

# Glucagon treatment in type 1 diabetes

-with focus on restoring plasma glucose during mild hypoglycemia

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<b>IOB</b>	Insulin on board
<b>I.v.</b>	Intravenous
<b>MDI</b>	Multiple daily injections
<b>Min</b>	Minutes
<b>PG</b>	Plasma glucose
<b>SAP</b>	Sensor augmented insulin pump
<b>S.c.</b>	Subcutaneous
<b>SD</b>	Standard deviation
<b>SEM</b>	Standard error of the mean
<b>SMBG</b>	Self-monitored blood glucose
<b>tAUC</b>	Total area under the curve
<b>T<sub>max</sub></b>	Time to maximal concentration
<b>TDD</b>	Total daily dose

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## THE 4 ORIGINAL PAPERS ARE:

- [1] Ranjan A, Schmidt S, Madsbad S, Holst JJ, Nørgaard K. Effects of subcutaneous, low-dose glucagon on insulin-induced mild hypoglycaemia in patients with insulin pump treated type 1 diabetes. *Diabetes Obes Metab.* 2016 Apr;18(4):410-8.
- [2] Ranjan A, Wendt SL, Schmidt S, Madsbad S, Holst JJ, Madsen H, Knudsen CB, Jørgensen JB, Nørgaard K. Relationship between Optimum Low-doses of Glucagon and Insulin Levels when Treating Mild Hypoglycaemia in Patients with Type 1 Diabetes - A Simulation Study. *Basic & Clinical Pharmacology & Toxicology* (Accepted Sept. 6th 2017)
- [3] Ranjan A, Schmidt S, Damm-Frydenberg C, Steineck I, Clausen TR, Holst JJ, Madsbad S, Nørgaard K. Low-Carbohydrate Diet Impairs the Effect of Glucagon in the Treatment of Insulin-Induced Mild Hypoglycemia: A Randomized Crossover Study. *Diabetes Care.* 2017 Jan;40(1):132-135
- [4] Ranjan A, Nørgaard K, Tetzschner R, Steineck I, Clausen TR, Holst JJ, Madsbad S, Schmidt S. Effects of Preceding Ethanol Intake on Glucose Response to Low-Dose Glucagon in Individuals With Type 1 Diabetes: A Randomized, Placebo-Controlled, Crossover Study. *Diabetes Care* 2018;41:1–10

## LIST OF ABBREVIATIONS

<b>AUC</b>	Area under the curve
<b>CGM</b>	Continuous glucose monitor
<b>CSII</b>	Continuous subcutaneous insulin infusion
<b>C<sub>max</sub></b>	Maximal concentration
<b>DCCT</b>	Diabetes Control and Complications Trial
<b>EDIC</b>	Epidemiology of Diabetes Interventions and Complications
<b>EGP</b>	Endogenous glucose production
<b>FPG</b>	Fasting plasma glucose
<b>GIR</b>	Glucose infusion rate
<b>GLP-1</b>	Glucagon-like peptide 1
<b>HbA1c</b>	Glycated hemoglobin A1c
<b>IAH</b>	Impaired awareness of hypoglycemia
<b>I.m.</b>	Intramuscular

## 1. INTRODUCTION

Since the discovery of insulin by Banting and Best in 1921, research in type 1 diabetes has mainly focused on optimizing insulin therapy and reducing late diabetes complications. There is no doubt that insulin treatment has extended and improved the quality of life for individuals with type 1 diabetes over the last several decades; and that the side effects involved with insulin treatment are preferable to the alternatives of diabetes complications and early death. However, only a minority achieves the treatment goal of near-normal glucose levels. Despite the recent advantages in insulin regimens with faster-acting insulin, insulin pumps, real-time continuous glucose monitors and hybrid closed-loop systems, the risk of hypoglycemia remains the key limiting factor in achieving optimal glycemic control with an insulin-only approach.

Hypoglycemia in type 1 diabetes is often caused by inappropriately high levels of insulin, and sometimes, in combination with failure of the counter-regulatory defense system to restore plasma glucose levels. One key counter-regulatory hormone is glucagon which is mainly secreted by the pancreatic  $\alpha$ -cells, and increases plasma glucose levels by stimulating glucose production in the liver. After many years in an “insulinocentric” era, the interest of glucagon in relation to type 1 diabetes has increased, with a focus on mainly two research areas. On one hand, research is focused on suppressing the paradoxically high glucagon levels observed in type 1 diabetes, as well as to improve the blunted glucagon response to hypoglycemia. On the other hand, research also focuses on using low-dose glucagon as an add-on to insulin therapy in order to avoid and treat hypoglycemia in type 1 diabetes, referred to as the “dual-hormone approach”. It remains unclear whether a suppression of endogenous glucagon levels, an addition of low-dose glucagon to prevent hypoglycemia, or both are required to optimize glucose control in type 1 diabetes. Nonetheless, targeting glucagon levels may therefore play a higher role in diabetes management than expected. Therefore more research is needed regarding the efficacy, safety and feasibility of glucagon therapeutics in individuals with type 1 diabetes.

This PhD thesis investigated the short-term effects and limitations of low-dose glucagon in the treatment of insulin-induced mild hypoglycemia in individuals with type 1 diabetes.

## 2. SCIENTIFIC BACKGROUND

### 2.1. Type 1 diabetes

Type 1 diabetes is caused by an autoimmune destruction of the insulin-producing  $\beta$ -cells in the pancreas [5], leading to a condition with insulin deficiency and hyperglycemia. The pathogenesis of type 1 diabetes is not fully understood and several factors may play a role. Furthermore, no curable treatment or prevention of the disease exists, and type 1 diabetes remains a chronic condition [6]. The discovery of insulin significantly changed the prognosis of type 1 diabetes from being a fatal condition with certain death within 2-3 years after diagnosis to a treatable chronic condition with an almost similar life-expectancy as healthy individuals [7,8]. However, the extended lifetime did not come without cost, and diabetes management has focused on reducing the late diabetes complications, in forms of nephropathy, neuropathy, retinopathy and cardiovascular disease.

As a result of the landmark study, the Diabetes Control and Complications Trial (DCCT), the late diabetes complications were found to be related to glycemic control in type 1 diabetes [9]. The DCCT proved that an intensive insulin therapy aiming for near-normal glucose levels could delay the development and progression of the microvascular diabetes complications; while its follow-up study, Epidemiology of Diabetes Interventions and Complications (EDIC), proved that it could also reduce the risk of cardiovascular disease - compared with former conventional insulin therapies with one or two insulin injections daily [9,10]. The beneficial effects of 6.5 years of the intensified therapy persisted for another 30 years, termed as the "legacy effect" [11]. Hence, individuals with type 1 diabetes are recommended to aim for near-normal plasma glucose levels with a glycated hemoglobin A1c (HbA1c) level below 7.0% (53 mmol/mol), a preprandial capillary plasma glucose level between 4.4 mmol/l and 7.2 mmol/l, and a postprandial capillary plasma glucose level below 10.0 mmol/l [12,13]. The benefits of the intensified therapy, however, came at the expense of an approximately 3-fold increased risk of severe hypoglycemia - causing severe discomfort, seizures, coma, or in worst cases death [14,15]. Even though DCCT showed that there was an inverse relationship between HbA1c and risk of hypoglycemia, later observational studies could not all replicate this [16,17]. Thus, risk of hypoglycemia may be independent of HbA1c, and rather be related to short-term glucose fluctuations [18]. The fear of hypoglycemia keeps most individuals with type 1 diabetes from using ideal insulin doses to achieve tight glycemic control. This in combination with relentless surveillance of the glucose level, comprises a substantial psychological burden in everyday life [19,20]. Thus, hypoglycemia appears to be the main limiting factor for achieving optimal glycemic control and preventing late diabetes complications, as well as alleviating disease burden for the individuals with type 1 diabetes [20,21].

### 2.2. Hypoglycemia

In healthy individuals, plasma glucose levels are tightly controlled by pancreatic  $\beta$ -cell secretion of insulin and  $\alpha$ -cell secretion of glucagon. Thus, a decrease in plasma glucose levels results in a decrease in insulin secretion and an increase in glucagon secretion - and vice-versa when plasma glucose levels are in-

creased [22]. There is a physiological hierarchy for preventing hypoglycemia and restoring plasma glucose levels (counter-regulation) that involves: 1) an decrease in insulin secretion, 2) an increase in glucagon secretion, and 3) an increase in sympathoadrenal catecholamine secretion [23]. Cortisol, growth hormone and FFA also participate in the process of counter-regulation during hypoglycemia [24,25]. The sympathoadrenal system is activated if endogenous glucagon and insulin responses fail to restore glucose levels [26]. Thus, healthy individuals do not experience hypoglycemia unless the counter-regulation is disrupted.

In type 1 diabetes, insulin deficiency impairs the relationship between the pancreatic  $\alpha$ -cell and  $\beta$ -cell, leading to rising glucagon levels as well as a blunted glucagon response to hypoglycemia [27,28]. The role of fasting and postprandial hyperglucagonemia is still highly debated and is beyond the scope of this thesis [27,29]. The blunted glucagon response to hypoglycemia, in combination with an impaired sympathoadrenal response, results in an impaired counter-regulation in type 1 diabetes. Notably, hypoglycemia in type 1 diabetes is always related to the use of exogenous insulin and results from a mismatch between insulin dose and insulin requirements, e.g. during and after exercise and in relation to a differing size of meals and irregular meal times. Hypoglycemia is defined as plasma glucose levels below 3.9 mmol/l, while severe hypoglycemia is a condition characterized by plasma glucose levels below 3.9 mmol/l in combination with cognitive impairment that requires external assistance for recovery [12,30]. Clinically significant hypoglycemia has recently been defined by the International Hypoglycaemia Study Group as glucose concentrations  $< 3.0$  mmol/L or  $< 2.8$  mmol/l detected by self-monitoring of blood glucose (SMBG) or continuous glucose monitoring (CGM) [30,31].

Around 20% of the population with type 1 diabetes is unable to recognize the symptoms of clinical hypoglycemia - a condition referred to as "impaired awareness of hypoglycemia" (IAH). It has been related to age, diabetes duration, recurrent hypoglycemia and strict glycemic control [32]. Importantly, IAH is associated with an increased risk of severe hypoglycemia, due to the loss of ability to perceive and act quickly on hypoglycemia symptoms, as well as a loss of effective counter-regulatory response [33]. A

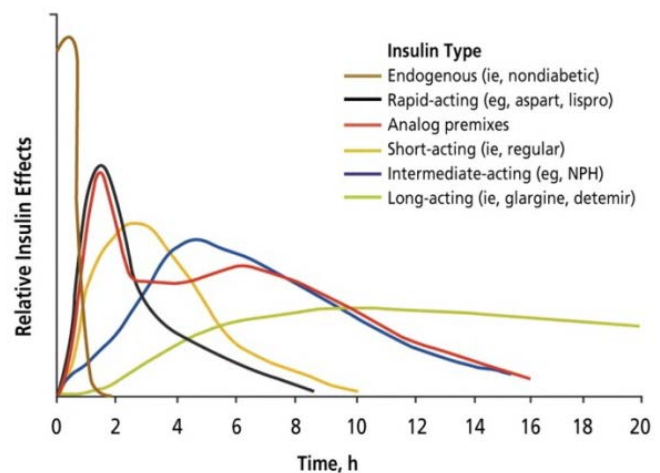


Figure 1: Illustration of insulin action profile for the endogenous and exogenous insulin. Graph extracted from Freeman JF (29).

vicious cycle may thus occur with recurrent hypoglycemia events leading to IAH that further increases the risk of hypoglycemia. Meticulous avoidance of mild hypoglycemia events has, however,

proven to partly restore awareness; leading to fewer severe hypoglycemic events, even though counter-regulation persists to be impaired [34,35].

Thus, hypoglycemia in type 1 diabetes is a combined result of iatrogenic hyperinsulinemia and defective counter-regulation of decreasing plasma glucose levels. Iatrogenic hyperinsulinemia could be prevented if the insulin dosing matched the secretion and clearance of endogenous insulin as seen in healthy individuals. However, even the most rapid-acting insulin analogues administered subcutaneously cannot match the kinetics of the physiological  $\beta$ -cell secreted insulin [36] (Figure 1). Other routes such as intraperitoneal insulin delivery may better match the kinetics, but are associated with infections at the administration site [37,38]. Compared with peripheral insulin delivery, intraperitoneal or intraportal insulin delivery may, however, effectively inhibit hepatic glucose production without increasing the glucose uptake in peripheral tissues (mainly muscles), and thereby prevent hyperglycemia without the risk of hypoglycemia [39,40]. The difference of peripheral glucose uptake between the routes of insulin delivery may partly explain why hypoglycemia occurs in type 1 diabetes [41,42]. Therefore, future insulin strategies should either focus on delivering insulin into the portal system or use liver-specific insulin analogues [43].

In addition to pharmacological progress with respect to fast-acting insulin analogues, several technological advances have been developed in attempt to mimic the physiological relationship of insulin and glucose, i.e. insulin pens (MDI) and bolus calculators, insulin pumps (CSII), real-time continuous glucose monitors (CGM), and automatic insulin delivery based on glucose levels (closed-loop systems). Even though the risk of hypoglycemia has been reduced with these technological advances, hypoglycemia will still occur and remains to be the limiting factor for tight glycemic control in type 1 diabetes. Importantly, hypoglycemia may be associated with increased cardiovascular mortality and morbidity [44–46].

### 2.3. Emerging anti-hypoglycemic therapeutics

Alternative adjunct therapies and technological solutions have been tested with the purpose of improving glycemic control and reducing the risk of hypoglycemia in type 1 diabetes. On the technological side, the use of continuous glucose monitors (CGM), the continuous subcutaneous insulin infusions (CSII) and bolus calculators has demonstrated improvement in glycemic control without an increase in events of hypoglycemia [47–51]. Further improvements were achieved when the next-generation CSII were upgraded with control algorithm integrating CGM values - referred to as sensor-augmented insulin pumps (SAP) [52]. The SAPs have further been developed to automatically suspend insulin infusion rates in response to low or predicted low CGM values. Thereby they are, respectively, capable of restoring (low glucose suspension) or preventing hypoglycemia (predicted low glucose suspension). Nonetheless, manual adjustments of insulin delivery are still required with these modalities to keep near-normal glucose levels. This manual control of glucose is termed an “open-loop system” as opposed to the automatic control of glucose that is a “closed-loop system”. Recent achievements with the optimized glucose control algorithm in combination with an increased CGM accuracy have paved the way to develop a closed-loop system - referred to as the “external artificial pancreas” [53]. The closed-loop system automatically infuses insulin s.c. in response to CGM values, trying to mimic the healthy pancreatic  $\beta$ -cell secretion of insulin [54]. The safety and feasibility of these

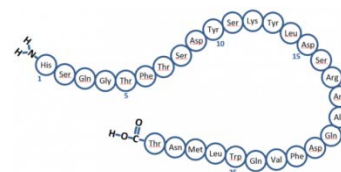
closed-loop systems have been demonstrated in outpatient settings - showing improvements in short-term glucose control and quality of life [55,56]. Furthermore, some studies show that the insulin-only closed-loop system may be outperformed by the dual-hormone closed-loop system with additional delivery of glucagon and/or amylin [57,58]. Even though closed-loop systems may alleviate the burden of diabetes management and improve glucose control, long-term effects are not known, and several shortcomings (including risk of hypoglycemia) still exist [59].

Regarding pharmacological approaches, drugs such as glucagon-like peptide 1, sodium-glucose co-transporter inhibitors, sulfonylureas, amylin and metformin have been investigated as potential adjuncts to the intensified insulin therapy in type 1 diabetes. Unfortunately, no clear clinical benefits have been seen with these drugs that outweigh their cost and side effects, nor have they succeeded in reducing the risk of hypoglycemia [60].

To summarize, no emerging therapeutics sufficiently improve glycemic control and at the same time fully prevent hypoglycemia risk. New treatment options are therefore needed in order to overcome these obstacles without adding further burden to diabetes management.

### 2.4. Glucagon

Soon after the discovery of insulin, researchers wondered why the first crude insulin extracts caused a brief hyperglycemic response prior to a decrease in plasma glucose [61]. The hyperglycemic response was initially misinterpreted as artifacts until Kimball and Murlin, in 1923, were able to isolate a fraction of the pancreas extract that caused an increase in plasma glucose [62]. The fraction was named “hyperglycaemic-glycogenolytic factor” or “the glucose agonist”, later abbreviated to glucagon [63]. Nevertheless, glucagon was first successfully crystallized 30 years after its discovery, and the 29-amino-acid polypeptide sequence of the hormone could be determined (Figure 2).



**Figure 2: The 29-amino-acid sequence of glucagon.**  
Source: [www.diapedia.org](http://www.diapedia.org) (46).

The healthy pancreatic  $\alpha$ -cell secretes glucagon in response to protein-rich meals, prolonged fasting, exercise and hypoglycemia [64]. Glucagon is primarily known to be a counter-regulatory hormone to insulin that stimulates glycogen breakdown (glycogenolysis) in the liver; leading to an increase in plasma glucose levels. Glucagon therefore soon became a potential drug in the treatment of severe hypoglycemia [65,66]. Outside hospital settings, intramuscular (i.m.) or s.c. 1000  $\mu$ g glucagon is the first treatment of choice for severe hypoglycemia [67]. Studies have shown that the ability of glucagon to raise plasma glucose is independent of the route of administration (i.e. intravenous (i.v.), i.m or s.c.), and that the success rate for glucose recovery was comparable to that achieved with a 25 g i.v. glucose bolus [68,69]. However in one study, only 41 of 100 patients with diabetes (type 1 and type 2) responded sufficiently to glucagon at the emergency department, while the remaining patients needed additional i.v. glucose to recover from severe hypoglycemia [70]. In contrast to that study, subsequent studies showed fewer non-responders to glucagon, but it was pointed out that glucagon under certain conditions may be ineffective when liver glycogen stores are

Name, company	Formulation	Reconstitution	Dose, mg	PG <sub>max</sub> , mmol/l	T <sub>max</sub> , min	Clinical Phase
<b>Glucagon, Eli Lilly &amp; co. [85]</b>	Recombinant DNA, Native	Manual reconstitution	1.0	9.44±0.29	43.6±4.2	Commercially available
<b>GlucaGen, Novo Nordisk [85]</b>	Recombinant DNA, Native	Manual reconstitution	1.0	9.73±0.33	34.6±3.2	Commercially available
<b>G-pen, Xeris Pharmaceutical [86]</b>	Dimethyl sulfoxide (DMSO)	No need for reconstitution	1.0	Mean±SD 8.22±1.38	Mean±SD 48.2±11.8 <sup>1</sup>	Phase III
<b>Dasiglucagon, Zealand Pharma [87]</b>	Peptide analog	No need for reconstitution	1.0	Mean±SD 11.61±2.22	Median(range) 150 (100-250)	Phase III
<b>SAR438544, Sanofi [88]</b>	Peptide analog	No need for reconstitution	0.15	7.21±0.50	69.0±13.2	Phase I
<b>BIODEL-961, Albiro Pharma [85]</b>	Lyophilized, Native	Automatic reconstitution	1.0	9.90±0.41	41.7±4.1	Phase II
<b>Intranasal glucagon, Locemia/Eli Lilly &amp; co. [89,90]</b>	Phospholipid +cyclodextrin	No need for reconstitution	3.0	Mean±SD 9.89±1.50	Median(range) 60 (30-90) <sup>2</sup>	Phase III

**Table 1: Available and emerging glucagon formulations for severe hypoglycemia.** Data presented as mean±SEM, if not otherwise stated. PG<sub>max</sub>: Peak plasma glucose. T<sub>max</sub>: Time to peak. <sup>1</sup>Data extracted from clinical trial.gov: NCT02081014. <sup>2</sup>Data extracted from clinical trial.gov: NCT01997411

depleted e.g. starvation, prolonged exercise, and alcohol abuse [71,72].

Besides using glucagon to treat severe hypoglycemia, Haymond and Schreiner proposed the off-label use of low-dose glucagon in treating mild hypoglycemia [73,74]. This regimen was initially used in type 1 diabetes children with gastroenteritis and inability to consume carbohydrates. Herein, all episodes of mild and impending hypoglycemia were completely managed by low-dose glucagon at home. Since these findings were published, there has been increasing awareness regarding the use of smaller doses of glucagon as a treatment option for mild hypoglycemia in individuals with type 1 diabetes regardless of age and concurrent illness [22,75]. The research focus has been on finding an optimal regimen for low-dose glucagon therapy and on making it feasible in daily-life outpatient conditions.

In contrast to other non-insulin adjunct therapies, low-dose glucagon was found to reduce events of mild hypoglycemia in type 1 diabetes. This has primarily been demonstrated in closed-loop studies in which the dual-hormone system with automatic insulin and glucagon delivery improved glucose control and hypoglycemia rates compared with the insulin-only system [57,76–78]. Similar results may be achievable in open-loop settings with low-dose glucagon as an alternative to oral fast-acting carbohydrates in treating mild and impending hypoglycemia [22,79]. As for now, no long-term studies have investigated the effects of the dual-hormone approach in an outpatient setting with regards to glucose control and risk of hypoglycemia in adults with type 1 diabetes.

Despite potential benefits, there may be limitations of using glucagon to treat mild hypoglycemia [80]. This PhD thesis provides an overview of the efficacy and limitations of using low-dose glucagon in individuals with type 1 diabetes.

### 2.5. Glucagon formulations

Glucagon as a drug for treatment of severe hypoglycemia was developed in the 1950s by Eli Lilly and Novo Nordisk. Both prod-

ucts exist in powder forms that need to be dissolved in saline or in a hydrochloride solution before use. Dissolved glucagon fibrillates and forms aggregations rapidly after the reconstitution; making it suitable for immediate use only. Furthermore, several steps are required to reconstitute glucagon from powder to liquid form (open the plastic cap; inject solvent to the powder vial; shake the vial until solution is clear; draw the solution into the syringe; and inject the solution s.c. or i.m.). For the experienced user, the procedure takes approximately two minutes to accomplish [81]. Non-medical persons would most likely need more time to inject glucagon, especially in stressful situations when their relative has hypoglycemia-induced seizures or unconsciousness. The formulation of glucagon may therefore be a major barrier for use in severe hypoglycemia. Several companies are currently developing soluble and stable glucagon products that soon could become commercially available (Table 1). In addition to the possible improvements in treating severe hypoglycemia, a stable glucagon formulation may allow for a prolonged use of low-dose glucagon without daily reconstitution in the treatment mild and impending hypoglycemia. The prospect of these new glucagon formulations has encouraged the development of dual-hormone therapy in an open-loop [75,79] and closed-loop settings [82–84].

## 3. HYPOTHESIS AND AIM OF THE PHD THESIS

### 3.1. Hypothesis

The hypothesis of this PhD thesis is that a low-dose glucagon given s.c. may be efficient in restoring plasma glucose levels during insulin-induced mild hypoglycemia in individuals with type 1 diabetes. The glucose response to low-dose glucagon may, however, be negatively influenced by ambient insulin levels, daily carbohydrate intake, and prior ethanol intake.

### 3.2. Aims

The aims of the PhD thesis were:



1) to determine the glucose response to various low doses of glucagon administered s.c. during insulin-induced mild hypoglycemia [1].

2) to determine the optimal glucagon dose at different ambient insulin levels, either estimated as serum insulin or as insulin-on-board [2].

3) to compare the glucose restoring effect of s.c. 100 µg glucagon after one week of low carbohydrate diet versus one week of high carbohydrate diet [3].

4) to compare the glucose restoring effect of s.c. 100 µg glucagon after preceding moderate ethanol intake versus non-ethanol intake [4].

#### 4. DETERMINING THE GLUCOSE RESPONSE TO GLUCAGON IN TYPE 1 DIABETES

In the first low-dose glucagon study performed by Haymond and Schreiner in 2001, children were given 10 µg glucagon per kg up to a maximum of 150 µg glucagon s.c. for each episode of impending or mild hypoglycemia [73]. The study was performed at home, and capillary blood glucose levels increased on an average from 3.4 mmol/l to 8.1 mmol/l after the first glucagon bolus. In some children, the glucose level did however not increase within 30 min, and as a consequence a second and even a third glucagon bolus were given to restore normoglycemia [75]. This study demonstrated that low-dose glucagon efficiently treated hypoglycemia, even though high intra- and interindividual variations in the glucose response to glucagon were seen. Consequently, the interest in finding an optimal glucagon dose to treat and prevent mild hypoglycemia was growing. Unfortunately, no consensus exists regarding methods for estimating the glucose response to s.c. low-dose glucagon in type 1 diabetes (Table 2). On one hand, the methods need to generate results that can be translated into clinical practice. On the other hand, several factors that may affect the glucose response need to be controlled and accounted for. Below, three of many methods to estimate glucose response to low-dose glucagon will be presented.

##### 4.1. Intravenous hyperinsulinemic-euglycemic clamp

In 1979, DeFronzo et al. described the highly-recognized glucose clamp technique to estimate insulin secretion and insulin sensitivity [91]. The hyperinsulinemic-euglycemic clamp is the “gold standard” for measuring insulin sensitivity and involves a fixed insulin infusion with varying glucose infusion to maintain euglycemia. When steady state is achieved, the glucose infusion rate (GIR) equals the rate of glucose uptake in the body tissues, which is equal to the overall insulin sensitivity. It also is possible to calculate substrate kinetics by adding isotopic (stable or radioactive) tracers in order to quantify the endogenous glucose production and rate of glucose disappearance [92]. Later, the clamp technique also became the “state of the art” for evaluating pharmacodynamics properties of new insulin compounds [93], since the GIR can compensate for the glucose lowering effect of insulin, and thus maintain target plasma glucose level (Figure 3). Parameters of the GIR are used to assess insulin pharmacodynamics, i.e. area under the curve, peak rate, and time to peak. Similar methods have been used to assess glucagon pharmacodynamics, in which it is determined how much GIR needs to be lowered after glucagon administration to maintain target plasma glucose level (Figure 3).

Applying the clamp and tracer methodology to measure the glucose response to glucagon may however be challenging [94–96]. First when administering a glucagon bolus, the immediate reduction in glucose infusion rate to maintain euglycemia represents a non-steady state condition. This necessitates the use of non-steady state equations to approximate the endogenous glucose production after glucagon boluses and may not be as accurate as intended [97]. Second, the glucose response to glucagon may be highly dependent on the insulin infusion rate. Even though some studies give individually adjusted insulin infusion rates, the risk of having too low insulin levels may lead to hyperglycemia, despite turning off the glucose infusion. Consequently, the quality of the glucose clamp will be poor with deviation from target levels [94]. Analysis of the results obtained with this method may therefore require mathematical modeling to account for the glucose deviation from target [95,96]. Finally, the glucose response is estimated from the GIR, and may only be of physiological interest, since it cannot be translated to a clinical outcome. This method is therefore quite challenging, and may not be ideal if plasma glucose cannot be maintained at a target level.

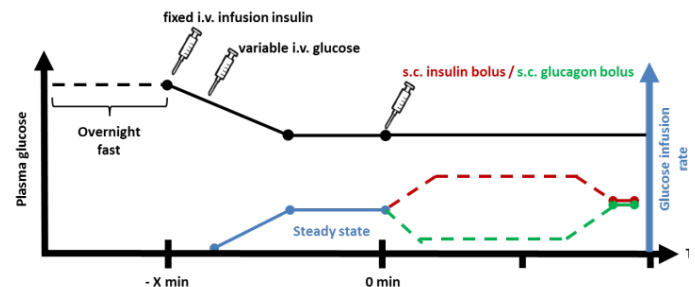


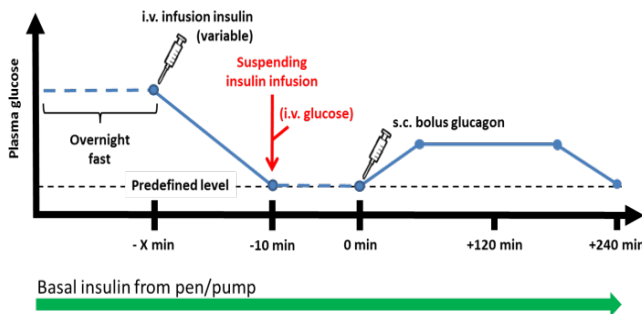
Figure 3: Schematic overview of the intravenous hyperinsulinemic-euglycemic clamp visit.

##### 4.2. Intravenous insulin infusion

Heise et al. demonstrated a method that keeps insulin concentrations fixed and meanwhile provides a clinical meaningful glucose response to glucagon [87]. In this study design, participants were admitted at a clinical center after an overnight fast and continued with their basal insulin as usual. Participants were then given a variable i.v. insulin infusion rate to establish a predefined plasma glucose level for at least 10 min. If plasma glucose went below the hypoglycemia threshold, i.v. glucose bolus was given and insulin rates were adjusted to maintain the target range for another 10 min. Thereafter, glucagon was administered s.c. and no adjustments in insulin infusion rate were carried out. Three other dose-finding studies used a modified approach by turning off the i.v. insulin infusion rate before glucagon administration [90,98,99]. The main outcome was AUC for plasma glucose after glucagon administration (Figure 4).

Even though the estimation of glucose response to glucagon is more clinically applicable than GIR from the clamp study, this method has some limitations to be considered. First, i.v. insulin has a different pharmacokinetic and bioavailability than s.c. insulin administration and the glucose response to glucagon may be different when insulin is given s.c. as seen in “real-life” settings. Furthermore, participants with type 1 diabetes rarely arrive with the same morning-fasting plasma glucose. In this study design, there was no attempt to achieve similar fasting plasma glucose levels, which could lead to different insulin exposures before glucagon administration. Therefore, there is a risk for differences in insulin exposures prior to glucagon administration. Thirdly, if

the plasma glucose level exceeds 10.0 mmol/l after glucagon administration, the glucose clearance from the kidneys should be accounted for, i.e. measuring glucose concentrations in urine before and after glucagon administration. However, from a clinical perspective, the glucose leaked into urine may not be relevant for estimating the glucose-restoring effect of glucagon during hypoglycemia since this obviously only occurs when glucose has been restored. Finally, two studies used a modified design by turning off the insulin infusion rate before glucagon administration in order to overcome the inhibitory effect of insulin [90,98]. However, this modified design is not advisable, since it overestimates the glucose response to glucagon as seen in post-hoc simulations studies [100]. Furthermore, the suspension of i.v. insulin rapidly affects glucose levels within 10 min in contrast to the suspension of s.c. insulin in insulin pumps where it last 60-120 min before an effect can be detected [101,102].



**Figure 4: Schematic overview of the intravenous insulin infusion study visit.**

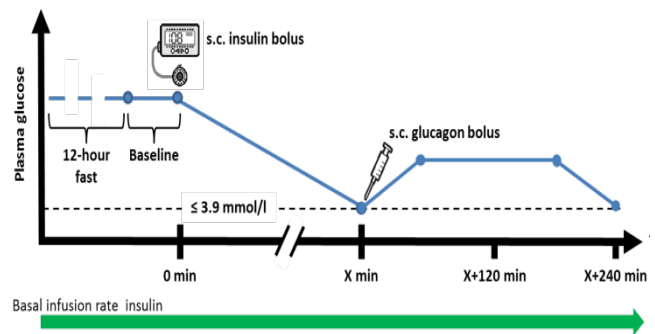
#### 4.3. Subcutaneous insulin infusion

We propose another design for estimating the glucose response to glucagon during a s.c. insulin-induced mild hypoglycemia. This study design has been used in Paper 1, 3 and 4 [1,3,4] and has later been modified by others [89,103]. The design requires a run-in period of two-four weeks before the study initiation to optimize insulin basal and insulin bolus settings for insulin pump-treated type 1 diabetes [104,105]. In the first 1-2 weeks of the run-in, participants are instructed to fast at certain periods per day: 8PM–12AM, 12AM–7PM, and 3PM–10PM. The basal rates are then adjusted accordingly to maintain blood glucose levels within the target range of 4.0-7.0 mmol/l. Fasting periods are repeated and additional basal insulin rate adjustments may be needed. In total patients have a minimum of six days to complete the basal rate adjustments; as we require two estimates for basal rates at each time period. Ideally, fasting over a whole day would provide the best estimation for the needed basal insulin rate, but prolonged fasting is not clinically feasible in type 1 diabetes. Once basal insulin rate settings are satisfactorily adjusted, bolus settings are adjusted for the next 1-2 weeks, involving adjustments of the insulin correction factor (= plasma glucose drop caused by one unit insulin) for three days, carbohydrate-insulin ratio (= amount of carbohydrates that by one unit of insulin keeps postprandial glucose level within target range) for three days, and insulin action time (= the duration of plasma glucose decrease after an s.c. insulin bolus) for 1-2 days [104].

Participants' glucose response to glucagon is estimated at study visits performed at the research unit. Participants are requested to eat >150 grams of carbohydrates daily (except for Paper 3), seven days prior to the study visit, and are instructed to

avoid excessive physical activity, ethanol intake and hypoglycemia (defined as CGM < 3.5 mmol/l or SMBG < 3.9 mmol/l) 24 hours before the study visit. Otherwise the study visit will be postponed for  $\geq 2$  days. After 12 hours of fasting, participants arrive in the morning aiming for a fasting PG level of 5.0-7.0 mmol/l. No change in basal insulin rate or bolus insulin is allowed 5 hours before arrival. Participants are instructed to check their capillary blood glucose level or CGM level at 2-3 AM to see whether insulin bolus or carbohydrate intake are needed to reach the target range. Upon arrival, a s.c. insulin bolus will be injected via the insulin pump. The insulin bolus is calculated via the insulin correction factor to lower the fasting plasma glucose level to 3.0 mmol/l. When plasma glucose reaches  $\leq 3.9$  mmol/l, a single s.c. bolus of glucagon is administered [1,3,4]. The individual s.c. insulin infusion basal rates are unchanged during the study visits (Figure 5).

The strength of our study design is that it tries to simulate "real-life" conditions with s.c. insulin bolus and basal administrations. However, there are shortcomings of the design that need to be addressed. First, participants will inevitably arrive with different fasting levels that require different insulin bolus doses to achieve hypoglycemia. Second, even though the basal insulin rate is supposed to keep plasma glucose in a steady state and the needed insulin dose to induce hypoglycemia is established, the outpatient insulin pump-adjustment may not fully apply to an inpatient setting. Individuals are less insulin sensitive and would require relatively higher insulin doses in hospital sedentary settings than in outpatient active settings. Finally, the risks of underestimating the insulin requirements in combination with the day-to-day variations of insulin absorption result in a relatively high failure rate to achieve target glucose level before glucagon bolus [106]. Therefore, investigators must be prepared to terminate and repeat the study visits.



**Figure 5: Schematic overview of subcutaneous insulin bolus study visit.**

#### 4.4. Glucose response to various low doses of subcutaneous glucagon [1]

We performed a dose-finding study in eight participants with insulin pump-treated type 1 diabetes [1]. Our participants served as their own control, were blinded for the intervention, and completed four study visits, each visit as described above (section 4.3). The glucose response to 100  $\mu$ g, 200  $\mu$ g and 300  $\mu$ g s.c. glucagon bolus were compared with a placebo bolus during s.c. insulin-induced mild hypoglycemia. We found that the glucose response to low-dose glucagon was dose-dependent, and that the response was independent of age, sex, and weight. It is noteworthy to mention that all of our participants were lean, and insulin levels were similar at all study visits.

Author	Year	Study aim for glucose response	Insulin regimen	Glucose intervention	Glucagon type (dose, µg)	PG when glucagon was injected	Estimation of glucose response	Strength	Main limitation
Haymond [73]	2001	Feasibility study	Usual care	None	Glucagon, Eli Lilly (20-150)	3.4 mmol/l	SMBG at 30 min	Outpatient	Not controlled No comparisons
Youssef [95]	2014	Effect of varying insulin levels	Fixed i.v.	Variable i.v. infusion rate	Glucagen, Novo (25-175)	Euglycemia	AUC of EGP over 60 min	Controlled	Risk of turning off glucose infusion
Blauw [98]	2015	Effect of varying glucose levels	Variable i.v.	Variable i.v. infusion rate	Glucagen, Novo (110-1000)	2.8, 4.0, 6.0 and 8.0 mmol/l	Incremental peak PG	None	Suspensions of infusions before glucagon injection
Ranjan [1]	2015	Dose-finding study	S.c. CSII basal/bolus	None	Glucagen, Novo (100-300)	≤3.9 mmol/l	Incremental peak PG	Performed with s.c.	Risk of repeating study visits
Castle [107]	2015	Effect of repeated glucagon doses	Fixed i.v.	I.v. bolus if PG ≤3.9 mmol/l	Glucagen, Novo (2.0 per kg)	5.0-7.2 mmol/l	AUC of PG for 90 min	Controlled	Accounting for glucose infusion
Ranjan [3]	2016	Effects of daily carbohydrate intake	S.c. CSII basal/bolus	None	Glucagen, Novo (200)	≤3.9 mmol/l	Incremental peak PG	Performed with s.c.	Risk of repeating study visits
Castle [99]	2016	PK/PD study	Fixed i.v.	I.v. bolus if PG ≤3.3 mmol/l	G-pump, Xeris (0.3-2.0 per kg)	4.5-7.8 mmol/l	AUC of PG 60, 120 and 150 min.	Controlled	I.v. and not s.c. insulin
Haymond [103]	2017	Dose-finding study	S.c. MDI basal/bolus	None	G-pen, Xeris (75-300)	6.1 mmol/l ≤3.9 mmol/l	Incremental PG at 60 min	Performed with s.c.	First dose given at euglycemia
Heise [87]	2017	Dose-finding study	Fixed i.v.	I.v. bolus if PG ≤2.8 mmol/l	Dasiglucagon, Zealand Pharma (100-1000)	≤3.5 mmol/l	AUC of PG for 30 min and for 360 min	Compared to different doses	Not accounting for urine glucose clearance
Rickels [108]*	2017	Effect of glucagon before exercise	S.c. MDI basal/bolus	None	G-pen, Xeris (150)	> 4.0 mmol/l	AUC of PG for 75 min	Compared to glucose tabs	Glucagon given at euglycemia
Ranjan [4]	2017	Effect of post-ethanol intoxication	S.c. CSII basal/bolus	None	Glucagen, Novo (100)	≤3.9 mmol/l	Incremental peak PG	Performed with s.c.	Risk of repeating study visits
Ekhlaspour [96]*	2017	Effect of acute ethanol intoxication	Fixed i.v.	Variable i.v. infusion rate	Glucagen, Novo (50)	Euglycemia	AUC of EGP over 60 min	Controlled setup	Risk of turning off glucose infusion
Haymond [79]	2017	Comparison to glucose tabs	S.c. MDI basal/bolus	None	G-pen, Xeris (150)	≤3.9 mmol/l	SMBG at 30 min CGM at 60-120 min	Outpatient	Not controlled

**Table 2: Open-loop studies investigating the glucose response to subcutaneous low-dose glucagon in type 1 diabetes.**

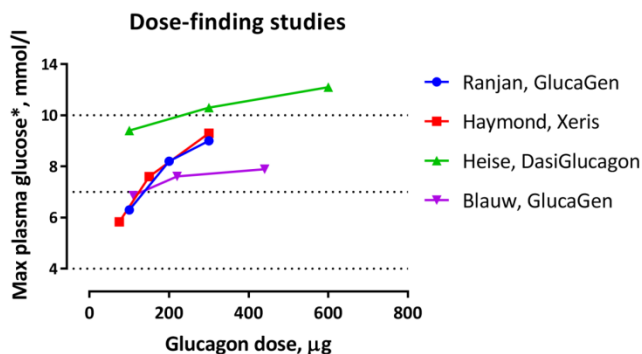
**AUC:** Area under the curve. **CSII:** Continuous Subcutaneous insulin infusion. **EGP:** Endogenous glucose production. **PG:** Plasma glucose measured by YSI.

**PK/PD:** Pharmacokinetic/pharmacodynamic. **I.v.:** Intravenous infusion. **MDI:** Multiple daily injections. **S.c.:** Subcutaneous. \* presented at the 77<sup>th</sup> scientific session of the American Diabetes Association, ADA, San Diego 2017. **SMBG:** self-monitored blood glucose.

Several other studies have later shown similar data on glucagon effectively increasing plasma glucose in a dose-dependent manner. As previously discussed only studies measuring the plasma glucose excursion may be suitable for determining the optimal glucagon dose to treat mild hypoglycemia.

However, no consensus exists regarding the optimal glucose excursion after glucagon bolus. In this dose finding study, we defined that the optimal glucagon dose had to restore normoglycemia (PG>4.9 mmol/l) and avoid rebound hyperglycemia (PG>7 mmol/l). In our controlled settings, 100 µg glucagon seemed to be an optimum dose to treat mild hypoglycemia.

Three other dose-finding studies have tested the ability of glucagon to increase plasma glucose during mild hypoglycemia (Figure 6). They suggested that the optimum doses would be in the range of 100-200 µg glucagon, especially when accounting for the risk of side effects that were accompanied with higher glucagon doses [1,103]. Furthermore, the maximum glucose excursion after glucagon administration did not differ significantly between doses of 200-300 µg s.c. [1] in our study, or between 250-2000 µg i.v. in another study in healthy individuals [69]. Whether these glucagon doses are effective in other conditions that might affect glucagon efficacy (i.e. low carbohydrate diet, exercise, ethanol consumption) will be discussed in the next section.



**Figure 6: Overview of subcutaneous glucagon dose-finding studies.** Mean peak glucose response to glucagon was calculated as incremental plasma glucose added to 3.9 mmol/l. Data was extracted from studies by Blauw with baseline glucose level of 4.0 mmol/l [98], Heise with glucose level of 3.2 mmol/l [87], and Ranjan [1] and Haymond [103] both with glucose level below 3.9 mmol/l.

## 5. POTENTIAL BARRIERS FOR THE USE OF GLUCAGON IN RESTORING PLASMA GLUCOSE

The aforementioned dose-finding studies were conducted in controlled settings, in which patients had restrictions regarding exercise, ethanol consumption, fasting glucose levels, and insulin levels. Glucagon might, however, in certain conditions have an attenuated glycemic effect, as observed in closed-loop dual-hormone studies [109–112]. Indeed, addition of glucagon in closed-loop systems has not been able to fully prevent hypoglycemia [77,113]. Glucagon failures have been suggested to be related to potential barriers that will be elucidated in the following section.

### 5.1. Glucose level

In dogs, the glucose response to glucagon has shown to be 3-fold increased during hypoglycemia compared with euglycemia [114]. This was explained by an additive effect of hypoglycemia and glucagon on the enzyme activity responsible for hepatic glycogenolysis. However, these findings were not reproducible in

humans. Blauw et al. performed a study in individuals with type 1 diabetes to investigate the glucose response to different glucagon doses at various plasma glucose levels. In their study, insulin and glucose were infused to achieve four successive predefined plasma glucose levels. At each glucose level, a s.c. glucagon bolus from 110 µg to 1000 µg was administered. They found that the pharmacokinetic and pharmacodynamic properties of glucagon were unchanged across different ambient plasma glucose levels of 8.0, 6.0, 4.0 and 2.8 mmol/l [98]. Similar findings were demonstrated by Hinshaw and colleagues that investigated the glucose response to three different i.v. glucagon doses during clamp settings that maintained plasma glucose at 5.0 or 3.3 mmol/l with variable infusion of glucose (50% glucose plus [3-<sup>3</sup>H] glucose) and constant infusion of insulin and somatostatin (modified version of method described in section 4.1) [115]. They found that the glucose response to i.v. glucagon, the plasma glucagon clearance, and the hepatic glucagon sensitivity did not differ between hypoglycemia and euglycemia levels in type 1 diabetes.

To sum up, the ability of glucagon to raise plasma glucose seems to be independent of ambient plasma glucose level in individuals with type 1 diabetes.

### 5.2. Insulin level [2]

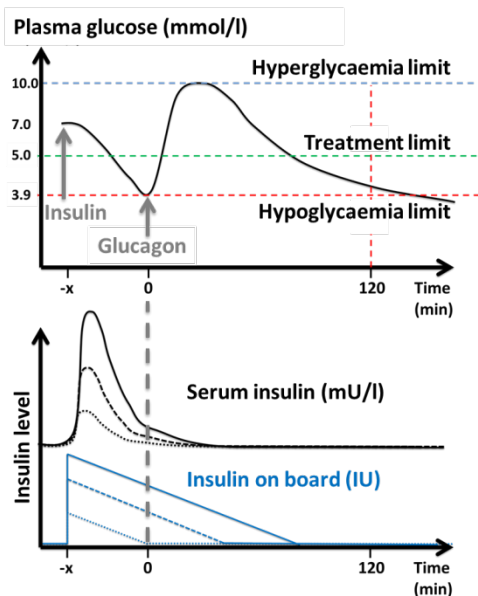
As previously documented, s.c. administered insulin cannot match the pharmacodynamics and pharmacokinetics of endogenous insulin. Compared to endogenous insulin, fast-acting insulin analogs have a delayed onset and offset leading to prolonged insulin actions for more than 4 hours after s.c. delivery (Figure 1). This leads to a predisposition to hypoglycemia, since the exogenous insulin cannot be retracted from the body. Hypoglycemia can only be prevented by timely insulin suspension [116], oral glucose intake or low-dose glucagon injections as seen with the closed-loop dual-hormone systems [57]. However, these approaches still fail to completely prevent or treat hypoglycemia. It is known that the hepatic glucose production is dependent on the ratio of glucagon and insulin [117]. In dual-hormone closed-loop studies, high ambient insulin levels were correlated with failure for glucagon to prevent and treat hypoglycemia [109–111]. When closed-loop systems accounted for the higher insulin levels, the glucagon failure rate could be reduced from 37 % to 20 % [109]. Youssef and colleagues confirmed these findings in a controlled hyperinsulinemic-euglycemic clamp study (section 4.1) investigating the relationship between i.v. insulin and s.c. glucagon on endogenous glucose production [95]. In eleven participants with type 1 diabetes, they found that high ambient insulin levels (mean±sem: 46.0±12.5 mU/L) suppressed endogenous glucose production (EGP) from increasing after s.c. administration of 25-175 µg glucagon doses. At lower insulin levels, similar glucagon doses increased EGP in a dose-dependent manner. However, one could argue that higher glucagon doses (>175 µg) still could have stimulated EGP at the high ambient insulin levels. In healthy individuals, the EGP is totally shut off when plasma insulin levels are above 50 mU/l [118]. In the same study by Youssef et al, they tried to extrapolate EGP at higher glucagon doses. The extrapolation may however not be valid since the actual glucagon doses used in the study were within the steep part of the glucagon-glucose saturation curve [69,119].

The data are not directly transferable to clinical use. First, the glucose response was shown as EGP rather than plasma glucose excursion. Second, insulin levels were presented as plasma insulin levels; while in clinical settings these concentrations are unknown. Third, even though a relationship has been presented, no



optimal glucagon dosing regimen was provided at varying insulin levels. This may not be an issue for closed-loop systems that have a computerized controller with a built-in complex algorithm automatically adjusting the insulin and glucagon delivery. In open-loop settings, simpler algorithms are needed for individuals to manually treat mild and impending hypoglycemia with a single bolus of low-dose glucagon - whether their insulin is given as a CSII or MDI. We performed a simulation study to propose a glucagon dosing regimen based on the ambient insulin levels [2]. The simulations were based on a gluco-regulatory model [120] that was validated using data from our previously mentioned dose-finding study [1]. The model was similarly successful in reproducing the data from the abovementioned study by Youssef et al [95] with simulations [100].

In our simulation study, seven virtual insulin-pump treated participants with type 1 diabetes went through three studies that differed regarding how insulin levels were estimated, i.e. serum insulin, insulin on board (IOB) or insulin on board adjusted for the total daily insulin dose (IOB/TDD). These alternative measurements for insulin levels were used, since no real-time insulin monitors currently exist. On the other hand, individuals with type 1 diabetes have for years used bolus calculators that roughly estimate the remaining activity of a prior s.c. insulin bolus (pharmacodynamics), so-called insulin-on-board (IOB) measured as units, IU [104]. Furthermore, previous studies have shown that IOB correlates highly with plasma insulin levels [121]. We designed this simulation study to determine the optimal glucagon dose at varying insulin levels, regardless of how insulin was estimated. No consensus however exists on the desirable glucose excursions after a glucagon bolus. We defined an optimal glucagon dose to increase PG from 3.9 mmol/l to a peak between 5.0 and 10.0 mmol/l, and sustain PG above 3.9 mmol/l for at least 120 minutes following the glucagon bolus (Figure 7).

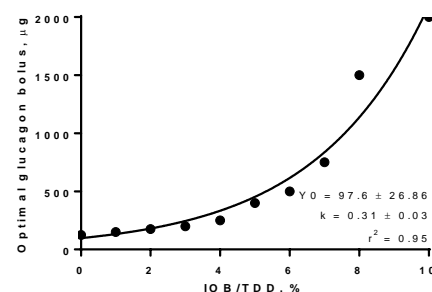
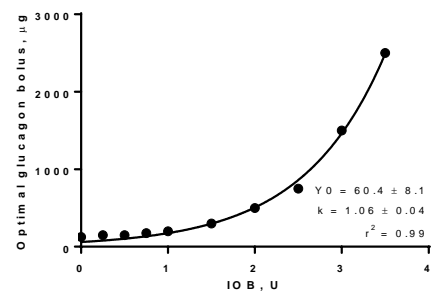
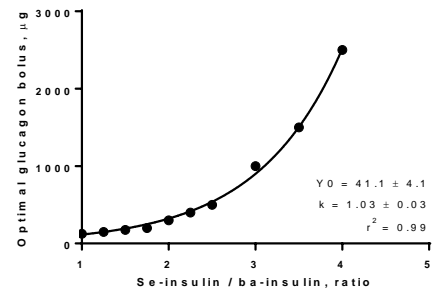


**Figure 7: Schematic description of study design and treatment assessment**

For each simulated experiment, one of ten different s.c. insulin boluses was injected to decrease PG from a baseline level of 7.0 to  $\leq 3.9$  mmol/l. The bolus sizes were chosen to reach predefined insulin levels when PG was 3.9 mmol/l. Once PG reached 3.9 mmol/l, one of 17 different s.c. glucagon boluses was adminis-

tered. Thus, in total each virtual participant went through 170 visits at each study, leading to 510 simulations per participant.

Regardless of how insulin levels were estimated, the optimal glucagon dose was exponentially related to ambient insulin levels (Figure 8). The lowest glucagon dose was 125  $\mu\text{g}$  to optimally treat mild hypoglycaemia when insulin levels were equal to the basal insulin levels. In contrast, glucagon doses above 500  $\mu\text{g}$  were needed when insulin levels were above 2.5 times the basal levels, when IOB were above 2.0 IU, or when IOB were above 6% of the total daily insulin dose. At these insulin levels, carbohydrates may be preferable due to the side effects caused by increasing glucagon doses.



**Figure 8. Optimal glucagon dose for treatment of mild hypoglycemia at varying insulin levels.** Optimum glucagon dose as a function of ambient insulin levels stratified by actual to baseline serum insulin concentration (upper panel), insulin on board (middle panel), and percentage of insulin on board to total daily insulin dose (lower panel). Exponential relations between the optimum glucagon dose and ambient insulin levels were found:  $Y=Y_0 \cdot e^{k \cdot \text{insulin}}$ . Parameters are presented as mean $\pm$ SD

The strength of simulation studies is their ability to simulate countless cross-over trials that would not have been feasible in clinical settings. Despite obvious limitations with our simulation study, this was the first study to suggest an alternative approach for glucagon dosing in open-loop systems. In contrast to the previously fixed glucagon dosing regimen [73,74,79], a simple insulin-dependent dosing regimen was presented. This may be relevant for future advanced bolus calculators that can guide

individuals with anti-hypoglycemic actions based on insulin on board and plasma glucose levels, and thus improve glucose control. Nevertheless, the predictive value of the model still needs to be evaluated in a real-world set-up including individuals treated at varying doses of insulin and in different clinical situations, i.e. during and after exercise, after different meals, and during a fasting state.

### 5.3. Hepatic glycogen depot

Any conditions depleting liver glycogen stores may potentially impair the glucose response to glucagon. Lockton et al. showed that the glucose response to a second 500 µg glucagon bolus was lower compared with the first 500 µg bolus in healthy individuals [122]. Repeated glucagon doses may therefore deplete liver glycogen stores and reduce the efficacy of glucagon to raise plasma glucose. In closed-loop studies, there was no evidence on glucagon having diminished hyperglycemic effect after prior multiple glucagon doses, even when receiving a total daily dose of glucagon above 1000 µg [109,110]. Castle and colleagues confirmed these findings by comparing the hyperglycemic effect of eight glucagon boluses each of 2 µg glucagon/kg (mean±sem dose: 140.7±8.2 µg. Total dose: 1125.8±65.5 µg over 16 hours). Regardless of participants being in a fed or fasting state, no differences were found on the glucose response to the first and last glucagon bolus. Glycogen stores were measured with a <sup>13</sup>C magnetic resonance spectroscopy. No correlations were found between liver glycogen stores and glucose response to glucagon or between liver glycogen stores and glucagon dosing.

In contrast to these findings, i.v. glucagon studies demonstrated that glucagon infusion initially increases plasma glucose levels but the effect wanes after only 30 min [115,123]. The sudden termination of glucagon efficacy has been described as the “evanescent effect” of glucagon, and may in insulin-dependent individuals be related to hepatic down-regulation of glucagon receptors [124,125]. This may therefore explain that pulsatile administrations of glucagon boluses, as seen in closed-loop studies and in the abovementioned study by Castle et al., remained effective. However, very frequently delivered glucagon boluses would be expected to behave as the constant glucagon infusion and result in evanescence. Nonetheless, the data support that the glucose response to glucagon differs between continuous glucagon infusion and pulsatile glucagon administration [126].

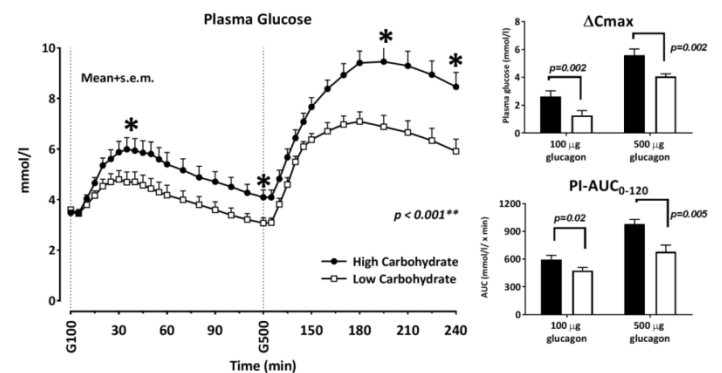
### 5.4. Carbohydrate intake [3]

Glycogen stores can be reduced during prolonged intake of low carbohydrate diets [127]. Dietary intervention and recommendations for type 1 diabetes are still massively debated, and extensive research has been done to determine an optimal diet composition for individuals with diabetes [128]. Carbohydrate-restricted diets are used among type 1 diabetes individuals due to the impression that this will result in a better control of the post-prandial glucose excursions as well as a reduction of the daily prandial insulin doses [129,130]. Not all individuals can, however, comply with or tolerate restricted diets [131]. Moreover there is no consensus on the optimal compositions of the carbohydrate restricted diets, making it difficult to compare previously reported dietary effects on for instance glycemic control, weight, and cardiovascular outcomes [132].

We investigated whether the daily carbohydrate consumption affects the anti-hypoglycemic effects of low-dose glucagon in ten insulin pump-treated participants. We compared the glucose responses to glucagon after one week of low carbohydrate diet (<

50 gram carbohydrate per day) with responses after one week of high carbohydrate diet (>250 gram carbohydrates per day). The low carbohydrate diet may be regarded as extreme but was successfully followed by our participants, as confirmed by the amount of carbohydrate registered in their insulin-pumps (47±10 vs 225±30 gram carbohydrate per day). After each dietary week, patients underwent a glucagon-glucose response study visit as described above in section 4.3.

The ten insulin pump-treated participants with type 1 diabetes had a lower glucose response (glucose peak and AUC) to 100 µg and 500 µg glucagon bolus after one week of low carbohydrate diet compared with after one week of the high carbohydrate diet (Figure 9). Meanwhile, insulin and glucagon profiles did not differ and could not explain the differential glucose response to glucagon after the two diets. Unfortunately, we were unable to measure hepatic glycogen stores or hepatic sensitivity to glucagon that might have explained the difference between the glucose responses to glucagon. In contrast, in the study by Castle et al. the glucose response to glucagon did not differ between participants in a fasting state with confirmed reduced glycogen stores compared with participants in fed state with confirmed increased glycogen stores [107]. In our study, fasting levels of glucagon, amino acids, free fatty acids and ketones were higher at end of the low carbohydrate diet week compared with the end of high carbohydrate diet week. We therefore speculated that the increase in glucagon and amino acid levels after the low carbohydrate diet might have down-regulated hepatic glucagon receptors, and suppressed the hepatic sensitivity to glucagon [29,124], see appendix 1.



**Figure 9: Plasma glucose after subcutaneous bolus of glucagon.** One study visit after a low (empty squares) and one after a high carbohydrate diet (filled circles). A s.c. insulin-induced hypoglycemia (t=0) was treated with 100 µg glucagon injection s.c. (G100) followed by a s.c. injection of 500 µg glucagon (G500) two hours later. The \* indicates a p < 0.05 while the \*\* indicates the p-value obtained by the repeated measurement ANOVA for time x study day.

In dual-hormone closed-loop systems, the controller may automatically adjust glucagon dosing according to individuals’ dietary and daily habits. Consequently, individuals eating low carbohydrate diets may need higher glucagon doses which probably increase the risk of side effects. On the other hand, low carbohydrate diets may reduce the frequency and time of hypoglycemia per day, resulting in less need for glucagon dosing in the first place [129].

To summarize, glucagon efficacy is impaired after one week of low carbohydrate diet and should be accounted for when glucagon is used for treatment of mild hypoglycemia. However, it

remains unknown whether these effects persist when the diet regimens are maintained over a longer period of time.

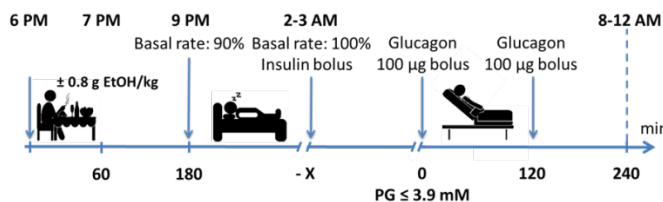
### 5.5. Ethanol intake [4]

Ethanol is commonly consumed in social context. Ethanol consumption is, however, a risk factor for severe hypoglycemia, and individuals with type 1 diabetes are recommended to limit their ethanol intake [128,133]. The pathogenesis of ethanol-associated hypoglycemia has been related to the inhibition of hepatic gluconeogenesis [134], impaired counter-regulation [135], impaired awareness of hypoglycemia symptoms [136], and impaired cognitive functions to take action for impending hypoglycemia [137].

As a result of ethanol inhibiting hepatic gluconeogenesis and thereby the hepatic glucose production, pharmaceutical companies have labeled a warning that glucagon as a rescue dose of 1000 µg may not be effective in treating ethanol-associated severe hypoglycemia [138,139].

As previously elucidated, glucagon mainly stimulates the breakdown of glycogen. Glucagon efficacy would theoretically be unaffected during acute ethanol intoxication in individuals with type 1 diabetes. This was also recently confirmed by Ekhlaspour and colleagues [96] who performed a combined ethanol infusion and hyperinsulinemic-euglycemic clamp experiment (section 4.1), in which 50 µg glucagon was administered during stable insulin, glucose and ethanol levels. The overall amount of glucose infusion to maintain euglycemia after s.c. 50 µg glucagon bolus did not differ between the ethanol visit and a placebo visit. Ethanol may therefore in the acute phase not affect the efficacy of low-dose glucagon.

However, ethanol-associated hypoglycemia typically occurs 8-12 hours after ethanol consumption. For that reason, we investigated whether the glucose response to low-dose glucagon was influenced in the period after ethanol intoxication [4]. Our study was conducted overnight in participants with type 1 diabetes who were served ethanol or an isovolemic-isotonic placebo non-ethanol drink with a dinner at 6PM (Figure 10). Participants slept from 9PM to 2-3AM, at which a s.c. insulin-induced hypoglycemia was performed as described previously (section 4.3). Once plasma glucose was 3.9 mmol/l, 100 µg glucagon was given followed by another 100 µg glucagon after two hours.



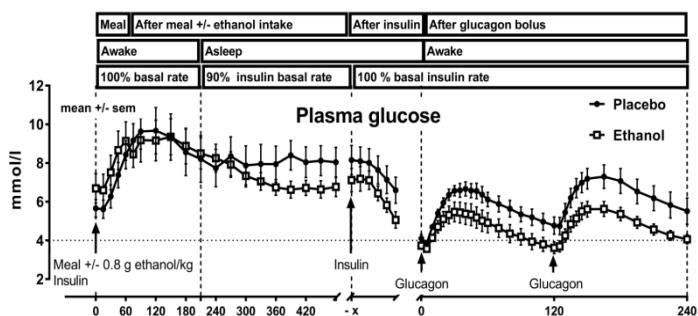
**Figure 10: Illustration of experimental design.** Ethanol (EtOH), meal and insulin bolus were given at 6PM. After 180 minutes, sleep was allowed. The insulin bolus to induce hypoglycemia was administered after 8-9 hours, once ethanol concentrations were undetectable. The first glucagon bolus was given when plasma glucose (PG) was below 3.9 mmol/l, followed by another glucagon bolus after 120 minutes.

In this study, the glucose response to glucagon tended to be lower on ethanol compared with the placebo visit ( $p=0.06$ ). The mean difference in incremental plasma glucose peak of 0.9 mmol/l was slightly lower than the predefined clinical relevant difference of 1.0 mmol/l. Our study might have been underpowered with 12 participants in order to obtain statistical significance. Furthermore, the time to reach hypoglycemia was shorter after

ethanol intake compared with the placebo. Insulin and glucagon levels did not differ between study visits. No correlations were observed between the time to achieve hypoglycemia and the glucose responses to the first glucagon bolus. The glucose response to glucagon was diminished in the late hours after ethanol intake, but glucagon was still able to restore normoglycemia with plasma glucose increase of 2.0 mmol/l (Figure 11).

In closed-loop systems, the controller would quickly compensate for the reduced glucagon effect by delivering additional glucagon boluses to prevent hypoglycemia. Even though we used low doses of glucagon, we would not expect the rescue dose of 1000 µg glucagon to be ineffective in restoring plasma glucose during severe hypoglycemia associated with ethanol intake.

In summary, the glucose response to low-dose glucagon may not be affected by concomitant ethanol intoxication, but may be attenuated in the period after ethanol intoxication.



**Figure 11: Plasma glucose concentrations on ethanol and placebo visit.** The concentration profiles after meal intake + ethanol consumption (empty squares) and after meal intake + placebo consumption (filled circles) until the ethanol before and after an insulin bolus was given to induce a mild hypoglycemia. A 100 µg glucagon was given once plasma glucose reached 3.9 mmol/l, followed by another 100 µg glucagon dose after two hours.

### 5.6. Exercise

The fear of exercise-induced hypoglycemia keeps individuals from performing the recommended amount of physical activity [128,140–142]. Exercise can vary from the high intensity anaerobic to low intensity aerobic training with different effects on glucose metabolism and plasma glucose levels [143]. The high intensity training typically increases plasma glucose acutely, but has a late hypoglycemic effect, while low intensity training has both an acute and a late hypoglycemic effect [144]. Furthermore, the individuals' prior physical activity level also influences the glucose response to exercise [145,146]. The pathogenesis for exercise-induced hypoglycemia is related to the combined effect of insulin independent glucose uptake in the muscles, the poor counter-regulatory response, and the inability to decrease ambient insulin levels, which are observed in healthy individuals [147,148]. Several strategies have been suggested to prevent mild hypoglycemia, i.e. fast-acting carbohydrate intake, insulin reduction or suspension, or a combination of both [143]. However, controlling the plasma glucose in relation to exercise is complex, which also has been the biggest hurdle for the insulin-only closed-loop systems [53]. Insulin infusion needs to be suspended hours before initiating the aerobic exercise, and no available system can predict exercise so far ahead [149–153]. Therefore, early planning of upcoming exercise is needed, and may not be practical. Carbohydrate intake before exercise may also be needed. We proposed that low-dose glucagon may be an alternative to carbohydrate

intake in preventing and treating exercise induced hypoglycemia [22].

Taleb and colleagues showed that the dual-hormone closed-loop system could outperform the insulin-only system during exercise by both significantly reducing the time spent in hypoglycemia and increasing the time in euglycemia [77]. In this study, exercise was however announced to the closed-loop system 20 min prior to the training session. The timing for announcement was considerably shorter than what might be needed with insulin suspension alone to prevent exercise-induced hypoglycemia. Another study by Rickels and colleagues showed that the glucose profile after 150 µg glucagon bolus was comparable with 16 g oral glucose tabs given just before a moderate exercise session of 45 min [108]. The timing of the announcement for exercise in closed-loop systems may therefore be even shorter if allowing the first glucagon dose, prior to the exercise, to be higher.

It remains unclear whether the glucose response to glucagon is affected after exercise compared with rest, and whether a glucagon bolus should be delivered before or after an exercise session. We are currently performing a three-arm study that investigates the glucose response to 200 µg glucagon after 45 min of moderate exercise compared with after 45 min of rest, as well as compared with glucagon given before the exercise session (clinicaltrial.gov NCT02882737).

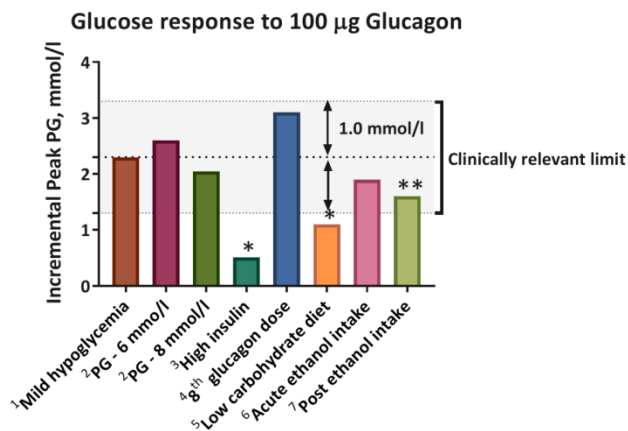
## 6. DISCUSSION

Low-dose glucagon added to an open-loop or a closed-loop system has been shown to sufficiently treat mild and impending hypoglycemia and increases plasma glucose in a dose-dependent manner in individuals with type 1 diabetes [1]. Nevertheless, glucagon-insulin therapy has not fully eliminated the occurrence of hypoglycemia in outpatient closed-loop studies. The reason could be that several factors may limit the efficacy of glucagon.

First, the commercially available powder form of glucagon is unstable and fibrillates rapidly after reconstitution, making it unsuitable for chronic use. However, new stable and soluble glucagon formulations are currently being tested in clinical trials. Second, the glucose response to glucagon is highly dependent on ambient insulin levels. In closed-loop systems, controllers may be able to dose glucagon based on predicted insulin levels [111]. For open-loop studies, only our simulation study has suggested an insulin-dependent glucagon dosing regimen for mild hypoglycemia [2]. Clinical studies are thus needed to confirm or adjust the proposed algorithm for open-loop glucagon dosing. Third, low carbohydrate diet (< 50 g per day) impairs the anti-hypoglycemic effect of glucagon [3]. Higher glucagon doses are therefore necessary to treat mild hypoglycemia for individuals practicing a low carbohydrate diet. Even though high glucagon doses increase the risk of side effects, frequencies of hypoglycemia may be lower during a low carbohydrate diet. Finally, preceding ethanol consumption may attenuate the glucose response to low-dose glucagon, but the plasma glucose increase after 100 µg glucagon was still of clinically relevant magnitude [4]. However, the glucose response to glucagon was not clinically affected by various glucose levels, prior repeated glucagon dosing or during ethanol intoxication (Figure 12).

Long-term studies are required for the assessment of the safety and efficacy of adding glucagon to insulin therapy. One major concern with glucagon is the acute side effects such as nausea, vomiting, dizziness and headache. The side effects have primarily been reported in studies with glucagon doses exceeding 500 µg. This was confirmed in our study [1] with lower glucagon

doses of 100, 200, 300 µg that showed similar side effects as compared to placebo treatment for mild hypoglycemia [1]. In closed-loop studies, the occurrence and severity of side effects did not differ between treatment arms of insulin plus glucagon and insulin plus placebo [77,78]. Furthermore, participants could only guess the correct treatment arm in 42% of the cases, i.e. almost similar prediction rate as for flipping a coin [154]. Another concern on the side effects is the potential risk of developing a migratory skin rash (necrolytic migratory erythema) characteristic for patients with glucagon producing tumors [155,156]. Necrolytic migratory erythema has not yet been reported in any short term dual-hormone studies, but has been observed in infants with congenital hyperinsulinemia treated with daily reconstituted native glucagon per day [157–159]. The skin rashes seem to however resolve shortly after removing glucagon exposure [160].



**Figure 12: Glucagon induced hyperglycemia at various conditions.** Data extracted from <sup>1</sup>[1], <sup>2</sup>[98], <sup>3</sup>[95], <sup>4</sup>[107] <sup>5</sup>[3], <sup>6</sup>[96] and <sup>7</sup>[4]. For each study, the proportion of glucose response to the intervention vs placebo were multiplied with the incremental peak plasma glucose (PG =2.3 mmol/l) caused by 100 µg glucagon from the Paper 1. The clinically significant relevant limit is ± 1.0 mmol/l. High ambient insulin levels (~41.7 mU/l) and one week of low carbohydrate diet (<50 g day) significantly impaired glucose response to low-dose glucagon in individuals with type 1 diabetes (\*indicates p<0.05). Preceding ethanol intake reduced the glucose response but the response was within the acceptable clinical margin (\*\*indicates p=0.06).

Glucagon doses exceeding 1000 µg, the maximal allowed dose per 24 hours [138,139], increase cardiac output by increasing blood pressure and pulse [161,162]. In our study, lower doses of glucagon to treat mild hypoglycemia, reduced heart rate and blood pressure compared with the placebo [1]. Whether low-doses of glucagon given during eu- or hyperglycemia may express chronotropic and inotropic effects have not been reported yet. Furthermore, glucagon stimulates lipid oxidation and ketogenesis without any increased incidence of diabetes ketoacidosis [163]. Free fatty acid and ketone levels are slightly elevated after glucagon administrations and may be augmented after low carbohydrate diets and diminished after ethanol consumption (appendix 8.1) [1,3,4]. As a result of the cardiovascular outcome trial of empagliflozin, EMPA-REG, we speculate that the glucagon induced hyperketonemia may not be as harmful as once thought [164].

Glucagon has shown to reduce calorie intake and increase energy expenditure that may be beneficial for individuals with type 1 diabetes who try to maintain or to reduce body weight [165,166]. Several mechanisms may explain weight reducing



effects of glucagon. First, the use of low-dose glucagon as an alternative to fast-acting carbohydrate for treatment of mild and impending hypoglycemia may indirectly reduce the daily calorie intake [167]. Second, glucagon may induce satiety as observed in studies with administration of 1000 µg glucagon given before meals. The effects on satiety seen in these studies could be explained by a glucagon specific effect on appetite and food intake, the side effects of glucagon, the inhibition of gastric motility by glucagon, or by a cross-reactivity of glucagon on GLP-1 receptors. Third, glucagon may increase the energy expenditure by stimulating thermogenesis in brown adipose tissue [168].

In general, glucagon exerts several extrahepatic effects (cardiovascular, gastrointestinal, pulmonary, renal and central nervous system) that should be monitored in future long-term studies to assess the risk-benefits of low-dose glucagon use [80,169].

### 6.1. Perspectives

The prospect of having stable glucagon formulations has in recent years motivated researchers to develop dual-hormone therapies for individuals with type 1 diabetes. The current formulations of native glucagon have shown to produce predictable glucose excursions that are comparable to oral carbohydrates in treating and preventing mild hypoglycemia. The use of glucagon may allow for an even more intensified insulin therapy, and thus improve glucose control without the need for additional calorie intake. As a result, glucagon as a non-caloric alternative may help individuals with type 1 diabetes to prevent weight gain.

As described, the glucose response to glucagon was impaired during high levels of insulin, after seven days of low carbohydrate diets, and maybe also 8-9 hours after ethanol intake. Closed-loop systems may automatically account for these conditions, since the controller adjusts the glucagon delivery based on prior glucose responses to glucagon. Ideally, the effect size of these conditions should be incorporated in the control algorithm to provide differential solutions for insulin and glucagon delivery to obtain optimal glucose control. Hence, next-generation controllers may account for additional inputs from e.g. accelerometers, and continuous insulin monitors. Nonetheless, the addition of glucagon to closed-loop systems, however, increases the cost and complexity which may outweigh the health benefits and may not be applicable for every individual with type 1 diabetes [83,170]. Furthermore, most closed-loop systems still require announcement of meal intakes and/or impending exercise sessions in order to work optimally [57]. Therefore, even though insulin and glucagon delivery are automatically controlled, the requirements to announce and troubleshoot system failures can be considered cumbersome. For that reason, low-dose glucagon may equally be used in open-loop systems [22].

Haymond et al. has recently studied the feasibility of a fixed glucagon dosing regimen in treatment of mild and impending hypoglycemia - showing that treatment success (proportion of rescues resulting in PG increase from 2.8 mmol/l to above 3.9 mmol/l after 30 min) of 150 µg glucagon was comparable to oral glucose tabs ( $p=0.99$ ) [79]. The fixed dosing regimen does however not account for factors such as exercise, hyperinsulinemia, circadian variability of insulin sensitivity and glucose trends that may impair glucagon efficacy. Moreover, individuals cannot be certain that the fixed dose always results in sufficient glucose recovery. A variable glucagon dosing regimen depending on these factors may be more appropriate. In paper 2, we proposed that a CGM-integrated advanced bolus calculator in an insulin pump or as a separate device could provide instructions for low-dose

glucagon dosing. Based on carbohydrate intake, CGM-values and insulin-on-board, this bolus calculator could calculate insulin dose, glucagon dose or the amount of carbohydrates needed to keep normal plasma glucose levels. This system differs from available bolus calculators by additionally providing advices on how to treat mild and impending hypoglycemia with glucagon [2]. This regimen will, however, not completely alleviate disease burden for individuals with type 1 diabetes. Even though future studies are needed to demonstrate feasibility, safety and efficacy of such CGM-integrated advanced bolus calculators, this approach may be much cheaper than dual-hormone closed-loop systems.

Reduction of postprandial glucose excursions plays a key role in improving glucose control [171,172] and may be achieved with timely and more appropriate dosing of meal insulin boluses [173,174]. Nevertheless, the novel faster-acting insulin (Faster Acting Insulin Aspart, FiAsp, Novo Nordisk®) delivered s.c. [175] or the short-acting insulin analogues delivered intraperitoneally cannot completely eliminate postprandial hyperglycemia [38]. Postprandial glucose excursions may be reduced by reducing postprandial hyperglucagonemia and by delaying the gastric emptying [176,177]. This could be achieved with co-administration of short-acting GLP-1 or amylin with meal insulin [178–180]. Yet, none of these drugs had significant effect on hypoglycemia [181]. A multi-hormone approach with co-administration of insulin plus amylin or insulin plus short-acting GLP-1 as well as the administration of low-dose glucagon may improve glucose control, but may be challenged with the increased cost and complexity of the therapy. Due to this complexity, a multi-hormone therapy would, after all, call for an automatic delivery system (multi-hormone closed-loop system).

### 6.2. Conclusions

The short-term use of glucagon seems to be safe, although the glucose response to glucagon may be impaired during hyperinsulinemia and after one week of low carbohydrate diet. Despite the fact that preceding ethanol intake may attenuate the glucose response to glucagon, glucagon may still sufficiently restore normoglycemia from an ethanol-associated hypoglycemia. The glucose response to glucagon is unaffected across various glucose levels, after moderate exercise, and during ethanol intoxication. Glucagon may potentially cause skin rashes, increase blood pressure and pulse rate, increase ketone levels, and promote weight loss. However, these effects have not been observed in the short-term studies with total daily glucagon dose below 1000 µg. The main obstacle for use of glucagon is still the stability of available glucagon as well as the lack of studies to confirm its long-term safety.

## 7. SUMMARY

Type 1 diabetes is a chronic disease caused by an autoimmune destruction of the insulin-producing cells in the pancreas, leading to a condition with insulin deficiency and elevated blood glucose levels. Individuals with type 1 diabetes are therefore recommended to frequently inject insulin subcutaneously to keep near-normal blood glucose levels, preventing the progression and onset of diabetes-related complications, i.e. kidney failure, blindness, amputation, stroke and heart attack. Unfortunately, the intensified insulin therapy is associated with risk of hypoglycemia—impeding individuals from reaching recommended treatment goals. In this PhD thesis, we hypothesized that low-dose glucagon may complement existing insulin therapy in improving glucose control by treating and preventing mild hypoglycemia.

The aim was to determine whether low-dose glucagon could treat insulin-induced mild hypoglycemia sufficiently, and to investigate conditions that might impair the efficacy of glucagon. We showed that the glucose response to low-dose glucagon was dose-dependent but was impaired during high blood levels of insulin, after one week of low carbohydrate diet and perhaps 8-9 hours after ethanol intake. These findings are clinically relevant when blood glucose levels are controlled through insulin and glucagon delivery.

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