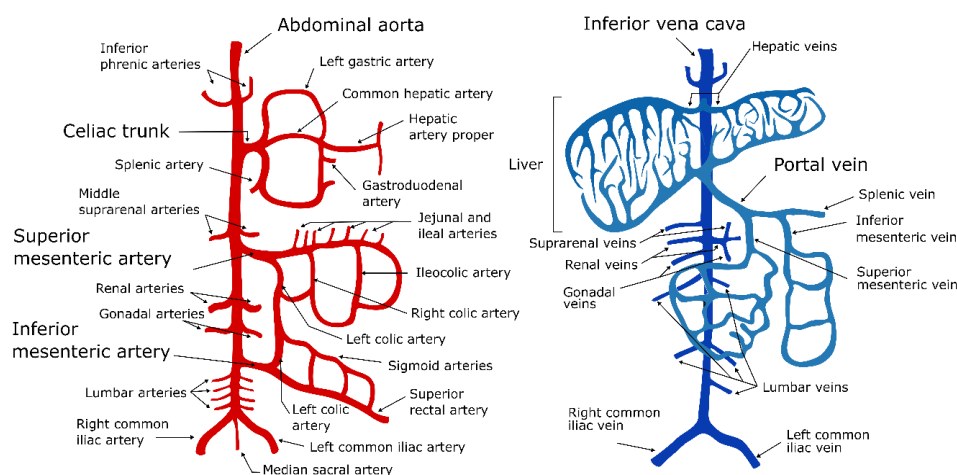
Although the volume of the IP space in humans is small in situ and under normal conditions, the surface of the IP space, the peritoneal lining, is for adults ~ 15 000 cm<sup>2</sup> (261) and covers the internal abdominal walls and most of the organs within the abdominal and pelvic cavity. The visceral peritoneum covers the visceral organs and accounts for ~80% of the total area, while the parietal peritoneum covers the inner abdominal and pelvic walls and accounts for ~18% of the total area (261). In males, the peritoneal space is a closed space, while in females the peritoneal lining is perforated by the Fallopian tubes.

The peritoneal cavity is subdivided into a supramesocolic compartment, inframesocolic compartment and the pelvic cavity, all further divided into smaller compartments (262,263). The supramesocolic compartment refers to the part of the peritoneal cavity above the transverse colon. It contains the liver, stomach, and spleen. The inframesocolic compartment refers to the part of the peritoneal cavity below the transverse colon, and contains the smaller intestines, pancreas, descending and ascending colon. The greater omentum is a fold of visceral peritoneal lining containing blood vessels, lymph vessels, and adipose tissue. It is attached to the greater curvature of the stomach and transverse colon and hangs down between the ventral abdominal wall and the intestines like a double folded apron. The pelvic cavity contains the bladder,

rectum and the female internal reproductive organs. Only parts of the bladder and uterus are covered by the peritoneal lining.

Three groups of arteries, all originating from the abdominal aorta, supply the abdominal organs and internal abdominal walls (Figure 10). The unpaired visceral arteries: celiac trunk, superior mesenteric artery, and inferior mesenteric artery supply the gastrointestinal tract, liver, spleen, and pancreas. The paired visceral arteries supply the kidneys, adrenal glands, and gonads, and finally, the lumbar arteries supply the musculoskeletal structure of the abdominal wall (264). In the basal fasting state, the mesenteric blood flow is approximately 10% of the cardiac output (265). Digestion of a meal leads to a rapid increase in abdominal blood flow, mainly through the mesenteric arteries supplying the intestines, liver, and pancreas (266). The postprandial blood flow in the superior mesenteric artery can increase fourfold compared with the blood flow in the resting and fasting state (267).

Blood from the small and large intestines, stomach, pancreas, spleen and the greater omentum drains into the portal vein and is transported to the liver (Figure 10). In the liver this venous blood from the portal vein is mixed with arterial blood from the hepatic artery in the sinusoids of the hepatic acinus (the smallest functional unit of the liver). The mixed blood then drains into the inferior vena cava and is transported to the right atrium of the heart. Blood from the inferior phrenic, the renal, testicular or ovarian, lumbar and common ileac veins all drains directly into the inferior vena cava and thus surpasses the liver (Figure 10).

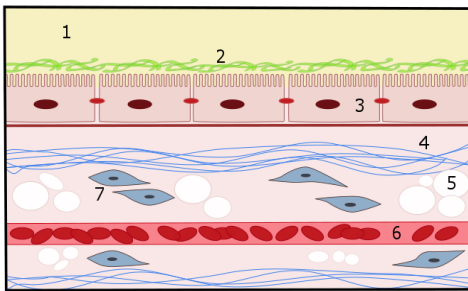


**Figure 10.** Overview of arterial and venous vessels of the abdominal cavity.

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## Histology

The peritoneal lining is the largest serosal membrane in the body (268). It consists of a single layer of mesothelial cells (mesothelium), a basal lamina and several layers of connective tissue embedded with capillaries, lymphatic vessels and nerves (submesothelial stroma) (Figure 11) (269,270). The mesothelial cells are flattened and have a diameter of  $\sim 25 \mu\text{m}$  (271). They have numerous microvilli at the peritoneal side (272), and are covered by a layer of glycocalyx which facilitate the formation of a stagnant layer of peritoneal fluid (271). Together the microvilli, glycocalyx, and layer of stagnant peritoneal fluid protects the surface against friction caused by movements of the abdominal organs and against damage from invading organisms (269).

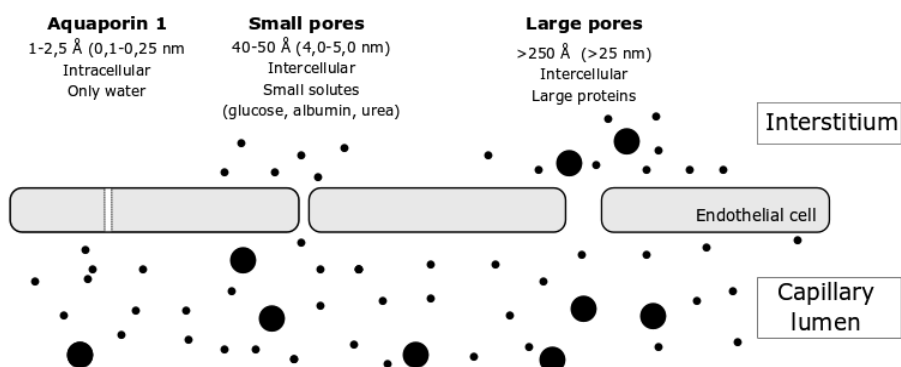


**Figure 11.** Sketch of the peritoneal lining. Peritoneal fluid (1), a layer of glycocalyx (2), mesothelium (3), collagen fibres (4), fat cells (5), capillary with red blood cells (6), fibrocytes (7). Image not drawn to scale.

The thickness of the peritoneal lining varies depending on age, being thicker in older children and adolescents compared with newborns and older adults (270). The blood capillary density also varies depending on age, with higher blood capillary density in young children and older adults compared with older children and adolescents (270). The key histological parameters of the peritoneal lining seem to be the same for different parts of the peritoneal cavity (269,270,273). The exception being the ovaries, which are covered with cuboidal epithelial cells and the mesothelium covering the lymphatic lacunae of the diaphragm, which is perforated by stomatas. These stomatas create a direct passage between the IP space and the lymphatic system through which peritoneal fluid is drained into the lymphatic circulatory system (274).

### 1.8.2.2. Physiology – transperitoneal water and solute transport

Transport of solutes and water across the peritoneal lining has been extensively studied in the field of peritoneal dialysis. Water and solutes must pass through several barriers between the lumen of the capillaries and the IP space. The mesothelium is not considered a major limiting factor for the diffusion of water and small solutes, and in fact, removing the mesothelium did not affect the transportation of water or small solutes from the IP cavity in rats (275). The endothelium of the capillaries, however, is considered the major barrier in the peritoneal lining, discriminating the transport of solutes based on their size. The peritoneal lining does not appear to have any significant charge-selective properties (276). The currently dominating theory, which has proved successful for modelling purposes, is known as the "three-pore-theory". This name refers to there being three types of pores in the endothelium through which water and solutes can diffuse (277,278): large pores, small pores, and aquaporin-1 (Figure 12). **Large pores** are intercellular and larger than 250 Ångström (Å) (25 nm). They allow for larger proteins like albumin, immunoglobulin M (IgM) and immunoglobulin G (IgG) to pass through (276,279). The large pores comprise 0.01 to 0.1% of the total pore area (280). **Small pores** are between 40–50 Å (4–5 nm) in size and are also intercellular. They let smaller solutes through, such as urea, creatinine, and glucose (276,279,281). The small pores comprise approximately 99.5% of the total pore area (280). **Aquaporin-1** are intracellular pores with a diameter of only 1–2.5 Å. Only water diffuses through these pores, and they play an important part in water transport over the peritoneal lining.



**Figure 12.** Schematic presentation of the "three-pore-model". Small circles represent molecules smaller than 50 Å, which can pass through the small pores. The larger circles

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*represent larger molecules and proteins that can only pass through the large pores. Water molecules are not drawn. Figure adapted from Devuyst et al. (280).*

The "three-pore-model" is a simple model and only takes into account the endothelial walls when modelling water and solute transport. The peritoneal lining include other barriers as well, and the extracellular matrix (282), endothelial glycocalyx (283) and the stagnant layer of peritoneal fluid on the mesothelium (284) also contribute to the transport dynamics across the peritoneal lining. These factors have not been investigated to the same extent as the endothelial pores. Inflammation will alter the properties of both endothelial walls (285,286) and endothelial glycocalyx (283,286), and thus alter the transport of water and solutes across the peritoneal lining. Acute inflammations will cause a reversible increase in peritoneal permeability and rate of diffusion of both water and solutes (285).

**Glucose** is a small molecule, 8.4–8.6 Å (0.84 x 0.86 nm) in size and has a molecule weight of 180 Da, and will easily pass through the peritoneal lining, both through the large and small pores (276,279,281,287). Transport of glucose through the peritoneal lining is passive, driven mainly by the concentration gradient and diffusion similar to other small molecules (288).

**Glucagon** is a peptide hormone consisting of 29 amino-acids. It has a molecular weight of 3485 Da (289) and is 47.1 Å (4.71 nm) in length (290). The composition of bovine, porcine and human glucagon is identical (291), and also identical to the recombinant glucagon available as emergency treatment (223,224). The molecule has a flexible structure and consists of an equilibrium of different conformers: random coil structure,  $\alpha$ -helix, and  $\alpha$ -helix stabilized either as trimers or by association with a receptor when it is in a solution of neutral pH (290). The glucagon molecule is too large to easily pass through the small pores in the endothelium of the peritoneal lining.

**Insulin** is a peptide hormone consisting of two chains of amino acids linked together by disulphide bonds. The chains are comprised of 21 and 30 amino acids, respectively. There are minor differences between human, animal and recombinant insulin with regards to amino acid composition and molecular weight. Human insulin has a molecule weight of 5808 Da (292) and has a radius of 1.3 nm (130 Å) (293). The insulin molecule is too large to pass through the small pores in the endothelium of the peritoneal lining.

### 1.8.2.3. Peritoneal fluid

The peritoneal fluid lubricates the peritoneal lining, reduces friction and facilitating movements of the abdominal organs. The peritoneal fluid has a specific gravity between 1.010–1.020, and contains mostly water, but also electrolytes, solutes, proteins and cells (294). The IP space is potentially large and can hold several litres of fluid. The amount of peritoneal fluid under normal conditions is not known, but is suggested to be between 15 to 100 ml (262,269,295,296). The fluid is distributed as a stagnant layer on the mesothelium, however, aggregates of up to 5 ml of free fluid have been detected by ultrasound and MRI in healthy persons (297,298). The IP fluid is suggested to be produced by the mesothelial cells, but this has not been determined. Women in reproductive age, not using hormonal contraceptives, have higher volumes of peritoneal fluid around the time of ovulation (299,300), and this increase in volume is speculated to result from oestradiol induced changes in vascular permeability and the addition of the fluid from the follicular rupture (299). The peritoneal fluid circulates within the abdominal space. Breathing movements and drainage of peritoneal fluid through lacunae in the diaphragm (274,301) creates a lower pressure in the upper part of the peritoneal cavity, moving the fluid cranially, while gravity moves the peritoneal fluid downwards in the standing position. These opposite forces, in addition to intestinal movement, create the movement and flow of the peritoneal fluid but it is the internal organs and anatomical structures that determine the flow pattern of peritoneal fluid (302,303).

### 1.8.2.4. Intraperitoneal glucose sensing

Utilizing peritoneal fluid for glucose measurements was first performed in 1982, in a study seeking to find a more suitable location for implantable glucose sensors than blood vessels (304). "Diffusion chambers" and capsules were inserted in the SC tissue, the IP space and the pericardial space of rabbits and baboons, from which fluid was extracted and used for glucose measuring. Rapid changes in glucose concentrations could not be detected by this method, but the study proved that glucose could be measured in other body fluids than blood.

The first experiments testing IP implanted glucose sensors were performed by Leland Clark et al. in the late 1980s (305,306). They were developing a long-lived implantable electrochemical glucose sensor utilizing a technique of immobilizing and stabilizing the glucose oxidase enzyme with glutaraldehyde vapour. To test this new sensor they chose

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to implant it in the IP space of rats "because it seems to be the most hostile environment for the sensor" (305). Sensors were extracted after weeks and months of implantation, immediately tested, and, if still functional, inserted into the IP space of another rat. The sensors remained functional for over 3 months despite being covered by a thin fibrous cap (305). This sensor technology was also investigated in vivo in the IP space of rabbits to investigate if the oxygen tension would be sufficient for the enzymatic reaction (306). Sensor output was compared to BG values showing high correlations between the measurements of the different compartments ( $r=0.87$ ). The sensor output was, however, delayed compared to BG values, and by adding a 10-minute time lag, the correlation between measurements increased ( $r=0.96$ ). The sensor output also reacted to falling BGLs after insulin injection in a diabetic rabbit.

Velho et al. was the first to publish a paper specifically investigating the IP glucose dynamics. They showed that the concentration of glucose in extracted peritoneal fluid correlated well with the BGL in both fasted and non-fasted rats. They also investigated an IP implanted electrochemical sensor in rats and showed that the sensors tracked the BG changes with an approximately 5 minutes delay, both after intravenous infusion of glucose and insulin (307). These first studies in the 1980s demonstrated that glucose could be measured in the IP space and that electrochemical sensors could potentially function for a long time in vivo.

Four papers exploring IP glucose sensing have been published since the 1980s, including Paper I of this thesis. They show results both superior and comparable to SC glucose sensing. Burnett et al. implanted modified Dexcom SEVEN (Dexcom, San Diego, CA) sensors in the IP space of pigs and compared them to commercially available Dexcom SEVEN sensors inserted in the SC tissue (308). They found faster glucose dynamics in the IP space compared with the SC tissue, with both significantly shorter time delay and time constant for the IP sensors compared with the SC sensors (308). The same research group also published preliminary results from an experiment on two sheep, which had a fluorescent glucose sensor (not further described) inserted in the IP space and flushed with saline to test performance over time. The results indicated that a sensor implanted in the IP space and regularly rinsed with saline, performs better over time compared with an identical sensor inserted in the SC tissue where flushing with saline is not applicable (309).

Our research group has published a paper where optical sensing technology was studied in pigs (310). This study forms the base for Paper II. The optical interferometric glucose



sensor, designed for intra-arterial (IA) use, was inserted in the IP space and the femoral artery of three pigs. Due to low mechanical robustness, this sensor could not be inserted in the SC tissue, and the identified glucose dynamics were not compared to the dynamics of SC glucose. The study identified fast IP glucose dynamics, and some of the measurements showed no delay between the IP sensor and the IA sensor, indicating that glucose can be detected simultaneously in the IP space and femoral artery.

#### 1.8.2.5. Intraperitoneal hormone delivery

**CIPII** is a treatment option currently used as a last resort for some patients with severe SC insulin resistance, frequent severe hypoglycaemia, lipoatrophy or skin disorders interfering with SC insulin delivery (4). A small number of patients currently use an abdominal port, the DiaPort (Roche, Switzerland), designed for CIPII. Infusion insulin in the IP space resembles the insulin secretion by the healthy pancreas as a portion of the hormone is absorbed by blood vessels in the greater omentum and intestinal and transported directly to the liver via the portal vein (311). A study on dogs demonstrated that the liver subtracted approximately 50% of IP infused insulin, by the so-called “first-pass-effect” (312). The degree of peripheral hyperinsulinemia is thus reduced after IP insulin delivery compared with SC insulin delivery (11). However, the introduction of an abdominal port or implantation of a pump brings with it a higher risk of serious complications compared with CSII. Local skin infections and clogging of the intra-abdominal catheters were the two main reasons for discontinuations of CIPII in some human studies (313).

CIPII has been studied in several clinical trials, mostly on patients with unstable diabetes or with serious complication related to CSII. Some publications show a reduction in mean HbA1c after CIPII treatment compared with SC insulin treatment (7,314–321), while other studies do not show a difference when comparing the two treatments (322–328). The studies also present conflicting results when looking at time spent in hypoglycaemia and incidents of severe hypoglycaemic episodes. Some studies observed less time spent below 4 mmol/L for the CIPII treatment compared to SC insulin delivery (316,317,329), while a study by Liebl et al. did not show reduced time in the hypoglycaemic range for CIPII treatment (324). Liebl et al., however, did observe a reduction in severe hypoglycaemic episodes for the CIPII treatment, an observation also found by Renard et al. (330). APT is working on a systematic review comparing CIPII and SC insulin treatment, and the preliminary results indicate better glycaemic control by CIPII treatment compared with SC insulin delivery. It should be mentioned that some of the

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publications included in the systematic review are of suboptimal design, which weakens the evidence base and hampers conclusive results.

IP delivery of **glucagon** has previously been studied in rats (331,332) and mice (333). These publications do not provide data suitable for system identification of the pharmacodynamics of glucagon and development of algorithms for a dual-hormone IP AP, but they demonstrate the glucose elevating properties of glucagon administered in the IP space. Loxham et al. found the glucose elevation to be higher and the glucose excursion to be of shorter duration after IP delivery compared with SC delivery in rats (331). The maximum BG value was observed after 20 minutes. Zlotnik et al. did not compare IP glucagon to SC delivery of glucagon in their study but showed that IP glucagon significantly raised the BGLs from baseline levels, with a peak value reached after 30 minutes (332). Both these studies only measured BG at a few time points, preventing closer identification of the glucose dynamics. The aim of Papers III and IV of this thesis was to identify the glucose dynamics after IP glucagon delivery by frequent blood sampling. The results from these publications will be used in the development of algorithms for a dual-hormone IP AP system, but this work is not within the scope of this thesis.

#### 1.8.2.6. Intraperitoneal AP

The development of an IP AP system is in its early stages. Several in-silico experiments have been published in which IP insulin delivery combined with either SC, IV or IP glucose monitoring have been investigated. They demonstrate that the IP space can potentially provide the fast insulin dynamics required for a fully automated AP system (334–337). Only a few in-vivo experiments on closed-loop with IP insulin delivery have been published. Matsuo et al. conducted a small study on alloxan-treated dogs with CIPII and SC glucose monitoring and ran the closed-loop for one day. The system was challenged with two unannounced meals. Although it was only tested on three dogs, the system kept the BGL within a range of 3.9 to 10.1 mmol/L (338). Three clinical trials on DM1 patients have investigated closed-loop with IP insulin delivery. Renard et al. conducted a trial on four people with DM1 for 48 hours with IV CGM and no meal announcements. There was no comparison to a control group with SC insulin delivery in this experimental setup, but the paper shows that the IV-IP closed-loop kept the BGL within the range of 80–240 mg/dL (4,5–13.3 mmol/L) for 85% of the time, with the majority of this time spent in the range of 120–240 mg/dL (6,7–13.3 mmol/L) (339). The algorithms were adjusted during the study, showing some improvement in time spent in the range of 80–

120 mg/dL (4.44–6.67 mmol/L). The same research group later conducted a similar study on eight people with DM1 and compared two-days of closed-loop to one-day of open-loop (340). Glucose levels were monitored by SC CGM and insulin boluses were manually given 15 minutes before meals in the closed-loop period. Unfortunately, the publication does not describe the regime for insulin bolusing in the control period. When looking at the whole trial period, the time spent within the target range of 4.4–6.6 mmol/L was approximately 40% for the closed-loop protocol compared with 30% for the open-loop protocol. The closed-loop, however, performed worse than the open-loop in the early postprandial period (18% vs 25% of time in target range, respectively). Dassau et al. published a study on ten adults with DM1 where they compared 24 hours of SC insulin closed-loop to a following 24-hour period of IP insulin closed-loop (341). Both protocols included SC CGM and three unannounced meals. The desired target range was set to 80–140 mg/dL (4.44–7.77 mmol/L). The IP closed-loop kept the BGLs within the target range for 40% of the overall time compared to 26% for a double SC AP system. The improvement in glucose control was mostly due to less time spent in hyperglycaemia for the IP approach. No papers have been published on in-vivo closed-loop trials with both IP glucose sensing and insulin delivery or with the inclusion of glucagon in an IP dual-hormone AP system.

The IP space is also under investigation as the location of an alternative “closed-loop” AP, which does not consist of a glucose sensor, control algorithms or an insulin pump. Instead, the system consists of a reservoir of insulin covered by a glucose-sensitive gel. The reservoir of insulin is refilled through a subcutaneous port by transcutaneous injections (342). Glucose binds to the gel surrounding the insulin reservoir, changing the permeability of the gel, thus releasing the contained insulin. Consequently, the correlation between the concentration of glucose and the permeability of the gel should permit increased release of insulin at concomitant high levels of glucose. The system has been investigated in rats and pigs (342,343). The implantation of the device was only successful in two of seven pigs (personal communication), and results were only presented from one of these pigs. The publication demonstrates that the device was capable of bringing BGL down to between 7 and 15 mmol/L for this pig but could not prevent the high glucose excursions (approximately between 18 and 33 mmol/L) after a 60 g oral glucose challenge. No further data from this AP approach have been published, and the status of the system is uncertain.

## 2. Aims of thesis

### 2.1. Overall aim

The main aim of this thesis was to determine whether glucose sensing in the peritoneum yields faster dynamics, improved robustness, and improved repeatability compared to subcutaneous glucose sensing for an artificial pancreas. The aim was also to identify the best site (i.e. fastest, most robust and reproducible dynamics) in the peritoneum for glucose sensing.

An additional aim for this thesis was to quantify the degradation and life expectancy of relevant glucose sensors when situated in the peritoneum. However, due to technical challenges and unavailable sensor technology appropriate for the specific use in the timeframe of this thesis, this issue could not be included in the thesis.

The second main aim of this thesis was to identify the pharmacokinetics and pharmacodynamics of intraperitoneal glucagon delivery, supporting our work towards an algorithm for an IP dual-hormonal AP system.

### 2.2. Specific aims

#### **Paper I**

The main aim of Paper I was to investigate potential differences in glucose dynamics in different parts of the peritoneal space. As comparator, glucose dynamics in the SC tissue was used.

#### **Paper II**

Paper II is a discussion of the large variation in the observed glucose dynamics within the peritoneal cavity, specifically from a set of animal experiments conducted by APT in 2014 using an interferometric glucose sensor developed for intravascular use. The paper presents a hypothesis that the positioning of the glucose sensor relative to the peritoneal lining has a major effect on the measured glucose dynamics.

#### **Papers III and IV**

The main aim of Papers III and IV was to identify the glucose-increasing effect of IP delivered glucagon and compare this to SC delivered glucagon. In Paper IV the

absorption of glucagon was also studied. Two different animal models were used for a broader evaluation of the topic.

## 3. Methods

### 3.1. Animals

#### 3.1.1. *Papers I and IV*

Pig models were chosen for Papers I and IV. Farm pigs (*Sus scrofa*, breed TN 70, mix between Norwegian Landswine and Yorkshire) were provided by a local farmer who is the sole provider of pigs to the animal facility of NTNU. The size of the animal was an important factor for deciding on a pig model for Paper I. The sensors needed to be separated sufficiently to examine glucose dynamics in different parts of the IP cavity, and the size of the FreeStyle Libre sensors themselves (35 mm in diameter) required an animal of a certain size. In Paper IV, a pig model was chosen to reproduce the experiments done in rats, as both the physiology and anatomy of pigs resemble humans more closely. We also plan to conduct closed-loop trials using a pig model in the near future. The pig model allowed for the insertion of tubes into the IP space providing a more controlled way of administrating glucagon compared to the rat study where glucagon was delivered by injections through the abdominal wall.

The pigs were acclimated to the staff and new environment for approximately one week before experiments. They were housed in groups as far as possible and given toys to stimulate their senses and intellect. They were fed with normal growth feed and provided water by drinking nipples ad libitum. Food was withdrawn for approximately 12 hours before the FreeStyle Libre-trials (Paper I) and nine–ten hours before the glucagon trials (Paper IV). The pigs were euthanized with an IP overdose of pentobarbital at the end of experiments under full anaesthesia.

#### 3.1.2. *Paper III*

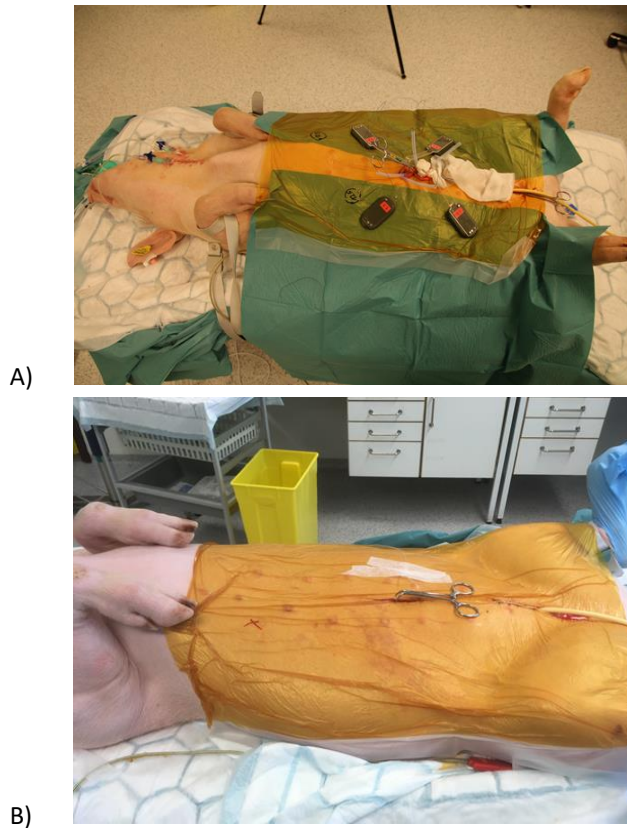
IP glucagon delivery was also studied using a rat (*Rattus norvegicus*) model. Ten male Sprague Dawley rats (470–615 g, Janvier Labs, France) were used in a pilot study to determine the final protocol and dose of glucagon to be used in the main study in which 20 rats were included. The rats were acclimated to the animal facility, housed in groups of three in plastic cages with solid bottoms (515x381x256mm, Techniplast, Italy) on sawdust and maintained on twelve hours light – twelve hours dark photoperiod at 20–24°C and relative humidity of 55% ± 5. They were fed expanded pellets (Special Diets Services RM1 for rats, UK) and provided fresh water ad libitum. Food was withdrawn

one to three hours before the experiments. The rats were trained to accept general handling and the use of a restrainer (Harvard apparatus, Holliston, USA) for three weeks before the start of the experiments. The rats were randomised to the different intervention groups, and as all intervention groups were represented in every cage, the individual rat was considered as the statistical unit. After the last intervention, the rats were anaesthetized with the same protocol used in the experimental protocol and euthanized with an IV overdose of pentobarbital.

### 3.2. Surgery and equipment

#### 3.2.1. *Papers I and IV*

The pigs were sedated while still in the pen, and after sedation carried into the operating room. An ear vein was cannulated and anaesthesia was induced by IV infusion. The pigs were intubated while in lateral recumbency and mechanically ventilated. A cut-down was made to access the carotid artery and jugular vein on the left side for the implantation of catheters for IV fluid infusion and blood sampling. The bladder was catheterized by a low laparoscopic cut-down, and the peritoneal cavity accessed through a cut-down around the umbilical area. Both abdominal cut-downs were made with a thermocauter to minimize bleeding. The length of the incision was approximately 6–8 cm for the glucose sensing trial (Paper I) and 2–3 cm for the glucagon trial (Paper IV) (Figures 13 A and B). FreeStyle Libre sensors and tubes for sampling peritoneal fluid were inserted and held in place by custom made restrainers (Paper I) (Figure 15). The tube of the glucagon pump was inserted with a pair of long forceps and externally fixed using tape (Paper IV) (Figure 13 B). The experiments lasted for approximately 8 hours and vital parameters were continuously monitored.



**Figure 13.** Photos of pig experiments. A) Pig with FreeStyle sensors inserted IP. The LimiTters and the custom-made holders are visible on the outside of the abdominal wall. B) Pig with a tube from a glucagon infusion pump inserted IP. The cut-down and bladder catheter can be seen in the far right of the photos.

### 3.2.2. Paper III

All rats were anaesthetized in a gas chamber with 5% isoflurane for induction of anaesthesia and put on a face mask with 2% isoflurane for further handling. The ten rats in the pilot study were anaesthetized for the entire experiments. We observed a steady increase in BGLs in these rats and suspected this to be a consequence of the anaesthesia protocol. We, therefore, reduced the amount of time the rats spent in anaesthesia and observed stable BGLs after this change of anaesthesia protocol. Two short periods of



anaesthesia were still required for animal welfare purposes and standardisation of procedures.

The rats used in the main study were used for three interventions with one week apart. A cut was made with a pointy scalpel over one of the lateral tail veins for blood sampling while the rats were anaesthetised. The rats were then placed in the restrainer where they would slumber. Two blood samples were taken before the rats were reintroduced into the gas chamber for a second round of anaesthesia. Glucagon or saline was injected according to the experiment protocol, and the rats were placed in a restrainer for the rest of the experiment and subjected to frequent blood sampling. A suture was required in some rats to close the wound in the tail and stop the bleeding at the end of the trial. We experienced that the wounds healed well after sampling regardless of the wound being sutured or not, and no wound infections were observed. The total volume of blood sampled from each rat was 525  $\mu$ l, in addition to the blood that was wiped off before each sample. This volume is less than the recommended maximum volume for repeated experiments with one week apart, which is 7.5% of total blood volume (for a rat of 350g = 1.68 ml). After the experiments, the rats were given NSAIDs (Metacam vet., Boehringer Ingelheim Vetmedica GmbH) IM for pain relief and allowed to fully awake before being reintroduced to their cage with their cage mates.





**Figure 14.** Photos from rat experiments. A) Rat in a restrainer. B) Close up of the tail and cut-down for blood sampling. An old wound can be seen to the right of the currently used cut-down. C) Blood was sampled in capillary tubes of 35  $\mu$ L.

### 3.3. Analysis of glucose, glucagon, and insulin

**Glucose** was analysed on a Radiometer ABL 725 (Radiometer Medical Aps, Brønshøj, Denmark) for all experiments described in this thesis. Arterial blood from the pig experiments was collected in heparinised syringes (LEO Pharma A/S, Ballerup, Denmark) and stored on ice before analysis. Venous blood from the rats was sampled in 35  $\mu$ L heparinised capillary tubes (Radiometer Medical Aps, Brønshøj, Denmark) (Figure 14 C). Peritoneal fluid from the pigs in Paper I was collected in 90  $\mu$ L heparinised capillary tubes (Radiometer Medical Aps, Brønshøj, Denmark) and also analysed on the Radiometer ABL 725 analyser. This bench-top analyser is only marketed for use on heparinized whole blood, plasma, and pleura fluid and not on peritoneal fluid. We contacted the manufacturer and received a confirmation the machine would be able to analyse peritoneal fluid samples.

Arterial blood for **glucagon** and porcine **insulin** analysis was collected in syringes without additives and immediately transferred to EDTA vacutainers (2 ml). The samples were stored in ice water for 10 minutes before the blood was separated by centrifugation and the plasma transferred to Eppendorf tubes and stored at  $-80^{\circ}\text{C}$  until analysis. **Glucagon** was analysed with Glucagon ELISA (10-1281-01 Mercodia, Uppsala, Sweden). The glucagon ELISA kit had a lower detection limit of 1.5 pmol/L and an upper detection limit of 172 pmol/L. Porcine **insulin** was analysed with Porcine Insulin ELISA (10-1200-01, Mercodia, Uppsala, Sweden). The manufacturer changed from an internal reference solution of porcine insulin to an international reference solution for humane insulin (66/304) in 2017 and changed the reported value from  $\mu\text{g/L}$  to mU/L. Mercodia informs that the kits themselves were not changed. The term “unit” was originally a measure of

biological activity, but the current definition of one U of insulin is the equivalent of 0.0347 mg of human insulin (344). Many studies report insulin results in units, but there has been expressed a need for reporting insulin in “Systeme Internationale” (SI) units (345). Merckodia recommends converting from mU/L to pmol/L by equation 1:

$$C_{kit} \left[ \frac{mU}{L} \right] \times 6 = C_{SI} \left[ \frac{pmol}{L} \right] \quad (1)$$

This method of conversion provided very different results when compared to analyses from previous experiments. Merckodia was informed of this discrepancy, and provided an alternative conversion method based on their internal reference solution of porcine insulin, explained in equation 2:

$$\frac{C_{kit} \left[ \frac{mU}{L} \right]}{115} \times 174 = C_{SI} \left[ \frac{pmol}{L} \right] \quad (2)$$

We chose to use the conversion method described in equation 2 so obtain data that are comparable to our previous experiments and other publications using the same kits and conversion method (346–348). Merckodia has not provided an explanation for the large difference in results depending on the method of conversion, but a possible explanation could be that human/recombinant insulin and porcine insulin differ in one amino acid(349,350) and molecular weight (5808 Da for human insulin (292) and 5778 Da for porcine insulin (351)). The porcine insulin ELISA kit had a lower detection limit of 2.3 mU/L and an upper detection limit of 173 mU/L.

### 3.4. Sensors and data collection

The sensors used in Paper I were commercially available FreeStyle Libre sensors (Abbot). The sensor is a flash glucose sensor which gives a glucose value when the user holds a reading device over the sensor. We did not manipulate or modify the sensors, but a modified “do-it-yourself” custom-made reading device, LimiTter (352), was constructed for automated collection of sensor data (353). This work was done by Patrick Bösch and is not explained further in this thesis. The LimiTter made it possible to obtain a read out from the sensors every 22 seconds, but a new glucose value was only provided approximately every minute. We could not retrieve raw ampere data from the sensors. We believe that the glucose values obtained with the LimiTter were not subjected to temperature correction, as this adjustment probably is done in the hand-held reader, which we did not use.

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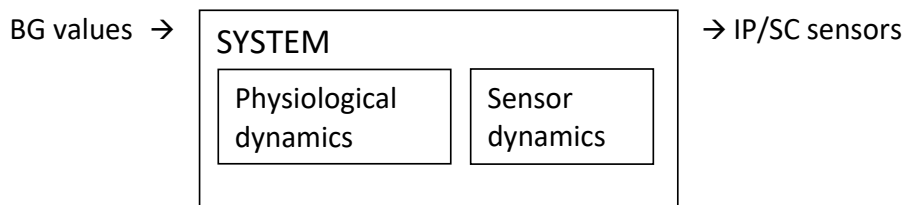
It became apparent during the first pigs experiments that the sensors reacted unexpectedly, and in vitro trials identified sensor signals with an immediate overshoot followed by declining readings, when subjected to sudden and large glucose elevations (354). For that reason, we only included data from simulated meals of glucose infusions and not large glucose boluses in Paper I.



**Figure 15.** Photo of the custom-made holder with the FreeStyle Libre sensor and LimiTter attached. The LimiTter is the dark green rounded shape and was fixed to the outside of the abdominal wall to collect sensor signals from the IP inserted FreeStyle Libre sensors. Photo by Patrick C. Bösch.

### 3.5. System identification

Mathematical modelling was applied to quantify and compare the dynamics of the experiments in Paper I. The latency between blood and the sensor location was modeled by a first-order transfer function, which contains two dynamic parameters; the time delay and the time constant. The time delay describes the lag after which a theoretical sudden pulse in the blood concentration first appears at the sensor location. The time constant quantifies the velocity with which the concentration at the sensor location reaches a constant value following a step-change in the blood (see section 1.7.4). These parameters were identified using the System Identification Toolbox in MATLAB (The MathWorks Inc., Natick, Massachusetts, U.S.A.).



**Figure 16.** Illustration of the identified system in Paper I.

Figure 16 illustrates the identified system. The BG values can be considered the input and the measurements at the sensor location the output. We were only interested in the physiological dynamics of the system, but as it was considered too complex to identify the dynamics of the sensors themselves, the results of the experiments in terms of time delays and time constants includes both the sensor dynamics and the physiological dynamics.

The scope of this thesis does not include system identification of glucose or glucagon dynamics. This work was carried out by a former member of APT, Konstanze Kölle, and is thus not included in the thesis.

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### 3.6. Statistical analysis

In Papers I, III and IV the different interventions were compared using a mixed linear model; a statistical method used in a variety of disciplines, including medical research. Mixed linear models are an extension of simple linear models and are particularly useful when analysing datasets with repeated measurements (longitudinal study), or where measurements are made on clusters of related statistical units. Another advantage of mixed linear models is their ability to deal with missing values. The models differ from simple linear models in that they also include random effects, factors influencing the results but not of major interest.

All statistical analyses for all papers were conducted in R (355). The choice of statistical methods was discussed with Associate Professor Øyvind Salvesen, who also wrote the scripts for the statistical analysis in R for Papers III and IV.

#### 3.6.1. Paper I

Dynamic parameters were analysed using a linear mixed-effect analysis with sensor location, amount of IV fluid infusion and direction of the sensor element (for IP sensors only) defined as fixed effects. To account for several measurements in each pig, pig ID was defined as a random effect.

#### 3.6.2. Papers III and IV

The relationship between BGL and time was analysed for all interventions with a linear mixed-effect analysis with the combination of time and treatments as the fixed effects. For Paper IV the bolus order was also defined as a fixed effect. The dependent variable was defined as log BGL to achieve normal distribution. The individual animal was defined as a random effect in both papers. An autoregressive model (AR(1)) was added as an error term to account for dependency within each time series. Comparisons of the glucose response at the different time points were done by the Wald's test. Comparing time to maximum glucose value and area under the curve (AUC) (Paper IV) for the different interventions were done using the Mann Whitney-test.



## 4. Summary of papers

### 4.1. Paper I

#### **Effect of sensor location on continuous intraperitoneal glucose sensing in an animal model**

Marte Kierulf Åm, Konstanze Kölle, Anders Lyngvi Fougner, Ilze Dirnena-Fusini, Patrick Christian Bösch, Reinold Ellingsen, Dag Roar Hjelme, Øyvind Stavdahl, Sven Magnus Carlsen, Sverre Christian Christiansen  
PLoS ONE. Public Library of Science; 2018;13(10):e0205447.  
doi: 10.1371/journal.pone.0205447

Most of the previous publications on IP glucose sensing lack a description of the precise positioning of the glucose sensing element, making assessments of potential differences in glucose dynamics within the IP space impossible. In Paper I, we aimed at investigating this potential spatial difference by inserting four FreeStyle Libre sensors in the different quadrants of the abdominal cavity of anaesthetized pigs and simulate meals by IV infusion of glucose. Frequent arterial blood and peritoneal fluid samples were collected for glucose reference. The dynamics between the intra-arterial glucose concentrations and the glucose values from the sensors are described by a two-compartment model. The time parameters of the different sensor locations were compared using a mixed linear model.

We found no apparent difference in glucose dynamics between the four quadrants of the peritoneal space. There was a statistically significant difference when comparing the estimated time constant between the caudal left sensor and the cranial right sensor, but the clinical significance is doubtful. The time delay of the IP sensors was significantly shorter compared with the SC sensors, but comparisons of the other time parameters did not show any significant differences.

We observed an accumulation of peritoneal fluid as the experiments prolonged, and that this affected the glucose dynamics. Although not statistically significant, the dynamics at the end of the experiments were slower when compared to the start of experiments for both IP and SC sensors. We reduced the amount of fluid given by IV infusion and although this consequently reduced the accumulation of peritoneal fluid and resulted in faster glucose dynamics, the improvement was not statistically significant.



The sensor elements of the FreeStyle Libre sensors were positioned against the abdominal wall in 28 of 84 sensor recordings (clamp or simulated meal), which resulted in slower glucose dynamics, but less noisy sensor signals. The difference in glucose dynamics between the positions of the sensing element, however, was not statistically significant.

#### 4.2. Paper II

##### **Why intraperitoneal glucose sensing is sometimes surprisingly rapid and sometimes slow: A hypothesis**

Marte Kierulf Åm, Anders Lyngvi Fougner, Reinold Ellingsen, Dag Roar Hjelme, Patrick Christian Bösch, Øyvind Stavadahl, Sven Magnus Carlsen, Sverre Christian Christiansen  
Medical Hypotheses 2019;109318.  
doi:10.1016/j.mehy.2019.109318

Contrasting results of the IP glucose dynamics have been described by our research group, from almost instant detection of changes in BGL in the publication by Fougner et al. (310) to results similar to SC glucose dynamics in Paper I of this thesis. In Paper II, we discuss the hypothesis that changes in BGL can be detected instantly when glucose is measured close to the peritoneal lining and how this approach can enable the fast sensing dynamic required for a fully automated AP system. The rate of diffusion of any molecule in a fluid depends on the properties of the molecule and the properties of the fluid. We discuss how the glucose molecules spend 750 seconds traveling only 1 mm by diffusion forces in water of 25°C, and how even small distances between the peritoneal lining and the sensing element would have a large impact on the sensing dynamics. Diffusion would be faster in water at 37°C, but slower in peritoneal fluid because of its higher viscosity. The paper concludes that the glucose sensing element of an IP AP system should be positioned very close to, or directly on the peritoneal lining to avoid any unnecessary volume of peritoneal fluid.

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The published paper includes a typing error in the last line of calculations of diffusion times for glucose in water on page 2. The last calculation should show:

$$\text{- For } x = 10 \mu\text{m}; \quad \Rightarrow \quad t_{25^\circ\text{C}, 10 \mu\text{m}} \approx 0.075 \text{ s}$$

The publisher has been made aware of this error and has published an erratum (356).

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#### 4.3. Paper III

##### **Intraperitoneal, subcutaneous and intravenous glucagon delivery and subsequent glucose response in rats: A randomized controlled crossover trial**

Ilze Dirnena-Fusini\*, Marte Kierulf Åm\*, Anders Lyngvi Fougner, Sven Magnus Carlsen, Sverre Christian Christiansen

(\*shared first authorship)

BMJ Open Diabetes Research and Care 2018;6:e000560.

doi:10.1136/bmjdr-2018-000560

The main aim of Paper III was to investigate, describe and compare the glucose dynamics after IP and SC delivery of glucagon in rats. We also investigated the glucose dynamics after IV delivery in five rats. The rats received glucagon doses of 5 µg/kg either IP, SC and an IP placebo injection of saline (n=15) or glucagon doses of 5 µg/kg IP, SC and IV (n=5). The different interventions were given one week apart, and the order of interventions was randomised. The rats were awake for most of the individual experiments but briefly anaesthetized for making a cut in the tail for blood sampling and the injection of glucagon/placebo.

We observed a faster glucose response when glucagon was injected into the IP space compared with the SC tissue, showing a statistically significant difference at 4 minutes after glucagon delivery. The glucose excursion was also of shorter duration after IP delivery, and the glucose values were statistically significantly lower at 40 and 50 minutes compared with SC delivery of glucagon. The maximum glucose value after IP delivery was observed after 25 minutes, ten minutes faster than for SC delivery, a statistically significant difference. Glucose values also increased in the placebo group, but the increase was small and we observed no clear peak in glucose values within the sampling period.

#### 4.4. Paper IV

##### **Intraperitoneal and subcutaneous glucagon delivery in anaesthetized pigs: Effects on circulating glucagon and glucose levels**

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(\* The manuscript was published after the completion of this thesis. Edited and published version, doi: 10.1038/s41598-020-70813-5)

The main aim of Paper IV was to identify the glucose dynamics after the IP delivery of glucagon and compare it to SC delivery in anaesthetised pigs. We also wanted to see if we could reproduce the results obtained from Paper III in a different animal model. Ten pigs received four repeated glucagon boluses; IP boluses of 0.3 µg/kg and 0.6 µg/kg and an SC bolus of 0.6 µg/kg. The order of these three glucagon boluses was randomised. The pigs also received an additional IP bolus of 1 mg as the end of experiments to test the maximal glucose response after IP delivery of glucagon.

We did not observe a faster or shorter-lived glucose response after IP delivery of glucagon compared with SC delivery as seen in Paper III, but IP delivery of 0.6 µg/kg glucagon gave a significantly higher glucose response compared with SC delivery from 14 to 30 minutes. An unexpected and unfortunate observation was the lack of glucose response in four pigs despite evidently high levels of glucagon in the general circulation. We suspect that this was caused by prolonged fasting and repeated glucagon bolusing resulting in depletion of glycogen storage for rapid glucose production by glycogenolysis.

## 5. Discussion

### 5.1. Ethics

The use of animals for research purposes is a controversial topic. A UK survey showed that the broad public opinion supported the use of animals, but only conditionally and only in situations where no alternative was possible. 26% of the people in the survey, however, wanted a total ban on animal use in research (357).

The use of animals in research is regulated both under national Norwegian law, “Forskrift om bruk av dyr i forsøk”, and under the “Directive 2010/63/EU on the protection of animals used for scientific purposes”. The legal regulations ensure a minimum standard for the use of animals in research, that the animals are used in experiments trying to answer purposeful scientific questions and defines what treatments the animals can receive. All projects which involve the use of animals must apply to and get approval from the appropriate authorities, which in Norway is the Norwegian Food Safety Authority.

“The **three Rs**” is the guiding principle in research using animals (358). The principle was defined by Russel and Burch in 1959 and is still the core principle in animal research. The three Rs stand for replacement, reduction, and refinement. Whenever possible the animal experiment should be **replaced**, and data should be obtained by methods not requiring the use of animals. Development of simulations and «fabricated» tissues are examples of technologies already substituting the use of animals in research and still holds great potential for further development. If the use of animals is required, the number of animals should be **reduced** and kept at a minimum. This does not necessarily mean that the number of animals should be kept low in absolute terms, but rather that as few animals as possible are used to obtain good and reliable data. Experiments on animals should be **refined** to both give the best possible data and minimize the load on the animals used in the experiments. Procedures in animal research will always have the potential for refinement. A supplement to the three Rs is the **three Ss** proposed by Professor Carol Newton in 1975 (359). She was a mathematician concerned about the unknown interactions between variables and large biological variation in the context of animal models. To ensure ethical and purposeful animal experiments Professor Carol Newtons defined three Ss: **good science** - conducting well-designed animal experiments, **good sense** - the use of a critical sense and “common sense” when scientific evidence is lacking and **good sensibility** - using our empathic abilities towards the animals we use in our research.

All the data forming the foundation of this thesis are obtained from animal experiments. There is little information on glucose sensing and glucagon delivery in the IP space available. The questions to be investigated involve complex anatomical features and physiological processes, and for that reason, animal experiments were considered necessary. All experiments were approved by the Norwegian Food Safety Authority (FOTS id 10922, 8606 and 12948). The number of animals used in our studies was kept low and the animal received proper anaesthetics and analgesics to minimize the stress and pain. Procedures were refined when possible. All pig trials were conducted as acute experiments, i.e. the pigs were under anaesthesia for the length of the seven–nine hours experiments and euthanized at the end of experiments while still under anaesthesia. The rats received analgesics after each intervention and were euthanized under full anaesthesia at the end of the study. All animals were euthanised with an IV overdose of pentobarbital.

## 5.2. Methodological considerations

### **Animal models**

We chose a pig model for two of the papers in this thesis, both because of the size of the animals but also because of the anatomical and physiological similarities between humans and pigs (360) (as mentioned in section 3.1.1). Although pigs resemble humans in many ways, or perhaps vice versa, some differences might affect the results obtained in our studies. One obvious difference is that pigs "lack" the greater omentum, which in humans lays as an "apron" between the ventral abdominal wall and the intestines. In the glucose sensing experiments in Paper I this implies that the sensors might have rested against the intestines, in contrast to the situation in humans where they would have rested against the greater omentum. It is difficult to decide how this has affected the results from the experiments, but it is reasonable to suggest some influence. The lack of the greater omentum in pigs would also potentially influence the results in glucagon kinetics and dynamics in Paper IV. Glucagon would in pigs be absorbed over the peritoneal lining of the intestines rather than the peritoneal lining of the greater omentum compared to humans. However, the venous blood from the omentum in humans drains to the portal vein in the same way as from the intestines in both pigs and humans. A potential difference in blood capillary density between the peritoneal lining of the intestines and the greater omentum would then be decisive if this anatomical difference is of importance. Rats have a relatively larger greater omentum compared with pigs, however, the procedure of blind IP injections of glucagon in Paper III probably induces larger uncertainties than the differences in abdominal anatomy. We don't know

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the exact location of the injected glucagon, and there is a chance that the glucagon was deposited in the lumen or walls of the intestines. The rats were anaesthetized and held by their hind legs to optimize the chances of successful IP injections.

#### **Anaesthesia and prolonged surgery**

Conducting experiments on anesthetized pigs for several hours is not without complications. Both medications used for sedation and anaesthesia and the unnatural inactivity and positioning of the animal will affect the physiology and possibly influence the obtained data. Under normal conditions, the intestines will constantly move and glide over each other. The speed and pattern of movement will depend on many factors, such as time since last meal, meal composition, diseases, and stress. General anaesthesia and abdominal surgery will reduce the peristaltic movements of the intestines (361–367). This is generally considered an unwanted side effect for the animal in the post-surgical period, but for our experiments, this reduced motility during general anaesthesia deviates from “normal” conditions, which we aim to study and cause reduced movement of peritoneal fluid.

During our first three pig experiments for Paper I, we experienced an increasing amount of peritoneal fluid over the time course of the one-day experiments. This problem is described by Schnoor et al. (368), and based on their recommendations we increased the size (age) of the pigs and reduced the volume of intravenous fluid infusions they were given. The correcting interventions had a marked effect with less accumulation of peritoneal fluid. However, we still consider the amount of peritoneal fluid at the end of our experiments to be greater than normal.

Anaesthetic drugs can also affect glucose metabolism. Of the drugs used in our experiments, azaperone (369), isoflurane (370–372) and thiopental (373–375) have been found to affect the BGL. Isoflurane affects the BGL by inhibiting insulin secretion (372), which we already inhibited by the use of somatostatin analogues in the experiments in Papers III and IV. The pigs in Paper I were not treated with somatostatin analogues so it is possible that their BGLs were affected by isoflurane, however isoflurane was only used as a complimentary anaesthetic drug and as we studied the rapid BG changes associated with IV infusions of glucose, the slow and stable increase in BGL by isoflurane would be of little importance.

We wanted to test the IP glucose dynamics for Paper I under near-normal post-prandial conditions and we simulated meals by stepwise IV infusions of glucose. The simulated

meals, however, were of shorter duration than normal meals, but the dynamics of the increasing and decreasing part of the glucose curve were prioritized and the length of the "meal" was reduced to obtain more data per experiment. Meals simulated by IV glucose infusion will also deviate in several important aspects from the normal digestion of food. Glucose elevations after IV glucose infusion will not result in secretion of incretins from gut cells, and not stimulate increased mesenteric blood flow.

#### **Endogenous insulin and glucagon suppression**

The animals in Papers III and IV were treated with somatostatin analogues, as these drugs suppress endogenous secretion of both glucagon and insulin (376,377). The pigs received both octreotide and pasireotide and the rats received only octreotide. Octreotide is a strong inhibitor of both glucagon and insulin secretion, while pasireotide predominantly inhibits glucagon secretion in rodents and humans (376).

The suppression of insulin secretion was not completely successful for all pigs (Paper IV), and the effect is unknown for the rats (Paper III) as we did not analyse for glucagon or insulin. As the ELISA kit cannot discriminate between exogenous or endogenous glucagon, the only way of testing the efficacy of glucagon suppression was to analyse samples for glucagon before and after the initial treatment of somatostatin analogues, i.e. before the first bolus of exogenous glucagon. In Paper IV we observed a decrease in glucagon after the initial somatostatin analogue treatment, indicating successful inhibition of endogenous glucagon secretion.

#### **Fasting of animals**

In Paper IV, we described that four of ten pigs became unresponsive to glucagon boluses, even though we detected elevated glucagon concentrations in peripheral blood. We suspect that the nine–ten hours of fasting, together with repeated glucagon boluses, caused a depletion of liver glycogen available for rapid glucagon-induced glucose production. Prolonged fasting has been shown to reduce the amount of available liver glycogen in humans after an overnight fast (248) and in pigs before slaughter (378). Glucagon also stimulates glucose production when no glycogen is available, through gluconeogenesis, but this glucose production is too slow to be identified in our experimental setup.

#### **The use of FreeStyle Libre sensors IP**

The FreeStyle Libre sensor is designed for use on the skin with only the sensing element inserted in the SC tissue. We placed the whole sensor in the IP space, subjecting it to a moist and warm environment. Although the sensors can be used while showering and

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swimming, the manufacturer informs that the sensor should not be submerged in water for more than 30 minutes. We cannot exclude the possibility that the suboptimal environment in the IP space affected the sensor results, however, we did not observe any loss in or reduction of signals from the sensors or increased signal noise towards the end of experiments after the sensors had been submerged in peritoneal fluid for several hours.

We initially wanted to investigate the glucose dynamics after both glucose boluses, i.e. rapid glucose elevations, and by simulated meals. We noticed, however, that the FreeStyle Libre sensor reacted with an overshoot followed by a declining output result when it was subjected to large and rapid glucose changes. The sensor was therefore investigated in-vitro with step-wise changes in glucose concentration (354) confirming the observations made in the very first pig experiment. We could not explain this sensor behaviour, but we suspect that this pattern in sensor output was caused by an internal algorithm of the FreeStyle Libre sensor. We, therefore, concluded that glucose boluses were not a suitable method for evaluating the physiological glucose dynamics, as the sensor signals could not be trusted.

The aim of Paper I was to identify the IP glucose dynamic. Ideally, we would have used a sensor with known dynamics and from which we could obtain raw values. That would have enabled us to remove the contribution of the sensor delay from the results. However, manufacturers do not publish the dynamics of their sensors and both economical and practical circumstances lead us to choose the FreeStyle Libre sensor for these experiments. It is difficult to say how a different sensor would have affected the results of the study (Paper I).

### 5.3. Discussion of main findings

#### *5.3.1. Spatial differences in glucose sensing (Paper I)*

In Paper I, we observed no overall statistically significant differences in time parameters when comparing the four quadrants of the IP space, except for the time constant between the cranial and caudal left quadrants. It is doubtful, however, if this difference is clinically significant as the results are only significant for one of multiple comparisons of time parameters. Paper I is the first publication investigating the potential spatial difference in glucose dynamics within the IP space. Previously published papers have focused on the feasibility of IP glucose sensing and do not describe the location of the



sensing element (305–309). However, Fougner et al. describes the placing of the sensors and briefly discusses how the positioning of the sensors might affect the results (310).

Although the sensors used for the study in Paper I might be sub-optimal for the experimental set-up as they are designed for SC use, the IP sensors were all subjected to the same environmental conditions. Hence, the comparison of glucose dynamics between the different sites within the peritoneal cavity should be valid. The results obtained in Paper I is in compliance with publications investigating the characteristics of the human peritoneal lining, which have not shown systematic differences in the key histological parameters, including microvascular density, in different parts of the peritoneum (270,273). However, given the large variation in data might we cannot exclude local differences in glucose dynamics.

### *5.3.2. IP vs. SC glucose sensing (Paper I)*

In Paper I, we found the time delay of the IP sensors to be significantly shorter for the IP sensors compared with the SC sensors. Comparisons of the other time parameters did not show a significantly better outcome for the IP sensors. Fast glucose dynamics in the IP space has been shown previously by Fougner et al. (310) and Burnett et al. (308). The latter publication compared modified amperometric sensors in the IP space with unmodified sensors of the same type in the SC tissue and showed that the mean time delay was significantly shorter for the IP sensors compared with the SC sensors. Although Fougner et al. did not directly compare IP and SC glucose dynamics, they observed time delays for the optical interferometric IP sensors to be between 0–26 seconds which is faster than what has been shown for any SC glucose sensing. The sensor technology used in the study by Fougner et al. differs from the amperometric sensors used by Burnett et al. and in Paper I which both used amperometric glucose sensors. We were not able to access the raw data (ampere) of the FreeStyle Libre sensors used in Paper I and we do not know how the sensor signals were processed from ampere to glucose values. The sensor data therefore include both the physiological latency and sensor latency, and although the sensor latency might be identical for both the IP and SC sensors, the fact that the IP sensors were used in a different environment than they have been designed for might influence the data.

We observed an accumulation of peritoneal fluid in the first pig experiments in Paper I. The speed of molecule movement by diffusion in water is slow, and accumulation of

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fluid will slow down the glucose dynamics, as the molecules must diffuse through a larger volume of fluid before reaching the sensing element. This is discussed in Paper II.

### *5.3.3. IP vs. SC glucagon delivery (Papers III and IV)*

Papers III and IV both show that glucagon delivered IP has a glucose elevating effect, confirming what has been shown in previous publications (331–333). The papers also show that the glucose response was faster after IP delivery in rats and gave a higher glucose response in anaesthetized pigs compared to SC delivery. Previous publications have not collected glucose values frequent enough for detailed description of glucose dynamics after IP delivery of glucagon. The results from Papers III and IV will be used in the development of algorithms for an IP AP system.

Although IP glucagon delivery does not give a much faster glucose elevation compared with SC delivery in Paper IV, the results suggest that small doses of glucagon will be sufficient to obtain the wanted elevation in BGL for an AP system. A larger proportion of glucagon will hit the liver after IP delivery as it is absorbed over the peritoneal lining and transported to the liver via the portal vein. This resembles normal glucagon secretion and distribution, while SC injected glucagon will be distributed in the systemic circulation before a smaller portion is transported to the liver. Reducing the peripheral concentrations in peripheral circulation will be an advantage as it can reduce the risk of potential side effects of glucagon treatment.

## **5.4 Strengths and limitations**

We have included a larger number of animals than comparable studies for both investigating IP glucose sensing and IP glucagon delivery. It is important, however, to acknowledge that our studies still should be considered as pilot studies and followed by additional experiments. The “Crisis of Reproducibility” of animal studies has been widely discussed in recent years, and suboptimal animal models, inadequate or inappropriate controls, and poor documentation practices are discussed as primary sources of this crisis (379). We have tried to accurately and comprehensively describe and explain our experiments to increase the reproducibility of the results and thereby to increase the values also for other researchers. We also describe all our obtained results including the data we have excluded from analysis and the reason for the exclusion, something not always done in comparable papers.

We investigated the glucose response after IP glucagon delivery in two different animal models. The rats in Paper III were awake for most of the experiments, and glucose dynamics were therefore minimally affected by anaesthesia. Both animal models showed fast glucose elevating effect of IP delivered glucagon and provide data for system identification and development of algorithms for an IP AP system.

The studies hold several limitations, which are discussed above in the methodical consideration (section 5.2).

The most obvious limitations are:

- Small sample sizes.
- Prolonged fasting of pigs in the study for Paper IV resulting in possible glycogen depletion.
- Prolonged anaesthesia and surgery for the studies in Papers I and IV affecting physiological parameters and accumulation of peritoneal fluid.
- Blind injections of glucagon in the IP-space of rats in Paper III.
- The use of sensors not designed for use in the IP space in Paper I, without the possibility to access raw data or to know how the signals were processed by the sensors.

## 6. Concluding remarks and implications for future research

For an AP system to fulfil the promise of fully automated BGL regulation, the system requires fast dynamics of both glucose sensing and hormone effect. The dynamics of the insulin effect, especially, but also glucose sensing in the SC tissue have proven too slow and therefore the SC tissue is suboptimal for an AP system. The work underlying this thesis aimed to investigate the IP space as an alternative location for an AP system, by investigating IP glucose sensing and glucagon delivery. IP insulin delivery will be covered by another doctoral thesis in APT.

This thesis shows that both glucose sensing and glucagon delivery in the IP space offer equally fast dynamics as the SC tissue in our experimental settings. Although the IP space was not conclusively superior to the SC tissue, the results are promising for the IP space as a potential location for an AP system. Paper I demonstrates that the IP space has a shorter time delay for glucose sensing compared with the SC tissue. This is essential because minimal delay in the detection of BGL changes might enable exclusion of meal announcements and provide the users with the freedom from interacting with their AP device. The results from Paper I also indicate that it does not matter where in the IP space the glucose sensing element is located.

Paper III shows how glucagon delivered in the IP space gives faster glucose elevating effect in rats and Paper IV shows how glucagon delivered IP gives a higher glucose elevation compared with SC delivery in pigs. Both the faster and larger effect after IP delivery of glucagon observed in our studies might be explained by how this route resembles the glucagon secretion by the healthy pancreas where glucagon is transported directly to the liver. The concentrations of glucagon in the peripheral circulation will be lower after IP delivery, possibly reducing or avoiding the common side effects of glucagon treatment.

The robustness of IP glucose sensing has not been investigated in this thesis as planned. The sensor technology used in Paper I was never meant to be further developed for IP use. Investigations of the life expectancy of relevant glucose sensors IP will be undertaken by APT once the appropriate sensor technology has been adequately developed.

Experiments on anaesthetized animals have several limitations, which are discussed in section 5.2., and the questions addressed in this thesis should also be investigated by

conducting experiments on awake animals. This refinement of the study procedure will provide better data as the effects of anaesthesia and the increased production of peritoneal fluid will be eliminated. Experiments on awake animals will also allow for the investigation of the scientific questions under normal physiological condition, especially regarding feeding with the subsequent increase in mesenteric blood flow and secretion of incretins observed after oral intake of food, but also with normal routines of sleep and activity.

Refinements of experimental procedures, in addition to the use of appropriate sensor technology, might demonstrate that the IP space will provide the fast dynamics required to develop a fully automated AP system, which will regulate the BGL adequately to avoid long-term side effects of both hyperglycaemia and hypoglycaemia. The development of an IP AP system is in its early stages and several aspects of the AP system must be investigated and developed before the system can be tested on human patients. APT is currently developing algorithms for the AP system and closed-loop experiments on anesthetized animals are under planning. Further, the development of a port through the abdominal wall and appropriate sensor technology is essential for a functional double IP AP system. This work is conducted by other members of APT.

The main argument for placing an AP system in the IP space might be the more physiological correct absorption and effect of insulin and glucagon. Decreasing peripheral hyperinsulinemia and hyperglucagonemia will, theoretically, reduce the unwanted side-effects of the hormones, and the faster absorption and high precision of delivery to the liver for both insulin and glucagon might allow for more aggressive actions by the IP AP system. The IP space is also a promising site for fast glucose dynamics. All together, this might enable the development of a fully automated IP AP system that normalizes or close to normalizes glucose levels without episodes of serious hypoglycaemia, and thereby also eradicate the long-term adverse effects of having DM1.

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## Appendices



# Paper I



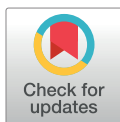
RESEARCH ARTICLE

# Effect of sensor location on continuous intraperitoneal glucose sensing in an animal model

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**Citation:** Åm MK, Kölle K, Fougner AL, Dirnena-Fusini I, Bösch PC, Ellingsen R, et al. (2018) Effect of sensor location on continuous intraperitoneal glucose sensing in an animal model. PLoS ONE 13 (10): e0205447. <https://doi.org/10.1371/journal.pone.0205447>

**Editor:** Clemens Fürsinn, Medical University of Vienna, AUSTRIA

**Received:** June 8, 2018

**Accepted:** September 25, 2018

**Published:** October 9, 2018

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**Data Availability Statement:** An Excel file with data used in statistical analysis, and all raw data from the sensors and blood and IP fluid analysis used in the system identification are available from the Dryad Digital Repository database (<https://doi.org/10.5061/dryad.5m1m755>)

**Funding:** The Double Intraperitoneal Artificial Pancreas project is part of Centre for Digital Life Norway and supported by the Research Council of Norway (NRC - [https://www.forskningradet.no/en/Home\\_page/1177315753906](https://www.forskningradet.no/en/Home_page/1177315753906)), grant number

## Abstract

### Background

In diabetes research, the development of the artificial pancreas has been a major topic since continuous glucose monitoring became available in the early 2000's. A prerequisite for an artificial pancreas is fast and reliable glucose sensing. However, subcutaneous continuous glucose monitoring carries the disadvantage of slow dynamics. As an alternative, we explored continuous glucose sensing in the peritoneal space, and investigated potential spatial differences in glucose dynamics within the peritoneal cavity. As a secondary outcome, we compared the glucose dynamics in the peritoneal space to the subcutaneous tissue.

### Material and methods

Eight-hour experiments were conducted on 12 anaesthetised non-diabetic pigs. Four commercially available amperometric glucose sensors (FreeStyle Libre, Abbott Diabetes Care Ltd., Witney, UK) were inserted in four different locations of the peritoneal cavity and two sensors were inserted in the subcutaneous tissue. Meals were simulated by intravenous infusions of glucose, and frequent arterial blood and intraperitoneal fluid samples were collected for glucose reference.

### Results

No significant differences were discovered in glucose dynamics between the four quadrants of the peritoneal cavity. The intraperitoneal sensors responded faster to the glucose excursions than the subcutaneous sensors, and the time delay was significantly smaller for the intraperitoneal sensors, but we did not find significant results when comparing the other dynamic parameters.

248872. The study is also supported by a scholarship from the Central Norway Regional Health Authority (<https://helse-midt.no/vart-oppdrag/vare-hovedoppgaver/forskning>) (grant number 2014/23166). MKÅ. The study is also supported by a grant from Norwegian Medical Association Johan Selmer Kvanes Endowment. (<http://legeforeningen.no/Emner/Andre-emner/Fond-og-legater/Andre-legater/Johan-Selmer-Kvanes-legat-til-forskning-og-bekjempelse-av-sukkersyke/>). The funding sources had no role in collection, analysis or interpretation of the data.

**Competing interests:** Author R. Ellingsen is board member of GlucoSet AS, a company in the glucose monitoring field. Authors D. R. Hjelme and R. Ellingsen are shareholders of GlucoSet AS.

## Introduction

Achieving tight glucose control in diabetes mellitus type 1 (DM1) treatment is a challenge, but crucial in preventing hyperglycaemia-related late complications. However, tighter glucose control is usually accompanied by an increased incidence of severe hypoglycaemia [1, 2]. In addition, patients face the burdens of constant self-surveillance of blood glucose levels, careful planning of exercise and carbohydrate consumption and deciding on dose and delivery of insulin. Fully automatic closed-loop delivery of insulin, i.e. a so-called artificial pancreas (AP), has the potential to revolutionise the way we treat diabetes, removing some of these burdens [3–5]. A well-functioning AP should mimic a healthy pancreas with regard to glucose regulatory function and keep the patients' blood glucose levels within the narrow safe range. Ideally, an AP obtains the glucose values seen in people without diabetes, thus providing DM1 patients with improved quality of life and longer life expectancy.

The majority of research on AP targets the subcutaneous (SC) tissue as the site for continuous glucose monitoring (CGM) and insulin delivery, the so-called “double SC approach”. Several clinical studies have explored the feasibility of this approach under free-living conditions [6, 7]. In 2016, the first hybrid AP was approved by the U.S. Food & Drug Administration (FDA) [8]. In contrast to a fully automatic closed-loop system, a hybrid AP requires the patients to administer pre-meal insulin boluses themselves [8].

Subcutaneous glucose measurements carry certain limitations, mainly due to slow glucose dynamics [9, 10]. At least 6–7 minutes are needed to transport glucose from the lumen of the capillaries to the SC interstitium [11, 12]. In addition, glucose dynamics in the SC tissue varies significantly between patients [13], and the performance of the CGM sensor is influenced by several factors; such as mechanical pressure [14–16], micro-haemorrhages at sensor site [17], certain drug interactions [18], temperature, fluctuations in tissue perfusion [19, 20] and local foreign body reaction [15, 21]. In addition to physiological factors, the sensor has its own internal dynamics, adding up to the total latency in CGM [10]. Both the delay and the variable dynamics make the SC tissue insufficient for glucose sensing in an AP. This paper uses the definitions of time delay, time constant etc. as earlier described by Ståvdahl et al. [10].

Given the delays in SC CGM, the use of the intraperitoneal (IP) space for CGM has been proposed. The glucose dynamics in the IP space has been shown to be fast [22–24]. The IP space also has other advantages compared to the SC tissue, providing a more mechanically and thermally stable environment.

To optimize the potential of a double IP artificial pancreas, it is important that the glucose sensing element be placed at the most appropriate site. To our knowledge potential differences in glucose dynamics within the peritoneal cavity has not previously been studied. The main aim of this study was therefore to explore and compare the glucose dynamics in four different locations within the peritoneal cavity of anaesthetised domestic pigs during sessions of IV glucose infusions. Secondly, we compared the performance of IP CGM to SC CGM. In order to resemble normal physiological glucose excursions, glucose challenges were infused as simulated meals of 30 minutes duration, rather than boluses.

## Materials and methods

### Ethical approval

The animal experiments were approved by the Norwegian Food Safety Authority (FOTS numbers 8606 and 12948), and was in accordance with «The Norwegian Regulation on Animal Experimentation» and «Directive 2010/63/EU on the protection of animals used for scientific purposes».

### Animals and animal handling

Between September 2016 and October 2017, twelve juvenile, non-diabetic farm pigs of both genders (1 male, 11 females) weighing 31–44 kg, were brought to the animal research facility approximately one week prior to experiments and acclimatised to the staff and new environment. They were housed together in a common pen, in groups of two or three whenever possible, provided wood chips as nesting material and toys to keep them occupied. The lighting condition was standardised with a 16 hours light period followed by an 8 hours dark period. They were fed standard commercial growth feed twice a day and provided water *ad libitum*. Food was removed 16 hours before the experiments.

### Anaesthesia

The pigs were premedicated with an intramuscular injection of 4 mg diazepam (Actavis Group, Hafnarfjordur, Iceland), 160 mg azaperone (Eli Lilly Regional Operations GmbH, Austria) and 750 mg ketamine (Pfizer AS, Norway), while in the pen. An auricular vein was cannulated and anaesthesia was induced with intravenous (IV) injections of 1 mg atropine (Takeda AS, Asker, Norway), 150–250 µg fentanyl (Actavis Group, Hafnarfjordur, Iceland), 75–125 mg thiopental (VUAB Pharma AS, Roztoky, Czech Republic) and 150–250 mg ketalar (Pfizer AS, Norway).

The pigs were intubated in the lateral position and mechanically ventilated and monitored on an anaesthesia machine (Aisys, GE Healthcare Technologies, Oslo). Anaesthesia was maintained by IV infusion of midazolam (0.5 mg/kg/h) (Accord Healthcare Limited, Middlesex, UK) and fentanyl (7.5 µg/kg/h) (Actavis Group, Hafnarfjordur, Iceland) and by inhalation of isoflurane (0–2%) (Baxter AS, Oslo, Norway). Room temperature was around 20 degrees Celsius. The body temperature of the pigs was monitored, and a heating blanket used when necessary.

The pigs received IV infusion of antibiotics (Cefalotin, Villerton Invest SA, Luxembourg), 2 g immediately after the pigs were anaesthetised and 1 g after 4 hours. Heparin (150 IE) (LEO Pharma A/S, Ballerup, Denmark) was injected in the peritoneal space at the same time points.

Fluid balance was achieved by continuous IV infusion of Ringer's acetate with individual adjustments to achieve stable blood pressure. To reduce the amount of IP fluid accumulating in the pigs, the amount of Ringer's acetate was reduced in consecutive experiments, from 5–9 ml/kg/h initially to 2.5 ml/kg/h in the last four pigs. The pigs also received IV fluid through antibiotics, glucose and when the catheters were flushed after every blood sample. Total fluid loss during experiments is not known, but estimates suggest that the pigs were in positive fluid balance, even at the lower infusion regimen.

### Surgical procedure

An intra-arterial line was placed in the left carotid artery for blood sampling and monitoring of physiological parameters and an IV line was placed in the left internal jugular vein for glucose and fluid infusions. Both catheters were inserted through the same cut-down.

The IP sensors were inserted through a 6–8 cm long cranio-caudal incision in the abdominal wall, 2–3 cm caudally to the umbilicus. The bladder was exposed through a small, low laparotomy for the insertion of a bladder catheter. Both cuts were made with a thermocauter to minimise bleeding into the abdominal cavity.

At the end of the experiments, and under full anaesthesia, the pigs were euthanised with an IV overdose of pentobarbital (minimum 100 mg/kg) (pentobarbital NAF, Apotek, Lørenskog, Norway).



One additional pig was used to refine the experimental protocol before the start of the study. The results from this pig are not included in this article.

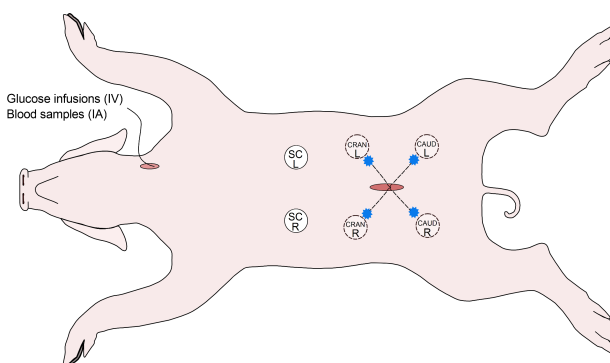
### Sensors and sensor placement

Four unmodified FreeStyle Libre (Abbott Diabetes Care Ltd., Witney, UK) sensors were positioned 10 cm into the abdominal cavity in four different directions, corresponding to the four quadrants of the abdominal cavity as shown in Fig 1. Two FreeStyle Libre sensors were inserted subcutaneously, 5 cm on each side of the ventral medial line at the height of the first pair of nipples, avoiding visual SC blood vessels.

Custom made retainers, made of PMMA, were used to hold the sensors in place and also hold the corresponding reading devices (LimiTTer) close enough to the sensors on the outside of the abdominal wall for registration of sensor signals (Fig 2). Data was transferred to an xDrip application for further handling [25]. IP fluid samples from the sensor locations were drawn using the same retainer as described above.

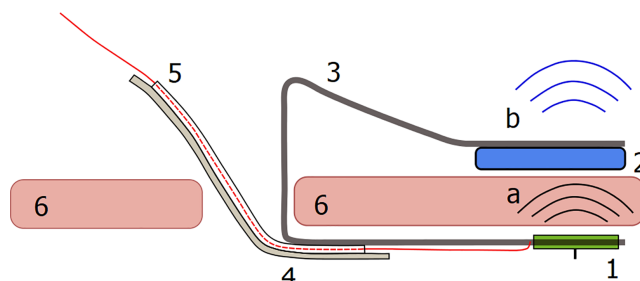
In six of the experiments, two of the four IP-sensors were positioned with the sensor element of the FreeStyle Libre sensor pointing towards the abdominal wall (total 28 sensor recordings). The other sensors were positioned with the sensor element pointing towards the visceral peritoneum (total 84 sensor recordings). Four pigs had one of the SC sensors inserted the day before the start of the experiment (24 hours). The other SC sensors were inserted after the pigs had been anaesthetised and allowed at least 1 hour to settle before the glucose infusions. The IP sensors were submerged in phosphate-buffered saline (Phosphate buffered saline tablet, Sigma-Aldrich Co., St. Louis, USA) with 3mmol/l glucose for approximately 1 hour before insertions. This ensured optimal conditions for the IP sensors in the 1-hour start-up phase, and we could also ensure that the sensor elements were completely exposed to glucose containing fluid, check the set-up functionality, and confirm sensor readings before inserting the sensors into the abdomen of the pigs.

After insertion into the IP space, the sensors were allowed at least 30 minutes to settle before glucose infusions were started. Several in vitro experiments were performed prior to the pig experiments in order to characterise the dynamics of the FreeStyle Libre sensor when subjected to changes in glucose concentration and to test the communication protocols. See publication by Bösch et al. for further details on the in vitro trials of the FreeStyle Sensors [25].



**Fig 1. Schematic presentation of sensor placement and sites for intraperitoneal fluid sampling.** Blue stars indicate sites for IP fluid sampling. SC = subcutaneous, IP = intraperitoneal, CRAN = cranial, CAUD = caudal, L = left, R = right, IA = intra-arterial, IV = intravenous.

<https://doi.org/10.1371/journal.pone.0205447.g001>



**Fig 2. Sketch of retainer for sensor and reading device.** The FreeStyle Libre was held in place on the inside of the abdominal wall, while the reading device (LimiTTer) was held tightly to the outside of the abdominal wall [25]. 1 = FreeStyle Libre sensor, 2 = LimiTTer, 3 = Retainer, 4 = IP fluid sampling tube, 5 = Guide wire with guide tube, 6 = Abdominal wall, a = Near-field communication, b = Bluetooth low energy.

<https://doi.org/10.1371/journal.pone.0205447.g002>

### Glucose infusions

Meals (IV meals) were simulated by stepwise IV infusions of glucose (Glucos B. Braun 200 mg/ml, B. Braun, Melsungen AG, Germany). The total amount of glucose per IV-meal was 245 mg/kg body weight infused over 30 minutes with a glucose rise of 3.5–5.5 mmol/L. IV glucose clamps (lasting 80–110 minutes achieving a glucose rise of 4–6 mmol/L) were performed to calibrate the IP sensors in 8 of the pigs. Table 1 presents the order in which the different glucose infusions were given.

### Glucose analysis of arterial blood and IP fluid

Arterial blood and IP fluid samples were analysed on a Radiometer ABL 725 blood gas analyser (Radiometer Medical ApS, Brønshøj, Denmark). Blood was collected in heparinised syringes (LEO Pharma A/S, Ballerup, Denmark) and IP fluid was collected in heparinised capillary tubes (Radiometer Medical ApS, Brønshøj, Denmark).

Due to the large quantity of IP fluid samples, some samples needed to be stored on ice for several hours before analysis. Some of them were tested immediately after harvesting and again after being stored for 6 hours. A mean change of + 0.1 mmol/L (SD 0.1 mmol/L) was observed.

### Data processing

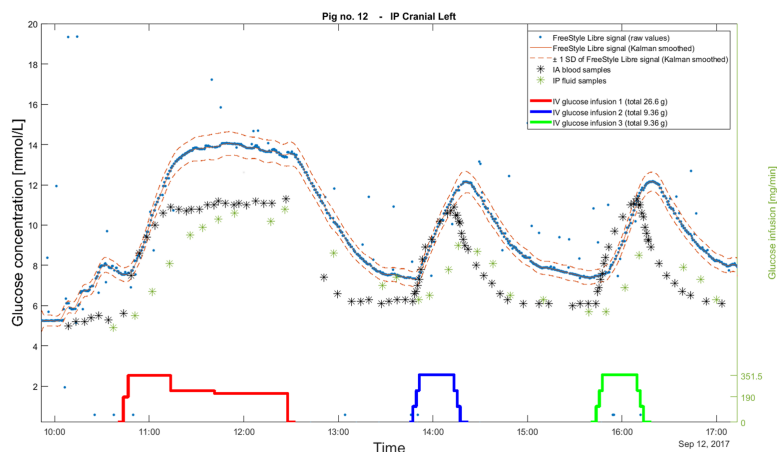
The open source devices LimiTTer (LimiTTer by JoernL @ GitHub) were used to relay the data transmitted by the FreeStyle Libre sensors to tablet computers running the open source Android application xDrip (xDrip by stephenblackwasalreadytaken @ GitHub). The

**Table 1. Order of glucose infusions for all pigs.**

Pig ID	First glucose infusion	Second glucose infusion	Third glucose infusion
2	Bolus <sup>a</sup>	IV meal (943 mg/kg)	IV meal (313 mg/kg)
3	Bolus <sup>a</sup>	IV meal (245 mg/kg)	IV meal (245 mg/kg)
4, 5	IV meal (245 mg/kg)	IV meal (245 mg/kg)	IV meal (245 mg/kg)
6, 8, 9	Clamp	Bolus <sup>a</sup>	Bolus <sup>a</sup>
7, 10, 11, 12, 13	Clamp	IV meal (245 mg/kg)	IV meal (245 mg/kg)

<sup>a</sup> Data not included in the study

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**Fig 3. Example of raw data and Kalman smoothed data.** The figure shows data from the entire experiment from the cranial left IP sensor in pig 12. The blue points represent raw data read by the LimiTter, the red solid line represents the Kalman smoothed data and the dashed line represents  $\pm 1$  standard deviation of the Kalman smoothed curve. The black stars (\*) represent the IA glucose samples and the green stars (\*) represent the IP glucose samples of the cranial left IP location. The glucose infusions are seen as solid lines at the bottom part of the graphics.

<https://doi.org/10.1371/journal.pone.0205447.g003>

LimiTTers were customised to read the FreeStyle Libre sensors approximately every 22 seconds, however, the FreeStyle Libre sensors only update the transmitted glucose values in one-minute intervals. The xDrip app was not customised and was only used to store the transmitted glucose values for later processing and to display the received data in order to supply a mean of monitoring the function of the set-up [22].

The raw data from the FreeStyle Libre sensors was denoised using a median filter with a window size of 5 samples, i.e. circa 130 seconds to remove single outliers. The thickness of the abdominal wall caused some transmission noise, and the communication protocol of the LimiTter was not flawless. The Kalman smoothing method by Staal et al. was then applied to the median-filtered glucose measurements producing interpolated series with a sampling rate of 1 s [26]. The smoother also removes outliers. Fig 3 illustrates how the raw measurements are affected by these methods for denoising and smoothing. Table 2 summarises the setting and tuning parameters.

Intra-arterial blood glucose analysis was performed manually in intervals ranging from 30 seconds to 10 minutes during the glucose infusions, and therefore less frequently than the FreeStyle Libre data. Equally distributed sampling intervals were necessary in order to use the data for system identification. Thus, blood glucose values were processed by a shape-preserving piecewise cubic interpolation to get the same sampling intervals as for the FreeStyle Libre data.

**Model identification.** As described above, continuous glucose sensors were placed in the IP cavity and the SC tissue. The dynamics between the intra-arterial glucose concentrations ( $G_{IA}$ ) and the sensed glucose concentrations are described by a two-compartment model [22]:

$$\frac{dG_{sens}(t)}{dt} = \frac{1}{\tau} (K \cdot G_{IA}(t - \Theta) - G_{sens}(t)) \quad (1)$$

with the sensor glucose concentration  $G_{sens}$ , the intra-arterial glucose concentration  $G_{IA}$ , the time constant  $\tau$ , the time delay  $\Theta$ , and the model gain  $K$ . The intra-arterial glucose

**Table 2. Settings and tuning parameters used for Kalman smoothing of FreeStyle Libre data [26].**

	FreeStyle Libre
Dynamic model	Model 2: Central-remote rate model
Model parameter	$T_d = 600\text{ s}$
Process noise covariance	$Q = \begin{bmatrix} 0 & 0 & 0 \\ 0 & 10 \Delta t & 0 \\ 0 & 0 & 0 \end{bmatrix}$ with $\Delta t = 1\text{ s}$
Measurement noise covariance	$R = (0.83/2)^2$ for $G \leq 5.55\text{ mmol/L}$ $R = (0.15/2)^2 \cdot G^2$ for $G > 5.55\text{ mmol/L}$
Initial covariance	$P_0 = \begin{bmatrix} 10 & 0 & 0 \\ 0 & 1 & 0 \\ 0 & 0 & 1 \end{bmatrix}$
Outlier removal	Based on smoothed data (outlierRemoval = 1)

The worst-case variances for self-monitoring blood glucose devices fulfilling ISO 15197:2013 were used.

<https://doi.org/10.1371/journal.pone.0205447.t002>

concentration was obtained from arterial blood samples. The sensor glucose concentration is measured by the FreeStyle Libre sensors. Thus, the modelled dynamics include both the physiological dynamics from blood to the sensor site and the internal sensor dynamics.

The time-domain Eq (1) was transferred into the frequency domain where a first-order transfer function with time delay was identified using the System Identification Toolbox in MATLAB (The MathWorks Inc., Natick, Massachusetts, U.S.A.). The optimal input delay was found by repeatedly determining the transfer function with a fixed delay. Time delays between 0 and 900 seconds with 1-second intervals were considered. The time delay that resulted in the lowest mean squared error (MSE) between the modelled and the measured sensor glucose was chosen.

The MATLAB-internal function *tfest* requires that the data starts at 0. Therefore, the stationary glucose value (called baseline in this article) at the beginning of the glucose infusion was subtracted to correct for the offset different from 0. This stationary value was determined for each sensor, site and clamp because it was not guaranteed that the same glucose level was reached between the glucose infusions. The stationary (baseline) values were determined as follows:

1. For intra-arterial glucose values, the sample at the start of the glucose infusion ( $t = 0\text{ min}$ ) and the three preceding samples (over a period of approximately 15 minutes) were averaged, i.e.

$$G_{IA,stat} = \frac{BGA(t = -15\text{ min}) + BGA(t = -10\text{ min}) + BGA(t = -5\text{ min}) + BGA(t = 0\text{ min})}{4}$$

2. For the sensed glucose values (SC and IP), the average over the last 3 minutes preceding the glucose infusion start was taken.

### Statistical analysis

The combined physiological and sensor dynamics were analysed based on the smoothed data from the section Data Processing.

**Inclusion of sensor recordings.** Sensor measurements that fulfilled the following criteria were included in statistical analysis:

1. Stable at beginning of glucose challenge

The sensor value was stable in the 3 min before the glucose infusion. Stable was defined as a glucose rate of change less than 0.1 mmol/L/min, i.e. if;

$$\left| \frac{dG_{sens}}{dt} \right| < 0.1 \frac{\text{mmol}}{\text{L} \cdot \text{min}}$$

2. Model fit

The identified model from section Model identification had a goodness of fit of more than 70%.

The following properties were investigated:

1. Time delay of the model identified in section Model identification.
2. Time constant of the model identified in section Model identification.
3. Time to 50% of maximum value from start of glucose challenge.
4. Time to 50% return to baseline levels, counted from start of glucose challenge.

All statistical analyses were conducted in R [27]. Dynamic parameters were analysed using a linear mixed effect analysis with maximum likelihood estimation with the sensor location, the amount of IV fluid infusion and the direction of the sensor element (for IP sensors only) defined as fixed effects. To account for several measurements in each pig, pig ID was defined as a random effect [28]. P-values for pairwise comparisons of the different IP sensor locations and comparison of all IP sensors against all SC sensors, were obtained by t-tests using the Satterthwaite approximation to the effective degrees of freedom, automatically calculated with the lmerTest and lme4 packages in R [28, 29]. The statistical threshold was set to 0.01 to account for multiple comparisons.

## Results

168 sensor recordings were recorded from 7 glucose clamps and 20 IV-meals. All SC sensor recordings (56 out of 56) and 56% (63 out of 112) of the IP sensor recordings were included in the statistical analysis (Fig 4). The mean percentage of model fit for the included sensors was 93.4% (SD 4.1%) for the SC sensors and 85.7% (SD 7.5%) for the IP sensors. Fig 5 shows examples of included and excluded sensor recordings based on the criterion of model fit.

Table 3 presents the mean and standard deviation of the dynamic parameters for the different IP sensor locations, as well as for all SC and IP sensors. The IP cranial left sensors and the IP caudal right sensors seemed to react faster than the IP cranial right and IP caudal left sensors. However, the only pairwise comparison to show a significant difference, was the smaller time constant of IP cranial left sensors compared to the IP caudal left sensors ( $p = .0075$ ) (Fig 6 and S1 Table). The estimated mean time delay of all IP sensors was significantly smaller than the mean time delay for all SC sensors ( $p = .0091$ ) (Fig 7 and S2 Table). Comparisons of SC and IP sensors for the other dynamic parameters did not show significant differences (Fig 7 and S2 Table). Fig 8 shows the mean and standard deviation for all included IP and SC sensors during IV-meals, and indicates that the IP sensors react faster than the SC, but also shows a larger variation.

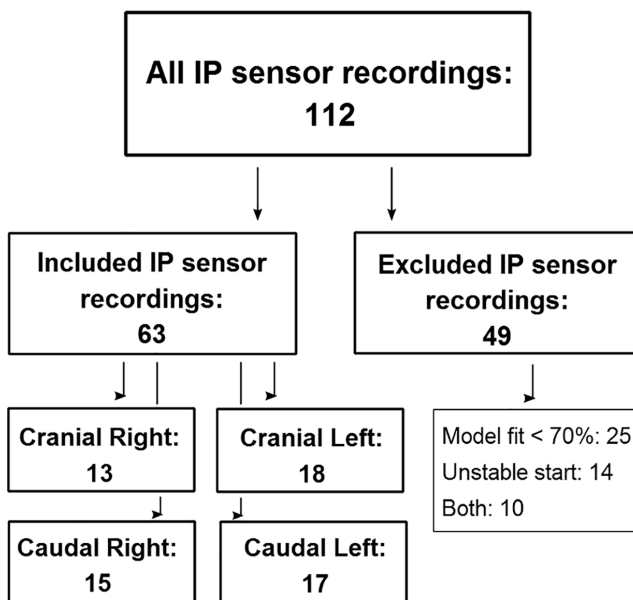


Fig 4. Scheme of included and excluded IP sensor recordings.

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The IP sensors positioned with the sensor element against the abdominal wall (75% included) show more stable signals than the sensors with the element towards the peritoneal space/visceral lining (50% included). Positioning of the sensor element also affects the glucose

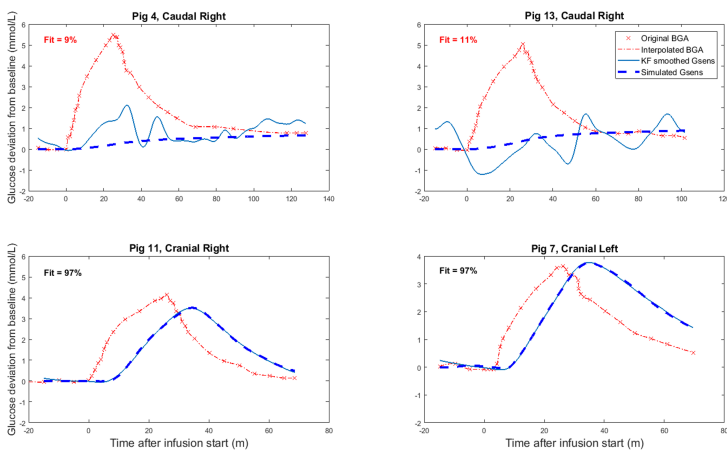


Fig 5. Examples of excluded (top panels) and included (bottom panels) sensor recordings. The red curves represent the intra-arterial blood glucose values, the solid blue lines represent the Kalman smoothed sensor signals and the dashed dark blue lines represent the sensor signals predicted by the model. The percentage of model fit is displayed in the top right corner of each panel. BGA = blood glucose analysis, Gsens = sensor glucose concentration.

<https://doi.org/10.1371/journal.pone.0205447.g005>

Table 3. Summary of results for the four IP sensor locations and mean of the IP and SC sensors.

Sensor location	Mean time delay $\pm$ SD (s) <sup>a</sup>	Mean time constant $\pm$ SD (min) <sup>a</sup>	Mean model fit $\pm$ SD (%) <sup>a</sup>	Mean time to 50% max (min) <sup>b</sup>	Mean time to 50% decline to baseline (min) <sup>b</sup>
IP Cranial Right	175.2 $\pm$ 139.2	13.2 $\pm$ 11.1	85.4 $\pm$ 8.0	18.0 $\pm$ 4.4	59.1 $\pm$ 15.6
IP Cranial Left	145.9 $\pm$ 127.8	8.6 $\pm$ 4.32 <sup>1</sup>	85.8 $\pm$ 7.3	16.9 $\pm$ 2.7	55.8 $\pm$ 6.7
IP Caudal Right	155.6 $\pm$ 139.8	11.9 $\pm$ 11.0	82.8 $\pm$ 7.6	17.3 $\pm$ 3.9	53.0 $\pm$ 11.1
IP Caudal Left	204.3 $\pm$ 160.4	15.6 $\pm$ 9.1 <sup>1</sup>	87.5 $\pm$ 6.4	18.0 $\pm$ 2.9	57.9 $\pm$ 11.2
All IP sensors	170.0 $\pm$ 140.8 <sup>2</sup>	12.2 $\pm$ 9.2	85.5 $\pm$ 7.3	17.5 $\pm$ 3.4	56.3 $\pm$ 11.1
All SC sensors	241.1 $\pm$ 149.9 <sup>2</sup>	9.5 $\pm$ 5.3	91.0 $\pm$ 6.5	19.0 $\pm$ 2.7	57.2 $\pm$ 10.1

<sup>a</sup> Calculations are done on identified model parameters using both glucose clamps and IV meals.

<sup>b</sup> Calculations done on Kalman smoothed data, exclusively from IV meals.

<sup>1</sup> Time constant of IP cranial left significantly smaller than IP caudal left,  $p = .0075$ .

<sup>2</sup> Time delay of IP sensors significantly smaller than SC sensors,  $p = .0091$ .

<https://doi.org/10.1371/journal.pone.0205447.t003>

dynamics, being slower for the sensors facing the abdominal wall (Fig 9). The difference is, however, not statistically significant (S2 Table).

IP fluid was frequently sampled during the experiments and shows increasing glucose values after IV infusions of glucose. A basic comparison with the IP sensor recordings at the same location showed no significant dynamic differences (Fig 10). The analysis using the MATLAB function *delayest* revealed time delays of 0 to 37 seconds between each IP sensor and its corresponding IP fluid samples (mean 10 s, SD 14 s). For 19 of the 30 included comparisons, the time delay was less than or equal to 2 seconds. This indicates that the internal sensor dynamics are very small compared to the physiological dynamics from blood to the peritoneal fluid. For the remaining 11 comparisons, the time delay was larger than 10 s; and the IP samples showed almost no increase. Thus, only the sensor recordings were further analysed by means of model identification.

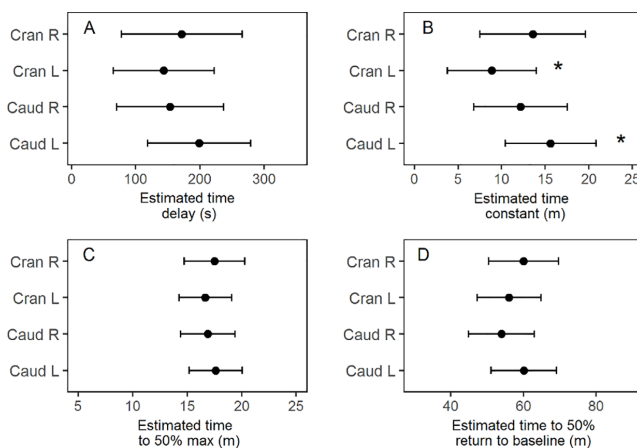
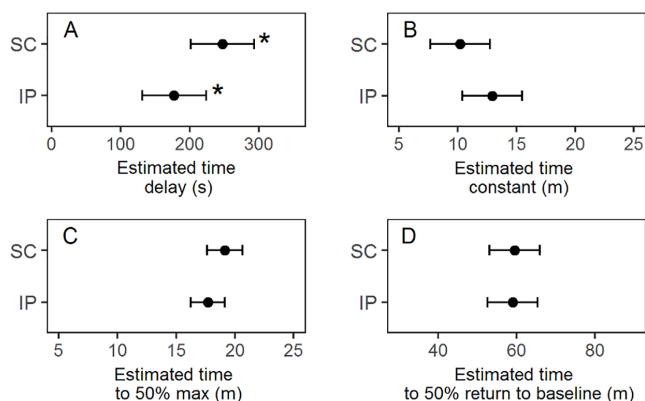


Fig 6. Point estimates and 95% confidence intervals for time delay (A), time constant (B), time to 50% max (C) and time to 50% decline to baseline level (D) for all IP sensors. Sensor location is set as fixed effect in the lmerTest-model. \* indicates a significant difference ( $p < 0.01$ ).

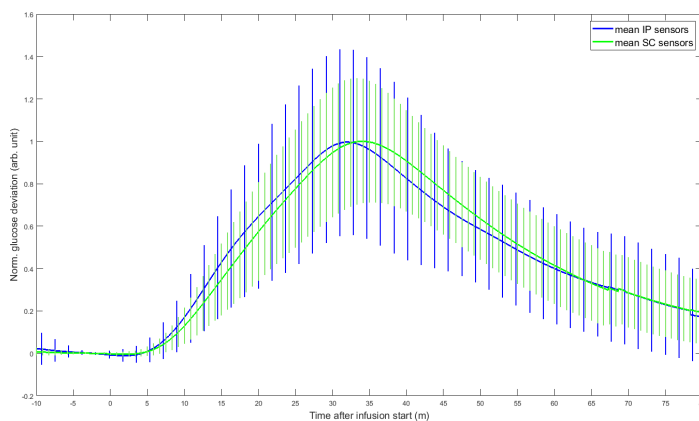
<https://doi.org/10.1371/journal.pone.0205447.g006>



**Fig 7. Point estimates and 95% confidence intervals for time delay (A), time constant (B), time to 50% max (C) and time to 50% decline to baseline level (D) for IP and SC sensors.** The mean parameter values for the IP sensors compared to those for the SC sensors with sensor location (SC or IP) as fixed effect in the lmerTest-model. \* indicates a significant difference ( $p < 0.01$ ).

<https://doi.org/10.1371/journal.pone.0205447.g007>

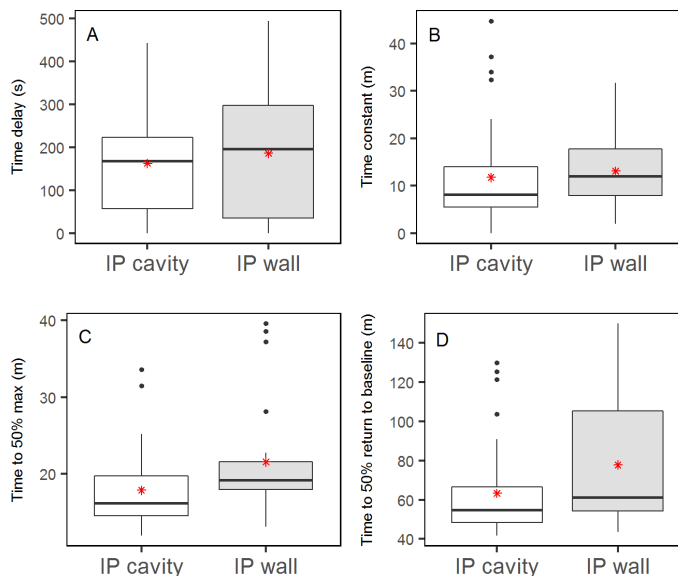
The amount of IP fluid increased in all pigs during the experiments, i.e. during the eight hours on the operation table. Pigs 2–9 received 5–9 ml/kg/h, and pigs 10–13 received approximately 2.5 ml/kg/h of Ringer’s acetate IV. The amount of accumulated IP fluid at the end of experiments was reduced from approximately 200 ml to approximately 50 ml after changing the IV-infusion regimen. The volume of infused IV fluid affected the glucose dynamics, with the higher infusion regimen resulting in slower dynamics for both SC and IP sensors, with the exception of the time to 50% max for IP sensors (Fig 11). The differences in dynamic parameters for the two infusion regimens were not significantly different (S2 Table).



**Fig 8. Distribution of sensor recordings from the included IP and SC sensors during IV-meals.** The blue curve represents the mean of the normalized IP sensor signals, and the green curve represents the mean of the normalized SC sensor signals. The sensor values were not calibrated and thus do not necessarily show the real glucose values. We therefore compare the normalized glucose deviation from the baseline. The mean was calculated by averaging at each sample over all IP and SC sensors, respectively. The average deviation was then scaled to the range [0, 1]. The same scaling factor was used to normalize all sensor readings from the sensing site under consideration before the standard deviation was determined.

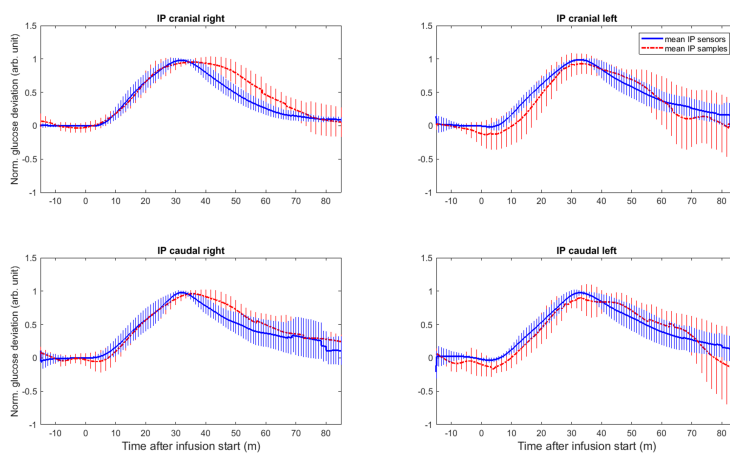
<https://doi.org/10.1371/journal.pone.0205447.g008>





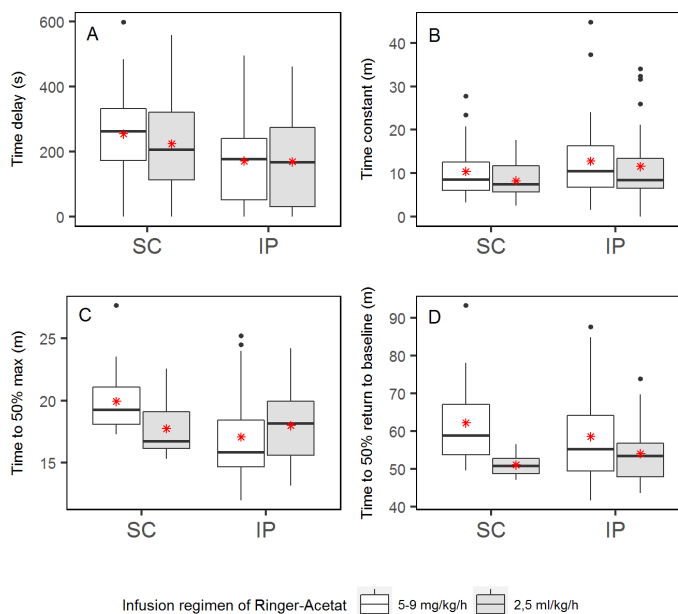
**Fig 9. Boxplots of time delay (A), time constant (B), time to 50% max (C) and time to 50% decline to baseline level (D) for IP sensors with different positioning of sensor element. Mean values are presented as a red \*.**

<https://doi.org/10.1371/journal.pone.0205447.g009>



**Fig 10. Distribution of included sensor recordings from the four different IP sensor locations compared to corresponding IP fluid samples during IV-meals. The blue curves represent the mean of the normalized sensor signals, and the red curves represent the mean of the normalized IP fluid samples. The hatched areas indicate  $\pm 1$  SD of the included data. Data was normalized by means of offset correction and scaling to the range [0, 1]. arb. unit = arbitrary unit.**

<https://doi.org/10.1371/journal.pone.0205447.g010>



**Fig 11. Boxplots of time delay (A), time constant (B), time to 50% max (C) and time to 50% decline to baseline level (D) for IP sensors with the different IV fluid regimens for both IP and SC sensors. Mean values are presented as red \*.**

<https://doi.org/10.1371/journal.pone.0205447.g011>

## Discussion

No obvious difference in glucose dynamics was found between four different locations in the peritoneal cavity of anaesthetised pigs. The difference in time constant between cranial left and caudal left was statistically significant. The clinical significance of this, however, is doubtful when only one of several comparisons shows statistical significance. Comparing IP sensors to SC sensors showed a significantly smaller time delay for the IP sensors, but we did not find significant results when comparing the other dynamic parameters. As secondary findings, we observed that the glucose dynamics were affected both by the amount of IV fluid given during experiments and the positioning of the sensor element (for the IP sensors), but these differences were not significant.

Glucose sensing in the peritoneal space has been studied in rabbits [30], rats [31, 32] and pigs [22, 33]. Burnett et al. reported the mean time delay and mean time constant to be 40.8 (SD 34.8) seconds and 5.6 (SD 2.9) minutes, respectively [22]. Fougner et al. have previously found a mean time delay of 9.7 (SD 9.5) seconds and mean time constant of 4.7 (SD 2.9) minutes in pigs [33]. The IP glucose dynamics (time delays and time constants) in the present investigation are slower compared to these previous studies.

Burnett et al. compared glucose sensing in the IP space to the SC tissue and found the glucose dynamics in the IP space to be significantly faster than in the SC tissue. The present investigation showed the time delay of the IP sensors to be significantly shorter than that of SC sensors. The other comparisons revealed, however, no statistically significant differences.

Comparing our present results to the previous published results is difficult for several reasons. First of all, different glucose sensors were used in the three studies: Burnett et al. used

modified Dexcom sensors in their trials, which is an amperometric sensor like the FreeStyle Libre sensor, but from a different manufacturer (Dexcom, San Diego, USA). Fougner et al. used optical interferometric sensors to measure glucose [33]. Secondly, the studies handle the measured glucose dynamics in different ways. Measured glucose dynamics is composed of two parts: the glucose diffusion from the circulation to the sensing location, and the internal sensor dynamics. Fougner et al. previously used sensors with known dynamics, and identified the actual dynamics related to the sensing location [33]. In both the present study and the study by Burnett et al., the overall dynamics, i.e. the physiologic and sensor delays, are analysed together. Thus, possible differences between sensing locations may be harder to identify if the sensor has slow dynamics that hides the minor contribution of the sensing location. That way, the potential of the sensing location is not assessed, but rather the combination of the sensing location and the particular sensor. As already pointed out, internal sensor dynamics appear to be very small compared to the physiological dynamics from blood to the peritoneal fluid. Given that we could not detect differences in dynamic contributions from the different sensing locations themselves, any such contributions are also likely to be small (i.e. the resulting dynamics are fast) compared to the gross physiological dynamics from blood to peritoneal fluid. Finally, we delivered glucose as simulated meals and clamps in the presented study, while glucose was given as boluses in the previous studies. Different speeds of glucose delivery might affect the observed dynamics. It is noteworthy that in the present study, simulating physiologic glucose excursions, the overall IP glucose dynamics are slower than previously reported and not significantly faster than the SC glucose dynamics.

The peritoneal lining is made up of a single layer of mesothelial cells with an underlying layer of connective tissue embedded with capillaries, other blood vessels, nerves and lymphatic vessels [34]. According to the “three pore model” referred to in the field of peritoneal dialysis, the endothelium of the capillaries is considered to be the major barrier for water and solutes crossing the peritoneal lining [35], although the interstitium is also believed to contribute [36]. Glucose is a small molecule, and passes easily through the small pores in the endothelium, mainly by diffusion [37, 38]. It should be mentioned that the greater omentum in pigs does not cover the whole front of the intestines in the way it does in humans. The sensor elements pointing into the peritoneal space of our pigs might be positioned against the visceral peritoneal lining of the intestines, whereas they would have been facing the greater omentum in humans. However, in humans there is no systematic variation in the key histological parameters, including microvascular density, in different parts of the peritoneum [39, 40]. This fits with our observation of no evident spatial difference in glucose dynamics between the four quadrants of the peritoneal cavity. However, the observed variation in data obtained within each of the four IP quadrants might indicate local differences in glucose dynamics. More knowledge of these possibly small-scale local differences is needed, as this might lead to considerable improvements of the glucose dynamics by more accurate placing of the sensors.

The positioning of the sensor element affected both the glucose dynamics and the quality of sensor signals. The glucose dynamics of the sensors with the sensor element towards the abdominal wall was slower than the dynamics of the sensors pointing towards the visceral peritoneum (Fig 9). This might be because the sensor elements were pressed against the wall and IP fluid surrounding the sensor element was kept stagnant. The quality of sensor signals was also affected by positioning of the sensor element. The signals were less noisy when the sensor element lay against the parietal peritoneum, which could be because the sensor element was pressed against the abdominal wall in a stable position. The sensors with the element pointing inwards in the peritoneal space might be exposed to a more unstable environment of moving fluid and organs. We frequently sampled IP fluid and this most likely contributed to circulation of IP fluid. It is, however, not possible to conclude whether the fluctuating signals,

especially seen in the excluded sensor recordings, are caused by actual changes in glucose concentration in IP fluid or should be considered as artefacts (e.g. due to movements of organs and IP fluid), or a combination of the two.

The Kalman smoothing we performed on the sensor data assumes dynamics by using a process model. It could be argued that the Kalman smoothing modifies the dynamics of the FreeStyle Libre data. However, the ratio of measurement to process noise was chosen low and by that, we trust the sensor values more than the process model. Our simulations showed that the smoothed glucose values follow the sensed values with good accuracy (Fig 3). The smoothing ensures that subsequent outliers that have not been eliminated by the median-filter are removed and subsequently replaced with missing values. Moreover, the original sampling intervals of the LimiTTer (reader for FreeStyle Libre sensors), vary in the range 20–22 seconds. For the identification procedure, we needed equal sampling intervals, which is one of the available output features of the Kalman smoother algorithm.

Blood glucose measurements were sampled less frequently, and they were less prone to outliers. They are interpolated assuming cubic curves. In that way, the actual blood glucose measurements are preserved and the values between the samples are estimated. We omitted the use of a Kalman smoother to better resemble the true blood glucose values: dependent on the tuning, a smoother either flattens the steep slope in the beginning of glucose infusions, or results in an overall poor fit. The interpolation, however, preserves the overall shape of the blood glucose curve. The timing of the blood glucose sampling was logged manually. This is one of the reasons why the identified time constants and time delays are not accurate to the single second.

The data was fitted to a model that has been developed to describe the SC glucose dynamics, but it has also been used previously to model IP glucose dynamics [22]. The model describes the glucose diffusion dynamics from the circulation to the sensing location. The model was fitted to the whole data curve, although the dynamics might differ between increasing and decreasing glucose concentrations. The physiologic glucose excursions in this study were slower compared to previous studies with IV glucose boluses [22, 33]. When glucose was infused as simulated meals, the pigs might have started to utilise glucose concomitantly as the IP glucose values were increasing. Thus, the two effects of glucose increasing and glucose decreasing, might overlap. The result on the model identification might be that in order to guarantee an overall good fit, the time delays and constants are overestimated to compensate for the glucose that has already been removed from the IP and SC sensing sites at the end of the glucose infusion. This effect might be less pronounced when glucose was given as boluses. A possible reason why the model achieved a higher fit for the SC sensors is that the model describes the SC dynamics better, whereas it is less suited to model the IP dynamics. However, it is out of the scope of this study to evaluate the model. Overall, Eq 1 resulted in adequately described IP glucose sensing, and it fits the purpose of this study to use the same model structure for comparison of IP and SC glucose sensing dynamics.

During several glucose infusions, the blood glucose values showed a steadily rising curve, but some IP sensors seemed to oscillate around this presumably actual value. The resulting model fit is low, although the model might actually fit the dynamics quite well. The percentage of fit of the identified models was used as an inclusion criterion. For this reason, some curves that described the actual dynamics of the sensor location well may have been excluded due to oscillations of unknown origin.

An AP requires adequate sensor technology, algorithms and an optimal site for the sensor and hormone administration. The IP space has previously been shown as a promising site for CGM due to its appearing fast glucose dynamics, but the IP glucose dynamics in the current experimental set up was equivalent to the glucose dynamics of the SC tissue. The overall

advantage of IP CGM must be considered against the obvious disadvantages; entering the IP space through a port imposes a risk for a more serious infection than in the SC space. Patients may also consider the port as too invasive. Real fast glucose dynamics is therefore essential for an IP glucose sensor to be a realistic alternative for diabetic patients, and the results shown in the presented study does not justify moving the glucose sensing into the abdomen, i.e. the clinical significance of a shorter time delay in IP glucose sensing has still to be proven, and it has to be weighed against the cost of surgical efforts and patient inconvenience. However, if combined with intraperitoneal insulin delivery with an external pump, a glucose sensor can be added using the same port as the insulin tube with no additional inconvenience for the patient.

Development of alternative sensor technology might improve the performance of CGM in the peritoneal space considerably. Amperometric sensors like the FreeStyle Libre used in the presented study and the modified Dexcom sensors used by Burnett et al. [22] measure glucose in the peritoneal fluid directly surrounding and in contact with the sensor element. Glucose in the peritoneal fluid must travel from the peritoneal lining and to the location of the sensing element to be detected, and the performance of sensors measuring glucose in the peritoneal fluid will therefore be highly influenced by the amount of fluid surrounding the sensor element and the movement of the IP fluid. The diffusion coefficient of glucose in water at 25°C is  $6.7 \times 10^{-6} \text{ cm}^2$  per second [41], and by using Fick's second law of diffusion [42] this implies that glucose will travel 1 mm in water in approximately 750 seconds. Optical sensor technology, such as near-infrared, mid-infrared and Raman spectroscopy however, might enable glucose measurements directly on the peritoneal lining or even in the capillaries embedded in the tissue, in addition to the ability to measure in the peritoneal fluid. Fougner et al. used optical interferometric sensors in their experiments and identified fast glucose dynamics in the peritoneal space [33]. They questioned if their sensors actually measured glucose directly in contact with the capillaries in the peritoneal lining, i.e. with only a short distance, consisting of the peritoneal lining and capillary wall and sensor membrane, and suggested this as an explanation of the fast IP glucose dynamics identified in the study [33].

Nevertheless, the IP space has other advantages compared to the SC tissue; it is more protected against changes in temperature and mechanical pressure and it can prove as an acceptable solution in combination with intraperitoneal delivery of insulin, even without superior glucose dynamics compared to SC tissue.

This study has several limitations. The FreeStyle Libre sensor is designed to operate in the SC tissue. Our use of the sensor in an alternative environment might affect the presented glucose values in ways we are not fully aware of. Variations in pressure, oxygen tension, excessive moisture, bowel movements and possibly other factors might influence the measurements and cause the heavy fluctuations and unpredicted signals seen in some of the IP-sensors. These signal fluctuations are not considered to be due to disturbances in signal transmission, as digital transmission errors cause artefacts in the received signal that are qualitatively different from the ones observed here.

Unlike most other CGM devices, the FreeStyle Libre sensor is factory calibrated [43]. The sensor system presents glucose values without initial calibration by the user. Unlike some of the other CGMs, it is not possible for the user to access the raw data. The sensor is preprocessing the data before presenting the glucose value, but information about the preprocessing is not available in the open literature. The FreeStyle Libre sensor also includes a temperature sensor, likely used for a control algorithm using the temperature in the surroundings of the sensing element to adjust the glucose value. We believe this temperature adjustment might be done in the FreeStyle Libre hand held reader, and if so, the glucose values we obtain using a Limit-Ter will be without this temperature correction. The glucose clamps were intended to be used for calibration of the sensors, but in this paper uncalibrated sensors recordings were used for

dynamic parameter comparisons since there was a risk of the calibration method affecting the estimation of such parameters. We also observed an overshooting of the sensor response when it was exposed to large and sudden differences in glucose concentrations in *in vitro* trials [25]. Such sensor behaviour was not observed when the sensors were exposed to gradual increase in glucose concentration. This sensor behaviour was unexpected, and for this reason, the IV boluses were excluded from further analysis.

The experiments were performed on non-diabetic pigs, and the presented glucose dynamics can be different from a diabetic animal model, and the results are by no means directly transferable to diabetic patients. This study also faces the challenge of being conducted on anaesthetised pigs undergoing long-lasting surgical procedure. This might influence the pigs' physiology and consequently the glucose dynamics we are examining, and is the main argument for the experiments to be of relatively short duration. For instance, we did observe an increase in IP fluid volume during the individual experiments. The total amount of IP fluid in humans or pigs is not well studied. There is no available information on the normal amount of IP fluid in pigs, and for humans the reported estimates range from 15 to 100 ml [34, 44–46]. We reduced the IV infusion rate of Ringer's acetate (see *Methods*), which resulted in a reduction of produced IP fluid. However, the volumes are still suspected to be unphysiologically large. It is likely that large amounts of IP fluid during the experiments will slow both the increasing and decreasing glucose dynamics because of a diluting effect and because more fluid consequently leads to greater distances for the glucose molecules to travel before being detected by the sensor element. The sensors in the pigs receiving the lower infusion rate did react faster than the sensors in pigs receiving the higher infusion rate, but the difference was not significant. Viewing the graphical comparisons of sensor signals to sampled IP fluid (Fig 10) suggest that there is no significant difference.

Fluid also accumulated in the SC tissue in pigs receiving large volumes of IV fluid, causing slower dynamics in the SC tissue especially the time to 50% return to baseline as can be seen in Fig 11. Interestingly, this may also be the case in humans. If present, the effect is probably minor but the physiologic delays may have a diurnal variation with the lowest values in the morning before having had anything to drink. It may also increase delays in patients with heart failure. The unphysiological IP fluid volumes might make the comparisons between SC and IP sensors problematic, but we assume equal conditions for the IP sensors making the comparisons of glucose dynamics within the IP space valid.

Research on glucose sensing in the peritoneal space is at its early stage. Long-term studies will examine the glucose dynamics in awake pigs and not during prolonged anaesthesia and surgery. The amount of IP fluid will probably be normalised, the wound in the abdominal wall will have healed and the effects of anaesthesia are removed. Most importantly, however, IP glucose dynamics will be investigated during normal physiologic excursions following meals and normal fasting.

In addition to more refined and physiologic animal experiments, development of the sensor technology is crucial in examining the IP glucose dynamics. The sensor's own dynamics should be rapid, its lifetime must be long, its sensitivity high and it should be robust.

## Conclusions

The present study found no major differences in glucose dynamics between the four quadrants of the intraperitoneal cavity of pigs. The time delay of the IP sensors was significantly smaller than that of the SC sensors (170 s vs. 241 s), but no other significant differences in subcutaneous and intraperitoneal glucose dynamics were identified.

## Supporting information

**S1 Table. Output from statistical analysis in R for comparisons of IP sensors.**

(DOCX)

**S2 Table. Output from statistical analysis in R for comparison of SC and IP sensors.**

(DOCX)

## Acknowledgments

The animal experiments were performed at the Comparative medicine Core Facility (CoMed) at Norwegian University of Science and Technology (NTNU). We would like to thank Oddveig Lyng especially, for her invaluable contribution. We also thank Associate Professor Øyvind Salvesen for helpful discussions of the statistical analysis.

Posters with the experiment set-up and some preliminary results were presented at the international conference on Advanced Technologies & Treatments for Diabetes (ATTD) 2017.

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## Paper II





## Why intraperitoneal glucose sensing is sometimes surprisingly rapid and sometimes slow: A hypothesis



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### ABSTRACT

The artificial pancreas requires fast and reliable glucose measurements. The peritoneal space has shown promising results, and in one of our studies we detected glucose changes in the peritoneal space already at the same time as in the femoral artery. The peritoneal lining is highly vascularised, covered by a single layer of mesothelial cells and therefore easily accessible for proper sensor technology, e.g. optical technology. We hypothesize that the rapid intraperitoneal glucose dynamics observed in our study was possible because the sensors were located directly at the peritoneal lining, at the point where the glucose molecules entered the peritoneal space. Glucose travels slowly in fluids by diffusion, and a longer distance between the sensor and the peritoneal lining would consequently result in slower dynamics. We therefore propose to place the glucose sensor in an artificial pancreas as closely to the peritoneal lining as possible, or even utilize appropriate sensor technology to measure glucose in the peritoneal lining itself.

### Introduction

Automatic closed-loop glucose control, i.e. an artificial pancreas (AP) system, has the ultimate aim of providing stable glucose control in the normal or near normal range and thereby improve the long-term outcomes for patient with diabetes mellitus type 1 (DM1). This requires precise, reliable glucose measurements as close to real time as possible. The intraperitoneal (IP) space is a possible site for real time glucose sensing in an AP, and animal studies indicate both superior and similar results compared to subcutaneous glucose sensing [1–3].

DM1 is a life-long disease in which the pancreas no longer produces insulin, resulting in loss of blood glucose (BG) regulation and increasing BG levels. Thus, these patients are dependent on external supply of insulin to control their BG levels. This is done almost exclusively by daily multiple subcutaneous (SC) injections or continuous SC infusion of insulin. Although the treatment of DM1 has seen incredible improvements over the last 100 years, and in particular during the last decades, the disease still leads to marked reduction in life expectancy and quality of life [4–6]. Several AP systems are under development and hold the promise of stabilizing BG levels in most patients with

DM1. An AP consists of three major components; a glucose sensor, an insulin infusion pump and a controller that calculates the appropriate dose of insulin (and glucagon if a bi-hormonal approach is chosen) based on the continuous glucose sensor data. Fast glucose sensing dynamics, i.e. glucose levels measured as close to real time as possible, is crucial to achieve a fully automated and well-functioning AP. Almost all groups working with AP use what can be called the double SC approach, i.e. they both measure glucose and deliver insulin in SC tissue. However, slow glucose dynamics of the SC tissue imposes challenges to all these AP systems [7]. Investigating the peritoneal space as an alternative site for an AP, i.e. a double IP approach is therefore warranted.

Glucose sensing in the IP space has only been sparsely studied [1–3,8–12]. However, it has been demonstrated that IP glucose sensing can sometimes be surprisingly rapid; reacting to intravenous (IV) glucose boluses almost as fast as intra-arterial (IA) sensors (time delays of 0–26 s between IA and IP sensor locations) [1]. This study used interferometric sensors (GlucoSet AS, Trondheim, Norway) and the observed sensors gave varying results. This variance might be explained by the location of the sensor, the proximity to the peritoneal lining, and

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<https://doi.org/10.1016/j.mehy.2019.109318>

Received 20 December 2018; Accepted 19 July 2019  
0306-9877/ © 2019 Published by Elsevier Ltd.

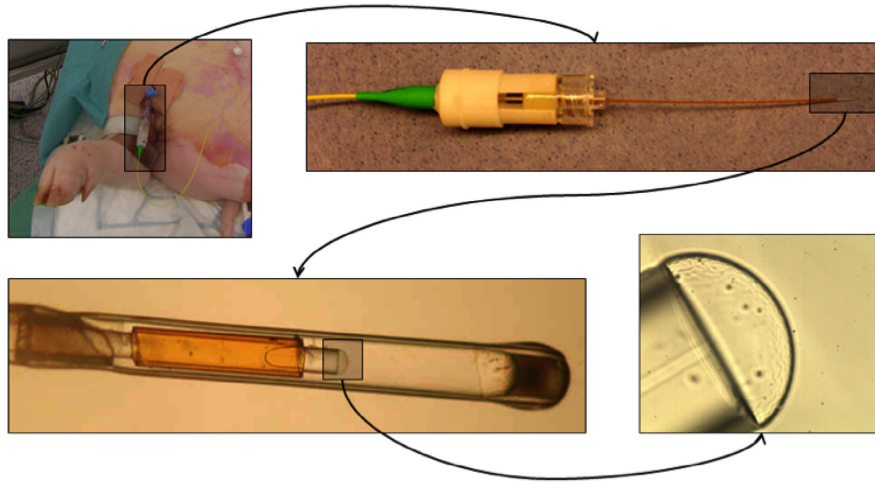


Fig. 1. The GlucoSet sensor at increasing magnification and its localisation in the femoral artery [1,14].

varying amounts of peritoneal fluid. This paper uses the definitions of time delay, time constant etc. as previously described by Ståvdahl et al. [13].

#### The hypothesis

We hypothesize that glucose changes can be detected as quickly in the abdominal cavity as in arterial blood only by locating the glucose sensor at the surface of, and in direct contact with, the peritoneal lining.

#### Evaluation of the hypothesis

Studies on IP glucose sensing has only been performed on animals [1–3,8–12]. Three studies report dynamic parameters, such as time delay and time constants on IP glucose dynamics, and with differing results [1–3]. It is difficult to compare these studies due to the use of different sensor technologies and system identification methods, as well as the lack of information on sensor dynamics in two of the studies. We will therefore discuss the results from one of our pig studies in which we used an interferometric glucose sensor (Fig. 1) [1]. This sensor was developed for intravascular use [14,15]. Glucose reversibly binds to receptors in a sphere-shaped hydrogel on the tip of an optical fibre, causing the hydrogel to expand or contract depending on the glucose concentration. The change of the optical length of the hydrogel alters the reflection of light, which is then translated to glucose values. In the article, the sensor dynamics was identified and excluded, and only the dynamics from the intra-arterial to the IP space was reported [1]. The sensors were placed in different locations in the ventral parts of the peritoneal cavity of pigs. The nature and the histological structures of the surrounding peritoneal lining were unknown, and the sensors could have been positioned against the peritoneal lining or in a compartment of fluid (Fig. 2). Pigs lack the greater omentum which in humans covers the intestines, so the sensors could have been resting against the visceral peritoneal lining of the intestines or the parietal peritoneal lining of the inner abdominal wall.

The peritoneal lining is made up of a single layer of mesothelial cells (mesothelium) with an underlying layer of connective tissue embedded with capillaries, other blood vessels, nerves and lymphatic vessels (submesothelium) [16,17]. Glucose is a small molecule (180 Da,  $8.6 \text{ \AA} \times 8.4 \text{ \AA}$ ), and passes easily through the small pores in the endothelium of the capillaries and into the peritoneal space and vice versa, mainly by diffusion [18,19]. Further transport of glucose in the

peritoneal fluid will also be by diffusion, although there is some movement of peritoneal fluid [16,20,21]. Convection forces also contribute to the movement of glucose from the capillaries to the IP space [22], but are not included in our calculations.

The diffusion coefficient for glucose in peritoneal fluid is not known, but we can make a short-cut calculation based on the diffusion time of glucose in water (25 °C).

**Fick's first law** describes the diffusion flux  $J$  for a solute as a function of the concentration gradient of the solute in a medium:

$$J = -D(\delta c / \delta x) \quad (1)$$

where  $D$  is the diffusion coefficient,  $\delta c / \delta x$  expresses the solutes change in concentration per unit of length in the diffusion direction. **Fick's second law** describes the time dependency of the change in concentration:

$$\delta c / \delta t = D \delta^2 c / \delta x^2 \quad (2)$$

where  $t$  is time. By combining Eqs. (1) and (2), it is possible to calculate the concentration as a function of time and position.

We are interested in an estimate of the time it takes a given molecule to diffuse an average distance in one direction. This diffusion time ( $t$ ) can be approximated by [23]:

$$t \approx x^2 / 2D \quad (3)$$

This gives the following diffusion times ( $D = 6.7 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$  for glucose in water at 25 °C [24]):

$$\text{– For } x = 1 \text{ mm; } \Rightarrow t_{25^\circ\text{C}, 1\text{mm}} \approx 750 \text{ s}$$

$$\text{– For } x = 100 \text{ } \mu\text{m; } \Rightarrow t_{25^\circ\text{C}, 100\mu\text{m}} \approx 7.5 \text{ s}$$

$$\text{– For } x = 10 \text{ } \mu\text{m; } \Rightarrow t_{25^\circ\text{C}, 10\mu\text{m}} \approx 750 \text{ s}$$

These calculations are indicative and based on the diffusion coefficient of glucose in water at 25 °C. According to the Stokes-Einstein equation [23] the diffusion coefficient may be estimated to be roughly 40% higher at 37 °C compared to the one at 25 °C due to increased thermal molecular motion and lower viscosity. Although the glucose diffusion coefficient in peritoneal fluid is unknown, and glucose probably will diffuse more rapidly in water due to its lower viscosity compared to that of IP fluid, we argue that it is likely that at 37 °C it will be of quite similar value to the one in water at 25 °C, given the apparent similarity of the fluids in this context.

For the IP sensors in our first study we estimated time delays between 0 and 26 s [1]. This implies a distance between the sensor and the

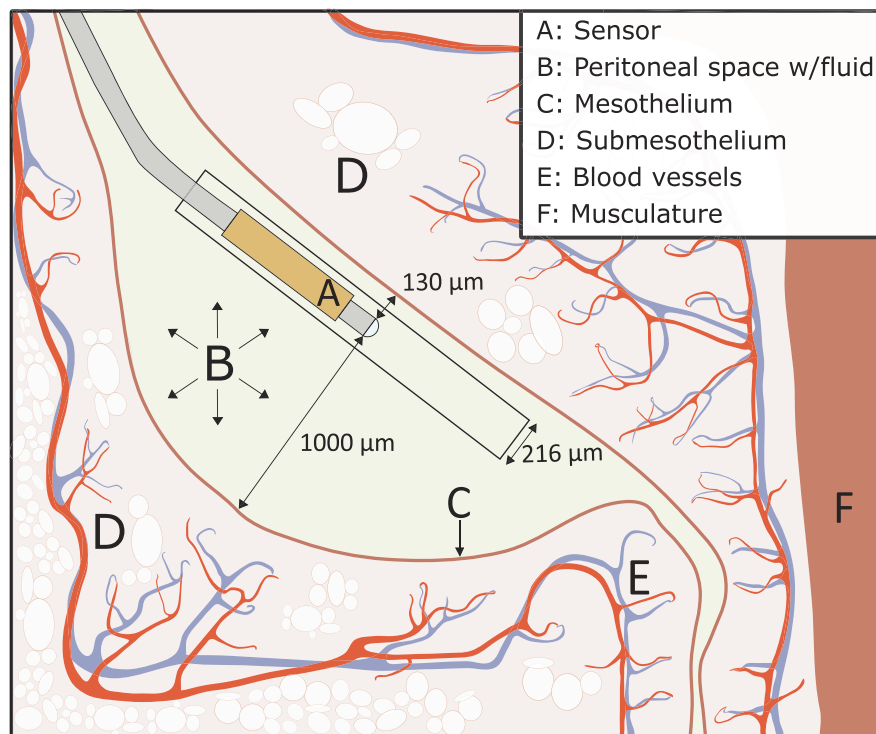


Fig. 2. Sketch of the mesothelium (A), submesothelium (D) with adipocytes and capillaries (E) and the GlucoSet sensor (A) in the peritoneal space (B) illustrating how different diffusion lengths between the sensor and the peritoneal lining/mesothelium could affect the glucose dynamics of intraperitoneal glucose sensing. Assuming Fickian diffusion, we estimate the glucose diffusion time to be approximately 13 s and 12.5 min for 130  $\mu\text{m}$  and 1000  $\mu\text{m}$  diffusion distances, respectively.

glucose source (capillaries) considerably less than 1 mm, under the prerequisite of mass transport being dominated by diffusion. A time delay of 26 s corresponds to a diffusion distance of approximately 190  $\mu\text{m}$ .

The average IP lining area in adult humans is 1.5 m<sup>2</sup> [25], probably somewhat less for the pigs in our study. The large area of the peritoneal lining compared to the small volume of free IP-fluid justifies the assumption that diffusion is the dominant force on glucose transportation into the IP space. It is possible to assume that active convection would affect and give equally fast responses, but we do not know if such a mechanism was present in the conditions of our pig experiments. It is unlikely, however, that changes in convective fluid transport can explain the marked differences observed in time delays.

The outer diameter of the membrane was 216  $\mu\text{m}$ , and the diameter of the fibre and hydrogel was 125  $\mu\text{m}$  [15], resulting in an approximate distance from the membrane to the hydrogel of 45  $\mu\text{m}$ .

### Consequences of the hypothesis

Minimizing time delays and time constants in an AP might eliminate the need for patients to calculate and administer insulin meal boluses, achieving the aim of fully automatic glucose regulation.

Thus, if our hypothesis is confirmed, intraperitoneal glucose sensors should ideally measure glucose as close to the peritoneal lining as possible, or even in the capillary network immediately below the peritoneal lining, in the peritoneal lining itself or where glucose emerges from the lining but before it enters the peritoneal fluid. This can be achieved by choosing sensor technology that minimizes the distance between the peritoneal lining and the active sensor site (be it electrochemical, optical or any other sensing technology) and with

membranes facilitating rapid diffusion of glucose. The latter is a well-known fact that all sensor manufacturers likely strive to achieve, but the relative importance of a suitable membrane increases as the other parts of the dynamics become faster.

Optical sensor technology might enable glucose sensing in or just below the peritoneal lining instead of in the peritoneal fluid, using mid-infrared (MIR), near-infrared (NIR) or Raman spectroscopy [26]. Transdermal, non-invasive optical glucose sensing using NIR spectroscopy has shown promising results in pre-clinical trials, but no products have made it to commercialization. The IP space should provide a more suitable environment for this type of sensor technology as the peritoneal lining is much thinner than the dermis and thus the capillary network is closer to the organ surface and in theory more accessible for glucose measurements. Less tissue between the sensor and the sensing site of glucose in the capillary network should also reduce the effect of interfering substances making the glucose sensing more reliable. By measuring into the capillary network rather than in the peritoneal fluid, real-time sensing can also be achieved. By measuring glucose in the peritoneal lining or below, one also avoids the effect of temperature variations, that may have a substantial influence on the subdermal blood flow and the SC glucose delays. Other epithelial or mesothelial surfaces in the human body might also be feasible for glucose sensing, as the capillaries are more accessible with optical sensing technology at these surfaces compared to the skin. Potential locations include, but are not limited to, the nasal mucosa, pleural cavity or the epithelium in the ear channel. Sensing glucose on, in or just below the peritoneal lining, will standardize the measured glucose dynamics within the peritoneal cavity as the differences in diffusion lengths are minimized. Reducing the diffusion length with only 0.5 mm will reduce the diffusion time by several minutes. Fixation of the sensor might be needed to ensure



glucose sensing in the proper environment, but exactly how this fixation of the sensor element is to be done, is yet to be determined. A possible solution might be to apply negative pressure to the area around the sensor element to both fixate the sensor and move any surrounding IP fluid.

Minimizing time delays and time constants is also important in insulin dynamics. The slow glucose lowering effect after SC insulin delivery, even with fast acting insulins [27], is considered the greatest challenge to a subcutaneous AP system. Delivering insulin in the IP space provides a faster effect compared to SC delivery [28], and resembles the normal physiologic situation when pancreas secretes insulin into the portal vein [29–32]. By moving both the glucose sensing and hormone delivery of the AP into the IP space, it is possible to improve both glucose sensing and insulin dynamics.

## Conclusion

Research is still needed in the field of IP glucose sensing to determine glucose dynamics, the best location of the sensor and the optimal sensor technology. However, we hypothesize that measuring glucose directly on the surface or in the peritoneal lining, and not in the peritoneal fluid, is crucial to optimize glucose sensing for an IP artificial pancreas. This technological approach might hold the promise of near real-time glucose measurements which seem to be crucial to be able to achieve normal non-diabetic glucose levels by means of an AP in patients with DM1. Thereby long-term complications may be avoided, normal life expectancy established and adverse effect of DM1 on quality of life reversed.

## Funding

The Double Intra-peritoneal Artificial Pancreas project is part of Centre for Digital Life Norway and supported by the Research Council of Norway (grant number 248872). The study is also supported by a scholarship from the Central Norway Regional Health Authority (grant number 2014/23166). The funding sources had no role in collection, analysis or interpretation of the data.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.mehy.2019.109318>.

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## Erratum Paper II





Contents lists available at ScienceDirect

Medical Hypotheses

journal homepage: [www.elsevier.com/locate/mehy](http://www.elsevier.com/locate/mehy)



Erratum

## Erratum to “Why intraperitoneal glucose sensing is sometimes surprisingly rapid and sometimes slow: A hypothesis” [Med. Hypotheses 132 (2019) 109318]



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The publisher regrets that there is an error in one of the calculations of diffusion times for glucose in water on page 2. The correct equation is;

– For  $x = 10 \mu\text{m}$ ;  $\Rightarrow t_{25^\circ\text{C}, 10\mu\text{m}} \approx 0.075 \text{ s}$

The publisher would like to apologise for any inconvenience caused.

DOI of original article: <https://doi.org/10.1016/j.mehy.2019.109318>

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<https://doi.org/10.1016/j.mehy.2019.109411>

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## Paper III



# Intraperitoneal, subcutaneous and intravenous glucagon delivery and subsequent glucose response in rats: a randomized controlled crossover trial

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**To cite:** Dirnena-Fusini I, Åm MK, Fougner AL, *et al*. Intraperitoneal, subcutaneous and intravenous glucagon delivery and subsequent glucose response in rats: a randomized controlled crossover trial. *BMJ Open Diab Res Care* 2018;6:e000560. doi:10.1136/bmjdr-2018-000560

► Additional material is published online only. To view please visit the journal online (<http://dx.doi.org/10.1136/bmjdr-2018-000560>).

ID-F and MKÅm are joint first authors.

Data from this study were presented as an abstract and poster at the 11th International Conference on 'Advanced Technology and Treatment for Diabetes', February 14–17, 2018, Vienna, Austria.

Received 28 May 2018  
Revised 31 August 2018  
Accepted 6 October 2018



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## ABSTRACT

**Objective** Hypoglycemia is a frequent and potentially dangerous event among patients with diabetes mellitus type 1. Subcutaneous glucagon is an emergency treatment to counteract severe hypoglycemia. The effect of intraperitoneal glucagon delivery is sparsely studied. We performed a direct comparison of the blood glucose response following intraperitoneally, subcutaneously and intravenously administered glucagon.

**Research design and methods** This is a prospective, randomized, controlled, open-label, crossover trial in 20 octreotide-treated rats. Three interventions, 1 week apart, in a randomized order, were done in each rat. All 20 rats were given intraperitoneal and subcutaneous glucagon injections, from which 5 rats were given intravenous glucagon injections and 15 rats received placebo (intraperitoneal isotonic saline) injection. The dose of glucagon was 5 µg/kg body weight for all routes of administration. Blood glucose levels were measured before and until 60 min after the glucagon/placebo injections.

**Results** Compared with placebo-treated rats, a significant increase in blood glucose was observed 4 min after intraperitoneal glucagon administration ( $p=0.009$ ), whereas after subcutaneous and intravenous glucagon administration significant increases were seen after 8 min ( $p=0.002$  and  $p<0.001$ , respectively). In intraperitoneally treated compared with subcutaneously treated rats, the increase in blood glucose was higher after 4 min ( $p=0.019$ ) and lower after 40 min ( $p=0.005$ ) and 50 min ( $p=0.011$ ). The maximum glucose response occurred earlier after intraperitoneal compared with subcutaneous glucagon injection (25 min vs 35 min;  $p=0.003$ ).

**Conclusions** Glucagon administered intraperitoneally gives a faster glucose response compared with subcutaneously administered glucagon in rats. If repeatable in humans, the more rapid glucose response may be of importance in a dual-hormone artificial pancreas using the intraperitoneal route for administration of insulin and glucagon.

## INTRODUCTION

Patients with diabetes mellitus type 1 (DM1) are treated with either repeated or continuous subcutaneous delivery of insulin to counteract hyperglycemia. Improved glucose control

## Significance of this study

### What is already known about this subject?

- Glucagon is usually given subcutaneously in patients with diabetes mellitus type 1 to treat severe hypoglycemia.
- Glucagon has also been used in dual-hormone artificial pancreas with some improvement in glucose control.

### What are the new findings?

- Glucagon injected intraperitoneally gives a higher glucose response 4 min after administration and affects blood glucose for a shorter period compared with subcutaneous injection.

### How might these results change the focus of research or clinical practice?

- The present results should encourage research on the feasibility of combined intraperitoneal administration of insulin and glucagon as part of an artificial pancreas in humans.

is important, as chronic hyperglycemia may induce neuropathy, nephropathy, retinopathy, and cardiovascular diseases.<sup>1,2</sup> Achieving euglycemia is challenging due to slow absorption and delayed glucose-lowering effect of subcutaneously administered insulin. This makes it difficult to achieve optimal postprandial glucose control without the risk of subsequent hypoglycemia.<sup>3</sup> Repeated and frequent episodes of hypoglycemia are associated with impaired neuroendocrine counter-regulation and symptom perception and deterioration of cerebral functions and may lead to hypoglycemia unawareness.<sup>4</sup> Therefore, the central nervous system's adaptation to frequent short-term hypoglycemia may contribute to the increased incidence of severe hypoglycemia.<sup>5</sup> Despite many small improvements in the treatment of DM1 during the last decades,



hypoglycemia remains a challenge for many patients with DM1.<sup>6-8</sup>

Glucagon is used for treating severe hypoglycemia when patients with DM1 are unconscious and unable to consume carbohydrates. The standard treatment for adults is an intramuscular, intravenous or subcutaneous injection of 1 mg of glucagon. Whether this dose is optimal for all routes of administration has hardly been studied.<sup>9-10</sup> The glucose increasing effect depends on the dose of the injected glucagon,<sup>11</sup> amount of liver glycogen<sup>12</sup> and baseline blood glucose level.<sup>13</sup> When studied in healthy men, there seems to be no major difference in the glucose effect between intramuscular and subcutaneous administration.<sup>14-15</sup> Recently, smaller glucagon doses have been used with success to avoid mild or impending hypoglycemia in children and adults.<sup>6-11-16</sup> Glucagon used as an emergency nasal spray and nasal powder has been launched as an alternative route of administration, providing a success rate in treating hypoglycemia similar to intramuscular injections.<sup>17-19</sup>

Recent work on algorithm-steered insulin delivery (ie, artificial pancreas (AP)) provides improvements to glucose regulation. Unfortunately, this automatically controlled (closed loop) delivery of insulin carries certain limitations, as nearly all recent developments depend on a double subcutaneous approach, that is, both glucose measurements and insulin delivery are in the subcutaneous tissue. The limitations are due to slow subcutaneous glucose dynamics secondary to both delayed and slow subcutaneous insulin absorption, which unavoidably lead to alternating periods of either a lack or excess of circulating insulin.<sup>20</sup> To solve the challenge of relative insulin excess, some research groups have incorporated glucagon as a counter-regulator in the AP system, to counteract imminent hypoglycemia, that is, a dual hormonal AP.<sup>7-21-22</sup> Despite achieving as low as 3% of time in hypoglycemic range during day and night closed loop control, hypoglycemia still remains a substantial daytime problem also in this subcutaneous dual-hormone approach.<sup>23</sup> Therefore, new routes should be explored to find better solutions for prevention of hypoglycemia.

Intraperitoneal glucagon administration has only been reported from a few animal studies.<sup>24-25</sup>

The main aim of this study was to compare the glucose increasing effect after subcutaneous and intraperitoneal delivery of glucagon, and to investigate the potential for intraperitoneal delivery of small doses of glucagon in an AP. Intravenous delivery of hormones is less realistic in free-living conditions. Therefore, intravenous route was only included in the study as an additional route to obtain more information on glucose dynamics after glucagon delivery, and not included as a main outcome in the paper. We hypothesized that the glucose response is faster after intraperitoneal compared with subcutaneous administration of glucagon. To investigate this hypothesis, we compared the immediate glucose response after intraperitoneal, subcutaneous and intravenous administration of glucagon in an animal model.

## RESEARCH DESIGN AND METHODS

### Pilot study

A pilot study was performed on 10 rats to refine the experimental protocol and to determine the glucagon dose to be used in the main study. Detailed explanation is available in the online supplementary material.

### Animals

In the main study male Sprague Dawley rats (n=20) (initial weight 470–615 g; Janvier Labs, France), in groups of three, were kept in plastic solid bottom cages (515×381×256 mm, Tecniplast, Italy) on sawdust. The rats were acclimatized to the animal facility and maintained on 12-hour light–12-hour dark photoperiod at 20–24°C and a relative humidity of 55%±5%. They were fed expanded pellets (Special Diets Services RMI for rats, UK) and fresh water was available ad libitum. To reduce stress and the possible effect of stress on glucose levels, the rats were trained to accept general handling and use of a restrainer (Harvard Apparatus, Holliston, USA) for 3 weeks prior to the start of experiments.

### Intervention groups and randomization

The assignment to intervention groups (n=20) and the order of procedures in each rat were randomized by creating random permutations of treatment and intervention groups. The glucagon dose was 5 µg/kg body weight (BW) for all interventions except placebo. All rats (n=20) received intraperitoneal and subcutaneous injection of glucagon, 15 of the rats received placebo intraperitoneal injections of 1 mL/kg BW of isotonic saline. The volume of placebo injection (1 mL/kg BW) was similar to the intraperitoneal glucagon injection (approximately 500 µL). To obtain information also after intravenous delivery of glucagon, five of the rats were administered intravenous glucagon (see online supplementary tables 2a and b). There was at least 1 week between each test procedure on each rat. To avoid bias based on metabolic individualities, trials were performed in the 12-hour light period, and all procedures in each individual rat were done at approximately the same time as of the light cycle. Group size was determined by the resource equation method.<sup>26</sup>

### Technical challenges

The rats were monitored for the entire sampling period (70 min) and surveyed for signs of stress. Except when the rats were anesthetized, they were kept in restrainers to facilitate blood sampling. Restrainers of two different sizes were tested before the start of the experiment. For most of the rats, the restrainers were either too large or too small. Thus, the larger restrainer was used for all rats, and a paper tissue was rolled up and taped vertically to the inside of the restrainer at a level behind the rat's shoulder, to prevent the smaller rats from turning around inside the restrainer.

### Procedures

Food was removed 1 hour before the start of the procedure and water was available ad libitum. The individual

glucagon and octreotide doses were based on the animal's weight on the day of the procedure.

#### Endogenous glucagon secretion

To suppress the endogenous glucagon and insulin secretion during the procedures, all rats received two subcutaneous injections of 10 µg/kg BW octreotide (Sandostatin 200 µg/mL, Novartis Europharm, UK). The first injection was given approximately 30 min before the start of each procedure and the second at the time of glucagon/placebo injection. Octreotide was given subcutaneously in the neck, but not at the same location as the subcutaneous glucagon injection.

#### Anesthesia

To prevent accidental movements in the time of the procedure, the rats were anesthetized with isoflurane (Isoflurane, Baxter, Oslo, Norway; 5% IF, 95% air in chamber; 2% IF, 95% air on face mask) for two short intervals at the start of each procedure. During the first anesthesia period, a cut in the tail for collection of blood samples was made. During the second anesthesia period, an injection of glucagon or placebo was given. When required, additional anesthesia was provided to rats showing signs of stress while kept in the restrainer.

#### Glucagon challenge

Glucagon (Glucagon, Novo Nordisk, Denmark) was diluted by 0.9% NaCl to a concentration of 5 µg/mL and the rats were given 5 µg/kg BW. Glucagon solutions were kept in a refrigerator and used the same day they were made. Solutions were warmed to approximately body temperature just before administration. Subcutaneous glucagon was injected at the back of the neck, and intraperitoneal glucagon and placebo (an equal volume of 0.9% NaCl) in the lower part of the abdomen, with the rat held at an angle after its hind legs. Intravenous glucagon was given in the lateral tail vein that was not currently used for blood sampling.

#### Glucose measurement

After disinfecting the skin, a 6–9 mm cut was made with a straight-edged scalpel over the lateral tail vein two-thirds down the length of the tail for blood sampling. Samples were collected 10, 5 and 1 min prior to glucagon injection, and 2, 4, 6, 8, 10, 15, 20, 25, 30, 40, 50, and 60 min after the glucagon or placebo injections. Whenever needed, the vein was carefully reopened with the tip of the scalpel to ensure sufficient blood flow for sampling.

Blood for glucose analyses was collected directly in heparinized capillary tubes (35 µL, Clinitubes, Radiometer Medical ApS, Brønshøj, Denmark), and stored on ice for a maximum of 30 min before analysis on a blood gas analyzer (Radiometer ABL 725, Radiometer Medical ApS). To ensure sufficient blood flow for sampling, the vein was gently stroked from the base of the tail and toward the wound, and the first small drop of blood was removed. For the third intervention, both veins had been used for sampling at former trials, and the new cut was

made proximal to the older cut. Occlusion of the rat's tail vein occurred in only one rat, and in this case the vein on the other side of the tail was used.

#### Animal welfare

The rats were given non-steroidal anti-inflammatory drugs (Metacam vet, Boehringer Ingelheim Vetmedica) 1 mg/kg BW as a single subcutaneous injection at the end of the two first procedures. A suture, to close the wound and stop the bleeding at the end of the procedure, was necessary in 19 cases. The wounds healed well after sampling regardless of the wound being sutured or not, and no wound infections were observed. After the third procedure, the rats were euthanized with an intravenous injection of pentobarbital (100 mg/kg) (Norges Apotekerforening, Norway) under isoflurane anesthesia.

#### Statistical analysis

The relationship between glucose levels and time was analyzed for all interventions using a mixed linear model with the combination of time and treatment as the fixed effect. The dependent variable was defined as log glucose concentration to achieve normal distribution. To account for multiple measurement series on each rat, rat identification was included as a random effect. To account for dependence within each series, the error term for each series was specified as a first-order autoregressive process AR (1) series accounting for minutes between measurements. Mean changes in glucose concentrations from –1 min to 2–60 min for the four treatments were compared using the Wald test. Maximum concentration and time until maximum concentration of the estimated model for the treatments were compared using the Mann-Whitney U test. To eliminate the effect of placebo intervention on the glucose response, the mean value of the 15 placebo interventions was subtracted from the mean value of the 20 subcutaneous and intraperitoneal interventions and the mean value of the 5 intravenous interventions at the given time points. All interventions are compared as models; therefore, comparison between unequal groups is allowed.<sup>27</sup> The software package R was used to analyze the data.<sup>28</sup> All values in the text are given as mean±SE of the mean, unless stated otherwise. Differences between the group means were considered statistically significant at a threshold of  $p \leq 0.05$ .

#### RESULTS

In general, the rats stayed calm during the experimental procedures. Thirteen incidents occurred during 60 procedures, in which the rats turned around inside the restrainers or showed signs of stress and consequently were taken out of the restrainer and repositioned. A similar number of incidences were found in all interventions (four during the intraperitoneal, four during the subcutaneous and three during the intravenous intervention). These incidents included two rats in whom stress was observed during three procedures (intraperitoneal, subcutaneous and intravenous). Two incidents of stress

were observed during the placebo procedure; however, no increase in blood glucose levels from baseline was observed (data not shown).

Additional anesthesia during blood sampling was needed during 10 intraperitoneal, 10 subcutaneous, 5 intravenous, and 5 placebo interventions, and the mean±SD time in anesthesia was 13.8±5.5 min (14.05±5.47, 13.55±4.37, 20±9.43 and 12±4.85, respectively). The rats were conscious for the rest of the 70 min procedure. After individually analyzing data from the 16 rats which received the longest duration of anesthesia (time in anesthesia 15–31 min, subcutaneous n=5, intraperitoneal n=5, placebo n=3 and intravenous n=3), only two rats (subcutaneous intervention, n=2) showed prolonged elevated glucose levels and no decrease of glucose values at the end of the intervention (at 60 min) (data not shown).

#### Glucose level

For calculation of glycemic state for rats at the beginning of the interventions, a mean baseline glucose was calculated according to the mean of three measurements preceding the intervention (–10, –5 and –1 min), and in addition a mean±SD in each intervention group was calculated. Blood glucose levels at the beginning of the intraperitoneal, subcutaneous, intravenous and placebo interventions were 6.72±0.90, 6.47±0.81, 6.17±1.12 and 6.51±0.81 mmol/L, respectively (see online supplementary figure 1).

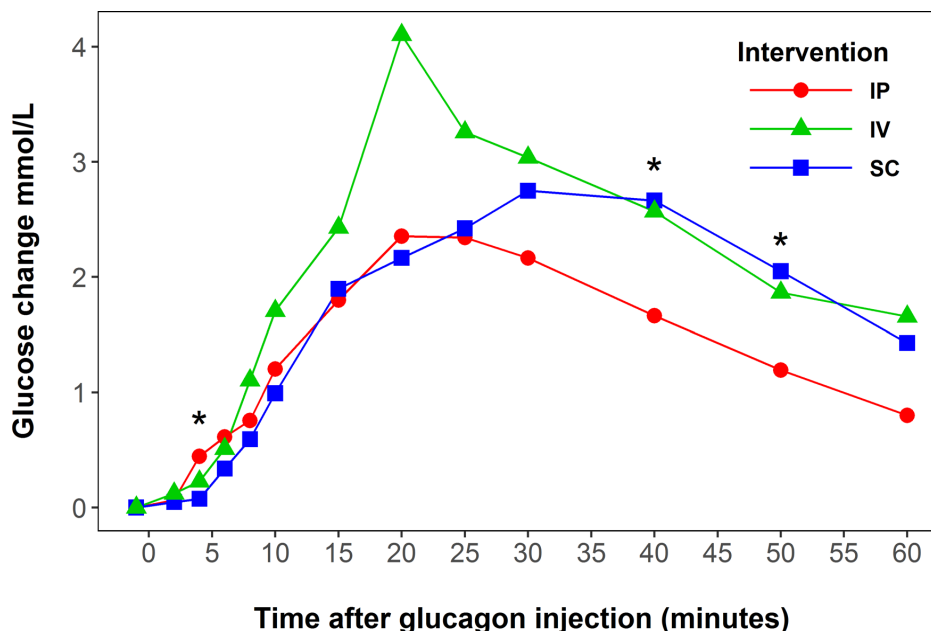
Compared with placebo, glucose was significantly increased 4 min after intraperitoneal glucagon injection ( $p=0.009$ ,  $n=20$ ), and 8 min after subcutaneous ( $p=0.002$ ,  $n=20$ ) and intravenous ( $p<0.001$ ,  $n=5$ ) injections (figure 1).

Comparing intraperitoneal glucagon injections with subcutaneous, the glucose increase after intraperitoneal glucagon was significantly higher at 4 min ( $p=0.019$ ) and significantly lower at 40 ( $p=0.005$ ) and 50 min ( $p=0.011$ ) (figure 1). Comparing intravenous glucagon injections with intraperitoneal, the glucose increase after intravenous injection was significantly higher at 20 min ( $p=0.001$ ). At the other time points, no significant differences were observed.

An increase in glucose levels was seen after all three routes of glucagon delivery, but there was no significant difference ( $p=0.52$ ) in absolute maximum blood glucose value after intraperitoneal glucagon injection (9.74 mmol/L) compared with subcutaneous injection (10.3 mmol/L). The estimated time until the maximum glucose value was significantly shorter ( $p=0.003$ ) after intraperitoneal glucagon injection (25 min) versus subcutaneous glucagon injection (35 min) (see online supplementary figure 1).

#### DISCUSSION

The results of this study indicate that the glucose response in rats comes earlier when glucagon is injected



**Figure 1** Estimated glucose delta values (mmol/L) after glucagon injection (5 µg/kg) in octreotide-treated rats. Green line represents intravenous (IV) intervention, blue line represents subcutaneous (SC) intervention, and red line represents intraperitoneal (IP) intervention. The glucose response for the placebo group has been subtracted from all groups presented in the figure. \*Represents significant difference between intraperitoneal and subcutaneous of glucagon delivery. Note: For one rat in intraperitoneal intervention, delta values were calculated using the –5 minute measurement (–1 minute for other rats).

intraperitoneally than when injected subcutaneously. Second, the maximum effect of glucose increase appears earlier, and the glucose response diminishes faster after intraperitoneal compared with subcutaneous glucagon injection.

The peritoneal lining is highly vascularized, and the blood capillary density in the peritoneal lining varies between individuals (higher amount in infants (0–1 year) and adults, and lower amount in children).<sup>29</sup> There is no systematic variation in histological parameters in different parts of the peritoneum.<sup>29,30</sup> Compared with subcutaneous absorption, peritoneal absorption may be faster due to a shorter distance to reach the capillaries and easier diffusion into the bloodstream. From animal studies, we know that most of the intraperitoneally injected insulin enters the portal vein and passes the liver before entering the systemic circulation.<sup>31–33</sup> Consequently, after intraperitoneal delivery, glucagon probably reaches the liver both earlier and at a higher concentration and thereby promotes hepatic gluconeogenesis earlier as compared with subcutaneously injected glucagon. This is compatible with our observation of faster glucose increase after intraperitoneal injection of glucagon compared with other injection routes. It also fits well with the observed earlier maximum glucose response after intraperitoneally injected compared with subcutaneously injected glucagon. Interestingly, time until maximum blood glucose increase after subcutaneous injection in our animal model is similar to what is observed in humans with diabetes.<sup>11</sup>

Our finding of an earlier rise in blood glucose after intraperitoneally injected glucagon, compared with subcutaneous injection, is difficult to compare with previous studies as blood glucose was measured at different time points and intervals. In previous studies, blood glucose was only measured 20<sup>24</sup> and 30<sup>25</sup> min after glucagon injection. Moreover, the study by Zlotnik *et al*<sup>25</sup> provided only data of intraperitoneal glucagon injection. In the present study, blood glucose was lower after intraperitoneal compared with subcutaneous injection of glucagon at time points 40 and 50 min. Fifty minutes after glucagon injection, a declining blood glucose was observed in all routes (intraperitoneal, subcutaneous and intravenous) of administration. This differs from a previous study in rats,<sup>25</sup> where after intraperitoneal injections of glucagon a significant rise in glucose levels was observed after 30, 60, 90 and 120 min. However, after 60–90 min, a flattening of the blood glucose curve was observed and at 120 min blood glucose subsequently decreased. This discrepancy between the previous and the present results may depend on the fact that in the previous study, rats were anesthetized during the whole procedure with isoflurane,<sup>25</sup> while in the present study we limited isoflurane use as much as possible (see online supplementary material). We also treated the rats with octreotide to inhibit endogenous release of insulin and glucagon during the experiments. Interestingly, in the previous study no difference in plasma glucose was

observed in the control group, while in our pilot study, with extended use of isoflurane, we observed an increase in glucose levels (see online supplementary material). A glucose increasing effect of isoflurane has been described previously.<sup>34</sup>

Loxham *et al*<sup>24</sup> demonstrated results similar to our study, where, after intraperitoneal injection of glucagon in non-diabetic rats, the glucose response after intraperitoneal administration was higher after 20 min and lower after 45 min compared with subcutaneous administration. Baseline glucose levels were also similar to ours. However, the authors did not mention whether anesthetics were used.<sup>24</sup> Noteworthy, Loxham *et al*<sup>24</sup> suggested that different strains of rats may react differently to a sudden rise in counter-regulators (in this case glucagon), making comparison between different strains of rats difficult.

Our glucagon dose of 5 µg/kg BW was only 2.5% of the dose used by Loxham *et al*<sup>24</sup> (200 µg/kg) and only 1% of the dose used by Zlotnik *et al*<sup>25</sup> (50 µg/100 g). We do not have information about why these particular doses were chosen. Another aspect is that previous authors used naïve rats, whereas the rats in the present study were treated with octreotide.<sup>35</sup> Our study provides information about possible doses of glucagon with which glucagon saturation is reached (see online supplementary material). It seems that in our study a glucagon dose of 5 µg/kg BW was appropriate (see online supplementary material) based on the observed blood glucose increase of around 3 mmol/L in all injection routes.

Our study is not the first to explore the effect of smaller doses of glucagon. The effect of smaller doses than the standard 1 mg of glucagon (commonly used in cases of serious hypoglycemia) has recently been examined in humans.<sup>6,11</sup> Glucagon may induce nausea and vomiting, and these side effects may be related to the size of the injected dose and the subsequent higher levels of glucagon in the systemic circulation. In patients with DM1 there seems to be a dose–response relationship between subcutaneous glucagon doses ranging from 0.11 mg to 1.0 mg and the glucose response.<sup>11</sup> Mini-doses of glucagon are effective in treating mild to moderate hypoglycemic episodes in both children<sup>6,36</sup> as well as adults.<sup>16</sup>

The motivation for performing this study was to investigate and compare different administration routes of glucagon and explore if glucagon administration intraperitoneally would provide some benefits compared with subcutaneous injection, aspects of importance for the development of a dual hormonal AP. Ideally, a dual-hormone AP should prevent hypoglycemia with small and, if necessary, repeated doses of glucagon.<sup>7,37</sup> Minimizing the amount of exogenous glucagon needed to counteract hypoglycemia is important to avoid the depletion of liver glycogen, to reduce the side effects, such as nausea and vomiting, and to avoid reactive hyperglycemia.<sup>11</sup>

A small dip in glucose values was observed prior to the injection of glucagon or saline, that is, during all procedures (see online supplementary figure 1). The reason

for this is unclear, but the equal response during all procedures is an indication of consistency of the experimental protocol for all the procedures during the first part of the experiment and thereby a sign of quality of the present study.

### Strengths and limitations of the study

The following are the strengths of the present study: (1) placebo-treated animals—the importance is illustrated by the fact that, although not significantly, the glucose levels tended to fluctuate also during the placebo procedures; (2) limited use of anesthesia, which may have major impact on glucose homeostasis; (3) systematic training of the rats to the procedures for weeks ahead of the test procedures (both pilot rats and trial rats) to minimize the stress response during the test procedures; and (4) randomization of the order of treatment in each rat.

The following are among the limitations of the present study: (1) With unguided injections into the abdominal cavity, we cannot be sure that all the glucagon or placebo was administered in the peritoneal space. However, glucagon and placebo saline were injected by the same procedure in anesthetized rats (injection could be done without experiencing unexpected movements of rat); therefore, possible deviation from intraperitoneal delivery should be equal between groups. (2) A few rats were stressed during the procedures, which might affect the blood glucose levels. However, in rats receiving placebo intervention, under signs of stress, the blood glucose level did not increase significantly (see online supplementary material). (3) Additional anesthesia was needed for some rats in all interventions; however, as it was described in the results, prolonged increased glucose levels were observed only in 2 out of the 16 rats which were exposed to the longest duration of anesthesia in the main study. Therefore glucose level increase at the end of the intervention can be individual response, not anesthesia-induced. (4) Rats were fasted differently (between 1 and 3 hours) depending on the order of performing the procedure (intraperitoneal, subcutaneous, intravenous or placebo). However, to avoid bias, all experiments on the same rat were conducted at approximately the same time as of the light cycle.

### CONCLUSION

Blood glucose increased faster when glucagon was injected in the peritoneal cavity compared with subcutaneous glucagon delivery in octreotide-treated rats. The maximum glucose response was reached earlier and the decline in glucose response was also faster. If repeatable in humans, a more rapid glucose response may be of importance in a dual-hormone AP using the intraperitoneal route for administration of insulin and glucagon.

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**Acknowledgements** The animal experiments were conducted at the Comparative Medicine Core Facility (CoMed), Norwegian University of Science and Technology (NTNU). CoMed is funded by the Faculty of Medicine and Health Sciences at NTNU and the Central Norway Regional Health Authority. We thank Associate Professor Øyvind Salvesen, Faculty of Medicine and Health Sciences, NTNU, for help with the statistical analysis, and Gita Nagarajah for her work on the glucose analysis.

**Contributors** ID-F and MKÅ completed the trial, collected and analyzed the data, wrote and edited the manuscript, and are the guarantors of the work. ALF, SMC and SCC contributed to the discussion, and reviewed and edited the manuscript. All authors contributed to the development of the protocol.

**Funding** The Norwegian Research Council (NRC) is funding the Double Intraperitoneal Artificial Pancreas project (project number 248872) as part of the Centre for Digital Life Norway (digitallifnorway.org). The study is also supported by a scholarship from the Central Norway Regional Health Authority (grant nr 2014/23166) and the Norwegian Medical Association Johan Selmer Kvanes Endowment. The funding sources had no role in the collection, analysis or interpretation of the data.

**Competing interests** None declared.

**Patient consent** Not required.

**Ethics approval** The study was approved by the Norwegian Food Safety Authority (FOTS-ID 10922) and was in accordance with 'The Norwegian Regulation on Animal Experimentation' and 'Directive 2010/63/EU on the protection of animals used for scientific purposes'.

**Provenance and peer review** Not commissioned; externally peer reviewed.

**Data sharing statement** Additional online data supplement is available.

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Paper IV





## **Intraperitoneal and subcutaneous glucagon delivery in anaesthetized pigs: Effects on circulating glucagon and glucose levels**

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Keywords: glucagon, intraperitoneal, subcutaneous, glucose, diabetes, pigs

## **ABSTRACT**

### **Background**

Glucagon has, in recent years, been incorporated in the dual hormone artificial pancreas, a device to automatically and continuously control the blood glucose levels of individuals with diabetes. The subcutaneous dual hormone artificial pancreases, however, has yet to fulfil its expectations of providing reliable and stable blood glucose levels. For this reason, the intraperitoneal space is investigated as an alternative location for an artificial pancreas.

### **Methods**

Glucose dynamics after subcutaneous and intraperitoneal glucagon delivery in ten anaesthetized pigs were investigated. The pigs received intraperitoneal boluses of 0.3 µg/kg and 0.6 µg/kg and a subcutaneous bolus of 0.6 µg/kg in randomized order. A bolus of 1 mg was delivered intraperitoneally at the end of the experiments to test the remaining capacity for rapid glucose production. Frequent blood samples were analysed for glucose and glucagon.

### **Results**

Six pigs responded with glucose elevations greater than 1 mmol/L after the 1 mg bolus and were included in further analysis. The intraperitoneal glucagon bolus of 0.6 µg/kg gave a significantly higher glucose response from 14 to 30 minutes compared with the 0.6 µg/kg subcutaneous bolus ( $p < 0.05$ ). The highest glucose value was observed around 20 minutes, regardless of the route of delivery.

### **Conclusions**

The results indicate that intraperitoneal glucagon delivery induces a larger glucose response compared with subcutaneous glucagon delivery. This might be explained by absorption of glucagon over the peritoneal lining and direct delivery to the liver and is encouraging for the incorporation of glucagon in an intraperitoneal artificial pancreas.

## **INTRODUCTION**

An artificial pancreas (AP) is a device that automatically and continuously regulates insulin delivery to control the blood glucose level (BGL) in people with diabetes, primarily patients with diabetes mellitus type 1 (DM1). The AP system holds great promise for relieving the patients of the potentially serious side-effects of the disease and the everyday burden of diabetes management <sup>1,2</sup>. Incorporating glucagon in the AP system, i.e. a dual hormone AP, might enable tighter glucose control <sup>3</sup>. However, the full potential of the AP is yet to be realized mainly due to the markedly delayed glucose

lowering response of subcutaneous (SC) delivered insulin. The intraperitoneal (IP) space is, for that reason, investigated as an alternative location for hormone delivery in an AP.

At present, glucagon is mainly used as an emergency treatment to counteract severe, insulin-induced hypoglycaemia and is injected either SC or intramuscularly (IM). The prescribed dose of 1 mg is large and has a single goal of promptly elevating the BGL. In an AP, however, the aim of glucagon delivery is not primarily to treat hypoglycaemia but to prevent imminent hypoglycaemia by small, and possibly repeated, doses. Mini-doses of SC glucagon are efficient in treating milder hypoglycaemia in children as well as in adults, and the glucose rising effect is the same regardless of hypoglycaemic or euglycaemic BGL<sup>4</sup>. Studies have shown that repeated mini-doses of glucagon did not deplete the liver of glycogen and that the glucose increasing effect was similar regardless of previous boluses<sup>4,5</sup>. This supports the use of glucagon in a dual hormone AP.

Both single hormone (insulin only) and dual hormonal (insulin and glucagon) AP systems show benefits compared with insulin treatment by multiple daily injections or by a pump, especially during night-time<sup>2,6</sup>. Dual hormone AP shows further improvement compared with single hormone AP as it shows less time spent in the hypoglycaemic range and prevention of hypoglycaemic episodes, especially in relation to exercise<sup>3</sup>. In addition, dual hormone AP seems to reduce carbohydrate intake as carbohydrates are often needed to correct mild hypoglycaemia<sup>3</sup>. Although the AP systems show improvement for patients, a fully automatic AP system is yet to be developed, as the only commercially available system still requires users to manage meal insulin boluses<sup>7,8</sup>. The slow dynamics of the SC tissue is probably the main obstacle that prevents these systems from being fully automated<sup>9</sup>. Moving the hormone delivery of an AP into the peritoneal space, can lead to faster absorption and improved glycaemic control, which has been shown for IP insulin delivery<sup>10-12</sup>. A large portion of IP injected insulin is transported by the portal vein directly to the liver, mimicking the normal physiological “first-pass effect”<sup>12,13</sup>. The faster and more appropriate insulin absorption is the main advantage of the IP AP system and adding glucagon as a second hormone in the IP AP system is only a minor adjustment and of interest even if the glucose response after IP delivery would be similar to that of SC delivery.

IP glucagon delivery has only been investigated in a few animal studies<sup>14-17</sup>, and if glucagon is to be incorporated in a dual hormone IP AP, the kinetics of glucagon absorption and effect on BGLs needs to be more closely identified. We have previously studied the glucose response after IP glucagon delivery in rats and found a faster effect compared with SC delivery<sup>17</sup>. The aim of the present study was to explore the glucose dynamics after IP delivery of glucagon and compare it to SC delivery in a pig model. We also wanted to investigate if the faster glucose response seen in the rat study could be repeated in a pig model.

## **MATERIALS AND METHODS**

### **Ethical approval**

The animal experiments were approved by the Norwegian Food Safety Authority (FOTS number 12948) and were in accordance with «The Norwegian Regulation on Animal Experimentation» and «Directive 2010/63/EU on the protection of animals used for scientific purposes».

### **Animals and animal handling**

Between February and June 2018, eleven female, juvenile, non-diabetic farm pigs (*Sus scrofa*), weighing 31-44 kg, were brought to the animal research facility approximately one week before experiments and acclimated to the staff and new environment. They were housed together in a common pen, in groups of two or three whenever possible, provided wood chips as nesting material and toys to keep them occupied. The lighting condition was standardized with a 16 hours light period followed by an 8 hours dark period. They were fed standard commercial growth feed twice a day and provided water ad libitum. Food was removed nine-ten hours prior to the experiments.

### **Anaesthesia**

The pigs were premedicated with an IM injection of 4 mg diazepam (Stesolid®, Actavis Group, Hafnarfjordur, Iceland), 160 mg azaperone (Stresnil®, Eli Lilly Regional Operations GmbH, Austria) and 750 mg ketamine (Ketalar®, Pfizer AS, Norway), while in the pen. An ear vein was cannulated, and anaesthesia was induced with an intravenous (IV) injection of 1 mg atropine (Takeda AS, Asker, Norway), 150-250 µg fentanyl (Actavis Group, Hafnarfjordur, Iceland), 75-125 mg thiopental (VUAB Pharma AS, Rostoky, Czech Republic) and 150-250 mg ketamine (Ketalar®, Pfizer AS, Norway).

The pigs were intubated in the lateral position and mechanically ventilated and monitored on an anaesthesia machine (Aisys, GE Healthcare Technologies, Oslo). Anaesthesia was maintained by IV infusion of midazolam (0.5 mg/kg/h) (Accord Healthcare Limited, Middlesex, UK) and fentanyl (7.5 µg/kg/h) (Actavis Group, Hafnarfjordur, Iceland) and by inhalation of isoflurane (0-2%) (Baxter AS, Oslo, Norway). The room temperature was around 20 degrees Celsius. The body temperature of the pigs was monitored, and a heating blanket applied when necessary.

The pigs received IV infusions of antibiotics (Cefalotin, Villerton Invest SA, Luxembourg), 2 g immediately after the pigs were anaesthetized and 1 g after 4 hours. Heparin (150 IE) (LEO Pharma A/S, Ballerup, Denmark) was injected in the peritoneal space at the same time points.

Fluid balance was achieved by continuous IV infusion of Ringer's acetate, approximately 1000 ml during the length of the experiment, with individual adjustments.

### **Surgical procedure**

An intra-arterial line was placed in the left carotid artery for blood sampling and monitoring of physiological parameters and an IV line was placed in the left internal jugular vein for fluid infusions. Both catheters were inserted through the same cut-down.

The catheter from an Animas Vibe insulin pump (serial number 80-26400-16, Animas Corp., West Chester, PA, USA) was inserted 10 -15 cm into the upper left part of the abdomen through a 2-3 cm long craniocaudal incision in the abdominal wall, 2-3 cm caudally to the umbilicus. The bladder was exposed through a small, low laparotomy for the insertion of a bladder catheter. Both cuts were made with a thermocauter to minimize bleeding.

At the end of the experiments, and under full anaesthesia, the pigs were euthanised with an IV overdose of pentobarbital (minimum 100 mg/kg) (pentobarbital NAF, Apotek, Lørenskog, Norway).

### **Suppression of endogenous glucagon secretion**

To inhibit endogenous insulin and glucagon secretion, the pigs were given IV injections of 0.4 mg octreotide (Sandostatin 200µg/ml, Novartis Europharm Limited, United Kingdom) every hour and SC injections of 0.3 mg pasireotide (Signifor 0.3mg/ml, Novartis Europharm Limited, United Kingdom) every third hour. To verify glycaemic stability, three blood samples were collected within the last 20 minutes before the first injection of somatostatin analogues. Suppression efficiency was evaluated by analysing glucagon and insulin levels for 30 minutes after somatostatin analogue injection (every 10 minutes). Blood samples were also analysed for porcine insulin at intervals throughout the experiments.

The effectiveness of the somatostatin analogues for the suppression of glucagon and insulin secretion is evaluated in the last paragraph of the Results section.

### **Glucagon boluses**

Glucagon (Novo Nordisk, Denmark) was mixed according to specification and placed in the pump. The volume of one unit by the pump was measured to 10 µl, and 1 unit was equivalent to 10 µg of glucagon. The glucagon in the pump was stored at room temperature for the length of the one-day experiments. Glucagon for the SC dose and the last 1 mg IP dose were mixed just before administration.

One pig was used to define the doses of glucagon to be used in the experimental protocol and not included in the analysis. We aimed at boluses which would raise the BGL with one to three mmol/L and chose to investigate three different IP doses of glucagon (0.3 µg/kg, 0.6 µg/kg and 1 mg) and one SC dose (0.6 µg/kg). The 0.3 µg/kg IP, 0.6 µg/kg IP and 0.6 µg/kg SC doses were delivered in a randomized order while the 1 mg IP bolus was given as the last bolus to test maximum glucose effect of IP glucagon

delivery. Two pigs, which did not show any post glucagon elevations in BGL, received a 1 mg IV dose at the end of experiments.

### **Glucose analysis**

Arterial blood samples for glucose analysis were collected every two to 15 minutes throughout the length of experiments, with the highest frequency from 15 before to 80 minutes after glucagon boluses. Samples were collected in heparinized syringes (LEO Pharma A/S, Ballerup, Denmark) and analysed on a Radiometer ABL 725 blood gas analyser (Radiometer Medical ApS, Brønshøj, Denmark). Most samples were analysed immediately, but some samples were stored on ice for a maximum 20 minutes before analysis.

### **Glucagon and porcine insulin analysis**

Three arterial blood samples within the last 20 minutes before glucagon boluses were analysed for glucagon and insulin in all pigs. Arterial blood samples, with the same intervals as glucose samples described above, were also collected after nine IP boluses and four SC boluses. Arterial blood was collected in empty syringes and immediately transferred to EDTA vacutainers (2 ml). The samples were stored in ice-water for 10 minutes before they were centrifugated and the plasma transferred to Eppendorfs and stored at -80°C until analysis. Glucagon was analysed with Glucagon ELISA (10-1281-01 Mercodia, Uppsala, Sweden) and porcine insulin was analysed with Porcine Insulin ELISA (10-1200-01, Mercodia, Uppsala, Sweden). The assay ranges for the glucagon and porcine insulin ELISA kits were 2-172 pmol/L and 2.3 – 173 mU/L, respectively.

To compare the porcine insulin results to former analysis, we chose to convert from mU/L to pmol/L using the conversion protocol provided by Mercodia using their internal porcine calibration solution. Procedure as follows:

$$(1) \frac{value [mU/L]}{115} \times 174 = value [pmol/L]$$

All glucagon samples were run in singles with a coefficient of variability (CV) <10%. Inter-assay CV were 8%, 8% and 6% for 42.6, 14.7 and 4.98 pmol/L standards, respectively.

### **Statistical analysis**

The relationship between BGL and time was analysed for all interventions using a mixed linear model with bolus order and the combination of time and treatments as the fixed effects. The dependent variable was defined as log BGL to achieve normal distribution. To account for multiple measurement series on each pig, pig identification was included as a random effect<sup>18</sup>. To account for dependence within each series, the error term for each series was specified as a first-order autoregressive process (AR (1)) series accounting for minutes between measurements. Estimated mean changes in BGL from time points 0 to 80 minutes for the 0.6 µg/kg IP, 0.3 µg/kg IP and 0.6 µg/kg SC boluses

and time points 0 to 20 minutes for the 1 mg IP bolus were compared using the Wald test. The area under the curve (AUC) from minute 0 to 80 and time until maximum BG change for the different boluses were compared using the Mann-Whitney U test. If the maximum value for BG change was observed for more than one time point, the first occurring time point was selected. The level of significance was set to 0.05. All statistical analyses were carried out in R <sup>19</sup>.

## RESULTS

Ten pigs received at least four glucagon boluses, except one pig which only received two boluses due to circumstances unrelated to the experiment.

### Glucose response

No apparent elevation in BGL was observed in four of nine 0.3 µg/kg IP boluses, three of ten 0.6 µg/kg IP boluses, one of ten 0.6 µg/kg SC boluses, and four of nine 1 mg IP boluses. Neither of the two IV boluses elevated the BGL. Figures and tables in the article are therefore presenting the pigs which showed a glucose response of at least 1 mmol/L after the last 1 mg IP dose (n=6). Results from all pigs (n=10) are presented as online supplementary materials.

The blood glucose (BG) changes for the different boluses are displayed in Figure 1. The IP glucagon boluses gave a faster glucose elevation compared with SC glucagon delivery and there were significant differences between the 0.6 µg/kg IP bolus and the 0.6 µg/kg SC bolus at the time points between 14 and 30 minutes ( $p < 0.05$ ) (Figure 1a). The 1 mg IP bolus gave significantly higher glucose elevations compared with all other boluses from 4 minutes to 20 minutes ( $p < 0.05$ ). We found no statistically significant differences comparing the 0.3 µg/kg IP and the 0.6 µg/kg SC bolus. When including all ten pigs in the analyses, we found no statistically significant differences in BG elevations between any of the different boluses for any of the different time points (online supplementary Figure 1). No statistically significant differences in AUC or time to maximum glucose response were found when comparing the different boluses (table 1 and online supplementary Table 1).



Table 1: Pharmacodynamic data of IP and SC glucagon boluses.

	Glucose			Glucagon			
	n <sup>†</sup>	CΔ <sub>max</sub> ± SD (mmol/l)	T <sub>max</sub> ± SD (min)	AUC <sub>0-80 min</sub> ± SD (mmol/l x min)	n <sup>†</sup>	C <sub>max</sub> ± SD (pmol/L)	T <sub>max</sub> ± SD (min)
0.6 µg/kg IP	6	2.4 ± 1.0	22.2 ± 4.5	688.1 ± 132.7	5	66.0 ± 62.0	13.3 ± 7.2
0.6 µg/kg SC	6	1.6 ± 0.9	19.3 ± 5.7	638.2 ± 101.5	3	23.3 ± 4.6	6.0 ± 2.0
0.3 µg/kg IP	5	2.2 ± 1.7	20.0 ± 7.4	625.8 ± 157.2	4	27.3 ± 19.7	38.5 ± 29.6

**Abbreviations:** CΔ<sub>max</sub>, maximum plasma concentration change; C<sub>max</sub>, maximum plasma concentration; T<sub>max</sub>, time to maximum plasma concentration. Data are arithmetic means ± standard deviations (SD).

(†) Samples for glucagon and insulin analysis were not collected from all boluses. For that reason, the numbers of included boluses differ between glucose and glucagon results.

Figure 2 displays the experiments on two different pigs illustrating the difference in glucose responsiveness. Figure 2a shows pig no. 10, which responded with BG increase after all four glucagon boluses. The glucose rise was approximately 2.2 and 3.7 mmol/L after the IP boluses and 1.3 mmol/L after the SC bolus. Figure 2b shows pig no. 11, which responded to the first glucagon bolus with a BG increase of 1.5 mmol/L but did not show apparent glucose responses to the last three boluses despite large measured glucagon levels in the circulation. Pig no. 11 also received an IV glucagon bolus at the end of the experiment without any detectable glucose response.

#### Glucagon absorption

An elevation in peripheral blood glucagon concentration was detected after 13 of 18 IP boluses and all five SC boluses from which blood samples were available for analysis, however only 14 of these doses resulted in glucose elevations larger than 1 mmol/L. Results from the included pigs are presented in Figure 1 and the results from all pigs are presented in online supplementary table 1. Mean maximum glucagon levels and mean time to maximum value for the different boluses for the included pigs are displayed in table 1. Glucose and glucagon curves from two experiments are shown in Figure 2. The maximum detection limit for glucagon values was 170 pmol/L and was reached in both pig 10 and 11 (Figures 2a and 2b).

### **Endogenous hormone production**

Endogenous insulin was detected in five of the six included pigs. The maximum concentration detected in these individual pigs were 5.3, 8.9, 11.5, 17.1, and 20.1 pmol/L. Of the four excluded pigs, only one pig had detectable levels of endogenous insulin with a maximum concentration of 6.8 pmol/L. Figure 2 presents the results obtained from two individual pigs throughout the one-day experiments. The mean  $\pm$  SD glucagon concentration before somatostatin analogue treatment was  $15.66 \pm 22.59$  pmol/L compared with  $8.38 \pm 8.45$  pmol/L after somatostatin analogue treatment.

### **DISCUSSION**

This study indicates that IP delivery of glucagon induces a larger glucose response after 14 to 30 minutes compared with SC delivery of the same glucagon dose (0.6  $\mu$ g/kg) in somatostatin analogue treated pigs. This indicates a faster effect by the IP route compared with the SC route. However, the time of maximal BG response is observed around 20 minutes regardless of the dose or route of delivery (Table 1 and online supplementary table 1). This is in line with previously published results for SC delivery (15-20 min) <sup>4,20,21</sup>.

In individuals with diabetes, glucagon is primarily used to treat serious hypoglycaemia and given as SC or IM injection. IP glucagon kinetics and effect have only been studied in a few animal experiments <sup>14-17</sup>. All but one study measured glucose only at a few time points, showing that IP glucagon delivery increase BGL but not documenting the full glucose response. We have previously conducted a study in rats with IP glucagon delivery and frequent blood sampling. That study showed that the glucose response after IP delivery was significantly higher after 4 minutes compared with SC delivery, indicating a faster glucose response after IP delivered glucagon <sup>17</sup>. The glucose elevation was also of shorter duration after IP delivery of glucagon. In the present study, we found a significantly larger glucose increase from 14 to 30 minutes after IP glucagon delivery, but we did not observe an earlier glucose peak, or a shorter duration of the glucose response compared with SC delivery of glucagon.

The peritoneal lining consists of a monolayer of mesothelial cells <sup>22</sup> that covers a layer of connective tissue containing several different cell types, blood and lymph vessels <sup>23</sup>. The peritoneal lining is permeable to water, small solutes, and proteins <sup>24</sup> and a portion of the glucagon will, after IP delivery, diffuse through the visceral peritoneum and be transported directly to the liver via the portal vein. This route of drug delivery has been described for insulin showing a fast and efficient absorption to the portal vein and a fast effect on the BGL <sup>25,26</sup>. We hypothesize that glucagon delivered by the IP route reaches the liver earlier and at a high concentration and induces a fast and large glucose response because the IP route resembles the normal glucagon secretion,

i.e. directly into the portal vein. This fits with our observation of a large glucose increasing effect even with the smallest IP dose (0.3 µg/kg), while hardly any increase in circulating glucagon levels was observed (Figure 2 a). Similar glucose elevations were also observed after the 0.6 µg/kg and 1 mg IP boluses but with concomitant significant increased levels of glucagon in the systemic circulation. The concentration of glucagon reaching the liver after SC administration will be equivalent to what is reaching the rest of the body, and high peripheral concentrations of glucagon can lead to unwanted side effects, such as nausea, vomiting, and increased heart rate.

In an AP system, glucagon will primarily be administered to avoid, not treat hypoglycaemia. Hence, we focused on glucagon doses that would elevate the BGL by one to three mmol/L. Small SC doses of glucagon to treat mild hypoglycaemia have been studied before in both children<sup>27,28</sup> and adults<sup>20,29,30</sup> with DM1 and in streptozotocin treated pigs<sup>31</sup>. Small doses of glucagon have also been studied in the context of an SC AP system<sup>4,5,32,33</sup>. There is no common definition of a “mini-dose” of glucagon or a consensus of optimal glucose response. Hence, the study protocols vary making comparisons between available data challenging. There is a dose-response relationship between glucagon and glucose response after SC delivery, with larger doses of glucagon resulting in higher glucose elevations<sup>4,20,32</sup>. However, this dose-response relation seems only to apply to smaller doses of glucagon and increasing doses over a certain level will not result in higher glucose responses<sup>4,20,34</sup>. This is in line with our results where the glucose elevations after the 1 mg IP bolus was only 0.5 mmol/L larger than the glucose elevation after the 0.3 µg/kg IP, even though the former dose is 80 times greater for a pig of 40 kg. It is acknowledged that glucagon binds to liver cells causing a non-linear increase of intracellular cAMP, where the half-maximum effect is reached already when 10 % of the receptors are bound<sup>35</sup>.

We observed a larger variation in blood glucagon concentrations and glucose responses after IP delivery compared with SC delivery of glucagon. This is in line with previously published results<sup>14,17</sup> and might be related to varying amounts of IP fluid. We also observed large variation in glucose responses both between pigs and between boluses in the same pig regardless of route of delivery. This might, to some extent, be explained by differences in available liver glycogen. Some pigs responded poorly or not at all to glucagon, even though glucagon was detected in peripheral blood (Figure 2b). El Khatib et al. conducted a study on streptozotocin treated pigs which were fasted for 20 hours before receiving a single dose of SC glucagon, but in contrast to our study the pigs had elevated fasting BGLs and received an initial insulin bolus and consequently a drop in BGLs<sup>31</sup>. Prolonged fasting has been shown to reduce the amount of available liver glycogen in humans after an overnight fast<sup>5</sup> and in pigs before slaughter<sup>36</sup>. Repeated glucagon boluses have not shown any statistically significant depletion of liver glycogen or less effect of the final glucagon boluses in people with DM1<sup>4,5</sup>. Contrasting results, however, were observed in healthy volunteers where the second

glucagon dose showed a marked lower effect compared with the first of two repeated glucagon doses of 0.5 mg<sup>37</sup>. In the present study we observed no statistically significant differences in glucose response comparing the different bolus orders. However, Figure 2b shows a pig responding to the first bolus but not to the later ones even though high levels of glucagon were detected in plasma. This lack of glucose response suggests depletion of hepatic glycogen stores available for fast glucose production by glycogenolysis in some of the pigs, and a possible explanation is prolonged fasting and repeated glucagon boluses. Hepatic glycogen depletion has been considered a potential, but not crucial, barrier for a successful dual hormone AP system in human trials<sup>33,38</sup>, and is a factor to be considered when studying glucagon dynamics using animal models.

The strength of the present study is frequent blood sampling and identification of the glucagon and glucose dynamics during and after IP glucagon boluses, which enables the development of algorithms for an IP dual hormone AP system. The study has, however, also several limitations. Even with repeated glucagon boluses in each pig the sample size of ten pigs is small. The absent glucose increasing effect in four pigs reduced the number of valid pigs in the study even further.

Experiments under anaesthesia will inevitably affect the pig's physiology and influence the data. Prolonged IV infusion of fluids can lead to increased production of IP fluid in pigs<sup>39,40</sup>. The IP space can potentially hold several liters, while under normal conditions it contains only small amounts of IP fluid<sup>41-44</sup>. Delivering glucagon into large volumes of IP fluid would dilute the glucagon and slow down the absorption and effect. We restricted the amount of IV fluid during the experiments to minimize the accumulation of IP fluid and observed only a moderate increase in the volume. Still, we believe that increased amounts of IP fluid may have influenced the results and expect accumulation of peritoneal fluid to be a lesser problem during long-term, free-living settings, both in animals and in humans.

The IP catheter was placed in the upper left quadrant of the abdomen. As the tip of the catheter was free-moving, the exact site for glucagon delivery is unknown, but this approach is equivalent to the current solution for IP insulin delivery by Accu-Check DiaPort (Roche). The absorption of glucagon over the peritoneal lining might differ throughout the IP space. We are not aware of any data related to this topic.

We measured small amounts of insulin in some of the pigs, indicating that endogenous insulin secretion was not completely suppressed. However, the amounts detected are small compared with insulin values of naive pigs undergoing a glucose tolerance test<sup>45</sup>. In the present study somatostatin analogues seemed to suppress endogenous glucagon secretion, as the concentration of glucagon was lowered after the initial somatostatin analogue administration. We observed some fluctuations in the

measured glucagon level within the same time series, which we cannot explain (e.g. example in Figure 2a).

In conclusion, glucagon gives a larger rise in BGL when delivered IP compared with SC in somatostatin analogue treated pigs under prolonged anaesthesia. These results imply that smaller doses of glucagon can be given IP with the same effect, potentially avoiding unwanted side effects of glucagon treatment.

#### **ACKNOWLEDGMENTS**

The animal experiments were performed at the Comparative Medicine Core Facility (CoMed) at Norwegian University of Science and Technology (NTNU). We would like to thank Oddveig Lyng especially, for her invaluable contribution to the animal experiments. We also thank Associate Professor Øyvind Salvesen for his help with the statistical analysis. We are very grateful to Tone Dypdalsbakk for conducting the ELISA analysis. The Double Intraperitoneal Artificial Pancreas project is part of the Centre for Digital Life Norway and supported by the Research Council of Norway (grant number 248872). The study is also supported by a scholarship from the Central Norway Regional Health Authority (grant number 2014/23166) and Norwegian Medical Association Johan Selmer Kvanes Endowment. The funding sources had no role in the collection, analysis or interpretation of the data.

#### **COMPETING INTERESTS**

None declared.

#### **PRIOR PRESENTATION**

Preliminary data were presented as an abstract and poster at the 12<sup>th</sup> International conference on “Advanced Technologies and Treatments for Diabetes” 20-23 February 2019, Berlin, Germany.

#### **ETHICAL APPROVAL OF RESEARCH**

The study was approved by the Norwegian Food Safety Authority (FOTS-ID 12948) and was in accordance with «The Norwegian Regulation on Animal Experimentation» and «Directive 2010/63/EU on the protection of animals used for scientific purposes».

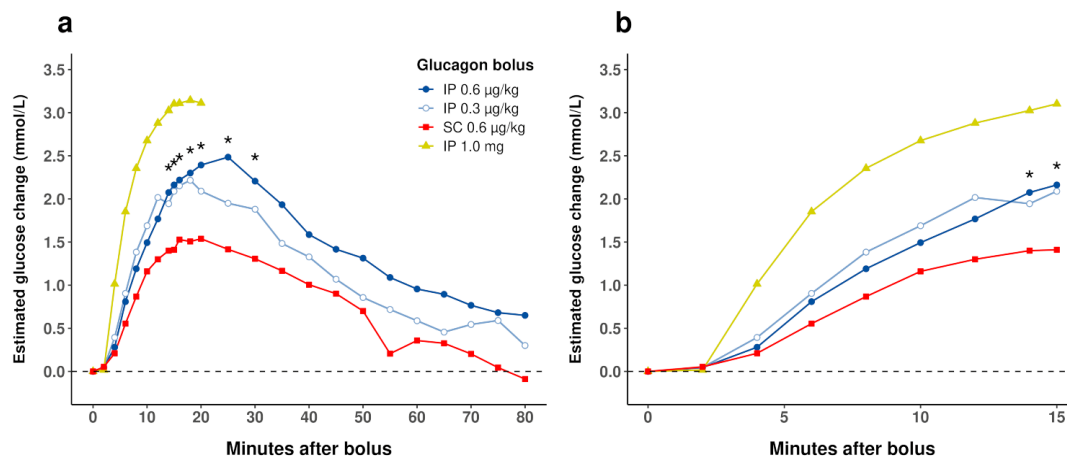
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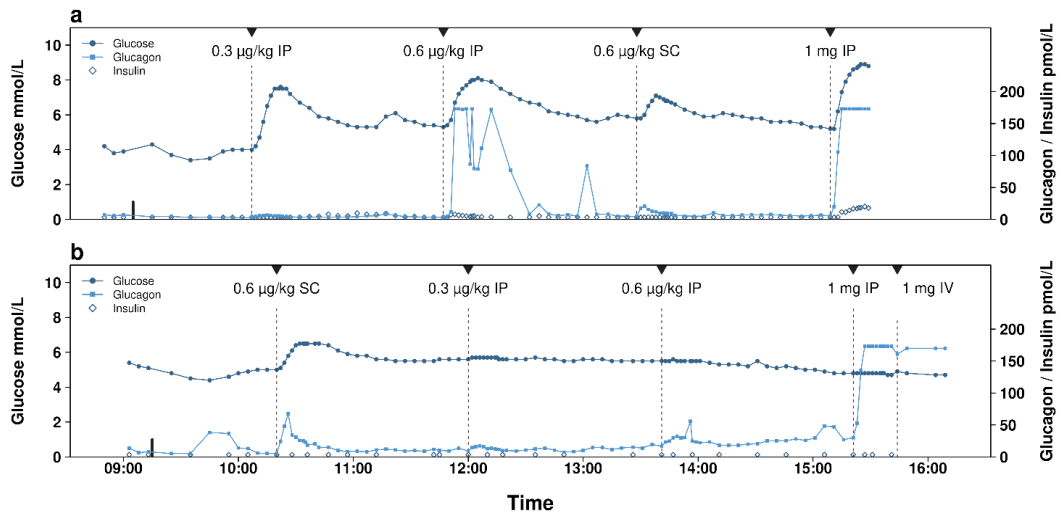
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**Figure 1:** Estimated glucose changes for the full 80 minutes (a) and for the first 15 minutes (b) after glucagon delivery in pigs which responded with a BG rise after the 1 mg IP bolus. The 0.6 µg/kg IP bolus gave significantly higher glucose elevations compared with the 0.6 µg/kg SC bolus from time points 14 to 30 minutes (marked with \*). 1 mg IP bolus gave significantly higher glucose elevations compared with all other boluses from 4 to 20 minutes (not marked in the graph). Data from the IV bolus are not presented in this graph.



**Figure 2:** Glucose, insulin, and glucagon profiles from two full experiments. Glucagon boluses are marked with triangles and vertical dashed lines. The top graph shows pig no. 10, which responded with a rise in BG values after every glucagon bolus (a), while the bottom graph shows pig no. 11, which only responded to the first glucagon bolus although high levels of glucagon were detected in peripheral blood after all boluses (b). Only the first octreotide and pasireotide treatments are marked as vertical solid bars around 9 o'clock.

