Dissertation

Glucagon Response and Endogenous Glucose Production during Hypoglycaemia in C-peptide Positive and C-peptide Negative Patients with Type 1 Diabetes Mellitus

submitted by

Dr. med. univ. Sabine Zenz

for the Academic Degree of

Doctor of Medical Science

(Dr. scient. med)

at the

Medical University of Graz

Department of Internal Medicine

Division of Endocrinology and Diabetology

under the Supervision of

Univ. Prof. Dr. Thomas R. Pieber

2025

Declaration of Academic Integrity

I hereby confirm that this thesis is the result of my own independent scholarly work. I also confirm that in all cases, where material from the work of others (in books, articles, essays, dissertations, and on the internet) is acknowledged, quotations and paraphrases are clearly indicated. No material other than that cited in the reference list has been used. I have read and understood the Medical University's regulations and procedures concerning plagiarism.

Furthermore, I hereby declare that if artificial intelligence (AI) tools were used for the generation and/or correction of certain text passages in the creation of this work, such employment was conducted in compliance with ethical principles, academic integrity, and the regulations of my university. Additionally, it was ensured that this usage was transparently disclosed and appropriately attributed.

Graz, 17.12.2025 Dr. med. univ. Sabine Zenz

Disclosures

Part of this thesis has been published in the following article:

Zenz S, Mader JK, Regittnig W, Brunner M, Korsatko S, Boulgaropoulos B, Magnes C, Raml R, Narath S.H., Eller P, Augustin T, Pieber TR. Impact of C-Peptide Status on the Response of Glucagon and Endogenous Glucose Production to Induced Hypoglycemia in T1DM. J Clin Endocrinol Metab. 2018 Apr 1;103(4):1408–17. doi: 10.1210/jc.2017-01836.

All of the following authors have explicitly agreed to the use of their data in this thesis:

Julia K. Mader[1], Werner Regittnig[1], Martina Brunner[3], Stefan Korsatko[1], Beate Boulgaropoulos[1,4], Christoph Magnes[4], Reingard Raml[4], Sophie H. Narath[4], Philipp Eller[5], Thomas Augustin[4], Thomas R. Pieber[1,4].

Authors' affiliations:

[1]Endocrinology and Diabetology, Department of Internal Medicine, Medical University of Graz, Graz, Austria

[2]Rheumatology and Immunology, Department of Internal Medicine, Medical University of Graz, Graz, Austria

[3]Clinical Trials Unit, Medical University of Graz, Graz, Austria

[4]Joanneum Research Forschungsgesellschaft mbH HEALTH – Institute for Biomedicine and Health Sciences, Graz, Austria

[5]Intensive Care Unit, Department of Internal Medicine, Medical University of Graz, Graz, Austria

This dissertation includes contributions from Reingard Raml (Joanneum Research Forschungsgesellschaft mbH, HEALTH—Institute for Biomedicine and Health Sciences, Graz, Austria), particularly regarding the calculation of the tracer-to-tracee ratio, including the analysis of glucose and [6,6-$^{2}H_2$]-glucose; from Sophie Narath (Joanneum Research Forschungsgesellschaft mbH, HEALTH—Institute for Biomedicine and Health Sciences, Graz, Austria), in relation to the calculation of endogenous glucose production and peripheral glucose disposal rate; and from Thomas Augustin (Joanneum Research Forschungsgesellschaft mbH, HEALTH—Institute for Biomedicine and Health Sciences, Graz, Austria), particularly for statistical support.

I confirm that I have obtained permission to reproduce figures and tables published in the related journals.

Graz, 17.12.2025 Dr. med. univ. Sabine Zenz

Acknowledgement

„Great things take time — and we used that time to create something special."

First and foremost, I would like to express my sincere gratitude to Univ.-Prof. Dr. Thomas R. Pieber for his professional support, scientific guidance, and for introducing me to the world of clinical research. His time and expertise made this work possible.

Special thanks go to Dr. Werner Regittnig, who always had an open ear for my concerns and supported me with great patience and outstanding scientific solutions, even in situations I perceived as particularly challenging.

I am especially grateful to Dr. Beate Boulgaropoulos for her invaluable support in scientific writing, characterized by remarkable patience, perseverance, and a deeply human approach.

I also thank Univ.-Prof. Dr. Philipp Eller and Dr. Reingard Raml for their support in carrying out this work, as well as the entire Clinical Trials Unit and the Joanneum Research Team for their commitment, enthusiasm, and humanity in implementing the study.

I would further like to thank my other co-workers who were involved in thesis related issues and the patients for their participation in the study.

My colleagues at the Division of Rheumatology and Immunology provided constant motivation and support, which was invaluable in bringing this work to completion.

The underlying study was conducted in 2014 and published in 2018. Building on this work, a further study was conducted and published in 2022. The work forming the basis of this dissertation was cited in *The Lancet* in 2018. Now, at the end of 2025, I am proud to complete this dissertation and close an important chapter of my life.

This dissertation steadily accompanied me over the years, even when my attention was sometimes on other matters. During this time, I completed my specialist training and obtained board certification in Internal Medicine and Rheumatology in 2021. Alongside my clinical work in rheumatology and night shifts, I have continued to be involved in scientific research and teaching activities in the field of Rheumatology.

During this period, I met my partner and, in 2023, was fortunate to become the mother of a wonderful daughter.

None of this would have been possible without the support of my family. My deepest gratitude goes to my partner Markus, my wonderful daughter Isabella, my parents Gabriele and Werner, my siblings Monika and Bernhard and my parents-in-law Stefanie and Maximilian.
This long journey has shown that perseverance pays off - or, as my grandmother always said:
„You only give up a letter."
„Aufgegeben wird nur ein Brief."

For completing my doctoral thesis and during my course of "medical science" I was enrolled in the doctoral school "sustainable health ". I received no financial funding for my doctoral thesis or the clinical study underlying this dissertation.

Table of Contents

List of Abbreviations

ADA: American Diabetes Association

AUC: Area under the curve

AUC_{EGP}: Area under the curve of endogenous glucose production

AUC_{GIR}: Area under the curve of gluco se infusion rate

$AUC_{glucagon}$: Area under the curve of glucagon

BMI: Body mass index

C-pos: C-peptide positive

C-neg: C-peptide negative

CRF: Case report form

ECG: Electrocardiogram

eCRFs: Electronical case report forms

EGP: Endogenous glucose production

ELISA: Enzyme linked immunosorbent assay

FPG: Fasting plasma glucose

GAD65: Glutamic acid decarboxylase 65

GC-MS: Gas chromatography–mass spectrometry

GIR: Glucose infusion rate

HAAF: Hypoglycaemia-associated autonomic failure

HbA1c: Hemoglobin A1c

HBsAg: Hepatitis B surface antigen

HIV: Human Immunodeficiency virus

IAA: Insulin autoantibodies

ICA: Islet cell autoantibodies

IFG: Impaired fasting glucose

IGT: Impaired glucose tolerance

I.V.: Intravenously

LOQ: Lower limit of quantification

PG: Plasma glucose

Ra: rate of glucose appearance

Rd: The rate of peripheral glucose disposal

RIA: Radioimmunoassy

SD: Standard deviation

SW: Shapiro-Wilk-Test

TTR: Tracer-to-tracee ratio

T1DM: Type 1 diabetes mellitus

T2DM: Type 2 diabetes mellitus

V: Visit

ZnT8: Zinc transporter 8

List of Figures

List of Tables

Abstract German

Einleitung:

Der Verlust der Betazellfunktion bei Typ-1-Diabetes (T1DM) erhöht das Risiko für schwere Hypoglykämien. Eine gestörte Signalübertragung zwischen Beta- und Alphazellen kann die Glukagonantwort während Hypoglykämien beeinträchtigen. Ziel der Studie war es, den Einfluss des C-Peptid-Status auf die Glukagonantwort und die daraus resultierende endogene Glukoseproduktion (EGP) bei einer Hypoglykämie in T1DM-Patient*innen zu untersuchen.

Material und Methoden:

Nach nächtlichem Fasten und intravenöser Insulininfusion zur Normalisierung der Plasmaglukose (PG), erhielten 10 C-Peptid-positive (C-pos) und 11 gematchte C-Peptid-negative (C-neg) T1DM-Patient*innen einen hyperinsulinämischen, stufenweise hypoglykämischen Clamp. Nach dem Steady-state (Baseline, Normoglykämie) durchliefen alle Patient*innen PG-Plateaus von 100, 63, 45 und 70 mg/dl. Die Glukagonkonzentrationen wurden mittels hochsensitiven Double-Sandwich-ELISA, die EGP mit einer stabilen Isotopen-Tracermethode ermittelt.

Ergebnisse:

Die Gesamt-Glukagonkonzentrationen waren in beiden Gruppen signifikant niedriger als in früheren Studien. Beim ersten PG-Plateau (100 mg/dl, high Insulin) waren die Glukagonspiegel supprimiert (C-pos: 3,3 ± 2,1; C-neg: 0,7 ± 0,8 pmol/l; Mittelwert ± SD). Während der Hypoglykämie zeigten C-pos bei den Plasmaglukose-Plateaus von 45 (C-pos: 9,9 ± 9,6; C-neg: 2,9 ± 3,0 pmol/l; p = 0,010) und 70 mg/dl (C-pos: 13,9 ± 5,9; C-neg: 5,7 ± 4,8 pmol/l; p = 0,002) eine signifikant stärkere Glukagonantwort als C-neg Patient*innen. Die EGP war ebenfalls bei C-pos Patient*innen bei beiden PG-Plateaus 45 (C-pos: 0,9 ± 0,6; C-neg: 0,5 ± 0,1 mg/kg/min; p = 0,037) und 70 mg/dl (C-pos: 2,1 ± 0,6; C-neg: 1,3 ± 0,7 mg/kg/min; p = 0,013) signifikant höher.

Schlussfolgerung:

Unsere Ergebnisse zeigen, dass beide Gruppen, während der normoglykämsichen Phase im Clamp, eine insulinabhängige Suppression der Glukagonspiegel hatten. Unter Hypoglykämie reagierten C-pos mit einer stärkeren Glukagonantwort und einer höheren EGP als die C-neg Patient*innen. Dies deutet darauf hin, dass die Alphazellen weiterhin auf Insulin und niedrige Glukosespiegel anspricht und dass eine verbleibende Betazellfunktion die Gegenregulation bei T1DM Patient*innen maßgeblich unterstützt.

Abstract English

Introduction:

Loss of beta-cell function in patients with type 1 diabetes mellitus (T1DM) is associated with an increased risk of severe hypoglycaemia. The resulting disruption of intra-islet signaling may lead to an insufficient glucagon response during hypoglycaemia. The aim of the study was to determine the impact of C-peptide status on the glucagon response and, as a consequence, on the endogenous glucose production (EGP) during hypoglycaemia in T1DM patients.

Material and Methods:

After normalisation of plasma glucose (PG) with an intravenously insulin infusion during an overnight fast, 10 C-peptide positive (C-pos) and 11 matched C-peptide negative (C-neg) T1DM patients underwent a hyperinsulinaemic, stepwise hypoglycaemic clamp. After steady-state (baseline, normoglycaemia), all patients underwent PG plateaus of 100, 63, 45 and 70 mg/dl. Glucagon concentrations were measured with a highly-sensitive double-sandwich ELISA. Endogenous glucose production (EGP) was assessed with a stable isotope tracer technique during the clamp.

Results:

In both groups, overall glucagon concentrations were significantly lower than previously reported. During the first PG plateau of 100 mg/dl, glucagon levels were suppressed in both groups (C-pos: 3.3 ± 2.1; C-neg: 0.7 ± 0.8 pmol/l; mean ± SD) due to high insulin concentrations. Hypoglycaemia triggered at PG plateau 45 mg/dl (C-pos: 9.9 ± 9.6; C-neg: 2.9 ± 3.0 pmol/l; $p = 0.010$) and at 70 mg/dl (C-pos: 13.9 ± 5.9; C-neg: 5.7 ± 4.8 pmol/l, $p = 0.002$) a significantly greater glucagon response in C-pos compared to C-neg patients. EGP was statistically significant higher at both PG plateaus 45 mg/dl (C-pos: 0.9 ± 0.6; C-neg: 0.5 ± 0.1 mg/kg/min, $p = 0.037$) and at 70 mg/dl (C-pos: 2.1 ± 0.6; C-neg: 1.3 ± 0.7 mg/kg/min, $p = 0.013$) in C-pos compared to C-neg patients.

Conclusion:

Our results revealed that during euglycaemia phase of the clamp, both groups had an insulin-dependent suppression of glucagon levels. Hypoglycaemia triggered a significantly more pronounced glucagon response and consequently, a higher EGP in C-pos compared to C-neg patients. This indicates that alpha-cells remain responsive to insulin and low glucose levels and that remaining beta-cell function may play a substantially role in counterregulation in T1DM patients.

1. Introduction

1.1. Type 1 Diabetes Mellitus

Type 1 diabetes mellitus (T1DM) is a chronic disease characterized by an immune-mediated loss of Langerhans' beta cells in the pancreas [1]. Progressive beta cell destruction leads to an absolute insulin deficiency, which occurs either within a few months or over several years [1]. Worldwide, the number of newly diagnosed individuals with T1DM is increasing [2,3], as is the number of patients dying from long-term consequences of this disease. Regular administration of subcutaneous insulin is therefore mandatory and essential for survival. However, insulin management is associated with a substantial risk of hypoglycaemia [2]. With increasing duration of T1DM, the risk of severe hypoglycaemia, marked by significant cognitive impairment, rises considerably and is critically linked to a substantially higher risk of premature death [4,5].

1.1.1. Epidemiology

The incidence of T1DM is globally increasing by about 2-3% per year [3,6,7]. It pathophysiology likely involves genetic, environmental, and immune factors [3,8]. More than 85% of T1DM cases are diagnosed under the age of 20 worldwide [9,10]. The incidence rate increases from birth, peaks at puberty between 10 and 14 years old, and stabilizes in young adulthood (between 15-29 years) [11]. However, T1DM can occur at all ages [12]. The destruction of beta cells is often slower in adults compared to youth, resulting in a delay in the need for insulin after diagnosis [13].
Although most common autoimmune diseases, such as systemic lupus erythematosus or Sjögren's disease, affect females more often than males, T1DM diagnoses are generally equally divided between females and males [14].

Various studies have described a correlation between genetic predisposition and the development of T1DM [15–21]. Specific HLA region genes (HLA-DQ, HLA-DR), which are involved in the pathogenesis of the autoimmune disease process, have been found to be associated with T1DM [15–17]. Classical twin studies have evaluated the impact of genetic factors in T1DM, and a genetic effect can be assumed, as the concordance rate is higher for

identical twins than for non-identical twins [22]. Despite this genetic predisposition, genetics alone do not account for the development of T1DM, as nearly 90% of newly diagnosed T1DM patients do not have a relative with diabetes [23].

Multiple trigger factors are associated with its development, as T1DM is a very heterogeneous disease [24]. Environmental factors, such as a seasonal pattern with increased rates in late autumn, winter, and early spring [25], as well as infant and adult diet, vitamin D deficiency, and reduced gut microbiome diversity, have been associated with T1DM [24]. Early-life exposure to infections with different viruses (e.g. enteroviruses) associated with islet inflammation is also suspected to play a role in the development of T1DM [26]. Additionally, reports of T1DM diagnoses are more common in countries with better public health systems and a greater diabetes infrastructure [27–29].

1.1.2. Development and Diagnosis of T1DM

The development of T1DM is a highly complex process that start years before first symptoms occur. This is supported by the fact, that typically, by the time of T1DM diagnosis, 40-70% of beta-cell function has already been destroyed [30,31]. As observed in other autoimmune diseases [32,33], specific autoantibodies are often detectable in T1DM patients years before the onset of symptoms [8,34]. Typical autoantibodies for T1DM are islet cell autoantibodies (ICA), autoantibodies to glutamic acid decarboxylase (GAD65), insulin autoantibodies (IAA), autoantibodies to tyrosine phosphatases IA-2 and IA-2b and autoantibodies to zinc transporter 8 (ZnT8) [1].
It has been shown that the risk of developing T1DM increases with the number of autoantibodies present [35]. Specifically, 15 % of children with a single autoantibody will develop T1DM during their lifetime, whereas the risk rises to 80% for children with two or more autoantibodies, who are likely to progress to T1DM within the next 15 years [35].

The pathogenesis of T1DM development has been described as a continuum in which different stages are passed through before the onset of symptoms (Figure 1) [36], leading to the following diagnostic criteria:

Stage 1 is defined as the presence of beta - cell autoantibodies (two or more islet autoantibodies) AND normoglycaemia,

Stage 2 is defined by the presence of beta-cell autoantibodies (≥ 2) AND dysglycaemia, without symptoms:

- Fasting plasma glucose (FPG) 100 - 125 mg/dl
- 2-hour plasma glucose (PG) 140 - 199mg/dl
- Hemoglobin A1c (HbA1c) 5.7-6.4% or ≥ 10% increase in HbA1C

Stage 3 is defined by the presence of beta-cell autoantibodies (≥ 2) AND clinical symptoms and signs of diabetes; typically leading to the clinical diagnosis of T1DM:

- FPG: ≥ 126 mg/dl OR
- 2-hour PG: ≥ 200 mg/dl OR
- HbA1c: ≥ 6.5% OR
- A random PG value: ≥ 200 mg/dl

Stage 4 is defined as long standing T1DM.

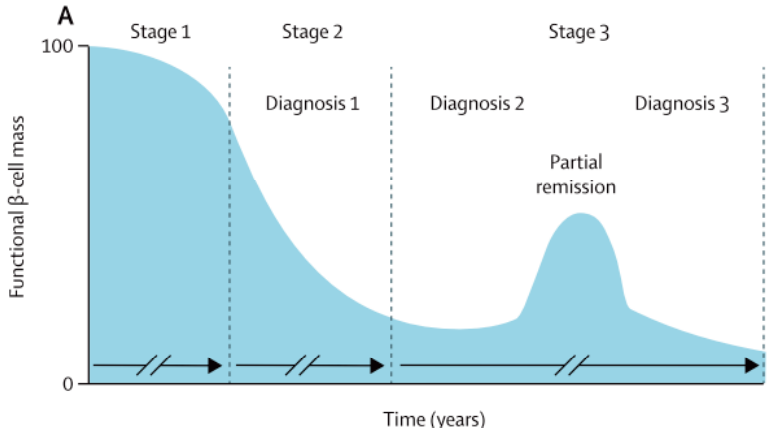

Figure 1 Staging of Type 1 Diabetes.
Reproduced from [3] with permission from the publisher Elsevier.

To assess endogenous insulin secretion and to support diagnostic differentiations, particularly between T1DM and type 2 Diabetes Mellitus (T2DM), the measurement of C-peptide concentration is frequently used. C-peptide is a 31-amino acid molecule cleaved from the precursor peptide proinsulin, making it a direct by-product of insulin biosynthesis. The

concentration of C-peptide correlates directly with endogenous insulin levels, and is therefore a useful marker for assessing endogenous insulin secretion.

In T1DM patients, C-peptide levels are typically reduced and progressively decline over the course of the disease and become extremely low or undetectable [37,38]. Additionally, fasting C-peptide measurement is a sensitive, well-accepted and clinically validated marker for evaluating beta-cell function in T1DM patients in clinical studies [5,37,39].

1.1.3. Pathogenesis

The pathogenesis of the pancreatic beta-cell destruction in T1DM remains not fully understood, but is believed to involve a complex interplay between beta-cells and both the innate and acquired immune systems [8,40,41]. In genetically predisposed individuals, it is assumed that an unknown trigger, likely involving one or more environmental factors, initiates an autoimmune process over several years that leads to the development of T1DM [42].

It is hypothesized that in the early phase the immune system triggers an insulitis, an inflammation of the pancreatic islets. Environmental factors such as viral infections may induce cellular stress in beta-cells. Innate immune cells detect molecular signatures of stress or infection through pattern recognition receptors. These receptors recognize either danger-associated molecular patterns released by stressed or dying beta-cells, or pathogen-associated molecular patterns derived from microbial pathogens such as viruses [42]. Recognition of these signals activates innate immune pathways, generating a proinflammatory microenvironment that facilitates subsequent activation of the adaptive immune response [42,43].

In response to these stimuli, beta-cells produce type 1 interferon, which triggers a proinflammatory cascade. This includes the recruitment of macrophages, neutrophils, and natural killer cells. Macrophages, as some of the first responders, secrete tumor necrosis factor, which activates NF-κB signaling pathway in beta-cells, a mechanisms known to promote apoptosis [42,44].

The inflammation in the pancreatic islets leads to increased vascular permeability, which facilitates the infiltration of naïve and non-islet-reactive T cells into the beta-cells. These T cells can become activated within the islets, further amplifying the autoimmune response [45].

Histologically, these infiltrates are located within and around the pancreatic islets, characterized by a predominance of CD8+ T lymphocytes and a lower presence of CD4+ T-cells [46].

Proinflammatory cytokines are released by these immune cells impair beta-cell metabolic function and viability [47].
As a counterregulatory response, beta-cells activate anti-inflammatory cytokines such as interleukin (IL)-10 and IL-4/IL-13, along with immune checkpoint molecules including PD-L1 and non-classical HLA class I molecules such as HLA-E. These protective adaptations persist throughout disease progression but ultimately fail to prevent ongoing beta-cell destruction [42,44,48].

1.1.4. Clinical Presentation of T1DM

The first clinical symptoms of T1DM are typically sudden and manifest as acute complications (within days to weeks) due to the beta-cell failure, leading to a loss of endogenous insulin secretion and resulting in hyperglycaemia (Stage 3). Over 95 % of newly diagnosed patients seek medical attention because of these symptoms, with only a minority being diagnosed during routine doctoral check-ups [34].
T1DM patients often present with polyuria, polydipsia, dehydration, weight loss, fatigue, visual disturbances, and an increased susceptibility to infections, all of which results from hyperglycaemia. Approximately one-third of the patients are diagnosed with life-threatening diabetic ketoacidosis [1,49].

After achieving normoglycaemia through exogenous insulin therapy, beta-cells can experience partial recovery in insulin secretion, leading to what is commonly referred to as the “honeymoon phase”. During this phase, patients may require very little, or even no, exogenous insulin for a period of time. The end of this partial remission is characterized by an increased need for exogenous insulin due to a more advanced beta-cell failure, resulting in absolute endogenous insulin deficiency. At this stage, exogenous insulin therapy then becomes essential and accompanies the patient for life [3].

A long-standing history of T1DM in these patients is associated with a higher risk of both microvascular and macrovascular complications, primarily due to the persistent hyperglycaemia [50,51]. Microvascular complications, which typically arise from the damage of the small blood vessels include nephropathy, neuropathy and retinopathy, which can lead to life-altering consequences such as kidney failure, loss of peripheral sensation or blindness. On

the other hand, macrovascular complications, resulting from damage of the lager blood vessels, can lead to a range of cardiovascular diseases such as peripheral vascular disease, cerebrovascular disease and ischemic heart disease, all of which contribute to an increased burden of morbidity and mortality [52].

Therefore, maintaining good glycaemic control remains a significant challenge for all patients with T1DM. At the same time, pursuing stringent glycaemic control can increase the frequency of hypoglycaemic events and, worse, can lead to life-threatening episodes of severe hypoglycaemia [53,54]. Additionally, the fear of hypoglycaemia often creates a barrier to achieve good glycaemic control, which, in turn, increases the risk of developing both micro- and macrovascular complications [55].

1.1.5. Hypoglycaemia

Iatrogenic Hypoglycaemia is a major limiting factor in achieving normoglycaemia in patients with T1DM [54]. The average T1DM patient has approximately two symptomatic hypoglycaemic events per week, which can lead to thousands of such episodes over a lifetime [56]. Additionally, patients experience an average of 0.2 to 3.2 severe hypoglycaemic episodes (requiring assistance due to unconsciousness) per year. While rare, some of these episodes can be fatal [54,57].

1.1.5.1. Definition and Classification

Hypoglycaemia is defined as "any episode of abnormally low PG concentration (with or without symptoms) that exposes the individual to potential harm" [58,59]. Although glycaemic thresholds for hypoglycaemia can vary among T1DM patients due to individual differences in symptom perception, it is generally agreed to classify hypoglycaemia into three levels based on blood glucose thresholds [60,61]:

- Level 1: Blood glucose level < 70 mg/dl but ≥ 54 mg/dl. This level should prompt the patient to initiate corrective action. It is considered the biochemical threshold, representing the lower limit of the physiological range of postabsorptive PG concentration and the point at which counterregulatory hormones are activated.

- Level 2: Blood glucose level < 54 mg/dl. This represents clinically significant hypoglycaemia and requires immediate action.
- Level 3: No specific blood glucose threshold. This level is classified as severe hypoglycaemia, an event that requires external assistance to actively administer carbohydrate, glucagon or other glucose-raising agents due to severe cognitive impairment.

Additionally, there are also clinical classification criteria for hypoglycaemia [58,62]:

1.) Documented symptomatic hypoglycaemia: An event characterized by typical symptoms of hypoglycaemia, with a measured glucose level of ≤ 70 mg/dl.

2.) Severe hypoglycaemia: An event characterized as hypoglycaemia that requires external assistance to treat due to severe cognitive impairment.

3.) Asymptomatic hypoglycaemia: An event characterized by the absence of typical hypoglycaemic symptoms, but with measured glucose levels of ≤ 70 mg/dl.

4.) Probable symptomatic hypoglycaemia: An event characterized with typical hypoglycaemic symptoms, but no glucose measurement was performed. The event is presumed to be caused by a PG level of ≤ 70mg/dl.

5.) Pseudohypoglycaemia: An event characterized with typical hypoglycaemic symptoms, but with measured glucose level of > 70 mg/dl.

1.1.5.2. Regulation of Glucose Homeostasis in Healthy Individuals

The brain depends almost entirely on glucose as its primary energy source and lacks the ability to synthesize or store it in any significant amount. Consequently, the glucose homeostasis in healthy individuals is carefully regulated through subtle, minute-to-minute changes in the secretion of the insulin and its counterpart, glucagon, to maintain stable blood glucose levels [55].

Insulin is secreted by the pancreatic beta-cells, which are surrounded by the glucagon-producing alpha cells, both located within the islets of Langerhans. These hormones act in opposition to each other to either increase or decrease the endogenous glucose production

(EGP), primarily throughout the liver (to a less extend by the kidneys) and decrease or increase the rate of peripheral glucose disposal (Rd) in the tissues, depending on the glucose need [56]. During overnight fasting, the liver of healthy individuals produces PG at a rate of approximately 2.2 mg/kg/min to maintain euglycaemia, mainly via glycogenolysis and gluconeogenesis [55,63]. It's production rate is equal to the body's Rd, with approximately 50-60% of the available glucose being consumed by the brain [55].

After meal ingestion, rising PG concentrations stimulate glucose entry into pancreatic beta-cells. Within these cells, the enzyme glucokinase phosphorylates glucose to glucose-6-phosphate, thereby acting as a glucose sensor and initiating the insulin secretion to lower blood glucose levels [64]. To restore normoglycaemia, insulin secretion suppresses the EGP by inhibiting glycogenolysis and promoting glycogen storage in the liver, which is the primary site of glucose uptake in healthy individuals. Insulin simultaneously enhances the Rd in the peripheral tissues, which is responsible for utilizing the remaining circulating glucose [65,66]. Additionally, insulin directly inhibits glucagon secretion by downregulating the glucagon gene expression in pancreatic alpha cells. The glucagon inhibition reduces EGP and enhances Rd, thereby contributing to the normalization of blood glucose levels and preventing from hyperglycaemia [67].

In contrast, to prevent or correct declining blood glucose in healthy individuals, such as during prolonged fasting, several physiological defence mechanisms have evolved [56,68]. The response of counterregulatory hormones begins even before the onset of hypoglycaemic symptoms [69].

Initially, within minutes, when PG declines in a physiological range (approximately 80 – 85 mg/dl), insulin secretion decreases (Table 1) [56,68].

Second, as PG reaches a threshold of approximately 70 - 75 mg/dl, glucagon secretion increases as the first-line counterregulatory hormonal defence. It increases the EGP through largely stimulating the glycogenolysis, and with little acute effect on gluconeogenesis and decreases the Rd [65,67]. The increase in glucagon secretion is triggered by a decrease in intra-islet insulin and potentially other beta-cell secretory products at low PG concentrations [56].

Third, when PG levels fall to approximately 65 to 70 mg/dl, concentrations of epinephrine and norepinephrine begin to increase. Elevated epinephrine levels stimulate the EGP through

glycogenolysis - the conversion of glycogen into glucose in the liver- as well as gluconeogenesis. It also decreases the Rd in peripheral tissues, suppresses insulin secretion and promotes lipolysis in adipose tissue, all contributing to the maintenance of blood glucose levels [68,70]. Norepinephrine levels support epinephrine levels during hypoglycaemia by also stimulating glycogenolysis, although to a lesser extent than epinephrine levels and additionally suppresses insulin secretion. Additionally, norepinephrine levels exert vasoconstrictive effects, which raise blood pressure to ensure adequate blood flow of vital organs during hypoglycaemia [65].

Table 1 Overview of Physiological Defence Mechanisms to Avoid Falling Plasma Glucose in Healthy Individuals.

Response	Glycaemic threshold (mg/dl [mmol/l])	Physiological effects	Role in prevention or correction of hypoglycemia (glucose counterregulation)
↓ Insulin	80 – 85 [4.4-4.7]	↑Ra, (↓ Rd)	Primary glucose regulatory factor, first defense against hypoglycemia
↑ Glucagon	65 – 70 [3.6-3.9]	↑Ra	Primary glucose counterregulatory factor, second defense against hypoglycemia
↑ Epinephrine	65 – 70 [3.6-3.9]	↑Ra, ↓ Rd	Involved, critical when glucagon is deficient; third defense against hypoglycemia
↑ Cortisol and growth hormones	65 – 70 [3.6-3.9]	↑ Ra, Rd ↓	Involved, not critical
Symptoms	50 – 55 [2.8-3.1]	↑ Exogenous glucose	Prompt behavioural defence (food ingestion)
↓ Cognition	< 50 [< 2.8]	-	(Compromises behavioural defense)

Source: Originally published in Cryer PE. Glucose homeostasis and hypoglycemia. In: Kronenberg HM, Melmed S, Polonsky KS, Larsen PR, editors. Williams Textbook of Endocrinology. 11th ed. Philadelphia: Saunders; 2008. p. 1503–1533 [71]. Reproduced in: Cryer PE. The Barrier of Hypoglycemia in Diabetes. Diabetes [56] and used with permission of the American Diabetes Association.
Abbreviations: Ra: rate of glucose appearance (endogenous glucose production); Rd: the rate of peripheral glucose disposal

If hypoglycaemia persists for a longer period, other counterregulatory hormones such as cortisol and growth hormones are released, further increasing EGP and inhibiting Rd to increase blood

glucose [56]. Additionally, fat mobilization increases and results in an excess of free fatty acids being delivered to the liver, where they are converted into ketone bodies from acetyl-CoA, a by-product of long-chain fatty acid metabolism. These ketone bodies are primarily used as fuel by skeletal muscles and the heart. Under extreme conditions, the brain can also rely for energy on them [72].

Healthy individuals start to ingest carbohydrates as a normal behavioural response to declining blood glucose levels. They experience typical neurogenic (autonomic) symptoms, which are primarily driven by the sympathetic nervous system. The brain detects low blood glucose levels and, as a defensive mechanism, induces hunger, sweating, anxiety, palpitations, and tremors — all signs that encourage the healthy individuals to ingest glucose, in order to normalize the blood glucose levels [65,73]. Thanks to these effective defense mechanisms, hypoglycaemia is an uncommon clinical event in healthy individuals [65].

1.1.5.3. Pathophysiology of Hypoglycaemia in T1DM

In patients with T1DM, the physiological defence mechanisms against falling PG levels are often impaired [56] (Table 2). Typically in T1DM, hypoglycaemia results from an interplay of iatrogenic insulin excess, often due to an overestimation of insulin requirements, combined with insufficient carbohydrate intake and increased physical activity [65,74].

The first mechanism to hypoglycaemia, suppression of insulin secretion, is absent in T1DM due to the autoimmune destruction of the pancreatic beta cells. As a result, exogenously administer insulin continues to circulate even when PG levels decline [75,76].
Secondly, the glucagon response to hypoglycaemia is impaired and can be even entirely absent in patients with T1DM [76]. This leads to an insufficient increase in EGP due to inadequate stimulation of glycogenolysis and gluconeogenesis, along with a failure to reduce the rate of glucose disposal (Rd), all essential mechanisms for restoring euglycaemia [75,76].
Finally, the third counterregulatory mechanism - the catecholamine response of epinephrine and norepinephrine - becomes increasingly important when insulin and glucagon responses are compromised. However, this counterregulatory response is also frequently blunted in T1DM patients, limiting the ability to restore normoglycaemia during hypoglycaemia [75,76]. A diminished epinephrine response to hypoglycaemia is also a key marker of reduced

sympathoadrenal activity and plays a central role in the development of impaired hypoglycaemia awareness [77]. T1DM patients affected by this condition have a sixfold increased risk of experiencing severe hypoglycaemia [56,78].

Table 2 Overview of Impaired Counterregulatory Response to Hypoglycaemia in T1DM Patients.

Response	Glycaemic threshold (mg/dl)	Impaired Response in T1DM	Impact on Patient Care
Insulin	80-85	Absent; exogenous insulin can't rapidly reduce	Prolongs hypoglycaemia
Glucagon	65-70	Impaired or even lost No increase of EGP No reduction of Rd	Major contributor to sever hypoglycaemia
Epinephrine	65-70	Impaired with recurrent hypoglycaemia (HAAF)	Impaired awareness; increasing risk of severe hypoglycaemia
Cortisol and growth hormones	65-70	Diminished response in C-neg patients	Affects recovery from prolonged hypoglycaemia and glucose stability
Symptoms	50 - 55	Lost; no exogenous glucose intake	Prolongs hypoglycaemia

Source: Table created based on data from Cryer PE. The Barrier of Hypoglycemia in Diabetes. Diabetes [56].
Abbreviations: EGP: endogenous glucose production; Rd: the rate of peripheral glucose disposal; HAAF: hypoglycaemia- associated autonomic failure

When all these physiological defense mechanisms against hypoglycaemia are impaired in T1DM patients, the risk of hypoglycaemic events rises substantially [74]. T1DM patients are not only at a higher risk of developing hypoglycaemia, but they also have a 25-fold increased risk of recurrent severe episodes. Such episodes are a serious threat to patient safety, carrying the potential for acute cognitive dysfunction, seizures, loss of consciousness, and, in extreme cases, death [79].

Although significant progress has been made in understanding the impaired counterregulatory mechanism during hypoglycaemia in T1DM, the underlying pathophysiological mechanisms remain not fully understood [75,76,80–82]. The loss of glucagon response to hypoglycaemia,

first described in 1973 [76], has been consistently observed in T1DM patients and is strongly associated with longer disease duration [75,80,83]. Evidence suggests that the glucagon response may already be impaired just months after the diagnosis of T1DM [81]. Hofman et al. demonstrated that even at the time of T1DM diagnosis, children and adolescents had already lost the glucagon response to hypoglycaemia, comparable to levels observed in patients with long-term T1DM [82].
However, it is still unclear when the glucagon response remains lost. This is of particular clinical importance, as the loss of glucagon response is thought to be irreversible; unlike to the epinephrine response and hypoglycaemia symptom awareness, which may improve with strict hypoglycaemia avoidance [84].

Paradoxically, patients with T1DM often exhibit elevated plasma glucagon levels during normoglycaemia, a phenomenon known as hyperglucagonemia, which correlates with higher blood glucose levels despite similar plasma insulin levels compared to healthy individuals [85,86]. Furthermore, inappropriate postprandial glucagon secretion following oral glucose intake has been observed to contribute to postprandial hyperglycaemia in T1DM patients [87,88].

The mechanisms underlying the impaired glucagon response during hypoglycaemia remain unclear. In healthy individuals, intra-islet signalling of Langerhans between alpha and beta cells tightly regulates insulin and glucagon secretion in response to changes in PG levels [89]. In T1DM, it is assumed that the beta-cell destruction disrupts this intra-islet communication, particularly the local insulin-mediated inhibition of glucagon release. This may contribute to an impaired ability of the alpha cells to respond appropriately to both normo- and hypoglycaemia [65,90]. Additionally, this dysfunction may involve a reduced sensitivity of alpha cells to both insulin and glucose levels [86,87,91].
Hyperinsulinaemic hypoglycaemic clamp studies [92,93] support the concept of insulin-insensitive alpha cells in T1DM, demonstrating that glucagon concentrations fail to be suppressed under normoglycaemic conditions despite high insulin levels (exogenous administered) — an effect that would be normally expected. These findings suggest that the alpha cells in T1DM no longer respond appropriately to insulin and may become functionally insensitive [86].

Therefore, adequate sympathetic nervous system activation, sufficient catecholamine release, and intact recognition of hypoglycaemia and its associated hypoglycaemic symptoms are crucial for protecting T1DM patients from hypoglycaemic episodes [94]. While newly diagnosed T1DM patients generally retain an intact epinephrine and norepinephrine responses to hypoglycaemia, these counterregulatory mechanisms may diminish over time, increasing the risk of cognitive dysfunction during hypoglycaemic episodes [73,80,95].

1.1.5.4. Clinical Manifestations and Hypoglycaemic Unawareness

Normally, declining blood glucose levels trigger typical hypoglycaemic symptoms in T1DM patients, prompting them to ingest carbohydrates to raise their blood glucose levels. Hypoglycaemic symptoms can be categorized in neuroglycopenic and neurogenic symptoms [96]. Neuroglycopenic symptoms are directly caused by glucose deprivation in the brain and can manifest as cognitive impairments, behavioural changes, and, at lower PG levels, seizure and coma. Neurogenic (or autonomic) symptoms arise from physical changes induced by the sympathoadrenal system (mostly the sympathetic neural activity) during hypoglycaemia. Neurogenic symptoms include palpitations, tremor, anxiety/arousal, while cholinergic neurogenic symptoms can be recognized by sweating, hunger and paraesthesia [96].

However, in T1DM patients, the awareness of hypoglycaemia is often impaired or even completely lost. They may not perceive or recognize low or declining blood glucose levels, which is particularly dangerous, as it increases the risk of severe hypoglycaemia occurring without any adequate behavioural response. Studies suggests [78,97] that approximately 50% of patients with T1DM experience significantly diminished perception of hypoglycaemic symptoms after 15 to 20 years of disease duration.

The aetiology of hypoglycaemic unawareness in T1DM is considered to be multifactorial [78]. One of the major risk factors is the recurrence of hypoglycaemic episodes, particularly those involving severe hypoglycaemic events, which progressively blunt both hypoglycaemic symptom awareness and the hormonal counterregulatory response. This, in turn, initiates a vicious cycle of recurrent hypoglycaemic episodes in affected patients [98].
This phenomenon is referred to as the hypoglycaemia-associated autonomic failure (HAAF), a clinical syndrome characterized by defective hormonal counterregulation and impaired

hypoglycaemia awareness, typically triggered by recurrent episodes of hypoglycaemia [73,90]. While the exact mechanisms are not fully understood, it is believed that disease progression attenuates the sympathoadrenal response—comprising catecholamine secretion and sympathetic nervous system activity—with the neural component being primarily affected [90]. Additionally, hypoglycaemic-induced alterations in central hypothalamic pathways have been proposed [90].

As a consequence, many T1DM patients develop a lower glycaemic threshold for sympathoadrenal activation and autonomic symptom onset. This shift leads to delayed or absent hypoglycaemic warning symptom and ultimately contributes to the clinical manifestation of hypoglycaemic unawareness [78,90].

Other risk factors for hypoglycaemic unawareness are a long diabetes duration and poorly controlled diabetes over time. Moreover, certain medications, such as beta-blockers and antidepressants, may also mask typically hypoglycaemic symptoms [8].

Interestingly, avoiding hypoglycaemia for 2-3 weeks by increasing the overall target blood glucose levels can improve hypoglycaemia unawareness and enhance the diminished epinephrine response in T1DM patients during hypoglycaemia [99]. Additional education, medical support, and technical devises such as continuous glucose monitoring devises, can also help improve hypoglycaemia unawareness [4].

1.1.5.5. Treatment of Hypoglycaemia

When using insulin, T1DM patients must be educated on how to recognize and appropriately treat hypoglycaemic episodes. In most cases, T1DM patients are able to self-manage mild to moderate hypoglycaemia. At the onset of hypoglycaemic symptoms, it is essential to confirm the condition by measuring blood glucose levels. If the blood glucose is below 70 mg/dl, treatment with 15-20 g of fast-acting carbohydrate is typically recommended to reduce symptoms and increase the blood glucose. After 15-20 minutes, blood glucose should be then re-evaluated, and the treatment repeated if hypoglycaemia persists. To prevent recurrence of hypoglycaemia, the intake of long-acting carbohydrate afterwards may be advisable, particularly if the next meal is not expected soon [100].

In cases of unconsciousness (e.g., due to severe hypoglycaemia) or when patients are unable to ingest glucose, parenteral therapy is necessary. In clinical settings, the preferred approach is the administration of intravenous glucose after establishing venous access. In out-of-hospital situations or when the iv. access is not possible, glucagon administration (via subcutaneous, intramuscular, or nasal route) is recommended, as it works by stimulating the EGP. Consciousness typically returns within 15 minutes after glucagon administration [74].

1.1.5.6. Risk Factors for Developing Hypoglycaemia in T1DM Patients

There are several risk factors contributing to the development of hypoglycaemia in patients with T1DM [56,74]. One important factor is incorrect insulin dosage. Another is a reduced supply of exogenous glucose, for example through inadequate, delayed or missed meals, or an overnight fast. Additionally, glucose utilization and insulin sensitivity may increase, such as after weight loss, improved blood glucose control, or during and shortly after physical activity. Further, EGP may be reduced due to alcohol consumption or liver dysfunction, and insulin clearance may be impaired due to renal dysfunction. Moreover, diabetic autonomic neuropathy significantly increases the risk of hypoglycaemia.
Various medications, as well as factors like older age, cognitive impairment including dementia, and poor diabetes education further contribute to increase the risk of hypoglycaemic events. However, hypoglycaemia rarely occurs due to a single risk factor; rather, it typically results from the interplay of multiple risk factors acting together [54,56].

Of particular clinical importance, as previously discussed, is recurrent hypoglycaemia, as it markedly increases the risk of subsequent severe hypoglycaemic episodes in T1DM patients and may contribute to a deterioration in overall metabolic control and quality of life [101]. In addition, there is a well-documented association between intensive glycaemic targets, lower individualized blood glucose thresholds, and an increased frequency of hypoglycaemic events in T1DM patients [56,57,73]. Behavioural factors, such as fear of hypoglycaemia, may also contribute to fluctuating blood glucose levels and increase the overall risk [56].
Interestingly, the absence of residual endogenous insulin secretion, reflected by C-peptide negativity, and a long duration of T1DM diabetes have been identified as risk factors for hypoglycaemic events [57].

1.1.5.7. Rationale for Performing the Study

Previous studies have demonstrated that the risk and severity of hypoglycaemic events in T1DM patients correlate with the disease duration [5,57,76,80]. The UK Hypoglycaemia Study Group observed that the duration of T1DM did not influence either the prevalence or incidence of mild hypoglycaemia, but patients with short disease duration (C-peptide positivity), had less severe hypoglycaemic episodes compared to those with long-standing T1DM (C-peptide negative) [57].

The duration of T1DM is correlated with a progressive decline in residual beta cell function, indicating that the risk of severe hypoglycaemia increases as beta cell function diminishes [5,101–103]. The progressive loss of beta cell function may contribute to the impairment of the essential glucagon response during hypoglycaemia, further exacerbating the risk of severe hypoglycaemic events. This impairment may also lead to a reduced EGP, compromising a critical counterregulatory mechanism to restore blood glucose levels. Moreover, C-peptide negativity is considered an important risk factor for the development of severe hypoglycaemic events [83]. However, the underlying mechanism by which residual beta-cell function provides protection against severe hypoglycaemia in patients with T1DM is not fully understood, and the potential influence of C-peptide status on the EGP during hypoglycaemia remains to be further investigated [5].

The interpretation of an impaired glucagon response during hypoglycaemia is further complicated by the variability and specificity of the available glucagon assays. Over time, different glucagon assays have been used to measure glucagon levels in T1DM. Analytical methods have improved, but commonly used assays are mostly nonspecific and also detect N-terminally extended or truncated forms of glucagon. These sometimes erroneously high glucagon levels may have compromised the interpretations of the role of glucagon inT1DM patients [5,104,105].

However, more advancement have led to development of a specific and highly sensitive glucagon assay that allows the specific detection of intact glucagon, which may enables a reassessment of glucagon dynamics in T1DM patients [104,106].

1.2. Aim

The aim of the study was to determine the impact of C-peptide status on the glucagon response and, as a consequence, on the EGP during hypoglycaemia in patients with T1DM [5].

For this purpose, we performed a standardised hyperinsulinaemic stepwise hypoglycaemic clamp in both C-pos and C-neg T1DM patients. Glucagon levels were measured using a specific and highly sensitive double-sandwich enzyme linked immunosorbent assay (ELISA). For assessing the EGP, a stable isotope tracer technique was applied during the hypoglycaemic clamp [5].

2. Material and Methods

2.1. Study design

It was an open, non-randomized, single-centre study initiated at the Division of Endocrinology and Diabetology, at the Medical University of Graz, Austria. The informed consent and the study protocol were written in accordance with Good Clinical Practice [107] and the Declaration of Helsinki [108] and approved by the local ethics committee of the Medical University of Graz (26-070 ex 13/14) [5].

2.2. Recruitment

An electronical diabetes database, created by the Division of Endocrinology and Diabetology at the Medical University of Graz, was used for recruitment. The database included both T1DM and T2DM patients, who were interested in potential participation in clinical studies, performed at the division and provided written informed consent for the storage of their personal data. From over 1000 potential individuals with T1DM, a total of 10 C-pos and 11 C-neg T1DM were selected. These groups were matched for demographic (age, gender) and metabolic characteristics [(weight, body mass index (BMI)] [5].

2.3. Subjects

2.3.1. Informed Consent

The informed consent (attached in the appendix) was obtained from all subjects before any study related activities were started. Potential subjects were provided with oral and written information about the study and the procedures involved, in accordance with local requirements. Subjects were fully informed of their responsibilities and their rights while participating in the study and of all the procedures involved as well as the possible risks and benefits of participation in the study. Subjects had to have the opportunity to ask questions and they had ample time to consider participation. If the subjects wished to participate in the study, they were asked to personally sign and date an informed consent form prior to any study-related activities. Likewise, the investigator had also to sign the informed consent form prior to any

study-related activities. All subjects were provided with a copy of their own signed and dated informed consent form. [5].

2.3.2. Inclusion Criteria at Screening Visit

1. Informed consent obtained before any study-related activities. Study-related activities are any procedures that were carried out as part of the study, including activities to determine suitability for the study.
2. Male or female, aged 18 – 64 years (both inclusive).
3. T1DM (as diagnosed clinically).

4a. Fasting basal C-peptide concentrations of ≥ 0.05 nmol/l for C-pos T1DM patients.

4b. Fasting basal C-peptide concentrations below the lower limit of quantification (LOQ = 0.017 nmol/l) [109] for C-neg T1DM patients.

5. BMI 18.0 - 28.0 kg/m2 (both inclusive).
6. HbA1c 42 – 80 mmol/mol (6.0-9.5 %).
7. Treated with daily insulin injections or continuous s.c. insulin infusion (CSII) [5].

2.3.3. Exclusion Criteria at Screening Visit

1. Known or suspected hypersensitivity to study product(s) or related products.
2. Recurrent severe hypoglycaemia (more than 1 severe hypoglycaemic event during the last 12 months) as judged by the investigator.
3. Hypoglycaemia unawareness as judged by the investigator.
4. Clinically significant abnormal haematology, biochemistry, lipids, or urinalysis or coagulation screening tests, as judged by the investigator and any of the following laboratory safety results:
    a. ASAT, ALAT, lipase, alkaline phosphatase > 2.0 times upper limit of reference range.
    b. Haemoglobin < 8.0 mmol/L (male) or < 6.4 mmol/L (female), total leukocyte count < 3.0 x 109/L, thrombocytes <100 x 109/L.
    c. Serum creatinine levels ≥ 126 μmol/L (male) or ≥ 111 μmol/L (female).
5. Suffer from or history of a life-threatening disease (e.g. cancer except basal cell skin cancer or squamous cell skin cancer), or any clinically significant respiratory, metabolic,

renal, hepatic, gastrointestinal, endocrinological (with the exception of diabetes mellitus and euthyroid struma), haematological, dermatological, venereal, neurological, psychiatric diseases or other major disorders as judged by the investigator.

6. Cardiac problems defined as decompensated heart failure (New York Heart Association class III and IV) at any time and/or angina pectoris within the last 12 months prior to screening and/or acute myocardial infarction at any time.
7. Supine blood pressure at screening (after resting for 5 min) outside the range of 90−140 mmHg for systolic or 50 − 90 mmHg for diastolic (repeated measurement on a second screening visit allowed to exclude white-coat hypertension). This exclusion criteria also pertained to subjects being on antihypertensive drugs.
8. Clinically significant abnormal electrocardiogram (ECG) at screening, as judged by the investigator.
9. Proliferative retinopathy or maculopathy and/or severe neuropathy, in particular autonomic neuropathy, as judged by the investigator.
10. Any disease or condition that, in the opinion of the investigator, would represent an unacceptable risk for the subject's safety.
11. Patients positive for hepatitis B surface antigen (HBsAg) or hepatitis C antibodies (or diagnosed with active hepatitis according to local practice).
12. Positive result of the screening test for Human Immunodeficiency Virus (HIV)-1 antibodies, HIV-2 antibodies and/or HIV-1 antigen according to locally used diagnostic testing.
13. History of multiple and/or severe allergies to drugs or foods or a history of severe anaphylactic reaction.
14. Subject who donated any blood or plasma in the past month or more than 500 mL within 3 months prior to screening.
15. Surgery or trauma with significant blood loss (more than 500 mL) within the last 3 months prior to screening.
16. Current treatment with systemic [oral or intravenously (i.v.)] corticosteroids, Monoamine Oxidase inhibitors, nonselective beta-blockers, growth hormone, herbal products or non-routine vitamins. Furthermore, thyroid hormones were not allowed unless the use of these has been stable during the past 3 months prior to screening.

17. Significant history of alcoholism or drug/chemical abuse as per investigator's judgement or a positive result in the drug/alcohol screen at the screening visit.
18. Smoker (defined as a subject who was smoking more than 5 cigarettes or the equivalent per day).
19. Not able or willing to refrain from smoking and use of nicotine gum or transdermal nicotine patches during the inpatient period.
20. Subject with mental incapacity or language barriers precluding adequate understanding or co-operation or who, in the opinion of the investigator, should not participate in the study.
21. Potentially non-compliant or uncooperative during the study, as judged by the investigator.
22. Any condition that would interfere with study participation or evaluation of results, as judged by the investigator.
23. Female of child-bearing potential who was pregnant, breast-feeding or intend to become pregnant or was not using adequate contraceptive methods (adequate contraceptive methods include sterilisation, hormonal intrauterine devices, oral contraceptives, sexual abstinence or vasectomised partner).
24. Use of drugs, which may interfere with the interpretation of study results or were known to cause clinically relevant interference with insulin action, glucose utilisation, or recovery from hypoglycaemia.
25. Severe acute and/or chronic diseases.
26. Diseases of the skin which could interfere with application of the catheters as judged by the investigator.
27. Receipt of any investigational medicinal product within 3 months before randomisation in this study [5].

2.3.4. Exclusion Criteria at Hypoglycaemic Clamp Day (Day 1, Visit 2)

1. Strenuous exercise later than 10:00 pm, 1 day before day 1, visit 2.
2. Consumption of alcohol later than 10:00 pm, 1 the day before day 1, visit 2.
3. Positive result of alcohol breath test on day 1, visit 2.

4. Consumption of coffee, tea, chocolate or beverages (such as cola and energy drinks) containing methylxanthine (caffeine, theophylline or theobromine) later than 10:00 pm, 1 day before day 1, visit 2.
5. Non-fasting (i.e. consumption of food or beverages, other than water, later than 05:00 pm on the day of the visit) expect if slight intake of rapidly absorbable carbohydrates (not more than 20g carbohydrates) has been necessary in order to prevent hypoglycaemia (if carbohydrates are ingested, subjects have to confirm by a blood glucose measurement that they are not hypoglycaemic).
6. Injection of long-acting insulin, Neutral Protamine Hagedorn insulin or any other intermediate-acting insulin products later than 08:00 am on day 1, visit 2.
7. Injection of short acting insulin later than 05:00 pm on day 1, visit 2.
8. Using subcutaneous infusion of insulin later than 05:00 pm for subjects using continuous subcutaneous insulin infusion on day 1, visit 2.
9. A hypoglycaemic event defined with PG ≤ 70 mg/dl later than 10:00 am on day 1, visit 2.
10. Not in stable glycaemic control at visit 2 as judged by the investigator.
11. Presence of any medical conditions that may confound the results of the study, as judged by the investigator.
12. Any use of systemic (oral, i.v. or inhaled) corticosteroids, monoamine oxidase inhibitors, prostaglandin blockers, systemic non-selective beta-blockers, growth hormone, herbal products or non-routine vitamins. Furthermore, thyroid hormones are not allowed unless the use of these has been stable during the past 3 months [5].

2.3.5. Stopping and Discontinuation Criteria

The subjects were advised in the informed consent forms that they had the right to withdraw from the study without prejudice and might be withdrawn at the investigator's discretion. A subject had to be withdrawn if the following applied:

1. Withdrawal of consent: subjects had the right to withdraw from the study at any time for any reason.
2. Pregnancy.
3. Intention of becoming pregnant.
4. The subject donated any blood during the study [5].

2.3.6. Study Schedule

The patients were invited to screening visit (visit 1), a hypoglycaemic clamp visit (visit 2: day1 and day 2) and a follow up visit (visit 3) (Table 3). The total study duration for each patient was approximately one month. Patients received a financial compensation for their time and participation on the study [5].

Table 3 Overview of Study Procedures

Study periods	**Informed Consent**	**Screening Visit**	**Hypoglycaemic Clamp Visit**		**Follow Up Visit**
			Day 1	Day 2	
Visit number		V1	V2		V3
Visit window		3-21d before V2	0		3 – 10 d after V2
SUBJECT RELATED INFO/ASSESSMENTS					
Informed consent	X				
In/exclusion criteria		X			
Clamp day exclusion criteria			X		
Demography		X			
Medical history		X			
Diagnosis of diabetes and diabetes treatment history		X			
Current diabetes treatment		X	X		X
Concomitant illness		X	X		X
Concomitant medication		X	X		X
Abuse of drugs, alcohol and smoking habits		X			
Hypoglycaemic clamp			X		
EFFICACY					
Plasma glucose			X	X	
Fasting C-peptide		X			
[6,6-$^{2}H_2$]-glucose				X	
Glucagon				X	

Epinephrine				X	
Norepinephrine				X	
Hypoglycaemic awareness				X	
Hypoglycaemic symptoms questionnaire				X	
SAFETY					
Hypoglycaemic episodes			X		X
Body measurements		X	X		X
Physical examination		X	X		X
Vital signs		X	X	X	X
ECG		X	X	X	X
Alcohol breath test		X	X		
Biochemistry		X			X
Haematology		X			X
Hepatitis		X			
Coagulation parameters		X			
HbA1c		X			
HIV		X			
Lipids		X			X
Urinalysis		X			X
Pregnancy test		X	X		X
Screen for drugs		X			
Fundoscopy		X			

Abbreviations: Visit: V; ECG: Electrocardiogram; Haemoglobin A1c: HbA1c; Human Immunodeficiency virus: HIV

2.3.7. Screening Visit (Visit 1)

The screening visit took place 3-21 days prior to visit 2. The subject was asked to attend the screening visit fasting (only water from 10 pm before the screening visit; minimum 9 hours). A rescheduling of the screening visit (within 1-7 days) was allowed once, in case the subject had failed to fast [5].

At screening visit, all inclusion and exclusion criteria were checked. Demographic data including date of birth, sex, ethnicity and race were obtained. The medical history, including

the diagnosis of T1DM, current medications (including diabetes treatment), and previous diabetes treatment history, were collected. History of drug abuse, alcohol and smoking habits were obtained. Data of body measurements including body weight, height and BMI, a physical examination, vital signs (blood pressure and heart beat), an ECG and an alcohol breath test were obtained from each patient. Blood was collected for laboratory examination for safety assessment (haematology, biochemistry, lipids, coagulation), and for analyses of HbA1c, fasting C-peptide concentrations, HBsAg, hepatitis C antibodies and HIV antibodies and HIV antigen. Urine was collected for urinalysis, for drug screening and in women of childbearing potential for pregnancy test. Additionally, patients had to bring a doctor's certificate of a fundoscopy, not older than 6 months, to exclude diabetic long-term complications, like diabetic retinopathy. Screened subjects who did not comply with all inclusion and exclusion criteria were excluded from the study [5].

2.3.8. Hypoglycaemic Clamp Visit (Visit 2)

At visit 2, patients underwent a hyperinsulinaemic, stepwise hypoglycaemic clamp with a stable isotope trace technique (Figure 2) [5].

The hyperinsulinaemic hypoglycaemic clamp is a widely accepted standardized methodology for investigating the effects of varying hypoglycaemia levels by setting predefined blood glucose thresholds to assess counterregulatory hormone responses in patients [110].

To assess whole-body glucose fluxes, including both EGP and Rd, during the hypoglycaemic clamp [5,111,112], a stable isotope tracer technique was applied according to Powrie et al. [113] and Steele et al. [114]. Tracer infusion rates were selected to maintain constant blood tracer enrichment at baseline and throughout the clamp, thereby avoiding errors caused by tracer mixing transients. The chosen tracer infusion rates allowed to achieve an average blood tracer enrichment of 4% both during baseline and the hypoglycaemic clamp procedure [5].

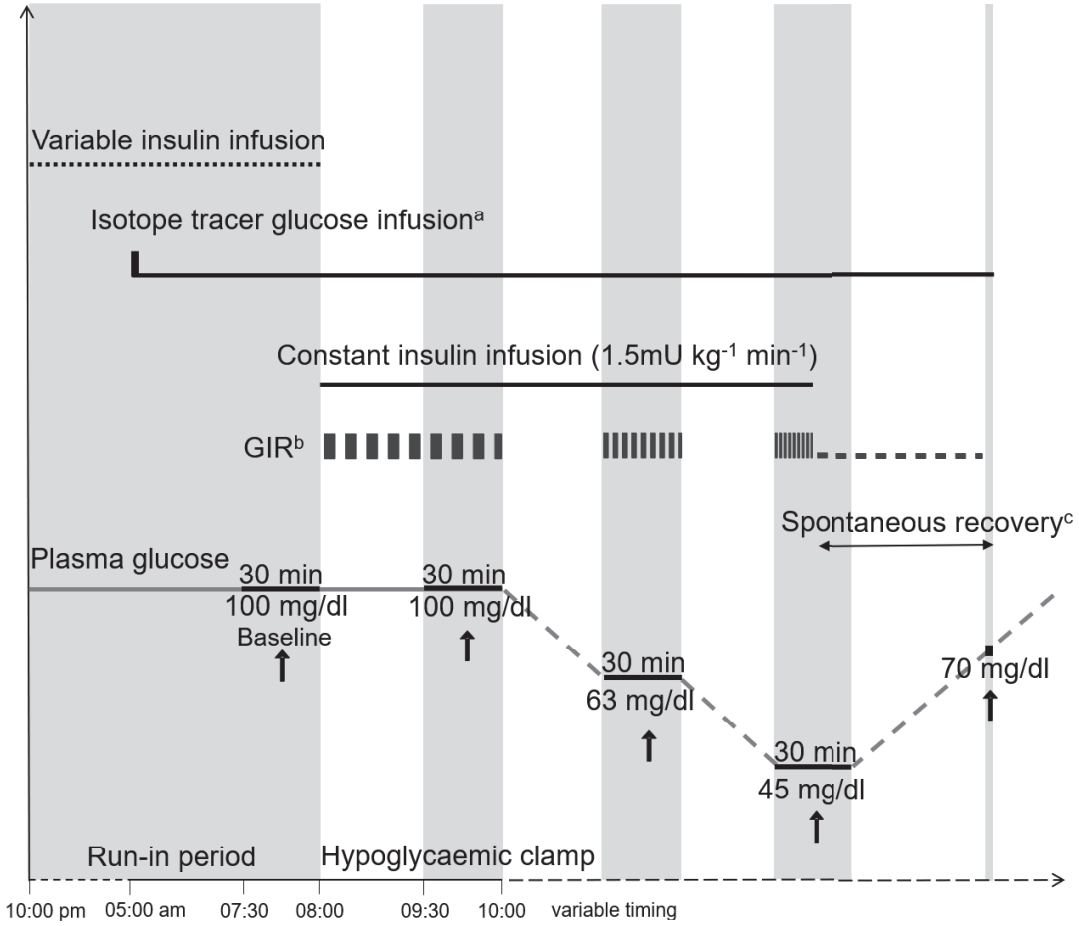

Figure 2 Hypogylcaemic Clamp Design with a Stable Isotope Tracer Technique.
Reproduced from [5] and adapted with permission of publisher Oxford University Press on behalf of the Endocrine Society.

a. Isotope tracer glucose infusion - 9.6 mg/kg/min of [6,6-2H2]-glucose was given i.v. for one minute and a constant rate of 0.08 mg/kg/min i.v. until the end of the clamp.
b. Glucose infusion rate (GIR) – was enriched with 4 mg [6,6-$^{2}H_{2}$]-glucose/ml.
c. Spontaneous glucose recovery – defined as the time at PG plateau 45 mg/dl – 15 min after insulin infusion (1.5 mU/kg/min) was stopped – until the end of the PG plateau 70 mg/dl, time point 10. 45 minutes was the maximum time to reach the next PG plateau 70 mg/dl, otherwise a glucose infusion was administered to increase the PG to the next PG plateau

↑ Hypoglycaemic response assessments were performed at each PG plateau and included blood sampling for glucagon and [6,6-$^{2}H_{2}$]-glucose concentrations, vital signs, hypoglycaemic awareness and hypoglycaemic symptoms tests.

On the evening (day 1) prior to the study day, each subject arrived at approximately 09:00 pm at the Clinical Trials Unit, at the Medical University of Graz, for an in-house stay for approximately 16 hours. All subjects had been advised no to inject long-acting or intermediate-acting insulin after 08:00 and not to inject short-acting insulin later than 05:00 pm on day 1. Last food intake was allowed at 05:00 pm, except from water at day 1. No hypoglycaemic events (PG level < 70 mg/dl) were allowed on day 1 [5].

Before any study procedures were started, hypoglycaemic clamp exclusion criteria were checked. Concomitant illness, current medications including diabetes treatment, and hypoglycaemic episodes since the last visit were assessed. Physical examination including documentation of vital signs (blood pressure, pulse and body temperature) and body weight were performed. ECG and an alcohol breath test were obtained. In women of childbearing potential, a urine analysis for pregnancy was done [5].

During the clamp, subjects remained fasting with the exception of water intake and stayed during the clamp in a supine or semi-supine position. A hand or antecubital vein of one arm was cannulated and the arm remained in a heating blanket throughout the clamp procedure for sampling of arterialised venous blood. The heating of the hand resulted in an arterialisation of the venous blood due to a reflective opening of arteriovenous shunts. The patency of the cannulation was maintained with a 154 mmol/l NaCl Infusion throughout the clamp procedure. A hand or antecubital vein in the contralateral arm was cannulated for a variable insulin infusion (human soluble insulin (40 IU Actrapid® [100 IU/ml], NovoNordisk, Copenhagen, Denmark; in 99.6 mL NaCl [154 mmol/l]) until 08:00 am, on the study day 2 or for safety reasons, a variable glucose infusion (10%, Fresenius Kabi, Graz, Austria) was applied, in order to obtain a PG level of 100 mg/dl overnight until 05:00 am, on the study day 2 [5].
The insulin infusion was stopped completely if glucose had to be infused and vice versa. Overnight, PG levels were measured every 5 to 30 minutes depending on the PG concentrations (PG target level: 100 mg/dl) and to avoid nocturnal hypoglycaemia (defined as PG level ≤ 70 md/dl). Blood samples were taken at scheduled time points (Table 4 and Table 5) [5].

At 05:00 am, day 2, a [6,6-$^{2}H_{2}$]-glucose solution (100 g/l, Euriso-Top, Saint-Aubin Cedex, France) was given i.v. as a primed infusion (9.6 mg/kg/min) for one minute and afterwards an

i.v. constant rate of 0.08 mg/kg/min [5,112]. It was administered, until the last blood samples were taken, at the PG plateau level of 70 mg/dl, on the study day 2, to assess the EGP and Rd during baseline and during the hypoglycaemic clamp. PG level deviation of ± 30% was allowed from 05:00 until 07:30 am. At 07:30 am, two and a half hours after [6,6-$^2H_2$]-glucose infusion was started, tracer glucose equilibration was reached (with the goal of a 4% enrichment) and defined as baseline (low insulin, duration of 30 minutes) [112]. A variable low insulin infusion was allowed for stable PG levels at 100 mg/dl with a PG deviation of ± 20% [5].

At approximately 08:00 am, on the study day 2, the variable low insulin infusion was increased to a constant high insulin infusion of 1.5 mU mg/kg/min and thereby the hyperinsulinaemic hypoglycaemic clamp was initiated (Figure 2). To keep the PG level stable for approximately 90 minutes at normoglycaemia, a controlled glucose infusion enriched with 4 mg [6,6-$^2H_2$]-glucose/ml (GIR = glucose infusion rate), to reduce changes in plasma enrichment of [6,6-$^2H_2$]-glucose, was administered [5,112]. Afterwards, the first PG plateau 100 mg/dl was initiated for 30 minutes (PG deviation of ± 10% was allowed). Then the glucose infusion was interrupted and the PG was allowed to fall to PG 63 mg/dl [5].

After reaching PG 63 mg/dl, the glucose infusion was re-started, in order to keep the PG stable for 30 minutes (PG plateau of 63 mg/dl; PG deviation of ± 10% was allowed). Subsequently, the glucose infusion was interrupted once again and PG was allowed to fall to 45 mg/dl (same procedure as described at PG plateau 63 mg/dl). When PG had been fallen to 45 mg/dl, or when PG had been reached a nadir at a higher PG concentration (if it was not possible to achieve lower PG concentrations/or when symptoms were unacceptable for the patients), a variable i.v. glucose infusion was re-started in order to keep PG stable at this plateau. No PG level below 40 mg/dl was allowed. After fifteen minutes at this PG plateau, the insulin infusion was stopped and the glucose infusion was tapered off to allow spontaneous glucose recovery from hypoglycaemia, if possible [5].

If the PG had not reached the next PG plateau 70mg/dl 45 minutes after termination of the insulin infusion, a constant i.v. glucose infusion (5.5 mg/kg/min) was initiated in order to increase PG to the last PG plateau 70 mg/dl (duration of 10 minutes). Therefore, the spontaneous glucose recovery was defined as: the time at PG plateau 45mg/dl – 15min after insulin infusion was stopped – until the end of the PG plateau 70mg/dl, time point 10). Finally, the PG was brought back to normoglycaemia and the hypoglycaemic clamp was terminated [5].

PG measurements were performed every 5-30 minutes from initiation of the clamp procedure until termination of the clamp according to Table 4. For safety reasons, PG measurements were allowed to be performed more frequent than every 5 min, if PG became ≤ 40 mg/dl or at the physician's discretion. Blood sampling for measurement of PG, [6,6-2H2]- glucose, counterregulatory hormones (glucagon, norepinephrine and epinephrine concentrations) were performed at baseline and during the PG plateau 100, 63 and 45 mg/dl at time point 0, 10, 20, 30 and at PG plateau 70 mg/dl at time point 0 and 10 minutes. Additionally, vital signs were assessed and an ECG was performed at each PG plateau [5].

Further, hypoglycaemic awareness was assessed by the question "Do you feel any symptoms of hypoglycaemia" with the possible answers of "yes" or "no" at each PG plateau at time points corresponding to specific PG concentrations according to Table 4 and Table 5 [5].
Additionally, hypoglycaemic response assessments were evaluated with the Edinburgh Hypoglycaemic Scale [115]. Symptoms of sweating, palpitations, shaking, hunger, confusedness, dizziness, strange behaviour, language difficulties, uncoordinated, headache and nausea were asked each patients at specific time points during the clamp (according to Table 4 and Table 5) and each patient had to give points: 1 (not at all) up to 7 points (completely agree). Further, potassium concentrations were measured at the discretion of the investigator and replaced, if necessary [5].

Table 4 Sampling Scheme for the Hypoglycaemic Clamp Visit

Approx. hour[1]	Variable i.v. insulin infusion (Actrapid®)[2]	Plasma glucose	Blood sampling	Hypoglycaemic response assessments
09:45 pm (day 1)		x		
10:00	Variable i.v. insulin infusion (Actrapid®) - PG target at 100 mg/dl	5 – 30 min		
11:00				
00:00 am (day 2)				
01:00				
02:00				
03:00				
04:00				
05:00			Sampling for [6,6-$^2H_2$] glucose[3]	
05:30				
06:00				
06:30				
07:00				
07:30		5 – 10 min	Sampling for [6,6-$^2H_2$]-glucose and counter-regulatory hormones[4]	
08:00	Start of the clamp with a constant i.v. insulin infusion (1.5 mg/kg/min) (See Table 5 and Figure 2)	5 min[6]	According to Table 5	According to Table 5
09:00				
10:00				
11:00				
12:00				
01:00 pm				
02:00				
03:00				
04:00				

[1] Actual starting time could vary but nominal timing should be followed in any case.
[2] Hypoglycaemia induction might be approx. at 09:30 am in the morning.
[3] Sampling for [6,6-$^2H_2$] glucose, before tracer infusion started (at 05:00 am in the morning, day 2)
[4] Tracer steady state should be reached; at 07:30 am (at time point 0, 10, 20, 30 minutes) blood sampling for [6,6-$^2H_2$] glucose and counterregulatory hormones were drawn.
[6] During development of hypoglycaemia, if PG was ≤ 3 mg/dl above the next PG threshold level, then PG measurements might be performed more frequent than every 5 min.

Table 5 Plasma glucose (PG) Target Profile and Sampling Scheme during the Hypoglycaemic Clamp

Approx. hour	Length of time period	PG level (mg/dl)	Insulin flow	Glucose flow[1]	Tracer ([6,6-$^2H_2$] Glucose)
09:45 pm (day 1)	-	100	variable	variable[2]	
05:00 am (day 2)	tracer enrichment		variable	no infusion	Bolus[4] + constant Infusion (0.08mg/kg/min)[5]
07:00					
07:15					
07:30	tracer steady state – baseline (30min)		stable	no Infusion	
From 08:00	1.5-2 hours		constant (1.5 mU/kg/min)	variable + tracer	
	First PG plateau[A] - (30 min)			variable + tracer	
	variable	decreasing to:		no infusion	
	PG plateau[A]- (30 min)	63		variable + tracer	
	variable	decreasing to:		no infusion	
	PG plateau[A]- (30 min)	45[3]		variable + tracer	
		variable	no infusion	variable + tracer	
	max 30 min	Spontaneous glucose recovery[6,7]	no infusion	only if needed	
	last PG plateau (30 minutes) [B]	70	no infusion	no infusion	
Hereafter the PG will actively be raised to 100 mg/dl					

[1] At 08:00 am, day 2, 10% glucose infusion will be enriched with 4mg D-[6,6-$^2H_2$] glucose /ml
[2] Variable i.v. glucose infusion (only for safety)
[3] If not possible to reach 45 mg/dl, nadir PG will be used
[4] A bolus of 9.6mg/kg/min for one minute of [6,6-$^2H_2$] -Glucose (tracer) will be given
[5] Constant i.v. tracer infusion of 0.08 mg/kg/min until the last [6,6-$^2H_2$]-glucose sample will be taken at the plateau of 70 mg/dl.
[6] PG will not be allowed to decline below 40 mg/dl.
[7] In case PG level 70 mg/dl is not reached 1 hour after nadir PG level has been reached, rescue glucose will be given to raise PG to 70 mg/dl.

[A] Assessments performed at 100, 63 and 45 mg/dl (prioritised):
Blood samples:

- [6,6-2H2]- glucose and glucagon measurements (at 0, 10, 20 and 30 minutes)
- Vital signs (at 0 and 30 minutes)
- Print out of 12-lead ECG

At PG levels 63 and 45 mg/dl: 1) between 0–5 minutes; 2) at 15 minutes; 3) between 25–30 minutes;

- Hypoglycaemic symptoms questionnaire and hypoglycaemic awareness
- Pkt 2–4 at appropriate time points (as specified in laboratory manual)

Potassium blood samples will be sampled as judged by the investigator.

[B] Assessments start to perform at 70 mg/dl (or higher) (prioritised):
(Independently of the further blood glucose levels)
Blood samples:

- [6,6-$^{2}H_{2}$]- glucose and glucagon measurements (at 0 and 10 minutes)
- Vital signs (at 0)
- Print out of 12-lead ECG

Hypoglycaemic symptoms questionnaire and hypoglycaemic awareness.
Potassium blood samples will be sampled as judged by the investigator.

After termination of the hypoglycaemic clamp, the fasting period ended and the subject was given a meal. Supervision on how to resume usual insulin therapy was given to the subject at the discretion of the investigator. Afterwards, the subject was released from the clinical site, if the investigator had no safety concerns. Before leaving the clinical site, the subject was informed about the potential symptoms of hypoglycaemia and about appropriate methods to treat hypoglycaemia [5].

2.3.9. Follow Up Visit (Visit 3)

The subject attended the visit 3, 3 – 10 days after visit 2. The subject had to be fasting (only water since 10:00 pm the evening before - minimum 9 hours before the visit was allowed). Changes in concomitant illness, medication, including current diabetes therapy, and hypoglycaemic episodes were recorded. Bodyweight, vital signs including blood pressure, pulse and body temperature, physical examination and an ECG were assessed. Blood was drawn for safety assessment (haematology, biochemistry and lipids) and a urinalysis including a pregnancy test in women of childbearing potential was performed [5].

2.4. Study Supplies

The medicinal product was supplied by the institutional pharmacy:

- Human insulin (Actrapid®) for i.v. infusion.

The non-investigational medicinal products during the study were also supplied by the institutional pharmacy:

- NaCl solution, 154 mmol/L for infusion.
- D-[6,6-$^{2}H_{2}$] glucose (100g/l), for i.v. infusion, was supplied by Euriso-Top GmbH and manufactured by the institutional pharmacy [5].

2.5. Study Monitoring and Source Data Verification

A qualified person of the Division of Endocrinology and Diabetology was monitoring in regular intervals the adherence to protocol and local requirements, to perform a case report form (CRF) verification and to assist the investigators in the study related activities.

The study electronical CRF (eCRFs) were prepared and used for data collection during the study. The investigator had to ensure the accuracy, completeness legibility and timeliness of data reported in the eCRFs and all required reports. Any change or correction to an eCRFs had to be dated, initialled and explained (if necessary) and had not to obscure the original entry, this applies to both written and electronic changes [5].

2.6. Blood Analyses and Biochemical Calculations

The planned total volume of blood to be drawn from each subject during the study was amount to less than 450 ml, taken over a period of approximately one month [5].

Blood analysis for glucagon concentrations was collected in P800 EDTA tubes (BD^{TM}, Becton Dickinson, Franklin Lakes, NJ) which contained protease inhibitors and DPP-IV inhibitors. These tubes were selected based on previously performed stability tests with a highly efficient inhibition of glucagon degradation [116]. Blood samples were collected at specific time points from the patient, immediately centrifuged for fifteen minutes and stored in a freezer at – 80°C. Glucagon concentrations were measured with a solid phase two-site ELISA (Mercodia Glucagon, Uppsala, Sweden), with the coefficient of variation for inter-assay variation with 7.3 – 9.4% and for the intra-assay variation with 3.3 – 5.1%. The detection limit of the

immunoassay was 1 pmol/l for glucagon and it had the following specificity for cross-reaction: Glicentin-Related Pancreatic Peptide < 0.0005%, Oxyntomodulin 4.4%, GLP-2 < 0.3%, GLP-1 < 0.3%, Mini-glucagon < 0.1%, and Glicentin 0.8%. All glucagon analyses were performed in a single run using kits from the same lot [5,104,117].

Blood samples for the analysis of natural glucose and [6,6-$^{2}H_2$]-glucose were collected in sodium fluoride tubes, immediately centrifuged at 4°C for 15 minutes and stored in a freezer at -80°C, as previously described [111,118]. Spiked dialysed plasma standards were prepared containing glucose (30 - 290 mg/dl) and [6,6-$^{2}H_2$]-glucose (0.5 - 5.8 mg/dl). Internal standards included [$^{13}C_6$]-glucose and [$^{13}C_6$, $^{2}H_7$]-glucose.

Glucose was analysed following protein precipitation and a two-step chemical derivatization. Protein precipitation was carried out using cold acetone. After centrifugation, the supernatant was evaporated to dryness. The first derivatization step involved the addition of an acetone/sulfuric acid mixture to obtain 1,2:5,6-di-O-isopropylidene-α-D-glucofuranose. For neutralization, sodium carbonate and sodium chloride were added. The resulting mixture was extracted with ethyl acetate, and the organic phase was evaporated to dryness. In the second derivatization step, an acetic anhydride/ethyl acetate mixture was added to obtain 3-acetyl-1,2:5,6-di-O-isopropylidene-α-D-glucofuranose.

Derivatized samples and standards were analysed by gas chromatography–mass spectrometry (GC-MS; 7890A GC, 7000B MS, Agilent Technologies, Santa Clara, USA) in split-less mode. A flow rate of 2 ml/min was used in the chromatography and a carrier gas with Helium using a HP-5MS 30m x 250 μm x 0.25 μm GC column (Agilent Technologies, Santa Clara, USA) was used. The temperature program was 110 °C (hold 0.5 min), followed by the Ramp 1: 25°C/min to 225°C.

Electron impact ionisation was used in single ion monitoring mode at the following mass-to-charge ratios (m/z): 287 (natural glucose), 288 ([$^{2}H_1$]-glucose), 289 ([$^{2}H_2$]-glucose), 293 ([$^{13}C_6$]-glucose) and 300 ([$^{13}C_6$, $^{2}H_7$]-glucose).

For calculating the tracer-to-tracee ratio (TTR), the peak areas of the analytes were used after quantifying the natural glucose and the [6,6-$^{2}H_2$]-glucose concentrations.

All steps, including sample processing, derivatization, GC-MS measurement, and tracer-to-tracee ratio (TTR) calculation of glucose and [6,6-$^{2}H_2$]-glucose were performed by Reingard

Raml of Joanneum Research Forschungsgesellschaft mbH HEALTH—Institute for Biomedicine and Health Sciences, Graz, Austria [5].

C-peptide concentrations were measured by using a two-site sandwich immunoassay (ADVIA Centaur, Siemens Healthcare Diagnostics, Camberley) with the lower limit of quantification of 0.017 nmol/l. For HbA1c, a HPLC-UV (Menarini HA-8160; Menarini Diagnostics, Florence, Italy) was used. PG concentrations were measured with a glucose analyser (Super GL; Dr. Müller Gerätebau GmbH, Freital, Germany) at bedside during the whole hypoglycaemic clamp [5].

Blood samples for norepinephrine and epinephrine concentrations were collected in EDTA-plasma tubes, immediately stored in the freezer at -80 °C and measured with a radioimmunoassay (DRG Instruments GmbH, Marburg, Germany). Unfortunately, only samples from 7 C-pos and 7 C-neg patients were available for analysis of norepinephrine and epinephrine concentrations [5].

2.7. Calculations and Statistical Analysis

All data were presented as mean ± standard deviation (SD). A p-value of < 0.05 was considered significant. Shapiro-Wilk test (SW) was used for testing normality in all parameters. For normally distributed data (SW-test: $p \geq 0.05$), a t-test was used and otherwise, for data with no normally distribution (SW-test: $p < 0.05$) a Mann-Whitney-U-test was applied [5].

Mean glucagon levels were determined for each patient and resulting data were compared between C-pos and C-neg patients at baseline and during each PG plateau (PG plateau 100, 63, 45, and 70 mg/dl). Primary parameter was defined as the mean glucagon concentration measured at PG plateau 45 mg/dl between C-pos and C-neg patients [5].

Glucagon suppression, between baseline and the first PG plateau 100 mg/dl, and glucagon increase, between PG plateaus 100 to 45 mg/dl, were calculated within each group by using a Wilcoxon-test [5].

The whole body glucose fluxes, EGP and Rd, were calculated according to Powrie et al.[113] and the modified equation of Steele [114] [5,111]. The calculation of the EGP and Rd levels were performed by Sophie Narath, of Joanneum Research Forschungsgesellschaft mbH HEALTH—Institute for Biomedicine and Health Sciences, Graz, Austria [5].

Mean EGP, Rd levels and the GIR were assessed for each patient and each plateau and compared between C-pos and C-neg patients. Within each group, EGP suppression, from baseline to first PG plateau 100 mg/dl, and EGP increase, between PG plateau 100 to 45 mg/dl, were calculated by using a paired t-test. To calculate the increase of the Rd from PG plateau 100 to 45 mg/dl within each group, a Wilcoxon- test was used. For each patient, mean PG, TTR, norepinephrine and epinephrine levels were assessed at baseline and at each PG plateau 100, 63, 45, 70 mg/dl and compared between C-pos and C-neg patients. Unfortunately, blood samples of only 7 C-pos and C-neg patients were available for calculation of norepinephrine and epinephrine levels. Overnight, every 30 minutes, mean PG levels were calculated and compared between C-pos and C-neg patients by using a Mann-Whitney-U-test [5].

The area under the curve (AUC) for GIR (AUC_{GIR}), for glucagon ($AUC_{glucagon}$) and for EGP (AUC_{EGP}) was calculated for each PG plateau by using the trapezoidal method [5].

The spontaneous glucose recovery was defined as the time at PG plateau 45 mg/dl – 15 min after insulin infusion was stopped – until the end of the PG plateau 70 mg/dl, time point 10 [5].

Statistical support was provided by the statistician Thomas Augustin from Joanneum Research Forschungsgesellschaft mbH HEALTH—Institute for Biomedicine and Health Sciences, Graz, Austria [5].

3. Results

3.1. Patients' Characteristics

This study involved 10 C-pos and 11 C-neg patients with T1DM. Table 6 presents the demographic and baseline characteristics of both groups. In C-pos patients, the mean fasting C-peptide concentration was 0.16 ± 0.1 nmol/l, while in C-neg patients, it was 0.0 ± 0.0 nmol/l. The mean diabetes duration was 2.5 ± 2 years for C-pos patients and 23.9 ± 10 years for C-neg patients. No patient was withdrawn during the study [5].

Table 6 Baseline Characteristics of C-pos and C-neg T1DM Patients.

Characteristic	**C-pos**	**C-neg**	***p* – value**
Subjects number (n)	10	11	-
Sex (male/ female)	5/5	5/6	NS
Age (years)	39.6 ± 13	37.4 ± 13	NS
Diabetes duration (years) (range)	2.5 ± 2 (1 - 8)	23.9 ± 10 (11 - 37)	**< 0.001**
BMI (kg/m²)	23.6 ± 1.8	25.0 ± 2.3	NS
C-peptide (nmol/l) (range)	0.16 ± 0.1 (0.05 – 0.36)	0.00 ± 0.0[a] (0.00 - 0.01)	**< 0.001**
HbA_{1c} (%)	7.3 ± 0.9	7.5 ± 0.8	NS
HbA_{1c} (mmol/mol)	56.3 ± 9.8	58.5 ± 8.7	NS
Daily basal insulin dose (U) (range)	9.1 ± 4 (0 - 15)	24.5 ± 12 (11 - 50)	**0.002**
Daily bolus insulin dose (U) (range)	17.7 ± 9 (5 - 36)	20.9 ± 4 (15 - 30)	NS
Total daily insulin dose (U) (range)	26.9 ± 12 (10 - 51)	45.4 ± 14 (26 - 75)	**0.017**

Reproduced from [5] with permission of publisher Oxford University Press on behalf of the Endocrine Society.
Data are mean ± SD, (min - max); p-value of <0.05 was considered significant;
NS, not significant. [a]LOQ of the assay was = 0.017 nmol/l for C-peptide measurements.

3.2. Run-In-Period

Overnight, PG levels were measured up to every 30 minutes, with no statistically significant differences observed between the two groups at any time point (Figure 3) [5].

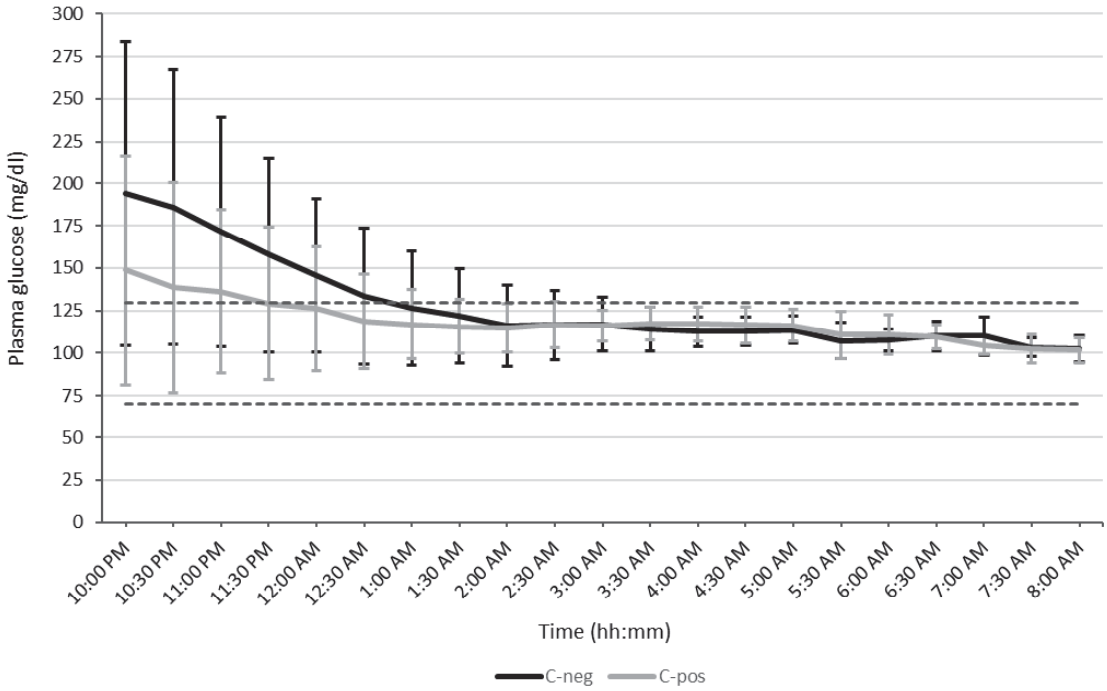

Figure 3 PG Levels during the Overnight Run-In Period in C-pos and C-neg Patients.
Reproduced from [5] and adapted with permission of publisher Oxford University Press on behalf of the Endocrine Society.
Mean PG levels, presented in mg/dl, measured every 30 minutes. Data are given as means ± SD; C-pos (grey); C-neg patients (black). Upper dash line (PG level of 130 mg/dl) and lower dash line (PG level of 70 mg/dl).

Insulin infusion rates for normoglycaemia were compared, revealing that, as expected, C-pos patients required less insulin than C-neg patients during the night (Figure 4). No hypoglycaemic events occurred during the run-in-period [5].

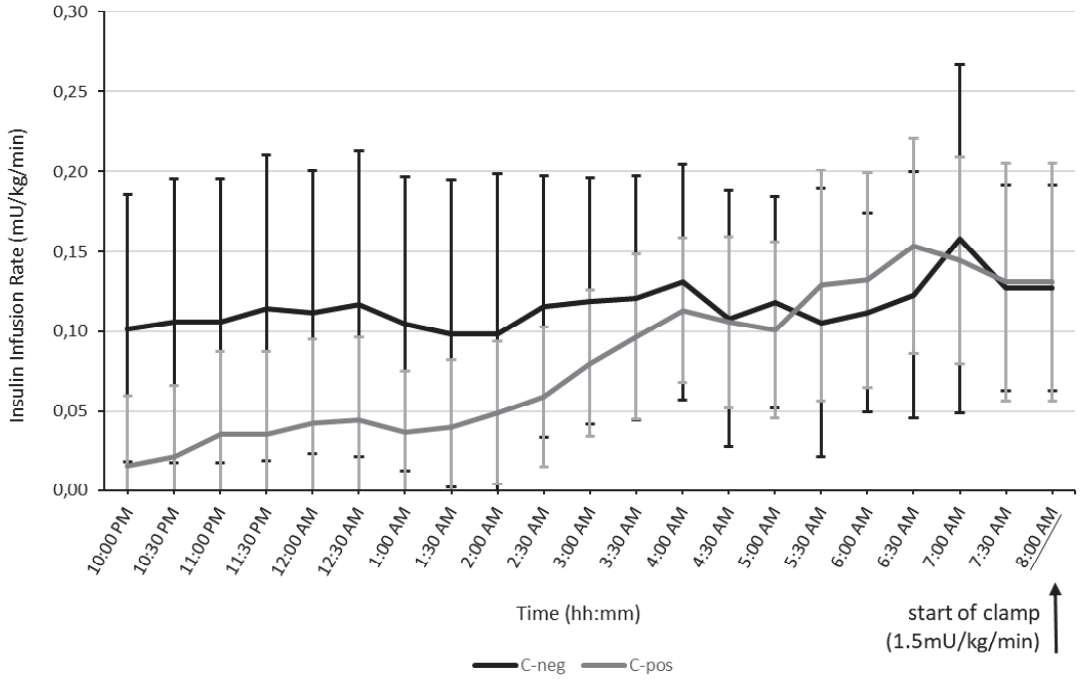

Figure 4 Insulin Infusion Rate (mU/kg/min) during the Overnight Run-In Period (every 30 minutes) in C-pos and C-neg Patients.
Reproduced from [5] and adapted with permission of publisher Oxford University Press on behalf of the Endocrine Society.
Data are presented as means ± SD; C-pos patients (grey); C-neg patients (black). Black arrow: high insulin infusion was started to start with the hypoglycaemic clamp at 8:00 am.

3.3. Hypoglycaemic Clamp

3.3.1. Plasma Glucose (PG) levels, Tracer-To-Tracee Ratio (TTR) and Glucose Infusion Rate (GIR)

The measured mean PG levels at baseline and at target PG plateaus of 100 and 70 mg/dl were comparable between C-pos and C-neg patients (Table 7 and Figure 5a). However, at PG plateaus 63 and 45 mg/dl, C-pos patients had significantly higher mean PG levels than C-neg patients [5].

Mean TTR were similar in both groups at baseline and across all PG plateaus during the clamp (Table 7 and Figure 5b) [5].

AUCGIR did not shown any significant differences between C-pos and C-neg patients at each PG plateau (Table 7 and Figure 5c). During spontaneous glucose recovery - defined as the time at PG plateau 45mg/dl - 15min after insulin infusion was stopped – until the end of the PG plateau 70mg/dl, C-pos patients had lower AUC_{GIR} compared to C-neg patients [5].

Table 7 Plasma Glucose (PG) Levels, Tracer-to-Tracee Ratio (TTR) and Glucose Infusion Rate (GIR) at Baseline and at each Target PG Plateau during the Hypoglycaemic Clamp in C-pos and C-neg Patients [5].

	Plasma Glucose (mg/dl)			**Tracer to Tracee Ratio (%)**			**Glucose Infusion Rate (AUC mg/kg)**		
target PG-plateau value	**C-pos**	**C-neg**	***p - value***	**C-pos**	**C-neg**	***p - value***	**C-pos**	**C-neg**	***p - value***
Baseline 100 mg/dl	101.9 ± 7.7	103.1 ± 6.5	0.694	3.8 ± 0.4	3.8 ± 0.5	0.984	-	-	-
100 mg/dl	99.4 ± 6.6	96.1 ± 5.2	0.108	4.5 ± 0.2	4.7 ± 0.3	0.251	302.5 ± 10	235.7 ± 74	0.107
63 mg/dl	64.5 ± 5.2	62.8 ± 3.2	**0.037**	4.9 ± 0.5	5.0 ± 0.4	0.422	232.7 ± 104	169.0 ± 60	0.098
45 mg/dl	48.8 ± 5.6	44.3 ± 3.2	**0.011**	4.8 ± 0.5	5.2 ± 0.4	0.061	137.7 ± 21	142.02 ± 20	0.654
70 mg/dl	77.6 ± 5.2	78.3 ± 4.6	0.719	3.8 ± 0.7	4.4 ± 0.6	0.086	18.4 ± 18	28.8 ± 19	0.197
Glucose Recovery*	-	-	-	-	-	-	139.9 ± 87	242.3 ± 76	**0.012**

Data are presented as means ± SD. P-value of <0.05 was considered significant. AUCGIR was calculated with the trapezoidal method.*: as the time at PG plateau 45 mg/dl – 15 min after insulin infusion was stopped – until the end of the PG plateau 70 mg/dl, time point 10.

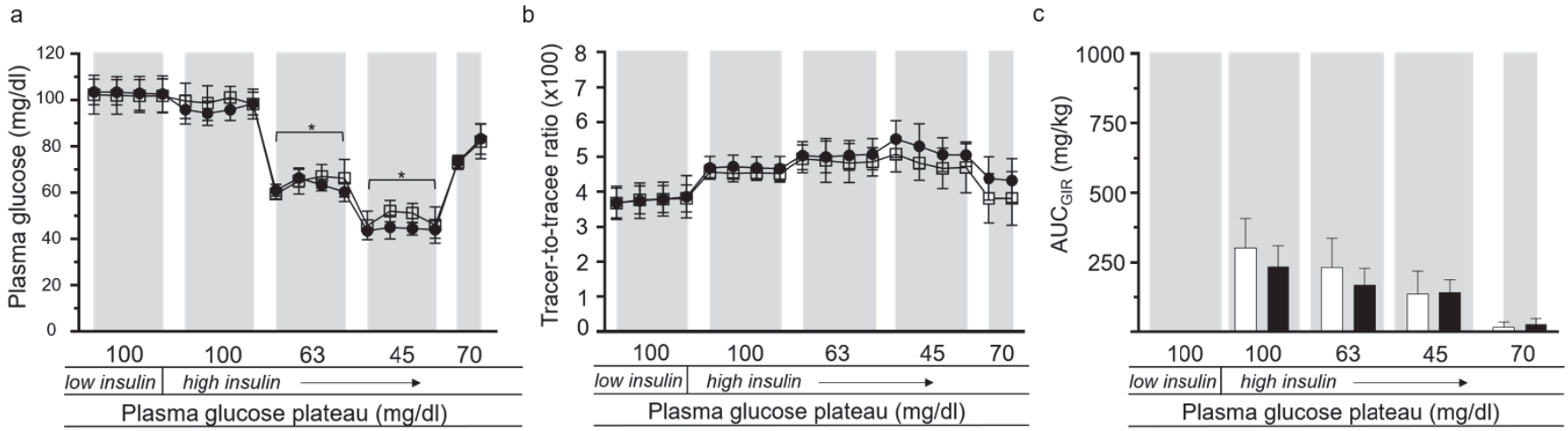

Figure 5a-c Plasma Glucose (PG) Concentrations (a), Tracer-To-Tracee Tatio (b), and Area Under the Curve for Glucose Infusion Rate (AUC_{GIR}) (c) during Hypoglycaemic Clamp at Baseline (100 mg/dl, low insulin) and at each Target PG Plateau in C-pos and C-neg Patients.

Reproduced from [5] and adapted with permission of publisher Oxford University Press on behalf of the Endocrine Society.

Data are presented as means ± SD; C-pos patients (empty squares/white bar); C-neg patients (full circles/black bar); grey shaded areas: baseline and each target PG plateau. AUC_{GIR} was calculated with the trapezoidal method; * $p < 0.05$.

3.3.2. Glucagon Concentrations, Endogenous Glucose Production (EGP) and Rate of Peripheral Glucose Disposal (Rd)

Overall glucagon levels were significantly lower in all T1DM patients during baseline and during the hypoglycaemic clamp compared to previous performed studies [5,82,92,119,120].

C-pos patients had statistically significant higher mean glucagon concentrations at baseline (normoglycaemia, low insulin) as well as during each PG plateau (100, 63, 45 and 70 mg/dl) compared to C-neg patients (Table 8 and Figure 6a).

Both groups showed a statistically significant suppression in glucagon concentrations from baseline to the PG plateau of 100 mg/dl (C-pos: baseline: 8.4 [± 4.6] pmol/l, PG plateau 100 mg/dl: 3.3 [± 2.1] pmol/l, $p < 0.002$; C-neg: baseline: 4.2 [± 2.4] pmol/l, PG plateau 100 mg/dl: 0.7 [± 0.8] pmol/l, $p < 0.001$).

From PG plateau 100 to 45 mg/dl and to recovery (PG plateau 70 mg/dl), C-pos patients had a three (3.3 to 9.9 pmol/l, difference of 6.6) to fourfold (3.3 to 13.9 pmol/l, difference of 10.6) increase in glucagon secretion.

In the C-neg patients, a fourfold to eightfold increase of glucagon secretion from the PG plateau 100 to 45 mg/dl (0.7 to 2.9 pmol/l, difference of 2.2) and to recovery PG plateau 70 mg/dl (0.7 to 5.7 pmol/l, difference of 5.0) have been revealed [5].

C-pos patients had significantly higher mean EGP rates at PG plateaus 45 and 70 mg/dl compared with C-neg patients (Table 8 and Figure 6b). At baseline, at PG plateaus 100 and 63 mg/dl mean EGP rate were comparable in both groups.

From baseline to PG plateau 100 mg/dl, a significant EGP suppression was observed within each group (C-pos: baseline: 2.1 [± 0.2] mg/kg/min, PG plateau 100 mg/dl: 0.5 [± 0.3] mg/kg/min, $p < 0.000$; C-neg: baseline: 2.0 [± 0.3] mg/kg/min, PG plateau 100 mg/dl: 0.3 [± 0.3] mg/kg/min, $p < 0.000$).

From PG plateau 100 to 45 mg/dl, an statistically significant EGP increase in both groups were seen (C-pos: PG plateau 100 mg/dl: 0.5 [± 0.3] mg/kg/min, PG plateau 45 mg/dl: 0.9 [± 0.6] mg/kg/min, $p < 0.012$; C-neg: PG plateau 100 mg/dl: 0.3 [± 0.3] mg/kg/min; PG plateau 45 mg/dl:

0.5 [± 0.1] mg/kg/min, $p < 0.012$). Additionally, a significant correlation between AUC_{EGP} and $AUC_{glucagon}$ was observed in all T1DM patients ($R2 = 0.512$; $p < 0.001$) (Figure 7) [5].

Mean Rd rates were comparable in C-pos and C-neg patients at baseline and at all PG plateaus (100, 63, 45 and 70 mg/dl) during the hypoglycaemic clamp (Table 8 and Figure 6c).
From PG plateau 100 to PG plateau 45 mg/dl, Rd significantly decrease in both groups (C-pos: PG plateau 100 mg/dl: 10.6 [± 3.3] mg/kg/min, PG plateau 45 mg/dl: 5.3 [± 2.0] mg/kg/min, $p < 0.005$; C-neg: PG plateau 100 mg/dl: 8.1 [± 2.4] mg/kg/min, PG plateau 45 mg/dl: 4.9 [± 1.4] mg/kg/min, $p < 0.003$) [5].

Table 8 Glucagon Levels, Endogenous Glucose Production (EGP) and the Rate of Peripheral Glucose Disposal (Rd) during the Hypoglycaemic Clamp at Baseline and at each PG plateau in C-pos and C-neg Patients [5].

	Glucagon levels (pmol/l)			**EGP (mg/kg/min)**			**Rd (mg/kg/min)**		
target PG-plateau value	**C-pos**	**C-neg**	***p - value***	**C-pos**	**C-neg**	***p - value***	**C-pos**	**C-neg**	***p - value***
Baseline 100 mg/dl	8.4 ± 4.6	4.2 ± 2.4	**0.016**	2.1 ± 0.2	2.0 ± 0.3	0.391	2.2 ± 0.2	2.1 ± 0.3	0.378
100 mg/dl	3.3 ± 2.1	0.7 ± 0.8	**0.000**	0.5 ± 0.3	0.3 ± 0.3	0.286	10.6 ± 3.3	8.1 ± 2.4	0.067
63 mg/dl	4.7 ± 3.3	1.4 ± 1.6	**0.003**	0.6 ± 0.3	0.5 ± 0.2	0.325	7.8 ± 3.0	5.7 ± 1.8	0.073
45 mg/dl	9.9 ± 9.6	2.9 ± 3.0	**0.010**	0.9 ± 0.6	0.5 ± 0.1	**0.037**	5.3 ± 2.0	4.9 ± 1.4	0.614
70 mg/dl	13.9 ± 5.9	5.7 ± 4.8	**0.002**	2.1 ± 0.6	1.3 ± 0.7	**0.013**	4.8 ± 1.6	5.0 ± 1.3	0.836

Data are presented as means ± SD. P-value of <0.05 was considered significant.

3.3.3. Norepinephrine and Epinephrine Concentrations

At baseline, mean norepinephrine levels were statistically significant higher in C-pos than in C-neg patients (Table 9 and Figure 6d). At PG plateau 100, 63, 45 and 70 mg/dl, no differences were observed between the groups [5].

Mean epinephrine levels were statistically significant higher in C-pos than in C-neg patients at PG plateau 100 and 70 mg/dl (Table 9 and Figure 6e). At baseline, at PG plateau 63 and at PG plateau 45 mg/dl, no differences were seen between the groups [5].

Table 9 Norepinephrine and Epinephrine Concentrations during the Hypoglycaemic Clamp at each PG Plateau in C-pos and C-neg Patients [5].

	Norepinephrine (pg/ml)			**Epinephrine (pg/ml)**		
target PG-plateau value	**C-pos**	**C-neg**	***p - value***	**C-pos**	**C-neg**	***p - value***
Baseline 100 mg/dl	153 ± 34	114 ± 74	**0.038**	7.3± 17.5	19.6 ± 8.9	0.315
100 mg/dl	168 ± 34	136 ± 58	0.226	36.7 ± 8.7	20.0 ± 9.2	**0.004**
63 mg/dl	229 ± 60	166 ± 73	0.107	55.0 ± 37.9	27.6 ± 9.7	0.073
45 mg/dl	261 ± 60	171 ± 106	0.128	96.6 ± 62.1	43.5 ± 20.1	0.227
70 mg/dl	246 ± 53	178 ± 88	0.103	79.4 ± 20.1	43.5 ± 20.1	**0.006**

Data are presented as means ± SD. P-value of <0.05 was considered significant. Blood samples for detecting norepinephrine were only available in 7 C-pos and 7 C-neg patients.

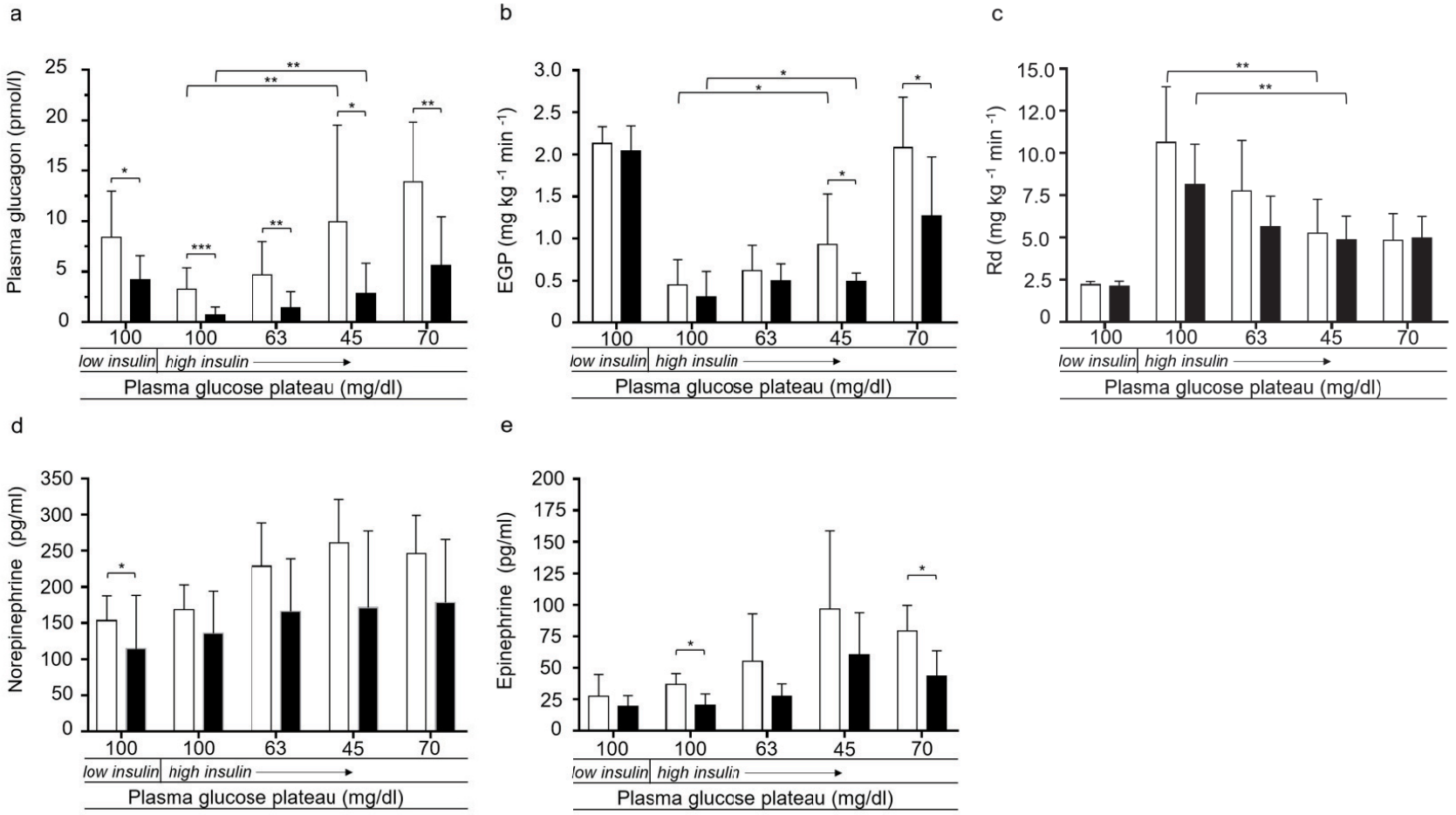

Figure 6a-e Plasma Glucagon Concentrations (a), EGP (b), Rd (c), Norepinephrine (d), and Epinephrine (e) at Baseline (100 mg/dl, low insulin) and at each Hypoglycaemic Clamp Plateau (high insulin) in C-pos and C-neg Patients.
Reproduced from [5] and adapted with permission of publisher Oxford University Press on behalf of the Endocrine Society.
Data are mean ± SD; C-pos patients (white bar); C-neg patients (black bar); * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$.

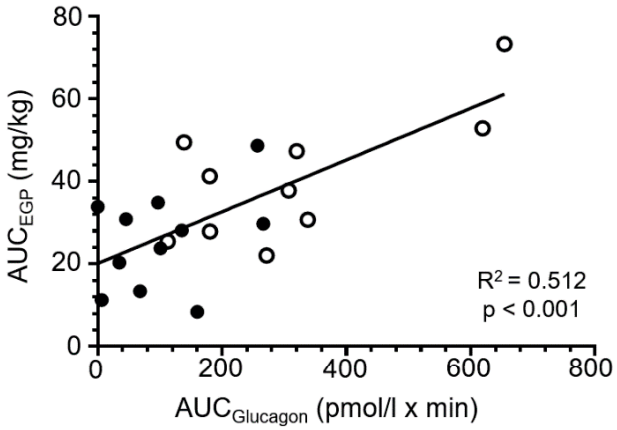

Figure 7 Linear Regression of $AUC_{glucagon}$ and AUC_{EGP} in all Type 1 Diabetes Patients. Reproduced from [5] with permission of publisher Oxford University Press on behalf of the Endocrine Society.
All patients (n: 21) were included. C-pos: empty circle (n = 10), C-neg: full circle (n = 11). AUCglucagon: defined as the sum of the AUCs of glucagon concentrations at the plateaus 100, 63, 45 and 70 mg/dl. AUC_{EGP} was defined as the sum of the AUCs of EGP at the plateaus 100, 63, 45 and 70 mg/dl.

3.3.4. Hypoglycaemic Awareness and Hypoglycaemic Symptoms Score

According to the hypoglycaemic awareness, all patient answered “no” to the question “Do you feel hypoglycaemia?” at PG plateau 100 mg/dl. At PG plateau 45 mg/dl, 12 patients (57.1%) of all patients answered with “yes”, regardless of their C-peptide status (Table 10) [5].
The hypoglycaemic symptom score increased from PG plateau 100 to 45 mg/dl in both groups, but was comparable at each PG plateau during the hypoglycaemic clamp between both groups [5].

Table 10 Hypoglycaemic Awareness at each PG plateau during the Hypoglycaemic Clamp in C-pos and C-neg Patients [5].

target PG-Plateau value	C-pos Yes (No)	C-neg Yes (No)
PG 100 mg/dl	0 (11)	0 (10)
PG 63 mg/dl	1 (10)	1 (9)
PG 45 mg/dl	7 (4)	5 (5)
PG 70 mg/dl	4 (7)	2 (8)

Descriptive data from all patients (n=21). Hypoglycaemic awareness was assessed with the Question “Do you feel any hypo?”.

4. Discussion

The main finding of our study was that C-pos T1DM patients had significantly higher glucagon levels during hypoglycaemia compared to C-neg T1DM patients. This likely led to a significantly greater EGP response in the C-pos group [5].
Additionally, significantly lower glucagon levels were observed in all patients, regardless of their C-peptide status, at baseline and during the hypoglycaemia clamp compared to previous performed studies [5,82,92,119,120].

At baseline (normoglycaemia, low insulin) and at each PG plateau during the hypoglycaemic clamp, glucagon levels were significantly higher in C-pos compared to C-neg patients [5]. Interestingly, glucagon levels decreased from baseline (normoglycaemia, low insulin) to the first PG plateau of 100 mg/dl (high insulin) in both groups, in response to the high insulin infusion. In the C-pos group, glucagon secretion then increased three to fourfold from the PG plateau 100 to 45 mg/dl (3.3 to 9.9 pmol/L, difference of 6.6) and to recovery PG plateau 70 mg/dl (3.3 to 13.9 pmol/L, difference of 10.6). The C-neg patients showed a fourfold to eightfold increase of glucagon secretion from the PG plateau 100 to 45 mg/dl (0.7 to 2.9 pmol/L, difference of 2.2) and to recovery PG plateau 70 mg/dl (0.7 to 5.7, difference of 5.0) [5].
The results of reduced (due to high insulin infusion) but responsive (due to low blood glucose) glucagon secretion during both, euglycaemia and hypoglycaemia in T1DM patients were in contrast with the commonly described literature [80,86,92]. They reported that T1DM patients had hyperglucagonaemia irrespective their glycaemic levels and C-peptide status [91,92].
Our results suggest that the alpha-cells from the Langerhans islets remained responsive to insulin and hypoglycaemia in all our patients with T1DM. This interpretation is supported by the greater glucagon levels revealed in our C-pos compared to the C-neg patients and by the small but still responsive glucagon response during hypoglycaemia in the C-neg patients [5].

In accordance with our results, Madsbad et al.[119] investigated the counterregulatory response to induced hypoglycaemia in T1DM patients with and without endogenous beta-cell function and found also a greater glucagon response in C-pos compared to C-neg patients during hypoglycaemia. However, overall glucagon levels were much higher in their patients compared to ours, and no differences were found between the two groups during glucose recovery (from

hypoglycaemia to normoglycaemia). Additionally, neither EGP nor the Rd were analysed during the hypoglycaemic clamp [5].

In contrast, in our study, the C-pos patients had a statistically significant greater glucose recovery, as shown by a reduced AUC_{GIR}, compared to C-neg patients. Moreover, we applied a stable isotope tracer technique during the hypoglycaemic clamp, allowing us to calculate both the EGP and the Rd. We observed in our patients that C-pos patients exhibited higher EGP at both targeted PG plateaus of 45 and 70 mg/dl compared to C-neg patients which likely explains the reduced AUC_{GIR}, observed in the C-pos group [5].

One explanation for these differences may lie in the varying experimental setups used to induce hypoglycaemia. Madsbad et al. [119] administered a high insulin infusion, but stopped it when patients showed symptoms of hypoglycaemia, irrespective of their PG levels. In contrast, we induced stepwise hypoglycaemia using a constant high insulin infusion and a variable glucose infusion to achieve target glucose plateaus of 100, 63, 45 and 70 mg/dl. As a result, our clamp and the duration of the hypoglycaemia for each T1DM patient, was much longer, which may explain the differences in the findings [5].

Interestingly, at baseline (normoglycaemia, low insulin), C-pos patients had higher glucagon levels than C-neg patients after an overnight of normoglycaemia (run-in-period), maintained with a low insulin infusion as needed [5]. One might expect that under euglycaemic conditions, T1DM patients with increased C-peptide levels compared to C-neg patients would have lower plasma glucagon levels due to the effect of insulin-induced suppression of glucagon secretion, but that was not the case. This result was in line with a previous described study, which showed that healthy subjects have higher glucagon levels than T1DM patients during an euglycaemic state [121].

In healthy individuals, the regulation of glucagon secretion is a complex interplay between direct signaling within the alpha-cells themselves and indirect, reciprocal signaling from beta-cells to alpha-cells [89]. One hypothesis regarding the disruption of this alpha-beta axis in T1DM patients is that the loss of beta-cell signalling leads to an absence of a beta-cell “switch-off” trigger, resulting in an increase of intra islet basal glucagon release [122]. Other possible contributors for the disturbed alpha-beta cell axis may be impaired PG sensing in the alpha-cells [76] and/or autonomic dysfunction [123].

In general, healthy individuals release endogenous insulin directly into the portal circulation, resulting in portal vein insulin levels that are approximately four times higher than systemic insulin levels [124]. However, with exogenous insulin therapy in T1DM patients, the mechanism is different, because the natural ratio of portal-to-systemic insulin is lost. This means that the direct actions of insulin on the liver to suppress EGP might be lessened. Therefore, in C-neg patients higher exogenous insulin doses are needed during euglycaemia to have a similar suppressive effect on the EGP in comparison to C-pos patients, but they result in higher systemic insulin concentrations. However systemic higher insulin concentrations in C-neg patients have greater effects on the alpha-cells to suppress glucagon secretion, thus resulting in lower glucagon concentration in C-neg compared to C-pos patients. This explanation is supported by our finding that C-neg patients needed more i.v. insulin during the night at euglycaemia prior to the hypoglycaemic induction than C-pos patients. This mechanism could be the reason, why our C-pos patients had higher glucagon levels during euglycaemia than the C-neg patients [5].

Further, we analyzed the relationship between the glucagon response and the EGP response during hypoglycaemia in our T1DM patients and found, that patients with a greater glucagon response had a higher EGP response, further emphasizing the relationship between glucagon and EGP.
Two subjects had particularly high glucagon levels compared to the other patients, suggesting an even greater response to hypoglycaemia. While one might assume these two subjects are outliers, the glucagon response in healthy subjects is even higher than those two highest found in our study [125]. Therefore, these two data points should not be considered as outliers. However, the question arises as to how the study results would differ with a wider range of glucagon responses to hypoglycaemia in T1DM patients. [5]

In comparison to previous performed studies, we observed much lower glucagon levels in all of our patients during the study [82,92,119,120], which can likely be attributed to the use of the highly sensitive double-antibody sandwich ELISA (Mercodia) for measuring glucagon [5]. Commonly used assays often detect extended or truncated glucagon forms, rather than solely measuring intact glucagon concentrations, which can lead to artificially elevated glucagon levels, potentially distort the interpretation of glucagon’s role in T1DM patients [126,127].

Additionally, it would be interesting to know what glucagon levels measured with the sensitive assay look like in healthy individuals during hypoglycaemia, as current evidence is based mainly on older studies [81,119]

Brunner et al. [117] compared fasting plasma glucagon levels in healthy individuals, T1DM and T2DM patients by using two different glucagon assays: an ELISA (Mercodia – the same we used in our study) and a MP Biomedicals radioimmunoassy (RIA). The results showed that the ELISA produced significantly lower overall glucagon levels compared to the RIA, with the glucagon levels measured using the ELISA aligning closely with those of the C-pos patients in our study [117].

Consistent with our finding, Holst JJ's research group validated the glucagon ELISA from Mercodia against other assays [104] and generally observed also similar low glucagon concentrations.

These findings emphasize the importance of carefully selecting precise laboratory methods for glucagon measurement [5].

We found no significant differences in the Rd between the groups across the PG plateaus, although C-pos patients exhibited a trend lower the Rd at PG plateaus 100 and 63 mg/dl compared to C-neg patients.

Additionally, C-pos patients tended to have slightly lower HbA_{1c} levels than C-neg patients (7.3 ± 0.9 vs. 7.5 ± 0.8, mean ± SD). Since lower HbA_{1c} values are generally associated with higher insulin sensitivity indices [128], the observed trend towards higher Rd at PG plateau 100 and 63 mg/dl in C-pos patients may be explained by a possibly higher insulin sensitivity in C-pos compared to C-neg patients [5].

Both groups showed an increase in the Rd from baseline (normoglycaemia, low insulin) to the first PG plateau 100 mg/dl (high insulin), reflecting the effect of high insulin levels during normoglycaemia in the patients. Furthermore, a statistically significant decrease in Rd was observed within both groups when PG levels dropped from 100 to 45 mg/dl [5].

Another potentially disturbed pathophysiological mechanism against hypoglycaemia in T1DM is the response of epinephrine and norepinephrine concentrations. In healthy individuals, the release of epinephrine and norepinephrine concentrations in response to hypoglycaemia enhances the EGP and reduces the Rd, helping to increase low PG levels [69,129].

It has been reported that patients with newly diagnosed T1DM had an intact hormonal response of epinephrine and norepinephrine levels to hypoglycaemia. However, in patients with long-standing T1DM, the response can be impaired [73,80,95].

In our patients, overall norepinephrine and epinephrine concentrations during the hypoglycaemic clamp tended to be lower in C-neg patients than in C-pos patients, but were only statistically significant in epinephrine concentrations at PG plateaus of 100 and 70 mg/dl and in norepinephrine concentrations at baseline (normoglycaemia, low insulin).

Our results suggest that the responses of epinephrine and norepinephrine concentrations may also contribute to the higher EGP response in C-pos compared to C-neg patients during hypoglycaemia. Of note, this analysis was limited by a smaller samples size, with only 7 C-pos and 7 C-neg patients available. A larger sample size would be necessary for further clarification [5].

Notable, during the hypoglycaemic clamp, both C-pos and C-neg patients had similar hypoglycaemic awareness and similar hypoglycaemic symptoms [5].

In this study, a standardized hyperinsulinaemic stepwise hypoglycaemic clamp was performed to induce hypoglycaemia in both, C-pos and C-neg patients [5].

To calculate the EGP and Rd in both groups, a stable isotope tracer technique was applied during the clamp. The TTR, as shown in Figure 5b, differed between the PG plateaus, but were stable within each PG plateau, allowing us to apply the modified equation of Steele to calculate the whole body glucose fluxes (EGP and Rd) in both groups [5,113,114].

Mean PG levels were similar in both groups, at baseline and at target PG plateaus of 100 and 70 mg/dl. However, at PG plateaus 63 and 45 mg/dl, C-pos patients had higher mean PG values than C-neg patients. These differences highlight how challenging it was to achieve low blood glucose levels during the clamp in the C-pos patients compared to C-neg patients, even though the high insulin infusion (1.5 mU/kg/min) was administered [5].

The C-pos patients, as previously described, had significant higher glucagon levels and showed a trend towards increasing norepinephrine and epinephrine levels. These results might contribute to a more pronounced EGP response than in the C-neg patients, resulting in the statistically significant higher PG levels at those plateaus (PG plateaus 63 and 45 mg/dl), while the GIR was similar in both groups.

Thus, we assume that our C-neg patients had a greater impaired counterregulation to hypoglycaemia than the C-pos patients, which indicates that preserved beta-cell function may contribute to counterregulation during hypoglycaemia in patients with type 1 diabetes [5].

Unfortunately, insulin concentrations were not measured during the clamp. However, the influence of subcutaneously administered insulin during the clamp can be ruled out, as patients' own subcutaneous insulin therapy was adjusted in advance, if necessary.

None of the patients used ultra-long-acting insulin, and all patients were instructed not to administer long-acting or intermediate-acting insulin after 08:00 in the morning on the study day 1. Additionally, the last short-acting insulin injection was administered together with the last meal at 05:00 pm on the study day 1. Upon arrival at the study center, all exclusion criteria, particularly the insulin administration including the required adjustments prior the study, were carefully controlled to eliminate possible interfering subcutaneous insulin levels during the study. Furthermore, during the overnight run-in phase, insulin infusion rates showed, as expected, that C-pos patients needed less exogenous insulin than C-neg patients, while both groups had similar blood glucose levels throughout the night. Based on this observation, we assume that subcutaneous insulin did not affect either the run-in phase or the hypoglycaemic clamp [5].

Hother-Nielsen et al. [130] and Bell et al. [131] demonstrated that a high insulin infusion, comparable to the one we used in our study, almost entirely suppressed endogenous insulin secretion in healthy subjects during a hyperinsulinaemic clamp at normoglycaemia.

Therefore, we suppose a similar suppressive effect in our clamp procedure due to the high insulin infusion (1.5 mU/kg/min). We assume that the endogenous insulin secretion for our C-pos patients was suppressed at normoglycaemia with the high insulin infusion, resulting in similar insulin concentrations in both groups at PG plateau 100 mg/dl.

Although insulin concentrations were not measured during the clamp, we can assume that due to the high insulin infusion during the clamp and the most likely suppression of the endogenous insulin secretion in C-pos patients, the insulin levels were comparable in both groups. Thus, we suppose that the results for EGP, Rd and GIR were not influenced by different insulin concentrations of both groups but were due to glucagon stimulation [5].

In order to ensure that only T1DM patients were included in the study, all patients had a history of acute hyperglycaemia with ketonuria at the time of initial T1DM onset, required daily subcutaneous insulin and had a BMI between 21.8 and 27.3 kg/m^2 at screening visit [5].

The C-pos patients were identified based on residual endogenous insulin secretion, defined as a fasting C-peptide level of 0.05 nmol/l or higher at screening visit. The C-neg patients were identified with a fasting non- detectable C-peptide level (lower limit of quantification was 0.017 nmol/l) at screening visit [5].
Finding C-pos patients for the study was challenging, as C-peptide positivity typically persists for only a short period of time during the course of the disease. Nevertheless, we successfully found 10 C-pos patients with a mean fasting C-peptide value of 0.16 nmol/L (range: 0.05-0.36 nmol/l). These C-peptide levels in our C-pos patients are consistent with the relatively short mean diabetes duration (2.5 ± 2 years), as well as a significantly lower total daily insulin requirement (26.9 ± 12 IU) compared to our C-neg patients (diabetes duration 23.9 ± 10 years; total daily insulin requirement: 45.4 ± 14 IU) [5].

To minimise confounding factors during the study, several exclusion criteria were established in the study protocol. No severe hypoglycaemic event was allowed within one month prior to screening visit (V1) and to the hypoglycaemic clamp visit (V2). No patient was allowed to have more than one severe hypoglycaemic event during the last 12 months and T1DM patients with hypoglycaemic unawareness were excluded from the study. Additionally, no hypoglycaemic event was allowed to take place later than 10:00 in the morning prior to the study day 1 (V2), otherwise the patient´s visit would have been rescheduled. As reported in Figure 3, no patient experienced a hypoglycaemic event during run-in-period overnight.
Regarding these protocol criteria and observations, we suppose that our study results were not influenced by previously occurred hypoglycaemic events prior the study and by hypoglycaemic unawareness of the patients [5].

Based on our results, we gained a better pathophysiological understanding of the differences between C-pos and C-neg patients during hypoglycaemia in T1DM. These findings provide valuable insights into how residual beta-cell function may influence counterregulatory mechanisms during hypoglycaemia.

We hypothesise that the glucagon concentrations and EGP may be even more pronounced during hypoglycaemia in newly diagnosed T1DM patients or those with a shorter duration of diabetes and higher C-peptide secretion [5].
Furthermore, we have developed a methodology at the Clinical Trials Unit at the Medical University of Graz for the accurate assessment of EGP and Rd during hypoglycaemia in T1DM patients. This approach allows us to explore the underlying processes occurring during hypoglycaemic episodes and to evaluate the effects of potential therapeutic interventions, such as the administration of a GLP-1 analogue, on these mechanisms [5].

In 2015, a randomized, double-blind, placebo-controlled, crossover dose-finding study was conducted at the Clinical Trials Unit [92]. The study involved a four-week subcutaneous liraglutide therapy, a GLP-1 analogue, as add on to insulin therapy (with varying doses across three different arms) versus placebo in patients with long-standing T1DM. A hyperinsulinaemic stepwise hypoglycaemic clamp was performed at the end of the 4-week treatment period. The results revealed that patients treated with liraglutide had a similar counterregulatory response to hypoglycaemia as the placebo group, addressing the concern of whether liraglutide may negatively affect counterregulation during hypoglycaemic episodes.
Interestingly, however, patients treated with liraglutide had a significantly lower GIR during the clamp compared to the placebo group. This finding raised the question of why liraglutide-treated patients had a one-third lower GIR during the clamp despite comparable blood glucose levels. One hypothesis proposed that this may be due to an increase in EGP and/or a reduction in Rd induced by liraglutide treatment. However, at the time, the investigation of EGP and Rd was not part of the study's primary research focus [92].
The measurement of EGP and Rd during a standardized hypoglycaemic clamp in T1DM patients enabled us to investigate this question in greater detail, as explored in the study by Zenz et al. in 2022 [132]. However, the results did not replicate those observed in C-neg T1DM patients in the 2014 study [92], as no significant differences in GIR, EGP, or Rd were found between the liraglutide and placebo treatment groups during hypoglycaemia. Nevertheless, the study provides additional insight into the metabolic effects of a 3-month liraglutide treatment in C-peptide-positive T1DM patients [132].

This methodological approach holds great potential for improving our understanding of diabetes-related complications and for the development of more targeted treatment strategies [5].

This dissertation has several limitations that should be emphasized.
We did not perform a glucagon stimulation test to stimulate endogenous insulin secretion for quantification of C-peptide in our patients. However, measuring fasting C-peptide is a validated method [5,37,39].
Additionally, it would have been valuable to recruit C-pos patients with a broad range of glucagon responses during hypoglycaemia and to compare these groups with a healthy control group to further investigate our research question [5].
The sample size in this study is relatively small, and a larger patient cohort would be of interest. However, finding C-pos patients with residual endogenous insulin function was challenging for the study [5].
Another limitation is that insulin concentrations and C-peptide levels were not measured during the clamp. While insulin infusion rates during the run-in period were available and provided insights into the two patient groups, with C-pos patients requiring less exogenous insulin than C-neg patients as expected, these data do not offer information regarding the experimental setup during hypoglycaemia [5].
Furthermore, measuring cortisol and growth hormone would have been of interest [5].
Finally, assessing diabetes autoantibodies at the screening visit would have been informative. However, due to the inclusion and exclusion criteria, only patients with typical T1DM onset history and clinical symptoms were enrolled. Therefore we assume that only "true" T1DM patients we included [5].

In conclusion, C-pos T1DM patients had significantly higher glucagon concentrations during induced hypoglycaemia and, as a result, a higher EGP compared to C-negative T1DM patients. However, C-neg T1DM patients also exhibited a smaller but still notable increase in glucagon concentrations and EGP during hypoglycaemia.
Additionally, our findings revealed that during euglycaemia phase (high insulin), an insulin-dependent suppression of glucagon levels occurred in both groups. Furthermore, we observed

significantly lower overall glucagon concentrations throughout the hypoglycaemic clamp, most likely due to use of a specific and highly sensitive glucagon ELISA.

These results indicate that alpha-cells in the islets of Langerhans remain responsive to insulin and low glucose levels, and that preserved beta-cell function may play a significant role in counterregulation during hypoglycaemia in patients with T1DM. Further studies are needed to investigate the role of C-peptide status in the glucagon response and its impact on EGP in T1DM patients during hypoglycaemia [5].

5. References

[1] Care D, Suppl SS. 2. Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes—2020. Diabetes Care 2020;43:S14–31. https://doi.org/10.2337/dc20-S002.

[2] Xia Y, Xie Z, Huang G, Zhou Z. Incidence and trend of type 1 diabetes and the underlying environmental determinants. Diabetes Metab Res Rev 2019;35:e3075. https://doi.org/10.1002/dmrr.3075.

[3] Quattrin T, Mastrandrea LD, Walker LSK. Type 1 diabetes. Lancet (London, England) 2023;401:2149–62. https://doi.org/10.1016/S0140-6736(23)00223-4.

[4] Rickels MR. Hypoglycemia-associated autonomic failure, counterregulatory responses, and therapeutic options in type 1 diabetes. Ann N Y Acad Sci 2019;1454:68–79. https://doi.org/10.1111/nyas.14214.

[5] Zenz S, Mader JK, Regittnig W, Brunner M, Korsatko S, Boulgaropoulos B, et al. Impact of C-Peptide Status on the Response of Glucagon and Endogenous Glucose Production to Induced Hypoglycemia in T1DM. J Clin Endocrinol Metab 2018;103:1408–17. https://doi.org/10.1210/jc.2017-01836.

[6] Maahs DM, West NA, Lawrence JM, Mayer-Davis EJ. Epidemiology of type 1 diabetes. Endocrinol Metab Clin North Am 2010;39:481–97. https://doi.org/10.1016/j.ecl.2010.05.011.

[7] Mayer-Davis EJ, Lawrence JM, Dabelea D, Divers J, Isom S, Dolan L, et al. Incidence Trends of Type 1 and Type 2 Diabetes among Youths, 2002-2012. N Engl J Med 2017;376:1419–29. https://doi.org/10.1056/NEJMoa1610187.

[8] DiMeglio LA, Evans-Molina C, Oram RA. Type 1 diabetes. Lancet (London, England) 2018;391:2449–62. https://doi.org/10.1016/S0140-6736(18)31320-5.

[9] SEARCH for Diabetes in Youth Study Group, Liese AD, D'Agostino RB, Hamman RF, Kilgo PD, Lawrence JM, et al. The burden of diabetes mellitus among US youth: prevalence estimates from the SEARCH for Diabetes in Youth Study. Pediatrics 2006;118:1510–8. https://doi.org/10.1542/peds.2006-0690.

[10] Vandewalle CL, Coeckelberghs MI, De Leeuw IH, Du Caju M V, Schuit FC, Pipeleers DG, et al. Epidemiology, clinical aspects, and biology of IDDM patients under age 40 years. Comparison of data from Antwerp with complete ascertainment with data from Belgium with 40% ascertainment. The Belgian Diabetes Registry. Diabetes Care

1997;20:1556–61. https://doi.org/10.2337/diacare.20.10.1556.

[11] Haller MJ, Atkinson MA, Schatz D. Type 1 diabetes mellitus: Etiology, presentation, and management. Pediatr Clin North Am 2005;52:1553–78. https://doi.org/10.1016/j.pcl.2005.07.006.

[12] Thunander M, Petersson C, Jonzon K, Fornander J, Ossiansson B, Torn C, et al. Incidence of type 1 and type 2 diabetes in adults and children in Kronoberg, Sweden. Diabetes Res Clin Pract 2008;82:247–55. https://doi.org/10.1016/j.diabres.2008.07.022.

[13] Leslie RDG, Williams R, Pozzilli P. Clinical review: Type 1 diabetes and latent autoimmune diabetes in adults: One end of the rainbow. J Clin Endocrinol Metab 2006;91:1654–9. https://doi.org/10.1210/jc.2005-1623.

[14] Soltesz G, Patterson CC, Dahlquist G, EURODIAB Study Group. Worldwide childhood type 1 diabetes incidence--what can we learn from epidemiology? Pediatr Diabetes 2007;8 Suppl 6:6–14. https://doi.org/10.1111/j.1399-5448.2007.00280.x.

[15] Todd JA, Bell JI, McDevitt HO. HLA-DQβ gene contributes to susceptibility and resistance to insulin-dependent diabetes mellitus. Nature 1987;329:599–604. https://doi.org/10.1038/329599a0.

[16] Hu X, Deutsch AJ, Lenz TL, Onengut-Gumuscu S, Han B, Chen W-M, et al. Additive and interaction effects at three amino acid positions in HLA-DQ and HLA-DR molecules drive type 1 diabetes risk. Nat Genet 2015;47:898–905. https://doi.org/10.1038/ng.3353.

[17] Lenz TL, Deutsch AJ, Han B, Hu X, Okada Y, Eyre S, et al. Widespread non-additive and interaction effects within HLA loci modulate the risk of autoimmune diseases. Nat Genet 2015;47:1085–90. https://doi.org/10.1038/ng.3379.

[18] Noble JA, Johnson J, Lane JA, Valdes AM. HLA class II genotyping of African American type 1 diabetic patients reveals associations unique to African haplotypes. Diabetes 2013;62:3292–9. https://doi.org/10.2337/db13-0094.

[19] Black MH, Lawrence JM, Pihoker C, Dolan LM, Anderson A, Rodriguez B, et al. HLA-Associated Phenotypes in Youth with Autoimmune Diabetes. Pediatr Diabetes 2013;14:121–8. https://doi.org/10.1111/j.1399-5448.2012.00905.x.

[20] Rich SS. Genetics and its potential to improve type 1 diabetes care. Curr Opin Endocrinol Diabetes Obes 2017;24:279–84. https://doi.org/10.1097/MED.0000000000000347.

[21] Jerram ST, Dang MN, Leslie RD. The Role of Epigenetics in Type 1 Diabetes. Curr Diab Rep 2017;17. https://doi.org/10.1007/s11892-017-0916-x.

[22] Salvetti M, Ristori G, Bomprezzi R, Pozzilli P, Leslie RD. Twins: mirrors of the immune system. Immunol Today 2000;21:342–7. https://doi.org/10.1016/s0167-5699(00)01658-3.

[23] Turtinen M, Härkönen T, Parkkola A, Ilonen J, Knip M, Finnish Pediatric Diabetes Register. Characteristics of familial type 1 diabetes: effects of the relationship to the affected family member on phenotype and genotype at diagnosis. Diabetologia 2019;62:2025–39. https://doi.org/10.1007/s00125-019-4952-8.

[24] Rewers M, Ludvigsson J. Environmental risk factors for type 1 diabetes. Lancet (London, England) 2016;387:2340–8. https://doi.org/10.1016/S0140-6736(16)30507-4.

[25] Ostman J, Lönnberg G, Arnqvist HJ, Blohmé G, Bolinder J, Ekbom Schnell A, et al. Gender differences and temporal variation in the incidence of type 1 diabetes: results of 8012 cases in the nationwide Diabetes Incidence Study in Sweden 1983-2002. J Intern Med 2008;263:386–94. https://doi.org/10.1111/j.1365-2796.2007.01896.x.

[26] Goldberg E, Krause I. Infection and type 1 diabetes mellitus - a two edged sword? Autoimmun Rev 2009;8:682–6. https://doi.org/10.1016/j.autrev.2009.02.017.

[27] Karvonen M, Viik-Kajander M, Libman I, LaPorte R, Tuomilehto J, Moltchanova E, et al. Incidence of Childhood Type 1 Diabetes. Diabetes Care 2000;23:1516–26. https://doi.org/10.2337/diacare.23.10.1516.

[28] DIAMOND Project Group. Incidence and trends of childhood Type 1 diabetes worldwide 1990-1999. Diabet Med 2006;23:857–66. https://doi.org/10.1111/j.1464-5491.2006.01925.x.

[29] Bach J-F. The effect of infections on susceptibility to autoimmune and allergic diseases. N Engl J Med 2002;347:911–20. https://doi.org/10.1056/NEJMra020100.

[30] Greenbaum CJ, Anderson AM, Dolan LM, Mayer-Davis EJ, Dabelea D, Imperatore G, et al. Preservation of beta-cell function in autoantibody-positive youth with diabetes. Diabetes Care 2009;32:1839–44. https://doi.org/10.2337/dc08-2326.

[31] Oram RA, Sims EK, Evans-Molina C. Beta cells in type 1 diabetes: mass and function; sleeping or dead? Diabetologia 2019;62:567–77. https://doi.org/10.1007/s00125-019-4822-4.

[32] Theander E, Jonsson R, Sjöström B, Brokstad K, Olsson P, Henriksson G. Prediction of Sjögren's Syndrome Years Before Diagnosis and Identification of Patients With Early Onset and Severe Disease Course by Autoantibody Profiling. Arthritis Rheumatol

2015;67:2427–36. https://doi.org/10.1002/art.39214.

[33] Jonsson R. Autoantibodies Present Before Symptom Onset in Primary Sjögren Syndrome. JAMA 2013;310:1854. https://doi.org/10.1001/jama.2013.278448.

[34] Katsarou A, Gudbjörnsdottir S, Rawshani A, Dabelea D, Bonifacio E, Anderson BJ, et al. Type 1 diabetes mellitus. Nat Rev Dis Prim 2017;3:17016. https://doi.org/10.1038/nrdp.2017.16.

[35] Ziegler AG, Rewers M, Simell O, Simell T, Lempainen J, Steck A, et al. Seroconversion to multiple islet autoantibodies and risk of progression to diabetes in children. JAMA 2013;309:2473–9. https://doi.org/10.1001/jama.2013.6285.

[36] Insel RA, Dunne JL, Atkinson MA, Chiang JL, Dabelea D, Gottlieb PA, et al. Staging presymptomatic type 1 diabetes: a scientific statement of JDRF, the Endocrine Society, and the American Diabetes Association. Diabetes Care 2015;38:1964–74. https://doi.org/10.2337/dc15-1419.

[37] Palmer JP, Fleming GA, Greenbaum CJ, Herold KC, Jansa LD, Kolb H, et al. C-Peptide Is the Appropriate Outcome Measure for Type 1 Diabetes Clinical Trials to Preserve β-Cell Function: Report of an ADA Workshop, 21-22 October 2001. Diabetes, vol. 53, 2004, p. 250–64. https://doi.org/10.2337/diabetes.53.1.250.

[38] Oram RA, Jones AG, Besser REJ, Knight BA, Shields BM, Brown RJ, et al. The majority of patients with long-duration type 1 diabetes are insulin microsecretors and have functioning beta cells. Diabetologia 2014;57:187–91. https://doi.org/10.1007/s00125-013-3067-x.

[39] Besser REJ, Shields BM, Casas R, Hattersley AT, Ludvigsson J. Lessons from the mixed-meal tolerance test: Use of 90-minute and fasting C-peptide in pediatric diabetes. Diabetes Care 2013;36:195–201. https://doi.org/10.2337/dc12-0836.

[40] Maahs, David M; West, Nancy A; Lawrence, Jean M; Mayer-Davis EJ. Chapter 1 : Epidemiology of Type 1 Diabetes. Endocrinol Metab Clin North Am 2010;39:481–97. https://doi.org/10.1016/j.ecl.2010.05.011.Chapter.

[41] Pugliese A, Skyler JS. George S. Eisenbarth: insulin and type 1 diabetes. Diabetes Care 2013;36:1437–42. https://doi.org/10.2337/dc13-0753.

[42] Zajec A, Trebušak Podkrajšek K, Tesovnik T, Šket R, Čugalj Kern B, Jenko Bizjan B, et al. Pathogenesis of Type 1 Diabetes: Established Facts and New Insights. Genes (Basel) 2022;13. https://doi.org/10.3390/genes13040706.

[43] Lernmark Å. Etiology of Autoimmune Islet Disease: Timing Is Everything. Diabetes 2021;70:1431–9. https://doi.org/10.2337/dbi18-0034.

[44] Colli ML, Szymczak F, Eizirik DL. Molecular Footprints of the Immune Assault on Pancreatic Beta Cells in Type 1 Diabetes. Front Endocrinol (Lausanne) 2020;11:568446. https://doi.org/10.3389/fendo.2020.568446.

[45] Mallone R, Eizirik DL. Presumption of innocence for beta cells: why are they vulnerable autoimmune targets in type 1 diabetes? Diabetologia 2020;63:1999–2006. https://doi.org/10.1007/s00125-020-05176-7.

[46] Campbell-Thompson ML, Atkinson MA, Butler AE, Chapman NM, Frisk G, Gianani R, et al. The diagnosis of insulitis in human type 1 diabetes. Diabetologia 2013;56:2541–3. https://doi.org/10.1007/s00125-013-3043-5.

[47] Vilas-Boas EA, Carlein C, Nalbach L, Almeida DC, Ampofo E, Carpinelli AR, et al. Early Cytokine-Induced Transient NOX2 Activity Is ER Stress-Dependent and Impacts β-Cell Function and Survival. Antioxidants (Basel, Switzerland) 2021;10. https://doi.org/10.3390/antiox10081305.

[48] Colli ML, Ramos-Rodríguez M, Nakayasu ES, Alvelos MI, Lopes M, Hill JLE, et al. An integrated multi-omics approach identifies the landscape of interferon-α-mediated responses of human pancreatic beta cells. Nat Commun 2020;11:2584. https://doi.org/10.1038/s41467-020-16327-0.

[49] Dabelea D, Rewers A, Stafford JM, Standiford DA, Lawrence JM, Saydah S, et al. Trends in the prevalence of ketoacidosis at diabetes diagnosis: the SEARCH for diabetes in youth study. Pediatrics 2014;133:e938-45. https://doi.org/10.1542/peds.2013-2795.

[50] The Diabetes Control and Complication Trial Research Group. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group. N Engl J Med 1993;329:977–86.

[51] Schofield J, Ho J, Soran H. Cardiovascular Risk in Type 1 Diabetes Mellitus. Diabetes Ther 2019:773–89. https://doi.org/10.1007/s13300-019-0612-8.

[52] David M. Nathan, Margaret Bayless, Patricia Cleary, Saul Genuth, Rose Gubitosi-Klug, John M. Lachin, Gayle Lorenzi, Bernard Zinman for the DRG. Diabetes control and complications trial/epidemiology of diabetes interventions and complications study at 30 years: advances and contributions. Diabetes 2013;62:3976–86.

[53] Strandberg RB, Graue M, Wentzel-Larsen T, Peyrot M, Wahl AK RB. The relationships among fear of hypoglycaemia, diabetes-related quality of life and psychological well-being in Norwegian adults with Type 1 diabetes. Diabetes Res Clin Pract 2017;124:11–9.

[54] Bonaventura A, Montecucco F, Dallegri F. Update on strategies limiting iatrogenic hypoglycemia. Endocr Connect 2015;4:R37–45. https://doi.org/10.1530/EC-15-0044.

[55] Moore MC, Warner SO, Dai Y, Sheanon N, Smith M, Farmer B, et al. C-peptide enhances glucagon secretion in response to hyperinsulinemia under euglycemic and hypoglycemic conditions. JCI Insight 2021;6:1–12. https://doi.org/10.1172/jci.insight.148997.

[56] Cryer PE. The barrier of hypoglycemia in diabetes. Diabetes 2008;57:3169–76. https://doi.org/10.2337/db08-1084.

[57] UK Hypoglycemia Study Group. Risk of hypoglycaemia in types 1 and 2 diabetes: effects of treatment modalities and their duration. Diabetologia 2007;50:1140–7.

[58] Seaquist ER, Anderson J, Childs B, Cryer P, Dagogo-Jack S, Fish L, et al. Hypoglycemia and diabetes: A report of a workgroup of the american diabetes association and the endocrine society. J Clin Endocrinol Metab 2013;98:1845–59. https://doi.org/10.1210/jc.2012-4127.

[59] McCall AL, Lieb DC, Gianchandani R, MacMaster H, Maynard GA, Murad MH, et al. Management of Individuals With Diabetes at High Risk for Hypoglycemia: An Endocrine Society Clinical Practice Guideline. J Clin Endocrinol Metab 2023;108:529–62. https://doi.org/10.1210/clinem/dgac596.

[60] American Diabetes Association Professional Practice Committee. 6. Glycemic Targets: Standards of Medical Care in Diabetes—2022. Diabetes Care 2022;45:S83–96. https://doi.org/10.2337/dc22-S006.

[61] International Hypoglycaemia Study Group. Glucose Concentrations of Less Than 3.0 mmol/L (54 mg/dL) Should Be Reported in Clinical Trials: A Joint Position Statement of the American Diabetes Association and the European Association for the Study of Diabetes. Diabetes Care 2017;40:155–7. https://doi.org/10.2337/dc16-2215.

[62] Workgroup on Hypoglycemia ADA. Defining and reporting hypoglycemia in diabetes: a report from the American Diabetes Association Workgroup on Hypoglycemia. Diabetes Care 2005;28:1245–9. https://doi.org/10.2337/diacare.28.5.1245.

[63] Sherwin RS. Role of the liver in glucose homeostasis. Diabetes Care 1980;3:261–5. https://doi.org/10.2337/diacare.3.2.261.

[64] Raju B, Cryer PE. Loss of the decrement in intraislet insulin plausibly explains loss of the glucagon response to hypoglycemia in insulin-deficient diabetes: documentation of the intraislet insulin hypothesis in humans. Diabetes 2005;54:757–64. https://doi.org/10.2337/diacare.28.5.1124.

[65] Cryer PE. Minireview: Glucagon in the Pathogenesis of Hypoglycemia and Hyperglycemia in Diabetes. Endocrinology 2012;153:1039–48.

[66] Adeva-Andany MM, Pérez-Felpete N, Fernández-Fernández C, Donapetry-García C, Pazos-García C. Liver glucose metabolism in humans. Biosci Rep 2016;36:1–15. https://doi.org/10.1042/BSR20160385.

[67] Ramnanan CJ, Edgerton DS, Kraft G, Cherrington AD. Physiologic action of glucagon on liver glucose metabolism. Diabetes, Obes Metab 2011;13:118–25.

[68] Schwartz NS, Clutter WE, Shah SD, Cryer PE. Glycemic thresholds for activation of glucose counterregulatory systems are higher than the threshold for symptoms. J Clin Invest 1987;79:777–81. https://doi.org/10.1172/JCI112884.

[69] Cryer PE. Hypoglycaemia: The limiting factor in the glycaemic management of Type I and Type II diabetes. Diabetologia, vol. 45, 2002, p. 937–48.

[70] Cryer PE. Glucose counterregulation: prevention and correction of hypoglycemia in humans. Am J Physiol 1993;264:E149-55. https://doi.org/10.1152/ajpendo.1993.264.2.E149.

[71] Kronenberg HM. Williams Textbook of Endocrinology. Elsevier Saunders; 2008.

[72] Keller U, Lustenberger M, Stauffacher W. Effect of insulin on ketone body clearance studied by a ketone body “clamp” technique in normal man. Diabetologia 1988;31:24–9. https://doi.org/10.1007/BF00279128.

[73] Dagogo-Jack SE, Craft S, Cryer PE. Hypoglycemia-associated autonomic failure in insulin-dependent diabetes mellitus. Recent antecedent hypoglycemia reduces autonomic responses to, symptoms of, and defense against subsequent hypoglycemia. J Clin Invest 1993;91:819–28. https://doi.org/10.1172/JCI116302.

[74] Davis HA, Spanakis EK, Cryer PE, Siamashvili M, Davis SN. Hypoglycemia During Therapy of Diabetes. 2000.

[75] Cryer PE. Hypoglycemia in type 1 diabetes mellitus. Endocrinol Metab Clin North Am

2010;39:641–54. https://doi.org/10.1016/j.ecl.2010.05.003.

[76] Gerich JE, Langlois M, Noacco C, Karam JH, Forsham PH. Lack of glucagon response to hypoglycemia in diabetes: evidence for an intrinsic pancreatic alpha cell defect. Science 1973;182:171–3. https://doi.org/10.1126/science.182.4108.171.

[77] DeRosa MA, Cryer PE. Hypoglycemia and the sympathoadrenal system: Neurogenic symptoms are largely the result of sympathetic neural, rather than adrenomedullary, activation. Am J Physiol - Endocrinol Metab 2004;287:32–41. https://doi.org/10.1152/ajpendo.00539.2003.

[78] Gold AE, MacLeod KM, Frier BM. Frequency of severe hypoglycemia in patients with type I diabetes with impaired awareness of hypoglycemia. Diabetes Care 1994;17:697–703. https://doi.org/10.2337/diacare.17.7.697.

[79] White NH, Skor DA, Cryer PE, Levandoski LA, Bier DM, Santiago J V. Identification of type I diabetic patients at increased risk for hypoglycemia during intensive therapy. N Engl J Med 1983;308:485–91. https://doi.org/10.1056/NEJM198303033080903.

[80] Arbelaez AM, Xing D, Cryer PE, Kollman C, Beck RW, Sherr J, et al. Blunted glucagon but not epinephrine responses to hypoglycemia occurs in youth with less than 1 yr duration of type 1 diabetes mellitus. Pediatr Diabetes 2014;15:127–34. https://doi.org/10.1111/pedi.12070.

[81] Siafarikas A, Johnston RJ, Bulsara MK, O'Leary P, Jones TW, Davis E a. Early loss of the glucagon response to hypoglycemia in adolescents with type 1 diabetes. Diabetes Care 2012;35:1757–62. https://doi.org/10.2337/dc11-2010.

[82] Hoffman RP, Arslanian S, Drash AL, Becker DJ. Impaired Counterregulatory Hormone Responses to Hypoglycemia in Children and Adolescents with New Onset IDDM. J Pediatr Endocrinol Metab 1994;7:235–44. https://doi.org/10.1515/JPEM.1994.7.3.235.

[83] McCrimmon RJ, Sherwin RS. Hypoglycemia in type 1 diabetes. Diabetes 2010;59:2333–9. https://doi.org/10.2337/db10-0103.

[84] Bisgaard Bengtsen M, Møller N. Mini-review: Glucagon responses in type 1 diabetes – a matter of complexity. Physiol Rep 2021;9:1–9. https://doi.org/10.14814/phy2.15009.

[85] Kulina GR, Rayfield EJ. The role of glucagon in the pathophysiology and management of diabetes. Endocr Pract 2016;22:612–21. https://doi.org/10.4158/EP15984.RA.

[86] Unger RH. Glucagon physiology and pathophysiology in the light of new advances. Diabetologia 1985;28:574–8. https://doi.org/10.1007/BF00281991.

[87] Kramer CK, Borgoño C a., Van Nostrand P, Retnakaran R, Zinman B. Glucagon response to oral glucose challenge in type 1 diabetes: lack of impact of euglycemia. Diabetes Care 2014;37:1076–82. https://doi.org/10.2337/dc13-2339.

[88] Hare KJ, Vilsbøll T, Holst JJ, Knop FK. Inappropriate glucagon response after oral compared with isoglycemic intravenous glucose administration in patients with type 1 diabetes. Am J Physiol Metab 2010;298:E832–7. https://doi.org/10.1152/ajpendo.00700.2009.

[89] Cooperberg BA, Cryer PE. beta-Cell-Mediated Signaling Predominates Over Direct alpha-Cell Signaling in the Regulation of Glucagon Secretion in Humans. Diabetes Care 2009;32:2275–80. https://doi.org/10.2337/dc09-0798.

[90] Cryer PE. Mechanisms of Hypoglycemia-Associated Autonomic Failure in Diabetes. N Engl J Med 2013;369:362–72. https://doi.org/10.1056/nejmra1215228.

[91] Sherr J, Xing D, Ruedy KJ, Beck RW, Kollman C, Buckingham B, et al. Lack of association between residual insulin production and glucagon response to hypoglycemia in youth with short duration of type 1 diabetes. Diabetes Care 2013;36:1470–6. https://doi.org/10.2337/dc12-1697.

[92] Pieber TR, Deller S, Korsatko S, Jensen L, Christiansen E, Madsen J, et al. Counter-regulatory hormone responses to hypoglycaemia in people with type 1 diabetes after 4 weeks of treatment with liraglutide adjunct to insulin: a randomized, placebo-controlled, double-blind, crossover trial. Diabetes, Obes Metab 2015;17:742–50. https://doi.org/10.1111/dom.12473.

[93] Dagogo-Jack S, Askari H, Morrill B, Lehner LL, Kim B, Sha X. Physiological responses during hypoglycaemia induced by regular human insulin or a novel human analogue, insulin glargine. Diabetes, Obes Metab 2000;2:373–83. https://doi.org/10.1046/j.1463-1326.2000.00109.x.

[94] Amiel SA. The consequences of hypoglycaemia. Diabetologia 2021;64:963–70. https://doi.org/10.1007/s00125-020-05366-3.

[95] Bolli G, de Feo P, Compagnucci P, Cartechini MG, Angeletti G, Santeusanio F, et al. Abnormal glucose counterregulation in insulin-dependent diabetes mellitus. Interaction of anti-insulin antibodies and impaired glucagon and epinephrine secretion. Diabetes 1983;32:134–41. https://doi.org/10.2337/diab.32.2.134.

[96] Towler DA, Havlin CE, Craft S, Cryer P. Mechanism of awareness of hypoglycemia.

Perception of neurogenic (predominantly cholinergic) rather than neuroglycopenic symptoms. Diabetes 1993;42:1791–8. https://doi.org/10.2337/diab.42.12.1791.

[97] Pramming S, Thorsteinsson B, Bendtson I, Binder C. Symptomatic hypoglycaemia in 411 type 1 diabetic patients. Diabet Med 1991;8:217–22. https://doi.org/10.1111/j.1464-5491.1991.tb01575.x.

[98] Davis SN, Mann S, Galassetti P, Neill RA, Tate D, Ertl AC, et al. Effects of differing durations of antecedent hypoglycemia on counterregulatory responses to subsequent hypoglycemia in normal humans. Diabetes 2000;49:1897–903. https://doi.org/10.2337/diabetes.49.11.1897.

[99] Cranston I, Lomas J, Maran A, Macdonald I, Amiel SA. Restoration of hypoglycaemia awareness in patients with long-duration insulin-dependent diabetes. Lancet (London, England) 1994;344:283–7. https://doi.org/10.1016/s0140-6736(94)91336-6.

[100] Kaur J, Seaquist ER. Hypoglycaemia in type 1 diabetes mellitus: risks and practical prevention strategies. Nat Rev Endocrinol 2023;19:177–86. https://doi.org/10.1038/s41574-022-00762-8.

[101] Mühlhauser I, Overmann H, Bender R, Bott U, Berger M. Risk factors of severe hypoglycaemia in adult patients with Type I diabetes - A prospective population based study. Diabetologia 1998;41:1274–82. https://doi.org/10.1007/s001250051065.

[102] Steffes MW, Sibley S, Jackson M, Thomas W. Beta-cell function and the development of diabetes-related complications in the diabetes control and complications trial. Diabetes Care 2003;26:832–6. https://doi.org/10.2337/diacare.26.3.832.

[103] Michael R. Rickels, Amy J. Peleckis, Eileen Markmann, Cornelia Dalton-Bakes, Stephanie M. Kong, Karen L. Teff and AN. Long-Term Improvement in Glucose Control and Counterregulation by Islet Transplantation for Type 1 Diabetes. J Clin Endocrinol Metab 2016;101:4421–30.

[104] Wewer Albrechtsen NJ, Hartmann B, Veedfald S, Windeløv JA, Plamboeck A, Bojsen-Møller KN, et al. Hyperglucagonaemia analysed by glucagon sandwich ELISA: nonspecific interference or truly elevated levels? Diabetologia 2014;57:1919–26. https://doi.org/10.1007/s00125-014-3283-z.

[105] Holst JJ. Evidence that enteroglucagon (II) is identical with the C-terminal sequence (residues 33-69) of glicentin. Biochem J 1982;207:381–8. https://doi.org/10.1042/bj2070381.

[106] Mercodia Glucagon ELISA - Dircetions for Use (2013). Uppsala, Sweden n.d.

[107] Baber N. International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use (ICH). Br J Clin Pharmacol 1994;37:401–4. https://doi.org/10.1111/j.1365-2125.1994.tb05705.x.

[108] World Medical Association Inc. Declaration of Helsinki. Ethical principles for medical research involving human subjects. J Indian Med Assoc 2009;107:403–5.

[109] ADVIA Centaur C-Peptide ELISA - Direction for use (2012). Camberley. UK n.d.

[110] Fabricius TW, Verhulst CEM, Kristensen PL, Tack CJ, McCrimmon RJ, Heller S, et al. Hyperinsulinaemic-hypoglycaemic glucose clamps in human research: a systematic review of the literature. Diabetologia 2021;64:727–36. https://doi.org/10.1007/s00125-020-05361-8.

[111] Plank J, Bodenlenz M, Sinner F, Magnes C, Görzer E, Regittnig W, et al. A Double-Blind, Randomized, Dose-Response Study Investigating the Pharmacodynamic and Pharmacokinetic Properties of the Long-Acting Insulin Analog Detemir. Diabetes Care 2005;28:1107–12. https://doi.org/10.2337/diacare.28.5.1107.

[112] Rickels MR, Kong SM, Fuller C, Dalton-Bakes C, Ferguson JF, Reilly MP, et al. Insulin sensitivity index in type 1 diabetes and following human islet transplantation: Comparison of the minimal model to euglycemic clamp measuresß. Am J Physiol - Endocrinol Metab 2014;306:1217–24. https://doi.org/10.1152/ajpendo.00667.2013.

[113] Powrie JK, Smith GD, Hennessy TR, Shojaee-Moradie F, Kelly JM, Sönksen PH, et al. Incomplete suppression of hepatic glucose production in non-insulin dependent diabetes mellitus measured with [6,6-2H2]glucose enriched glucose infusion during hyperinsulinaemic euglycaemic clamps. Eur J Clin Invest 1992;22:244–53. https://doi.org/10.1111/j.1365-2362.1992.tb01458.x.

[114] Steele R, Wall JS, de Bodo RC, Altszuler N. Measurement of Size and Turnover Rate of Body Glucose Pool by the Isotope Dilution Method. Am J Physiol Content 1956;187:15–24. https://doi.org/10.1152/ajplegacy.1956.187.1.15.

[115] McAulay V, Deary IJ, Frier BM. Symptoms of hypoglycaemia in people with diabetes. Diabet Med 2001;18:690–705. https://doi.org/10.1046/j.1464-5491.2001.00620.x.

[116] Brunner M, Raml R, Mautner S, Zenz S, Lipp R PT. Latest News on Glucagon Stability. 77th Scientific Sessions, American Diabetes Association (ADA) 2017; June 9-13., San Diego, USA. [Abstract] n.d.

[117] Brunner M, Moser O, Raml R, Haberlander M, Boulgaropoulos B, Obermayer-Pietsch B, et al. Assessment of Two Different Glucagon Assays in Healthy Individuals and Type 1 and Type 2 Diabetes Patients. Biomolecules 2022;12:1–8. https://doi.org/10.3390/biom12030466.

[118] Hachey DL, Parsons WR, McKay S, Haymond MW. Quantitation of Monosaccharide Isotopic Enrichment in Physiologic Fluids by Electron Ionization or Negative Chemical Ionization GC/MS Using Di- O -isopropylidene Derivatives. Anal Chem 1999;71:4734–9. https://doi.org/10.1021/ac990724x.

[119] Madsbad S, Hilsted J, Krarup T, Sestoft L, Christensen NJ, Faber OK, et al. Hormonal, metabolic and cardiovascular responses to hypoglycaemia in Type 1 (insulin-dependent) diabetes with and without residual B cell function. Diabetologia 1982;23:499–503. https://doi.org/10.1007/BF00254298.

[120] Briscoe VJ, Ertl AC, Tate DB, Davis SN. Effects of the Selective Serotonin Reuptake Inhibitor Fluoxetine on Counterregulatory Responses to Hypoglycemia in Individuals With Type 1 Diabetes. Diabetes 2008;57:3315–22. https://doi.org/10.2337/db08-1000.

[121] Bischof MG, Bernroider E, Krssak M, Krebs M, Stingl H, Nowotny P, et al. Hepatic glycogen metabolism in type 1 diabetes after long-term near normoglycemia. Diabetes 2002;51:49–54. https://doi.org/10.2337/diabetes.51.1.49.

[122] Farhy LS, McCall AL. Optimizing reduction in basal hyperglucagonaemia to repair defective glucagon counterregulation in insulin deficiency. Diabetes Obes Metab 2011;13 Suppl 1:133–43. https://doi.org/10.1111/j.1463-1326.2011.01455.x.

[123] Taborsky GJ, Ahrén B, Havel PJ. Autonomic mediation of glucagon secretion during hypoglycemia: implications for impaired alpha-cell responses in type 1 diabetes. Diabetes 1998;47:995–1005. https://doi.org/10.2337/diabetes.47.7.995.

[124] Evans ML. Too Much or Too Little? The Double Jeopardy of Subcutaneous Insulin Therapy in Diabetes. Diabetes 2015;64:3353–4. https://doi.org/10.2337/dbi15-0003.

[125] Liu D, Adamson U, Lins PE, Clausen-Sjöbom N. An analysis of the glucagon response to hypoglycaemia in patients with type 1 diabetes and in healthy subjects. Diabet Med 1993;10:246–54. https://doi.org/10.1111/j.1464-5491.1993.tb00052.x.

[126] Wewer Albrechtsen NJ, Holst JJ, Cherrington AD, Finan B, Gluud LL, Dean ED, et al. 100 years of glucagon and 100 more. Diabetologia 2023;66:1378–94. https://doi.org/10.1007/s00125-023-05947-y.

[127] Holst JJ, Albrechtsen NJW. Methods and guidelines for measurement of glucagon in plasma. Int J Mol Sci 2019;20. https://doi.org/10.3390/ijms20215416.

[128] Borai A, Livingstone C, Abdelaal F, Bawazeer A, Keti V, Ferns G. The relationship between glycosylated haemoglobin (HbA1c) and measures of insulin resistance across a range of glucose tolerance. Scand J Clin Lab Invest 2011;71:168–72. https://doi.org/10.3109/00365513.2010.547947.

[129] Sprague JE, Arbeláez AM. Glucose counterregulatory responses to hypoglycemia. Pediatr Endocrinol Rev 2011;9:463–73; quiz 474–5.

[130] Hother-Nielsen O, Henriksen JE, Holst JJ, Beck-Nielsen H. Effects of insulin on glucose turnover rates in vivo: isotope dilution versus constant specific activity technique. Metabolism 1996;45:82–91. https://doi.org/10.1016/s0026-0495(96)90204-8.

[131] Bell PM, Firth RG, Rizza RA. Assessment of insulin action in insulin-dependent diabetes mellitus using [6(14)C]glucose, [3(3)H]glucose, and [2(3)H]glucose. Differences in the apparent pattern of insulin resistance depending on the isotope used. J Clin Invest 1986;78:1479–86. https://doi.org/10.1172/JCI112739.

[132] Zenz S, Regittnig W, Boulgaropoulos B, Augustin T, Brunner M, Korsatko S, et al. Effect of Liraglutide Treatment on Whole-body Glucose Fluxes in C-peptide-Positive Type 1 Diabetes During Hypoglycemia. J Clin Endocrinol Metab 2022;107:E3583–93. https://doi.org/10.1210/clinem/dgac369.

The following tool was used for the optimization of language and phrasing only: ChatGPT 4o, OpenAI, used in the period from 06.01.2025 to 14.11.2025, URL: https://chatgpt.com

6. Appendix

6.1. Informed Consent

PatientInneninformation[1] und Einwilligungserklärung zur Teilnahme an der klinischen Prüfung

Eine offene[2], monozentrische[3] Pilotstudie[4] um die endogene Glucose Produktion[5] während einer hypoglykämischen Reaktion[6] bei Typ 1 Diabetikern zu untersuchen

Sehr geehrte Patientin, sehr geehrter Patient!

Wir laden Sie ein an der oben genannten klinischen Prüfung teilzunehmen. Die Aufklärung darüber erfolgt in einem ausführlichen ärztlichen Gespräch.

Ihre Teilnahme an dieser klinischen Prüfung erfolgt freiwillig. Sie können jederzeit ohne Angabe von Gründen aus der Studie ausscheiden. Die Ablehnung der Teilnahme oder ein vorzeitiges Ausscheiden aus dieser Studie hat keine nachteiligen Folgen für Ihre medizinische Betreuung.

Klinische Prüfungen sind notwendig, um verlässliche neue medizinische Forschungsergebnisse zu gewinnen. Unverzichtbare Voraussetzung für die Durchführung einer klinischen Prüfung ist jedoch, dass Sie Ihr Einverständnis zur Teilnahme an dieser klinischen Prüfung schriftlich erklären. Bitte lesen Sie den folgenden Text als Ergänzung zum Informationsgespräch mit Ihrem Prüfarzt sorgfältig durch und zögern Sie nicht Fragen zu stellen.

Bitte unterschreiben Sie die Einwilligungserklärung nur

- wenn Sie Art und Ablauf der klinischen Prüfung vollständig verstanden haben,
- wenn Sie bereit sind, der Teilnahme zuzustimmen und
- wenn Sie sich über Ihre Rechte als Teilnehmer an dieser klinischen Prüfung im Klaren sind.

[1] Wegen der besseren Lesbarkeit wird im weiteren Text zum Teil auf die gleichzeitige Verwendung weiblicher und männlicher Personenbegriffe verzichtet. Gemeint und angesprochen sind – sofern zutreffend – immer beide Geschlechter

[2] Offen bedeutet, dass sowohl Sie als auch Ihr Studienarzt wissen welche Prüfmedikation Sie erhalten

[3] Monozentrisch: diese klinische Studie wird nur an einem Studienzentrum durchgeführt

[4] Eine Vorstudie mit vorausgehende Untersuchungen, in der alle in Betracht kommenden, wichtigen Faktoren zusammengetragen werden

[5] Endogene Glucose Produktion: Der vom Körper selbstständig produzierte Zucker

[6] Hypoglykämische Reaktion: Unterzuckerung

Zu dieser klinischen Prüfung, sowie zur Patienteninformation und Einwilligungserklärung wurde von der zuständigen Ethikkommission eine befürwortende Stellungnahme abgegeben.

1. Was ist der Zweck der klinischen Prüfung?

Der Zweck dieser Studie ist es die Methodik zur Bestimmung der endogene Glukoseproduktion während eines hypoglykämischen Clamps in Typ 1 Diabetes Mellitus Probanden zu bestimmen.

2. Welche anderen Behandlungsmöglichkeiten gibt es?

Zur Behandlung Ihrer Erkrankung können Sie mit Ihrer standardisierten Diabetestherapie fortfahren.

3. Wie läuft die klinische Prüfung ab?

Diese klinische Prüfung wird an der Univ.Klinik für Innere Medizin, Abteilung für Endokrinologie und Stoffwechsel unter der Leitung von Herrn Univ.Prof.Dr.Thomas R. Pieber durchgeführt.

Vor Aufnahme in diese klinische Prüfung wird die Vorgeschichte Ihrer Krankheit erhoben, und Sie werden einer umfassenden ärztlichen Untersuchung unterzogen.

Ihre Teilnahme an dieser klinischen Prüfung wird voraussichtlich 4 Wochen dauern.

Folgende Maßnahmen werden ausschließlich aus Studiengründen durchgeführt:

Bei der Clampvisite müssen Sie folgende Anweisungen einhalten:

- Vermeiden Sie starke körperliche Betätigung ab 10:00 (morgens), 1 Tage vor der Clampvisite, Visite 2
- Vermeiden Sie den Konsum von Alkohol ab 10:00 Uhr (morgens), 1 Tage vor der Clampvisite, Visite 2
- Vermeiden Sie den Konsum von Kaffee, Tee, Schokolademilch oder Energiegetränke, ab 10:00 (morgens), 1 Tage vor der Clampvisite, Visite 2
- Bitte erscheinen sie nüchtern (beinhaltet kein Essen und keine Getränke, außer Wasser) später als 17:00 Uhr am Tag 1 der Clampvisite, Visite 2
- Vermeiden Sie langwirksame Insulin, NPH insulin oder sonst ein mittelwirksames Insulinprodukt später als 08:00 morgen am Tag 1 der Clampvisite, Visite 2
- Vermeiden Sie kurzwirksames Insulin, später als 17:00 am Tag 1 der Clampvisite, Visite 2
- Vermeiden Sie subkutane Insulininfusion später als 17:00 für Probanden, welche Insulinpumpe (CSII) verwenden am Tag 1 der Clampvisite, Visite 2
- Vermeiden Sie Hypoglykämie (BG $\leq$ 63 mg/dl oder PG $\leq$ □70mg/dl) später als 10:00 Uhr morgens am Tag 1, der Clampvisite, Visite 2

Visite 1: Screening- (Einschluss-) Visite

Zur Einschlussuntersuchung kommen Sie bitte ins Clinical Research Centre der Medizinischen Universität Graz, Stiftingtalstrasse 24 /1. OG, 8010 Graz.

Bei dieser Visite wird eine Einschlussuntersuchung durchgeführt um festzustellen, ob Sie für diese Studie geeignet sind. Zu diesem Zweck werden Einschluss- und Ausschlusskriterien überprüft, medizinische Daten (Alter, Gewicht, Größe, etc.), Vorerkrankungen bzw. aktuelle Erkrankungen und aktuell eingenommene Medikamente erhoben. Nach einer körperlichen Untersuchung (inklusive Blutdruck-, Herzfrequenz- und Körpertemperaturmessung), werden ein EKG (Aufzeichnung des Herzrhythmus), ein Atemalkoholtest, sowie eine Urinanalyse durchgeführt und Serumwerte und Blutbild (inklusive C-Peptid, HbA1c, Hepatitis B und C, bestimmt. Weiters wird auch ein HIV und Drogentest durchgeführt. Sie werden über das HIV-Testergebnis nach den gesetzlichen Richtlinien informiert werden. Bei gebärfähigen Frauen wird ein Urin-Schwangerschaftstest durchgeführt. Bei dieser Untersuchung werden Ihnen insgesamt 23,5ml Blut entnommen. Das entspricht in etwa einem Esslöffel.

Visite 2: Hypoglykämische Clamp Visite

Um ca. 20:00 werden Sie gebeten sich in unser Clinical Research Centre zu begeben. Ungefähr um 22:00 Uhr wird Ihnen dann an jeder Hand ein venöser Zugang gelegt. Die erste Kanüle wird zur Blutabnahme verwendet um Blutzucker, deuterierte Glukose[7] und Glukagon[8] zu messen. Diese Hand wird während des Clamps in eine Heizdecke gewickelt. Die zweite Kanüle wird zur Infusion von Insulin (kurzwirksames Normalinsulin, Actrapid®) und Glucose verwendet. Danach wird eine variable intravenöse Infusion (Flüssigkeitszufuhr in eine Vene die mit unterschiedlicher Geschwindigkeit erfolgt) von Glukose und Humaninsulin verabreicht, um einen Plasma Glukose Level von 5,5mmol/L (100mg/dl) bis zum nächsten morgen ca. 08:00 Uhr zu erreichen (Tag 2).

Um 05:00 Uhr morgens, am Tag 2, erhalten Sie zusätzlich eine Glukoseinfusion, welche mit deuterierten Glukose versehen ist. Zu Begin werden Sie für eine Minute einen Bolus dieser Glukoselösung erhalten und danach werden Sie eine konstante Rate dieser Lösung intravenös verabreicht bekommen, bis zur letzten Blutabnahme nach dem hypoglykämischen Clamp.

An Tag 2, zwischen 08:00 und 10:00 Uhr morgens wird mit dem hypoglykämischen Clamp (Unterzuckerungsuntersuchung) begonnen. Indem Sie eine höhere Rate an i.v. Insulin (Actrapid) erhalten. Ihre Plasmaglukose soll ungefähr eine Stunde bei 100mg/dl liegen, welche mit einer variablen Glukoseinfusion i.v. erlangt wird. Diese Glukose ist abermals mit deuterierter Glukose vermischt. Nach ca. 1h wird die Glukoseinfusion beendet und infolge der Wirkung der Insulininfusion kann der Plasmaglukosewert auf 63 mg/dl sinken. Nach 30 Minuten stabiler Plasmaglukosewerte, kann der Wert weiter auf 45mg/dl fallen, wo die

[7] Eine Zuckerart die im Körper in sehr kleinen, vernachlässigbaren Mengen produziert wird. Bringt man diese künstlich hergestellte Zuckerart in den Körper ein, wird sie von den Körperzellen gleich wie der normale Zucker aufgenommen und verarbeitet. Die Messung der beiden Zuckerarten im Blut erlaubt die Bestimmung der Produktionsraten des normalen Zuckers im Körper.

[8] Glukagon ist ein Hormon, das vom Körper produziert wird, um den Blutzuckerspiegel zu erhöhen.

Plasmaglukose wieder für 15-30 Minuten stabilisiert wird. Die Insulininfusion wird nach 15 Minuten bei 45mg/dl abgeschalten und Ihre Blutglukose kann wieder spontan steigen. Wenn nach 60 Minuten bei 45mg/dl ihr Plasmaglukosewert nicht gestiegen ist, wird mit einer Glukoseinfusion begonnen. Bei einem Blutzuckerwert von 72mg/dl werden die letzten zwei Blutabnahmen statt finden. Sobald danach ein Wert von 100mg/dl erreicht ist, wird dieser Wert beibehalten bis es vom Prüfarzt als sicher erachtet wird den hypoglykämischen Clamp zu beenden. Nach der Beendigung des hypoglykämischen Clamps wird Ihnen eine Mahlzeit serviert und der Clamp ist beendet. Während des Clamps dürfen Sie wegen der strengen Blutglukose Kontrolle nichts essen und sich nur liegend in Ihrem Bett befinden. Jedoch dürfen Sie soviel Wasser und Mineralwasser trinken wie Sie wünschen.

Während der geplanten Unterzuckerung werden zu den genannten Blutzuckerlevels, Blutabnahmen stattfinden, um ihren Blutzuckerwert zu bestimmen und weiters werden Blutabnahmen stattfinden um Glukagon9 und die deuterierte Glukose10 in Ihrem Körper zu messen. Zusätzlich, zu Ihrer Sicherheit, werden hypoglykämische Symptome und Wahrnehmung, Blutabnahmen zur Messung Ihres Kaliums und Messungen des Blutdrucks und Pulses durchgeführt. Eine EKG (Untersuchung der Herzaktivität) Überwachung wird während der gesamten Zeit der Unterzuckerung durchgeführt.

Aus Sicherheitsgründen wird Ihr Studienarzt Sie über Dosierung und Verabreichung nach dem Clamp aufklären. Ihr betreuender Studienarzt wird Sie weiters über Änderungen Ihres Gesundheitszustandes und jeglicher neuen Medikation befragen bevor Sie nach dem Clamp, Tag 2, nach Hause entlassen werden. Sie sollten nun wieder Ihre gewohnte Insulinbehandlung fortsetzen und die Dosierung mit Ihrem Studienarzt besprechen. Während des gesamten Hypoclamps werden Ihnen 148 ml Blut entnommen. Das entspricht in etwa 10 Esslöffel.

<u>Visite 3: Follow-up Visite</u>

Die Abschlussvisite wird 3-10 Tage nach Visite 2 stattfinden. Nach einer körperlichen Untersuchung (inklusive Blutdruck-, Puls- und Temperaturmessung) wird ein EKG durchgeführt, das Körpergewicht ermittelt und eine Blut- sowie eine Harnprobe genommen um Ihre Blut- und Harnwerte zu ermitteln. Bei gebärfähigen Frauen wird ein Schwangerschaftstest anhand eines Urintests durchgeführt. Die Test Resultate der Einschluss- und Abschlussuntersuchung werden am Studienzentrum mit Ihren medizinischen Unterlagen aufbewahrt. Sie können diese Ergebnisse gemeinsam mit Ihrem Arzt besprechen. Bei dieser Untersuchung werden Ihnen 11ml Blut entnommen. Das entspricht in etwa einem Esslöffel.

Nach dieser Visite ist Ihre Teilnahme an dieser klinischen Studie beendet.

[9] Glukagon ist ein Hormon, das vom Körper produziert wird, um den Blutzuckerspiegel zu erhöhen.
[10] Eine Zuckerart die im Körper in sehr kleinen, vernachlässigbaren Mengen produziert wird. Bringt man diese künstlich hergestellte Zuckerart in den Körper ein, wird sie von den Körperzellen gleich wie der normale Zucker aufgenommen und verarbeitet. Die Messung der beiden Zuckerarten im Blut erlaubt die Bestimmung der Produktionsraten des normalen Zuckers im Körper.

4. Was ist Actrapid®?

Actrapid® ist ein von der Firma Novo Nordisk hergestelltes schnell wirkendes Humaninsulin. Actrapid® ist in Österreich ein bereits zugelassenes Arzneimittel, welches hohe Blutzuckerspiegel bei Patienten mit Diabetes mellitus (Diabetes) senkt.

5. Worin liegt der Nutzen einer Teilnahme an der Klinischen Prüfung?

Es ist nicht zu erwarten, dass Sie aus Ihrer Teilnahme an dieser klinischen Prüfung gesundheitlichen Nutzen ziehen werden. Die Ergebnisse dieser klinischen Prüfung sollen dazu beitragen, ein noch besseres Verständnis für die Mechanismen im Körper des Typ 1 Diabetes Mellitus zu bekommen und darauf in der Zukunft für Menschen mit dieser Erkrankung eine Erleichterung und Verbesserung der Blutzuckereinstellung zu ermöglichen.

6. Gibt es Risiken, Beschwerden und Begleiterscheinungen?

In dieser speziellen Clampstudie, wird versucht eine Hypoglykämie zu provozieren, was sehr wahrscheinlich zu Symptomen eines niedrigen Blutzuckers führen wird, die häufigsten Symptome sind weiter unten beschrieben. Während der normalen Clampuntersuchung kommt es zu einer kontrollierten Hypoglykämie und eine Hyperglykämie wird durch engmaschigen Blutzuckerkontrollen und die variable bzw. konstante Insulin- bzw. Glukoseinfusion verhindert. Falls es zu unakzeptablen hypoglykämischen Symptomen oder einem Fehler in der Clampuntersuchung kommt, wird eine Glukoseinfusion verabreicht und der Clamp abgebrochen.

Während der gesamten Studie werden Sie mehrere Venenpunktionen haben und erhalten zumindest sechs venöse Dauerkanülen, jede davon über einen Zeitraum von ungefähr 18 Stunden. In manchen Fällen können an der Stelle der Venenpunktion lokale Blutergüsse auftreten und in seltenen Fällen kann es zu Infektionen oder sogar zu Phlebitis (Venenentzündungen) kommen, welche lokal oder systemisch (z.B. mittels Antibiotika) behandelt werden müssten.

Insgesamt werden Ihnen bei dieser Studie über einem Zeitraum von zirka 4 Wochen 182,5 ml Blut entnommen (zum Vergleich: Bei einer Blutspenden werden Ihnen zirka 450 ml But entnommen.) Das entspricht in etwa 12 Esslöffel.

7. Zusätzliche Einnahme von Arzneimitteln?

Aufgrund Ihrer Studienteilnahme ist eine zusätzliche Einnahme von Arzneimitteln nicht notwendig.

8. Hat die Teilnahme an der klinischen Prüfung sonstige Auswirkungen auf die Lebensführung und welche Verpflichtungen ergeben sich daraus?

Ihre Studienteilnahme beinhaltet wie oben beschrieben drei Besuche am Studienzentrum mit einem stationären Aufenthalt von ca. 18 Stunden. Eine Änderung Ihrer Lebensführung oder sonstige Verpflichtungen ergeben sich daraus nicht.

9. Was ist zu tun beim Auftreten von Symptomen, Begleiterscheinungen und/oder Verletzungen?

Sollten im Verlauf der klinischen Prüfung irgendwelche Symptome, Begleiterscheinungen oder Verletzungen auftreten, müssen Sie diese Ihrem Prüfarzt mitteilen, bei schwerwiegenden Begleiterscheinungen umgehend, ggf. telefonisch (Telefonnummern, etc. siehe unten).

10. Versicherung

Als Teilnehmer an dieser klinischen Prüfung besteht für Sie der gesetzlich vorgeschriebene verschuldensunabhängige Versicherungsschutz (Personenschadenversicherung gemäß § 32 Arzneimittelgesetz), der alle Schäden abdeckt, die an Ihrem Leben oder Ihrer Gesundheit durch die an Ihnen durchgeführten Maßnahmen der klinischen Prüfung verursacht werden können, mit Ausnahme von Schäden auf Grund von Veränderungen des Erbmaterials in Zellen der Keimbahn.

Die Versicherung wurde für Sie bei der Wiener Städtische Allgemeine Versicherungs-AG, HF2 Haftpflicht Fachabteilung, Schottenring 30, 1010 Wien, Telefonnummer: 050 350, unter der Polizzennummer 08-N811.957 abgeschlossen. Auf Wunsch können Sie in die Versicherungsunterlagen Einsicht nehmen.

Im Schadensfall können Sie sich direkt an den Versicherer wenden und Ihre Ansprüche selbständig geltend machen. Für den Versicherungsvertrag ist österreichisches Recht anwendbar, die Versicherungsansprüche sind in Österreich einklagbar.

Zur Unterstützung können Sie sich auch an die Patientenanwaltschaft, Patientenvertretung oder Patientenombudsschaft wenden.

Um den Versicherungsschutz nicht zu gefährden

- dürfen Sie sich während der Dauer der klinischen Prüfung einer anderen medizinischen Behandlung nur im Einvernehmen mit Ihrem behandelnden Prüfarzt unterziehen **(ausgenommen davon sind Notfälle).** Dies gilt auch für die zusätzliche Einnahme von Medikamenten oder die Teilnahme an einer anderen Studie.
- müssen Sie dem behandelnden Prüfarzt - oder der oben genannten Versicherungsgesellschaft - eine Gesundheitsschädigung, die als Folge der klinischen Prüfung eingetreten sein könnte, unverzüglich mitteilen.
- müssen Sie alles Zumutbare tun um Ursache, Hergang und Folgen des Versicherungsfalles aufzuklären und den entstandenen Schaden gering zu halten. Dazu gehört ggf. auch, dass Sie Ihre behandelnden Ärzte ermächtigen, vom Versicherer geforderte Auskünfte zu erteilen.

11. Informationen für gebärfähige Frauen – Schwangerschaftstest

Schwangere und stillende Frauen dürfen an dieser klinischen Prüfung NICHT teilnehmen. Als gebärfähige Frau dürfen Sie an der klinischen Prüfung nur teilnehmen,

- wenn ein Arzt vor und einmal monatlich während der klinischen Prüfung das Nichtvorliegen einer Schwangerschaft (Schwangerschaftstest) feststellt. Es wird Ihnen weiters die Durchführung eines Schwangerschaftstests nach Abschluss der Studie empfohlen.

- wenn Sie sich verpflichten während der Dauer eine zuverlässige Art der Empfängnisverhütung (Pille, Spirale) zu praktizieren.

Sollten Sie dennoch während der klinischen Prüfung schwanger werden oder den Verdacht haben, dass Sie schwanger geworden sind, informieren Sie bitte umgehend Ihren Prüfarzt.

12. Wann wird die klinische Prüfung vorzeitig beendet?

Sie können jederzeit auch ohne Angabe von Gründen, Ihre Teilnahmebereitschaft widerrufen und aus der klinischen Prüfung ausscheiden ohne dass Ihnen dadurch irgendwelche Nachteile für Ihre weitere medizinische Betreuung entstehen.

Ihr Prüfarzt wird Sie über alle neuen Erkenntnisse, die in Bezug auf diese klinische Prüfung bekannt werden, und für Sie wesentlich werden könnten, umgehend informieren. Auf dieser Basis können Sie dann Ihre Entscheidung zur weiteren Teilnahme an dieser klinischen Prüfung neu überdenken.

Es ist aber auch möglich, dass Ihr Prüfarzt (oder gegebenenfalls der Auftraggeber dieser klinischen Prüfung) entscheidet, Ihre Teilnahme an der klinischen Prüfung vorzeitig zu beenden, ohne vorher Ihr Einverständnis einzuholen. Die Gründe hierfür können sein:

a.) Sie können den Erfordernissen der Klinischen Prüfung nicht entsprechen;

b.) Ihr Prüfarzt hat den Eindruck, dass eine weitere Teilnahme an der klinischen Prüfung nicht in Ihrem Interesse ist;

Sofern Sie sich dazu entschließen, vorzeitig aus der klinischen Prüfung auszuscheiden, oder Ihre Teilnahme aus einem der oben genannten Gründe vorzeitig beendet wird, ist es für Ihre eigene Sicherheit wichtig, dass Sie sich einer normalen Kontrolluntersuchung unterziehen. Diese besteht meistens aus einer körperlichen Untersuchung sowie aus Laboruntersuchungen.

13. In welcher Weise werden die im Rahmen dieser klinischen Prüfung gesammelten Daten verwendet?

Sofern gesetzlich nicht etwas anderes vorgesehen ist, haben nur die Prüfärzte und deren Mitarbeiter Zugang zu den vertraulichen Daten, in denen Sie namentlich genannt werden („personenbezogene“ Daten). Weiters können Beauftragte von in- und ausländischen Gesundheitsbehörden, der zuständigen Ethikkommission Einsicht in diese Daten nehmen, um die Richtigkeit der Aufzeichnungen zu überprüfen. Diese Personen unterliegen einer gesetzlichen Verschwiegenheitspflicht.

Die Weitergabe der Daten im In- und Ausland erfolgt ausschließlich zu statistischen Zwecken in verschlüsselter (nur „indirekt personenbezogener“) Form, das heißt, Sie werden nicht namentlich genannt. Auch in etwaigen Veröffentlichungen der Daten dieser klinischen Prüfung werden Sie nicht namentlich genannt.

Die Prüfärzte und ihre Mitarbeiter unterliegen im Umgang mit den Daten den Bestimmungen des österreichischen Datenschutzgesetzes 2000 in der jeweils geltenden Fassung.

Wenn Sie Ihre Einwilligung zurückziehen und damit Ihre Teilnahme vorzeitig beenden, werden keine neuen Daten mehr über Sie erhoben. Auf Grund gesetzlicher Dokumentationspflichten (Arzneimittelgesetz) kann jedoch weiterhin für einen gesetzlich festgelegten Zeitraum eine Einsichtnahme in Ihre personenbezogenen Daten zu Prüfzwecken durch autorisierte, zur Verschwiegenheit verpflichtete Personen erfolgen.

14. Entstehen für die Teilnehmer Kosten? Gibt es einen Kostenersatz oder eine Vergütung?

Durch Ihre Teilnahme an dieser klinischen Prüfung entstehen für Sie keine zusätzlichen Kosten. Für die Teilnahme erhalten Sie eine pauschale Aufwandsentschädigung von insgesamt € 600,--. Die Aufwandsentschädigung wird aliquot ausbezahlt und setzt sich folgendermaßen zusammen: € 25,-- € für die Einschlussuntersuchung, € 550,-- für die Clamp Visite 2, und € 25,-- für die Nachuntersuchung.

15. Möglichkeit zur Diskussion weiterer Fragen

Für weitere Fragen im Zusammenhang mit dieser klinischen Prüfung stehen Ihnen Ihr Prüfarzt und seine Mitarbeiter gern zur Verfügung. Auch Fragen, die Ihre Rechte als Patient und Teilnehmer an dieser klinischen Prüfung betreffen, werden Ihnen gerne beantwortet.

- Name der Kontaktperson: Dr.Stefan Korsatko
  - Ständig erreichbar unter: 0316-385-72385
- Name der Kontaktperson: Dr.Sabine Zenz
  - Ständig erreichbar unter: 0316-385-72837
- Wo erhalte ich mehr Information?
  - 24h Notfall Nummer: +43 316 385 80769

16. Wo kann ich weitere Informationen einholen?

- Univ.Prof.Dr.Thomas Pieber
  - Ständig erreichbar unter: 0316-385-82383

17. Sollten andere behandelnde Ärzte von der Teilnahme an der klinischen Prüfung informiert werden?

Ihr Hausarzt wird von Ihrer Studienteilnahme informiert.

18. Einwilligungserklärung

Name des Patienten in Druckbuchstaben:.....

Geb.Datum: ..... Code: .....

Ich erkläre mich bereit, an der klinischen Studie Pilot_EGP teilzunehmen.

Ich bin von Herrn/Frau (Dr.med.)_____ausführlich und verständlich über mögliche Belastungen und Risiken, sowie über Wesen, Bedeutung und Tragweite der klinischen Prüfung, die bestehende Versicherung sowie die sich für mich daraus ergebenden Anforderungen aufgeklärt worden. Ich habe darüber hinaus den Text dieser Patientenaufklärung und Einwilligungserklärung, die insgesamt 10 Seiten umfasst gelesen. Aufgetretene Fragen wurden mir vom Prüfarzt verständlich und genügend beantwortet. Ich hatte ausreichend Zeit, mich zu entscheiden. Ich habe zurzeit keine weiteren Fragen mehr.

Ich werde den ärztlichen Anordnungen, die für die Durchführung der klinischen Prüfung erforderlich sind, Folge leisten, behalte mir jedoch das Recht vor, meine freiwillige Mitwirkung jederzeit zu beenden, ohne dass mir daraus Nachteile für meine weitere medizinische Betreuung entstehen.

Ich bin zugleich damit einverstanden, dass meine im Rahmen dieser klinischen Prüfung ermittelten Daten gespeichert werden. Mir ist bekannt, dass zur Überprüfung der Richtigkeit der Datenaufzeichnung Beauftragte der zuständigen Behörden, der Ethikkommission und ggf. des Auftraggebers beim Prüfarzt Einblick in meine personenbezogenen Krankheitsdaten nehmen dürfen.

Sollte ich meine Teilnahme an dieser Studie widerrufen oder wird meine Teilnahme an der Studie durch den Sponsor oder den Prüfarzt vorzeitig beendet, so willige ich ein, dass die bis zu diesem Zeitpunkt erhobenen Daten weiterhin verwendet werden dürfen, soweit dies erforderlich ist, um

a) sicherzustellen, dass meine schutzwürdigen Interessen nicht beeinträchtigt werden

und

b) der gesetzlichen Pflicht zur Vorlage vollständiger Zulassungsunterlagen und den gesetzlichen Dokumentationspflichten zu entsprechen.

Beim Umgang mit den Daten werden die Bestimmungen des Datenschutzgesetzes 2000 beachtet.

Eine Kopie dieser Patienteninformation und Einwilligungserklärung habe ich erhalten. Das Original verbleibt beim Prüfarzt.

.....

(Datum und Unterschrift des Patienten)

.....

(Datum, Name und Unterschrift des verantwortlichen Prüfarztes)

(Der Patient erhält eine unterschriebene Kopie der Patienteninformation und Einwilligungserklärung, das Original verbleibt im Studienordner des Prüfarztes.)

6.2. Publications With First Authorship Related to This Thesis

CLINICAL RESEARCH ARTICLE

Impact of C-Peptide Status on the Response of Glucagon and Endogenous Glucose Production to Induced Hypoglycemia in T1DM

Sabine Zenz,[1] Julia K. Mader,[1] Werner Regittnig,[1] Martina Brunner,[2] Stefan Korsatko,[1] Beate Boulgaropoulos,[1,3] Christoph Magnes,[3] Reingard Raml,[3] Sophie H. Narath,[3] Philipp Eller,[4] Thomas Augustin,[3] and Thomas R. Pieber[1,3,4]

[1]Division of Endocrinology and Diabetology, Department of Internal Medicine, Medical University of Graz, 8036 Graz, Austria; [2]Center for Medical Research, Clinical Research Center, Medical University of Graz, 8010 Graz, Austria; [3]Joanneum Research Forschungsgesellschaft mbH HEALTH—Institute for Biomedicine and Health Sciences, 8010 Graz, Austria; and [4]Intensive Care Unit, Department of Internal Medicine, Medical University of Graz, 8036 Graz, Austria

Context: Complete loss of β-cell function in patients with type 1 diabetes mellitus (T1DM) may lead to an increased risk of severe hypoglycemia.

Objective: We aimed to determine the impact of C-peptide status on glucagon response and endogenous glucose production (EGP) during hypoglycemia in patients with T1DM.

Design and Setting: We conducted an open, comparative trial.

Patients: Ten C-peptide positive (C-pos) and 11 matched C-peptide negative (C-neg) patients with T1DM were enrolled.

Intervention: Plasma glucose was normalized over the night fast, and after a steady-state (baseline) plateau all patients underwent a hyperinsulinemic, stepwise hypoglycemic clamp with glucose plateaus of 5.5, 3.5, and 2.5 mmol/L and a recovery phase of 4.0 mmol/L. Blood glucagon was measured with a specific and highly sensitive glucagon assay. EGP was determined with a stable isotope tracer technique.

Main Outcome Measure: Impact of C-peptide status on glucagon response and EGP during hypoglycemia.

Results: Glucagon concentrations were significantly lower in C-pos and C-neg patients than previously reported. At baseline, C-pos patients had higher glucagon concentrations than C-neg patients (8.39 $\pm$ 4.6 vs 4.19 $\pm$ 2.4 pmol/L, $P = 0.016$, mean $\pm$ standard deviation) but comparable EGP rates (2.13 $\pm$ 0.2 vs 2.04 $\pm$ 0.3 mg/kg/min, $P < 0.391$). In both groups, insulin suppressed glucagon levels, but hypoglycemia revealed significantly higher glucagon concentrations in C-pos than in C-neg patients. EGP was significantly higher in C-pos patients at hypoglycemia (2.5 mmol/L) compared with C-neg patients.

Conclusions: Glucagon concentrations and EGP during hypoglycemia were more pronounced in C-pos than in C-neg patients, which indicates that preserved β-cell function may contribute to counterregulation during hypoglycemia in patients with T1DM. (***J Clin Endocrinol Metab*** **103: 1408–1417, 2018)**

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in USA

Received 17 August 2017. Accepted 29 January 2018.
First Published Online 1 February 2018

Abbreviations: AUC, area under the curve; AUC_{EGP}, area under the curve for endogenous glucose production; AUC_{GIR}, area under the curve for glucose infusion rate; $AUC_{GIR+EGP}$, area under the curve for total glucose infusion rate; $AUC_{glucagon}$, area under the curve for glucagon; BMI, body mass index; C-neg, C-peptide negative; C-pos, C-peptide positive; CV, coefficient of variation; EGP, endogenous glucose production; GC, gas chromatography; GIR, glucose infusion rate; HbA1c, hemoglobin A1c; LOQ, lower limit of quantification; MS, mass spectrometry; PG, plasma glucose; Rd, rate of peripheral glucose disposal; SD, standard deviation; SW, Shapiro-Wilk test; T1DM, type 1 diabetes mellitus; TTR, tracer-to-tracee ratio.

[1] Copy of "Impact of C-peptide Status on the Response of Glucagon and Endogenous Glucose Production to Induced Hypoglycemia in T1DM," by Zenz S, Mader JK, Regittnig W, Brunner M, Korsatko S, Boulgaropoulos B, et al. J Clin Endocrinol Metab 2018;103:1408–17. https://doi.org/10.1210/jc.2017-01836 [5]. No modifications were made. Copyright 2018 by Oxford University Press on behalf of the Endocrine Society. Reprinted with permission.

Tight glycemic control in patients with type 1 diabetes mellitus (T1DM) decelerates the progression of microvascular complications such as retinopathy, neuropathy, and nephropathy (1) and can also reduce the development of macrovascular complications such as ischemic heart disease, peripheral vascular disease, and cerebrovascular disease (2). However, achievement of more stringent treatment goals raises the risk of recurrent symptomatic and severe hypoglycemic events. Hypoglycemia is not only associated with an adverse clinical outcome but also has a negative impact on quality of life of affected patients (3).

In healthy people, glucagon and insulin produced from the α- and β-cells exert opposing effects on their target tissues, and their interaction plays a central role in glucose homeostasis (4). The defense mechanisms against hypoglycemia include reduction of endogenous insulin secretion and release of pancreatic glucagon, which in turn raises plasma glucose (PG) by increasing hepatic glucose output through stimulation of glycogenolysis and activating gluconeogenesis (5, 6).

In patients with T1DM, β-cells are destroyed by an autoimmune reaction leading to deficiency of endogenous insulin secretion. This might cause secondary abnormalities in the function of other pancreatic islet cells, like abnormal glucagon release by α-cells. Glucagon levels after oral food intake have been shown to be inappropriately high, which is associated with pronounced postprandial hyperglycemia in patients with T1DM (7, 8). In contrast, diminished glucagon response to hypoglycemia and insufficient stimulation of glycogenolysis and gluconeogenesis are major reasons for severe hypoglycemic events (9–11). Reasons for this disturbed glucagon response to hypoglycemia may be impaired PG sensing in the α-cells (11), autonomic dysfunction (12), or a loss of an insulin "switch off" signal from the β-cells (13), but the underlying mechanism is not yet fully understood.

Over time, different experimental setups, like various glucagon assays, have been used to gain a better understanding of glucagon levels in T1DM during hypoglycemia. Analytical methods for glucagon determination have been improving over the years, but commonly used assays are mostly unspecific and detect *N*-terminally extended or truncated forms of glucagon as well. The resulting and to some degree erroneous high glucagon concentrations may have compromised the interpretations of the role of glucagon in patients with T1DM (14, 15). Recently, a specific and highly sensitive glucagon assay that enables the specific detection of intact glucagon has become available (14, 16) and allows a reassessment of the role of glucagon during hypoglycemia in patients with T1DM.

The severity of hypoglycemia in patients with T1DM correlates with diabetes duration (17–19). Patients with short diabetes duration [(C-peptide positive (C-pos)] had as many hypoglycemic events as patients with long diabetes duration [C-peptide neg (C-neg)], but these hypoglycemic events were less severe (17). Diabetes duration is associated with a loss of residual β-cell function, which indicates that the risk of occurrence of severe hypoglycemia increases with the loss of β-cell function and that C-peptide negativity is a major risk factor for developing severe hypoglycemia (20, 21). However, the mechanism of how residual β-cell function protects against severe hypoglycemia remains unclear, and the impact of C-peptide status on glucose release from the liver is controversial (22, 23).

We aimed to assess the impact of the C-peptide status in patients with T1DM on the glucagon response during a hyperinsulinemic hypoglycemic clamp by measuring glucagon levels with a specific and highly sensitive glucagon assay and by assessing the endogenous glucose production (EGP) with a stable isotope tracer technique.

Materials and Methods

Trial design

We conducted an open, comparative trial in matched C-pos and C-neg patients with T1DM, applying a hyperinsulinemic, stepwise hypoglycemic clamp. Written informed consent was obtained from all patients before any trial-related activities were started. The trial was approved by the local ethics committee of the Medical University of Graz, Austria (26-070 ex 13/14) and performed in accordance with Good Clinical Practice (24) and the Declaration of Helsinki (25).

Participants

All enrolled patients with T1DM had a history of T1DM with acute hyperglycemia and ketonuria and had a daily insulin requirement with either multiple daily insulin injections or continuous subcutaneous insulin infusion. Inclusion criteria were age 18 to 64 years, body mass index (BMI) 18.0 to 28.0 kg/m^2, and hemoglobin A1c (HbA1c) 6.0% to 9.5% (42 to 80 mmol/mol). Exclusion criteria were any late complications of diabetes, including hypoglycemic unawareness, any severe hypoglycemic event within 1 month before screening, and any relevant health risk during the hypoglycemic clamp.

At the screening visit, the cutoff for C-neg was determined as a fasting C-peptide below the lower limit of quantification (LOQ) of the assay (LOQ = 0.017 nmol/L) (26), and the cutoff for C-pos was determined as a fasting C-peptide of ≥0.05 nmol/L. We used the electronical diabetes database built by our department for screening. It provides information about all patients with diabetes (type 1 and type 2) who have given written informed consent for potential participation in clinical trials. We screened 1000 patients with T1DM and invited all 40 consecutive C-pos patients for a screening visit. Finally, 10 C-pos patients with T1DM were enrolled, and 11 C-neg patients with T1DM, also screened from the database, were

matched with regard to age, sex, weight, and BMI. The recruitment phase lasted from January 2014 until October 2015. No study patient withdrew his or her informed consent during the study.

Stepwise hypoglycemic glucose clamp with stable isotope tracer technique

On the evening before the study day, participants arrived at the Clinical Research Center at 20:00 hours for an in-house stay for ≥16 hours. All participants had been advised not to inject long-acting or intermediate-acting insulin after 08:00 hours (no ultra-long-acting insulin was used) and not to do any strenuous exercise after 10:00 hours. The last short-acting insulin injection was administered with the last meal at 17:00 hours. No hypoglycemic event (PG ≤3.9 mmol/L) was allowed to take place after 10:00 hours in the morning on the day before the study day; otherwise, the participant's visit was rescheduled.

A hand vein was cannulated for sampling of arterialized venous blood and remained wrapped in a heating blanket throughout the clamp. To achieve normoglycemia overnight, a hand vein in the contralateral arm was cannulated for a variable human soluble insulin infusion [40 U Actrapid (100 U/mL) (NovoNordisk, Copenhagen, Denmark) in 99.6 mL NaCl (154 mmol/L)], until 08:00 hours on the study day, or for safety reasons a glucose infusion (10%, Fresenius Kabi, Graz, Austria) until 05:00 hours on the study day. Overnight, PG was measured every 5 to 30 minutes depending on the glucose concentrations needed to keep the PG stable (PG target level of 5.5 mmol/L) and to avoid nocturnal hypoglycemia (defined as PG level ≤3.9 mmol/L). At 05:00 hours [6,6-$^2H_2$]-glucose solution (100 g/L; Euriso-Top, Saint-Aubin Cedex, France) was given intravenously with a bolus of 9.6 mg/kg/min for 1 minute and a constant rate of 0.08 mg/kg/min until the end of the clamp. From 05:00 until 07:30 hours, a PG level deviation of ±30% was allowed. Two and a half hours after infusion start, tracer glucose equilibration phase was reached and defined as baseline (low insulin, duration 30 minutes), while a variable low-insulin infusion was administered for stable PG levels (5.5 mmol/L, ±20% of PG deviation). At 08:00 hours, the hyperinsulinemic clamp was initiated by increasing the insulin infusion to a constant rate of 1.5 mU/kg/min (Fig. 1). PG was kept stable at normoglycemia with a variable glucose infusion rate (GIR) enriched with 4 mg [6,6-$^2H_2$]-glucose/mL for ~90 minutes. Then, the first PG plateau of 5.5 mmol/L was started and lasted 30 minutes. Afterward, the GIR was turned off and the PG was allowed to drop to a plateau of 3.5 mmol/L and afterward to nadir (target 2.5 mmol/L). Each PG plateau was kept stable with the GIR for 30 minutes. For safety reasons, PG was not allowed to drop below 2.2 mmol/L. Fifteen minutes after having reached nadir, the constant insulin infusion was stopped, and the GIR was tapered off to enable spontaneous recovery from hypoglycemia, if possible. If PG had not recovered 45 minutes after the insulin infusion was terminated, a constant intravenous glucose infusion (5.5 mg/kg/min) was initiated to reach the last PG plateau of 4.0 mmol/L (10 minutes) and finally normoglycemia. Throughout the clamp, participants continued fasting

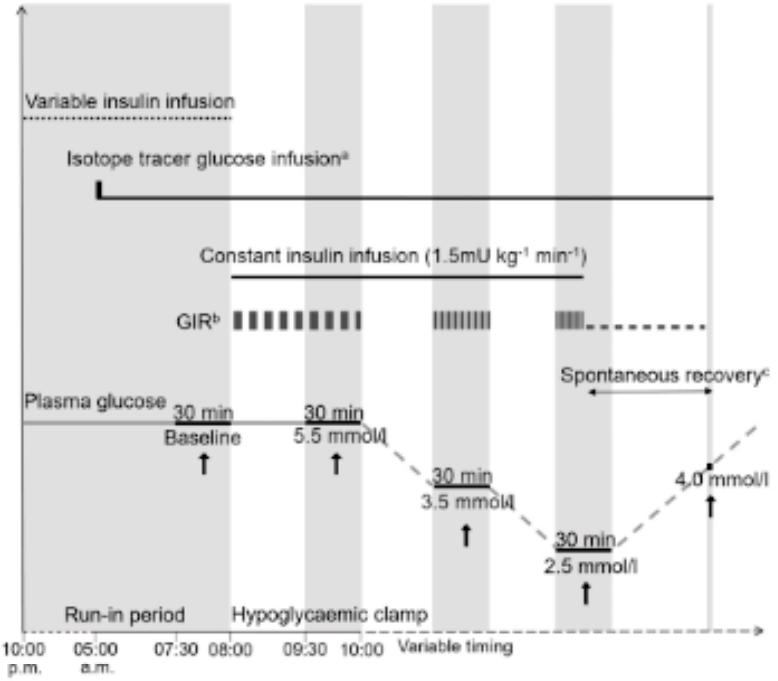

Figure 1. Hypoglycemic clamp with stable isotope tracer technique. [a]Isotope tracer glucose infusion: A bolus of 9.6 mg/kg/min of [6,6-$^2H_2$]-glucose was given for 1 minute and a constant rate of 0.08 mg/kg/min until the end of the clamp. [b]GIR was enriched with 4 mg [6,6-$^2H_2$]-glucose/mL. [c]Spontaneous recovery: After constant insulin infusion (1.5 mU/kg/min) was stopped, desired maximum time to reach plateau 4.0 mmol/L was 45 minutes. ↑, hypoglycemic response assessments include blood samples for glucagon and [6,6-$^2H_2$]-glucose, vital signs, hypoglycemic awareness, and hypoglycemic symptom tests.

(no food or beverages except water) and stayed in a supine or semisupine position. PG measurements were performed every 5 minutes from increasing insulin infusion (08:00 hours) until termination of the clamp. Blood sampling for measurement of [6,6-$^2H_2$]-glucose, glucagon, norepinephrine, and epinephrine concentrations was done at baseline and during the PG plateaus 5.5, 3.5, and 2.5 mmol/L at 0, 10, 20, and 30 minutes after the respective PG plateau had been reached. At the plateau 4.0 mmol/L, blood samples were taken at 0 and 10 minutes. Symptoms of hypoglycemia were evaluated with the Edinburgh Hypoglycemic Scale (27), and hypoglycemia awareness was assessed with the participants' responses (yes/no) to the question, "Do you feel any symptoms of hypoglycemia?"

Biochemical and hormonal blood analyses

P800 EDTA tubes (BD; Becton Dickinson, Franklin Lakes, NJ) were used to collect blood samples for the glucagon measurements. P800 EDTA tubes contain protease inhibitors and DPP-IV inhibitors and were selected based on the results of previously performed stability tests that showed a highly efficient inhibition of glucagon degradation (28). Samples were centrifuged for 15 minutes immediately after sample collection, stored at −80°C, and analyzed with a solid phase two-site enzyme immunoassay (Mercodia Glucagon ELISA; Mercodia, Uppsala, Sweden) (14, 16). The coefficient of variation (CV) for intra-assay variation was 3.3% to 5.1%, and the CV for interassay variation was 7.3% to 9.4%. The specificity for cross-reaction was with glicentin 0.8%, oxyntomodulin 4.4%, mini-glucagon <0.1%, glucagonlike peptide-1 <0.3%, glucagonlike peptide-2 <0.3%, and glicentin-related pancreatic peptide <0.0005%, and the detection limit was 1 pmol/L. All glucagon measurements were performed by blinded staff members in the laboratory.

EDTA plasma samples for detection of norepinephrine and epinephrine were stored at −80°C and were measured with a radioimmunoassay (DRG Instruments GmbH, Marburg, Germany). The plasma C-peptide concentrations were determined with a two-site sandwich immunoassay (ADVIA Centaur; Siemens Healthcare Diagnostics, Camberley, UK; LOQ 0.017 nmol/L). HbA1c was measured by high-performance liquid chromatography-ultraviolet (Menarini HA-8160; Menarini Diagnostics, Florence, Italy). No insulin measurements were done during the study.

Total PG concentrations for the clamp were measured at bedside with a glucose analyzer (Super GL; Dr. Müller Gerätebau GmbH, Freital, Germany; CV 2%). To determine natural glucose, [6,6-$^2H_2$]-glucose, and tracer-to-tracee ratio (TTR), blood samples were collected in sodium fluoride tubes, centrifuged immediately for 15 minutes at 4°C, and stored at −80°C, and plasma samples were prepared as described previously (29, 30). Standards were prepared by spiking the dialyzed plasma with well-known amounts of glucose (30 to 290 mg/dL) and [6,6-$^2H_2$]-glucose (0.5 to 5.8 mg/dL) as well as internal standard ([$^{13}C_6$]-glucose, [$^{13}C_6$, $^2H_7$]-glucose). The processed samples and standards (1 μL) were directly injected into the gas chromatography–mass spectrometry (GC-MS) (7890a GC, 7000b MS; Agilent Technologies, Santa Clara, CA) in splitless mode. Chromatography was performed with a flow rate of 2 mL/min and helium as carrier gas, with an HP-5MS 30 m × 250 μm × 0.25 μm GC column (Agilent Technologies) with the following temperature program: 110°C, hold 0.5 minutes, ramp 1: 25°C/min to 225°C. The analytes were detected with electron impact ionization in single ion monitoring mode at m/z 287 (natural glucose), m/z 288 ([$^2H_1$]-glucose), m/z 289 ([$^2H_2$]-glucose), m/z 293 ([$^{13}C_6$]-glucose), and m/z 300 ([$^{13}C_6$, $^2H_7$]-glucose). The natural glucose and [6,6-$^2H_2$]-glucose concentrations were quantified, and the TTR was calculated with the peak areas of the analytes. [6,6-$^2H_2$]-glucose measurement and calculation of TTR were also performed by blinded staff members.

Statistical analysis

Data are given as mean ± standard deviation (SD) unless indicated otherwise. The level of significance was set to $\alpha = 0.05$ for all tests. All parameters were tested for normality with a Shapiro-Wilk (SW) test. If data were distributed normally (SW test, $P \geq 0.05$), a t test was applied. Otherwise (SW test, $P < 0.05$), a Mann-Whitney U test was used. Average glucagon values were compared between C-pos and C-neg patients at baseline and at each PG plateau (5.5, 3.5, 2.5, and 4.0 mmol/L). Based on pilot data, the primary parameter was defined as average glucagon concentration measured at PG plateau 2.5 mmol/L. Within each group glucagon suppression was tested by calculating the individual difference between baseline and PG plateau 5.5 mmol/L, and the glucagon increase was assessed from PG plateau 5.5 to 2.5 mmol/L with a Wilcoxon test. Average PG, TTR, norepinephrine, and epinephrine were determined for each participant and each plateau, and resulting values were compared between the groups. Overnight average PG values were compared every 30 minutes between C-pos and C-neg patients with a Mann-Whitney U test.

Calculation for EGP and the rate of peripheral glucose disposal (Rd) were performed by blinded staff members to avoid a bias. EGP and Rd levels were calculated according to Powrie and the modified equation of Steele (29, 31, 32). Average EGP levels, Rd levels, and GIRs were calculated for each participant and each plateau in C-pos and C-neg patients, and the values from C-pos and C-neg patients were compared with each other. A paired t test was used to calculate EGP suppression within each group from baseline to plateau 5.5 mmol/L and to detect the EGP increase within each group between plateau 5.5 and 2.5 mmol/L. A Wilcoxon test was used within each group to assess the increase of the Rd from plateau 5.5 to 2.5 mmol/L. Norepinephrine and epinephrine blood samples were available only for 7 C-pos and 7 C-neg patients.

Additional parameters were the area under the curve (AUC) for glucose infusion rate (AUC_{GIR}), for glucagon ($AUC_{glucagon}$), and for endogenous glucose production (AUC_{EGP}). To calculate the AUC_{GIR}, $AUC_{glucagon}$, and AUC_{EGP} for each plateau, the trapezoidal method was applied. To calculate the area under the curve for total glucose infusion rate ($AUC_{GIRtotal}$), all AUC_{GIR} values at each PG plateau were added together. Linear regression was applied to $AUC_{glucagon}$ and AUC_{EGP}.

Results

Participant characteristics

Twenty-one men and women with T1DM were enrolled in the trial. Demographic and baseline characteristics are summarized in Table 1. Mean fasting C-peptide value was 0.16 ± 0.1 nmol/L for 10 C-pos patients and 0.0 ± 0.0 nmol/L for 11 C-neg patients.

Table 1. Demographic and Baseline Characteristics of C-Pos and C-Neg Patients With T1DM

Characteristic	C-Pos	C-Neg	*P*
Subjects, n	10	11	—
Sex, male/female	5/5	5/6	NS
Age, y	39.6 ± 13	37.4 ± 13	NS
Diabetes duration, y (range)	2.5 ± 2 (1–8)	23.9 ± 10 (11–37)	<0.001
BMI, kg/m^2	23.6 ± 1.8	25.0 ± 2.3	NS
C-peptide, nmol/L (range)	0.16 ± 0.1 (0.05–0.36)	0.00 ± 0.0[a] (0.00–0.01)	<0.001
HbA1c, %	7.3 ± 0.9	7.5 ± 0.8	NS
HbA1c, mmol/mol	56.3 ± 9.8	58.5 ± 8.7	NS
Daily basal insulin dose, U (range)	9.1 ± 4 (0–15)	24.5 ± 12 (11–50)	0.002
Daily bolus insulin dose, U (range)	17.7 ± 9 (5–36)	20.9 ± 4 (15–30)	NS
Total daily insulin dose, U (range)	26.9 ± 12 (10–51)	45.4 ± 14 (26–75)	0.017

Data are means ± SD (minimum–maximum). Kruskal-Wallis and Wilcoxon tests were used. Level of significance was set at $P < 0.05$.

Abbreviation: NS, not significant.

[a] LOQ of the assay = 0.017 nmol/L for C-peptide measurements.

PG levels, TTR, and GIR

No nocturnal hypoglycemic event occurred during the night before hypoglycemia was induced. C-pos patients needed less exogenous insulin during the night before hypoglycemia induction than C-neg patients. Mean PG levels did not differ at baseline (C-pos, 5.7 ± 0.4; C-neg, 5.7 ± 0.3 mmol/L), at glucose plateaus 5.5 and 4.0 mmol/L between the two groups (Fig. 2a). At glucose plateaus 3.5 and 2.5 mmol/L, mean PG levels were significantly higher in C-pos than in C-neg patients (difference in PG concentrations was 0.1, $P = 0.037$, and difference in PG concentrations was 0.2 mmol/L, $P = 0.011$, respectively). Mean TTRs were comparable in C-pos and C-neg patients at baseline and throughout the clamp at each glucose plateau (Fig. 2b). During the hypoglycemic clamp, AUC_{GIR} did not differ between C-pos and C-neg patients at all glucose plateaus (Fig. 2c). During recovery, which was defined as the time at glucose plateau 2.5 mmol/L (15 minutes after insulin infusion had been stopped) until the end of glucose plateau 4.0 mmol/L, AUC_{GIR} was significantly lower in C-pos than in C-neg patients (difference in AUC_{GIR} values was 102 mg/kg). $AUC_{GIR_{total}}$ was comparable in both groups.

Glucagon concentrations, EGP, and Rd

Mean glucagon concentrations were significantly higher in C-pos compared with C-neg patients at baseline (difference in glucagon concentrations was 4.2 pmol/L) and during all glucose plateaus (plateau 5.5 mmol/L, difference in glucagon concentrations was 2.6; plateau 3.5 mmol/L, difference in glucagon concentrations was 3.3; plateau 2.5 mmol/L, difference in glucagon concentrations was 7.0; and plateau 4.0 mmol/L, difference in glucagon concentrations was 8.2 pmol/L). At plateau 5.5 mmol/L, suppression of glucagon concentrations was observed in both groups after insulin infusion was increased. From glucose plateau 5.5 mmol/L to nadir, glucagon levels significantly increased in both groups (Fig. 3a).

Mean EGP rates did not differ in C-pos and C-neg patients at baseline and at glucose plateaus 5.5 and 3.5 mmol/L. At glucose plateaus 2.5 and 4.0 mmol/L, EGP response to hypoglycemia was significantly higher in C-pos compared with C-neg patients (differences in EGP were 0.4 and 0.8 mg/kg/min, respectively). At glucose plateau 5.5 mmol/L suppression of EGP occurred in both groups, after insulin infusion had been increased (Fig. 3b).

Mean Rd was similar in both groups at baseline and during all glucose plateaus. After insulin was increased to the constant rate, Rd increased for both groups at glucose plateau 5.5 mmol/L. Rd significantly decreased from glucose plateau 5.5 mmol/L to nadir glucose in both groups (Fig. 3c). Linear regression revealed a significant correlation between EGP and glucagon (Fig. 4).

Norepinephrine and epinephrine

Mean norepinephrine concentrations (Fig. 3d) did not differ between both groups except for lower concentrations of norepinephrine at baseline in C-neg compared with C-pos patients. Mean epinephrine concentrations (Fig. 3e) were lower in C-neg patients at plateau 5.5 and 4.0 mmol/L than in C-pos patients.

Hypoglycemic awareness and hypoglycemic symptom scores

At glucose plateau 5.5 mmol/L, all participants answered "no" to the question "Do you feel hypoglycemic?" and at glucose plateau 2.5 mmol/L, 57.1% of the patients answered "yes," regardless of their C-peptide status. Hypoglycemic symptom scores increased in response to hypoglycemia, with no differences in score according to the C-peptide status, at any PG plateau

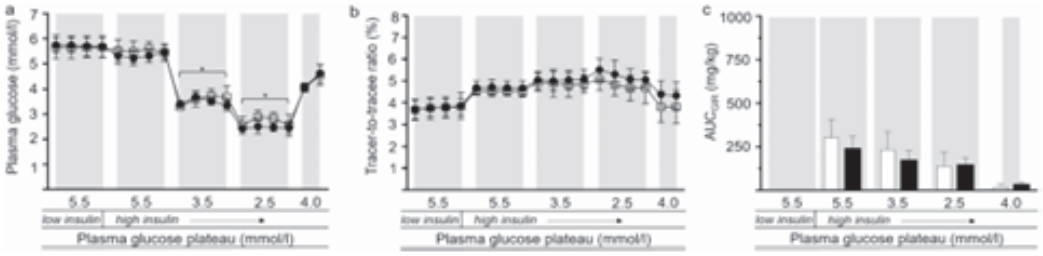

Figure 2. (a) PG concentrations, (b) TTR, and (c) AUC_{GIR} during hypoglycemic clamp at baseline (5.5 mmol/L, low insulin) and at each PG plateau (high insulin) in C-pos and C-neg patients. Four samples were taken at 10-minute intervals after patients reached baseline and the respective PG (5.5, 3.5, 2.5 mmol/L). Two samples were taken at 10-minute intervals at glucose plateau 4.0 mmol/L. Data are presented as means ± SD; C-pos patients, empty squares and white bars; C-neg patients, full circles and black bars; gray shaded areas, baseline and each PG plateau. AUC_{GIR} was calculated with the trapezoidal method. $^*P < 0.05$.

or when PG decreased from plateau 5.5 mmol/L to 2.5 mmol/L.

Discussion

Our main finding was that induced hypoglycemia revealed significantly higher glucagon concentrations in C-pos than in C-neg patients and therefore might contribute to more pronounced EGP in these patients. Furthermore, significantly lower glucagon concentrations were detected in all patients at baseline and at each PG plateau than those found in previous studies (22, 23, 33, 34).

Glucagon levels in C-pos patients were significantly higher in euglycemia and throughout the hypoglycemic clamp than in C-neg patients. Interestingly, we found an insulin-dependent suppression of glucagon secretion from baseline to PG plateau 5.5 mmol/L in both groups. Furthermore, we observed in our C-pos patients a hypoglycemia-induced threefold to fourfold increase in glucagon secretion from PG plateau 5.5 mmol/L to nadir (3.3 to 9.9 pmol/L, difference of 6.6) and to recovery (3.3 to 13.9 pmol/L, difference of 10.6). In our C-neg patients, hypoglycemia-induced fourfold to eightfold increases in glucagon secretion from PG plateau 5.5 mmol/L to nadir (0.7 to 2.9 pmol/L, difference of 2.2) and to recovery (0.7 to 5.7, difference of 5.0) were seen (Fig. 3a). The findings of reduced but responsive glucagon levels at euglycemia and hypoglycemia in T1DM are in contrast to previous reports describing hyperglucagonemia independent of C-peptide status and glycemic levels (34, 35). Although in the current experiments we cannot establish the mechanism of the plasma glucagon levels, it is likely that α-cells in the islets of Langerhans remain responsive to insulin

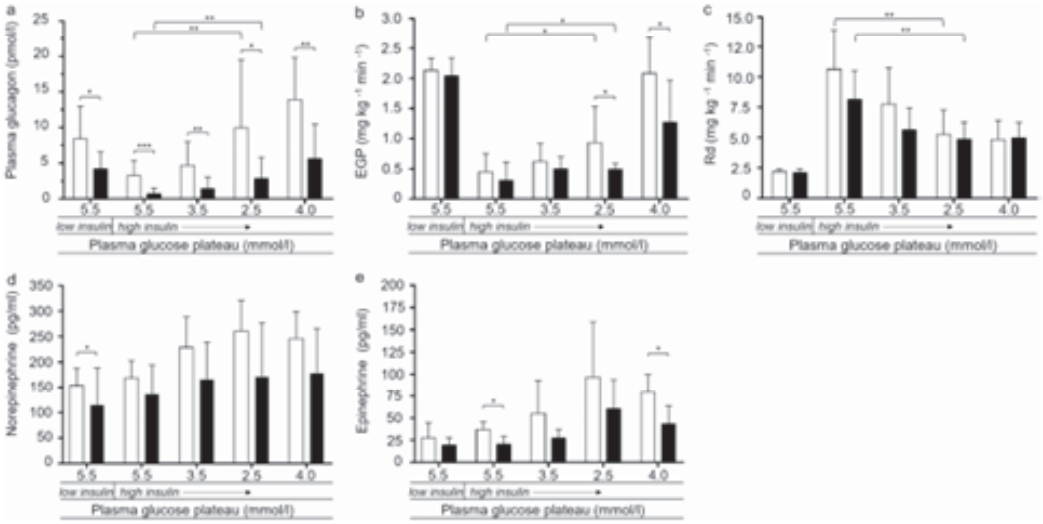

Figure 3. (a) Plasma glucagon concentrations, (b) EGP, (c) Rd, (d) norepinephrine, and (e) epinephrine at baseline (5.5 mmol/L, low insulin) and at each hypoglycemic clamp plateau (high insulin) in C-pos and C-neg patients. Data are means ± SD; C-pos patients, white bars; C-neg patients, black bars. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

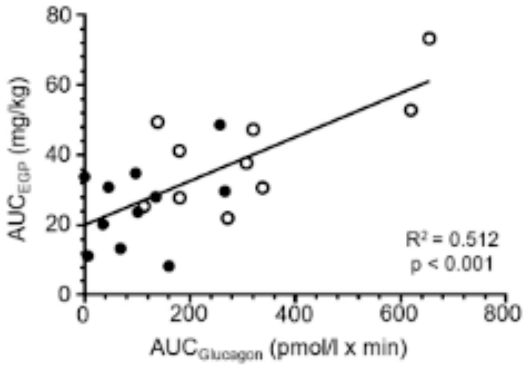

Figure 4. Linear regression of $AUC_{glucagon}$ and AUC_{EGP} in all patients with T1DM [n = 21; C-pos, empty circles (n = 10), C-neg, full circles (n = 11)]. $AUC_{glucagon}$, AUC of glucagon at plateaus 5.5, 3.5, 2.5 (standardized) and 4.0 mmol/L added together; AUC_{EGP}, AUC of EGP at plateaus 5.5, 3.5, 2.5 (standardized) and 4.0 mmol/L added together.

and to low glucose levels. This assumption is supported by our findings that C-pos patients exhibit higher glucagon levels than C-neg patients and by the positive glucagon levels staining in long-term patients with T1DM (36).

In line with our findings, Madsbad *et al.* (22) also observed higher glucagon levels with hypoglycemia in C-pos patients with T1DM compared with C-neg patients. However, they did not see any difference in glucose recovery between the two groups, and no EGPs were determined during their study. In contrast, we calculated the EGP and observed a greater EGP response at hypoglycemia (2.5 mmol/L) and recovery in C-pos than in C-neg patients. The reasons for these diverse results may lie in different hypoglycemic experimental setups. Madsbad *et al.* induced hypoglycemia with a constant insulin infusion, and they stopped the insulin infusion when patients had symptoms of hypoglycemia, regardless of their PG values. In contrast, we induced hypoglycemia stepwise depending on the patient's PG values (PG plateau 3.5, 2.5 mmol/L). Therefore, our clamp and the duration of hypoglycemia for each patient lasted longer, which could explain the different findings regarding glucose recovery.

In view of the anatomy of the pancreatic islets, one would expect in the basal euglycemic state that in C-pos patients with T1DM, the higher insulin concentrations at the level of the α-cell would suppress glucagon secretion, which would result in lower blood glucagon levels in C-pos T1DM compared with C-neg patients with T1DM. However, available published research comparing hormone levels between healthy subjects and patients with T1DM suggests that the reverse is the case (37–39). This research supports our findings of higher glucagon concentrations at baseline in C-pos than in C-neg patients with T1DM and suggests that in C-pos patients, the possibly higher insulin concentrations at the level of the α-cells may not suppress glucagon secretion.

Another impaired counterregulatory mechanism to decreasing PG in patients with T1DM is attenuated secretion of epinephrine and norepinephrine concentrations. In healthy subjects, the epinephrine response to hypoglycemia has also a stimulating effect on the EGP and limits glucose utilization by insulin-sensitive tissues (40, 41). It has been reported that the epinephrine response to hypoglycemia in healthy subjects and patients with new-onset T1DM remained intact, whereas patients with long-standing T1DM showed diminished epinephrine response (9, 42). However, we did not find a statistically significant difference between C-pos and C-neg patients regarding the epinephrine and norepinephrine response to hypoglycemia, but we found a tendency toward higher epinephrine and norepinephrine levels in C-pos compared with C-neg patients. Based on these results we assume that the epinephrine response might also contribute to the differences in the EGP response.

Several well-controlled clinical intervention studies have been performed aiming to prevent or postpone T1DM in people at risk and preserve residual β-cell function from autoimmune destruction (43–45). So far, these interventions have not been able to stop the autoimmune destruction of β-cells, but residual β-cell function has been preserved for a certain time (44–46). C-peptide status has been associated with severity of hypoglycemia in patients with T1DM, but the mechanism by which residual β-cell function and its impact on α-cells protect from severe hypoglycemia is still controversial (35, 37). It has been suggested that the inability of α-cells to produce adequate amounts of glucagon during hypoglycemia and the risk of severe hypoglycemic events is increasing with the loss of β-cell function (17, 18). In a previous study (23), the diabetes duration of patients with T1DM (7.8 ± 3.6 years) was comparable to that of our C-pos patients (range 1 to 8 years). However, our C-pos patients had higher glucagon levels and higher EGP values. We assume that the observed glucagon concentrations and the EGP might have been even more pronounced during hypoglycemia in newly diagnosed patients or patients with shorter diabetes duration.

The inclusion criteria for our C-pos patients was a fasting C-peptide level of ≥0.05 nmol/L, and it was challenging to find such patients for the study. Nevertheless, after recruitment the mean C-peptide level of our C-pos patients was 0.16 nmol/L. This level indicates a residual amount of endogenous insulin secretion that will remain for a certain period of time and that definitely is observed in all patients with T1DM. To ensure enrollment of patients with T1DM, the enrolled patients had a history

of T1DM with acute hyperglycemia and ketonuria, a daily insulin requirement, and a normal BMI.

We induced hypoglycemia by applying a hyperinsulinemic, stepwise hypoglycemic clamp and determined EGP with a stable isotope tracer technique. Although the tracer enrichment differed between plateaus, it remained stable within each plateau, which allowed us to apply the modified Steele equation to calculate EGP and Rd (31). Unfortunately, we cannot present data for insulin concentrations during the clamp, but based on results from Hother-Nielsen *et al.* (47) and Bell *et al.* (48), we assume that because of the high insulin infusion during the clamp and the likely suppression of endogenous insulin secretion, the insulin levels were comparable in both groups. During the hypoglycemic clamp, C-pos patients had higher mean PG levels at plateaus 3.5 and 2.5 mmol/L than C-neg patients. The fact that it was more difficult to reach glucose nadir in C-pos patients than in C-neg patients might be attributed to the greater counterregulation of higher glucagon levels and therefore higher EGP during hypoglycemia in C-pos than in C-neg patients. Furthermore, patients with T1DM with an increased glucagon release during hypoglycemia also had an increase of EGP, which strongly suggests a relationship between glucagon and EGP (Fig. 4). Two patients showed substantially high glucagon response in comparison with the other patients, but the glucagon response in healthy subjects is even higher than the two highest found in our study. C-pos patients spanning a wider range of glucagon response to hypoglycemia would have been desirable for the study. Of note, C-pos and C-neg patients showed similar glucose requirements, the same Rd, and the same hypoglycemic awareness and symptoms during hypoglycemia.

A limitation of the study is that it lacks a glucagon stimulation test to stimulate endogenous insulin secretion for C-peptide quantification. However, detecting fasting C-peptide concentrations is a validated standard. Another limitation of this study is the lack of a nondiabetic control group.

In conclusion, induced hypoglycemia revealed significantly higher glucagon concentrations and might contribute to more pronounced EGP in C-pos than in C-neg patients, which indicates that preserved β-cell function may contribute to counterregulation during hypoglycemia in patients with T1DM. Additional studies are needed to elucidate the role of the C-peptide status in the pathophysiology of glucagon secretion and its impact on the EGP to counteract hypoglycemia in patients with T1DM.

Acknowledgments

The authors thank Martina Urschitz and Michael Wolf (Division of Endocrinology and Diabetology, Medical University of Graz, Austria) for performing the hypoglycemic clamp, Andrea Berghofer (Division of Endocrinology and Diabetology, Medical University of Graz, Austria) for data monitoring, Petra Baumann (Joanneum Research Forschungsgesellschaft mbH HEALTH—Institute for Biomedicine and Health Sciences, Graz, Austria) for statistical assistance, Bernd Tschapeller and Christian Krainer (Joanneum Research Forschungsgesellschaft mbH HEALTH—Institute for Biomedicine and Health Sciences, Graz, Austria) for data management, Selma Mautner (Division of Endocrinology and Diabetology, Medical University of Graz, and Joanneum Research Forschungsgesellschaft mbH HEALTH—Institute for Biomedicine and Health Sciences, Graz, Austria) for editorial assistance and critical review of the manuscript, and the participants for their participation in the study.

Financial Support: This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Clinical Trial Information: ClinicalTrials.gov no. NCT02028078 (registered 6 January 2014).

Author Contributions: S.Z., W.R., and T.R.P. contributed to the conception and the study design. S.Z. and S.K. conducted the clinical study. S.Z., M.B., C.M., R.R., and S.H.N. contributed to the acquisition of data. S.Z. and T.A. contributed to the statistical analysis. S.Z., J.K.M., W.R., B.B., P.E., and T.R.P. interpreted the data. S.Z. drafted the manuscript. All authors reviewed and revised the manuscript for important intellectual content and gave final approval of the manuscript to be published. T.R.P. is the guarantor of this work. The data that support the findings of this study are available from the corresponding author upon request.

Correspondence and Reprint Requests: Thomas R. Pieber, MD, Division of Endocrinology and Diabetology, Department of Internal Medicine, Medical University of Graz, Auenbruggerplatz 15, 8036 Graz, Austria. E-mail: endo@medunigraz.at.

Disclosure Summary: The authors have nothing to disclose.

References

1. Nathan DM, Genuth S, Lachin J, Cleary P, Crofford O, Davis M, Rand L, Siebert C; Diabetes Control and Complications Trial Research Group. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N Engl J Med*. 1993;**329**(14):977–986.
2. Nathan DM, Bayless M, Cleary P, Genuth S, Gubitosi-Klug R, Lachin JM, Lorenzi G, Zinman B; DCCT/EDIC Research Group. Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Study at 30 years: advances and contributions. *Diabetes*. 2013;**62**(12):3976–3986.
3. Strandberg RB, Graue M, Wentzel-Larsen T, Peyrot M, Wahl AKRB, Rokne B. The relationships among fear of hypoglycaemia, diabetes-related quality of life and psychological well-being in Norwegian adults with type 1 diabetes. *Diabetes Res Clin Pract*. 2017;**124**(124):11–19.
4. Evans ML. Too much or too little? The double jeopardy of subcutaneous insulin therapy in diabetes. *Diabetes*. 2015;**64**(10):3353–3354.

5. Cryer PE. Minireview: glucagon in the pathogenesis of hypoglycemia and hyperglycemia in diabetes. *Endocrinology*. 2012;**153**(3):1039–1048.
6. Ramnanan CJ, Edgerton DS, Kraft G, Cherrington AD. Physiologic action of glucagon on liver glucose metabolism. *Diabetes Obes Metab*. 2011;**13**(suppl 1):118–125.
7. Kramer CK, Borgoño CA, Van Nostrand P, Retnakaran R, Zinman B. Glucagon response to oral glucose challenge in type 1 diabetes: lack of impact of euglycemia. *Diabetes Care*. 2014;**37**(4):1076–1082.
8. Hare KJ, Vilsbøll T, Holst JJ, Knop FK. Inappropriate glucagon response after oral compared with isoglycemic intravenous glucose administration in patients with type 1 diabetes. *Am J Physiol Endocrinol Metab*. 2010;**298**(4):E832–E837.
9. Bolli G, de Feo P, Compagnucci P, Cartechini MG, Angeletti G, Santeusanio F, Brunetti P, Gerich JE. Abnormal glucose counterregulation in insulin-dependent diabetes mellitus. Interaction of anti-insulin antibodies and impaired glucagon and epinephrine secretion. *Diabetes*. 1983;**32**(2):134–141.
10. Cryer PE. Hypoglycemia in type 1 diabetes mellitus. *Endocrinol Metab Clin North Am*. 2010;**39**(3):641–654.
11. Gerich JE, Langlois M, Noacco C, Karam JH, Forsham PH. Lack of glucagon response to hypoglycemia in diabetes: evidence for an intrinsic pancreatic alpha cell defect. *Science*. 1973;**182**(4108):171–173.
12. Taborsky GJ Jr, Ahrén B, Havel PJ. Autonomic mediation of glucagon secretion during hypoglycemia: implications for impaired alpha-cell responses in type 1 diabetes. *Diabetes*. 1998;**47**(7):995–1005.
13. Farhy LS, McCall AL. Optimizing reduction in basal hyperglucagonaemia to repair defective glucagon counterregulation in insulin deficiency. *Diabetes Obes Metab*. 2011;**13**(suppl 1[2]):133–143.
14. Wewer Albrechtsen NJ, Hartmann B, Veedfald S, Windeløv JA, Plamboeck A, Bojsen-Møller KN, Idorn T, Feldt-Rasmussen B, Knop FK, Vilsbøll T, Madsbad S, Deacon CFHJ, Holst JJ. Hyperglucagonaemia analysed by glucagon sandwich ELISA: nonspecific interference or truly elevated levels? *Diabetologia*. 2014;**57**(9):1919–1926.
15. Holst JJ. Evidence that enteroglucagon (II) is identical with the C-terminal sequence (residues 33–69) of glicentin. *Biochem J*. 1982;**207**(3):381–388.
16. *Mercodia Glucagon ELISA: directions for use*. Uppsala, Sweden: Mercodia; 2013.
17. UK Hypoglycaemia Study Group. Risk of hypoglycaemia in types 1 and 2 diabetes: effects of treatment modalities and their duration. *Diabetologia*. 2007;**50**(6):1140–1147.
18. The DCCT Research Group. Epidemiology of severe hypoglycemia in the diabetes control and complications trial. *Am J Med*. 1991;**90**(4):450–459.
19. Mühlhauser I, Overmann H, Bender R, Bott U, Berger M. Risk factors of severe hypoglycaemia in adult patients with type I diabetes–a prospective population based study. *Diabetologia*. 1998;**41**(11):1274–1282.
20. Steffes MW, Sibley S, Jackson M, Thomas W. Beta-cell function and the development of diabetes-related complications in the diabetes control and complications trial. *Diabetes Care*. 2003;**26**(3):832–836.
21. Rickels MR, Peleckis AJ, Markmann E, Dalton-Bakes C, Kong SM, Teff KL, Naji A. Long-term improvement in glucose control and counterregulation by islet transplantation for type 1 diabetes. *J Clin Endocrinol Metab*. 2016;**101**(11):4421–4430.
22. Madsbad S, Hilsted J, Krarup T, Sestoft L, Christensen NJ, Faber OK, Tronier B. Hormonal, metabolic and cardiovascular responses to hypoglycaemia in type 1 (insulin-dependent) diabetes with and without residual B cell function. *Diabetologia*. 1982;**23**(6):499–503.
23. Hoffman RP, Arslanian S, Drash AL, Becker DJ. Impaired counterregulatory hormone responses to hypoglycemia in children and adolescents with new onset IDDM. *J Pediatr Endocrinol Metab*. 1994;**7**(3):235–244.
24. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH harmonized tripartite guideline: guideline for good clinical practice. *J Postgrad Med*. 2009;**47**(1):45–50.
25. World Medical Association Inc. Declaration of Helsinki. Ethical principles for medical research involving human subjects. *J Indian Med Assoc*. 2009;**107**(6):403–405.
26. *ADVIA Centaur C-Peptide ELISA: direction for use*. Camberley, UK: Siemens Healthcare Diagnostics; 2012.
27. McAulay V, Deary IJ, Frier BM. Symptoms of hypoglycaemia in people with diabetes. *Diabet Med*. 2001;**18**(9):690–705.
28. Brunner M, Raml R, Mautner S, Zenz S, Lipp RPT. Latest news on glucagon stability [abstract]. In: 77th Scientific Sessions, American Diabetes Association (ADA); June 9–13, 2017; San Diego, CA.
29. Plank J, Bodenlenz M, Sinner F, Magnes C, Görzer E, Regittnig W, Endahl LA, Draeger E, Zdravkovic M, Pieber TR. A double-blind, randomized, dose-response study investigating the pharmacodynamic and pharmacokinetic properties of the long-acting insulin analog detemir. *Diabetes Care*. 2005;**28**(5):1107–1112.
30. Hachey DL, Parsons WR, McKay S, Haymond MW. Quantitation of monosaccharide isotopic enrichment in physiologic fluids by electron ionization or negative chemical ionization GC/MS using di-O-isopropylidene derivatives. *Anal Chem*. 1999;**71**(20):4734–4739.
31. Steele R, Wall JS, De Bodo RC, Altszuler N. Measurement of size and turnover rate of body glucose pool by the isotope dilution method. *Am J Physiol*. 1956;**187**(1):15–24.
32. Powrie JK, Smith GD, Hennessy TR, Shojaee-Moradie F, Kelly JM, Sönksen PH Jr, Jones RH. Incomplete suppression of hepatic glucose production in non-insulin dependent diabetes mellitus measured with [6,6-2H2]glucose enriched glucose infusion during hyperinsulinaemic euglycaemic clamps. *Eur J Clin Invest*. 1992;**22**(4):244–253.
33. Briscoe VJ, Ertl AC, Tate DB, Davis SN. Effects of the selective serotonin reuptake inhibitor fluoxetine on counterregulatory responses to hypoglycemia in individuals with type 1 diabetes. *Diabetes*. 2008;**57**(12):3315–3322.
34. Pieber TR, Deller S, Korsatko S, Jensen L, Christiansen E, Madsen J, Heller SR. Counter-regulatory hormone responses to hypoglycaemia in people with type 1 diabetes after 4 weeks of treatment with liraglutide adjunct to insulin: a randomized, placebo-controlled, double-blind, crossover trial. *Diabetes Obes Metab*. 2015;**17**(8):742–750.
35. Sherr J, Xing D, Ruedy KJ, Beck RW, Kollman C, Buckingham B, White NH, Fox L, Tsalikian E, Weinzimer S, Arbelaez AM, Tamborlane WV; Diabetes in Children Network. Lack of association between residual insulin production and glucagon response to hypoglycemia in youth with short duration of type 1 diabetes. *Diabetes Care*. 2013;**36**(6):1470–1476.
36. Foulis AK. The pathology of islets in diabetes. *Eye (Lond)*. 1993;**7**(Pt 2):197–201.
37. Siafarikas A, Johnston RJ, Bulsara MK, O'Leary P, Jones TW, Davis EA. Early loss of the glucagon response to hypoglycemia in adolescents with type 1 diabetes. *Diabetes Care*. 2012;**35**(8):1757–1762.
38. Rickels MR, Fuller C, Dalton-Bakes C, Markmann E, Palanjian M, Cullison K, Tiao J, Kapoor S, Liu C, Naji A, Teff KL. Restoration of glucose counterregulation by islet transplantation in long-standing type 1 diabetes. *Diabetes*. 2015;**64**(5):1713–1718.
39. Bischof MG, Bernroider E, Krssak M, Krebs M, Stingl H, Nowotny P, Yu C, Shulman GI, Waldhäusl W, Roden M. Hepatic glycogen metabolism in type 1 diabetes after long-term near normoglycemia. *Diabetes*. 2002;**51**(1):49–54.

40. Cryer PE. Hypoglycaemia: the limiting factor in the glycaemic management of type I and type II diabetes. *Diabetologia*. 2002; **45**(7):937–948.
41. Sprague JE, Arbeláez AM. Glucose counterregulatory responses to hypoglycemia. *Pediatr Endocrinol Rev*. 2011;**9**(1):463–475.
42. Arbelaez AM, Xing D, Cryer PE, Kollman C, Beck RW, Sherr J, Ruedy KJ, Tamborlane WV, Mauras N, Tsalikian E, Wilson DM, White NH; Diabetes Research in Children Network (DirecNet) Study Group. Blunted glucagon but not epinephrine responses to hypoglycemia occurs in youth with less than 1 yr duration of type 1 diabetes mellitus. *Pediatr Diabetes*. 2014;**15**(2):127–134.
43. Simmons KM, Gottlieb PA, Michels AW. Immune intervention and preservation of pancreatic beta cell function in type 1 diabetes. *Curr Diab Rep*. 2016;**16**(10):97.
44. Rigby MR, Harris KM, Pinckney A, DiMeglio LA, Rendell MS, Felner EI, Dostou JM, Gitelman SE, Griffin KJ, Tsalikian E, Gottlieb PA, Greenbaum CJ, Sherry NA, Moore WV, Monzavi R, Willi SM, Raskin P, Keyes-Elstein L, Long SA, Kanaparthi S, Lim N, Phippard D, Soppe CL, Fitzgibbon ML, McNamara J, Nepom GT, Ehlers MR. Alefacept provides sustained clinical and immunological effects in new-onset type 1 diabetes patients. *J Clin Invest*. 2015;**125**(8):3285–3296.
45. Pescovitz MD, Greenbaum CJ, Bundy B, Becker DJ, Gitelman SE, Goland R, Gottlieb PA, Marks JB, Moran A, Raskin P, Rodriguez H, Schatz DA, Wherrett DK, Wilson DM, Krischer JP, Skyler JS; Type 1 Diabetes TrialNet Anti-CD20 Study Group. B-lymphocyte depletion with rituximab and β-cell function: two-year results. *Diabetes Care*. 2014;**37**(2):453–459.
46. Orban T, Bundy B, Becker DJ, DiMeglio LA, Gitelman SE, Goland R, Gottlieb PA, Greenbaum CJ, Marks JB, Monzavi R, Moran A, Raskin P, Rodriguez H, Russell WE, Schatz D, Wherrett D, Wilson DM, Krischer JP, Skyler JS; Type 1 Diabetes TrialNet Abatacept Study Group. Co-stimulation modulation with abatacept in patients with recent-onset type 1 diabetes: a randomised, double-blind, placebo-controlled trial. *Lancet*. 2011;**378**(9789):412–419.
47. Hother-Nielsen O, Henriksen JE, Holst JJ, Beck-Nielsen H. Effects of insulin on glucose turnover rates in vivo: isotope dilution versus constant specific activity technique. *Metabolism*. 1996;**45**(1): 82–91.
48. Bell PM, Firth RG, Rizza RA. Assessment of insulin action in insulin-dependent diabetes mellitus using [6(14)C]glucose, [3(3)H]glucose, and [2(3)H]glucose. Differences in the apparent pattern of insulin resistance depending on the isotope used. *J Clin Invest*. 1986;**78**(6):1479–1486.

The Journal of Clinical Endocrinology & Metabolism, 2022, **107**, e3583–e3593
https://doi.org/10.1210/clinem/dgac369
Advance access publication 14 July 2022
Clinical Research Article

Effect of Liraglutide Treatment on Whole-body Glucose Fluxes in C-peptide–Positive Type 1 Diabetes During Hypoglycemia

Sabine Zenz,[1,2] Werner Regittnig,[1] Beate Boulgaropoulos,[1,3] Thomas Augustin,[3] Martina Brunner,[4] Stefan Korsatko,[1] Julia Münzker,[1] Sophie H. Narath,[3] Reingard Raml,[3] Christoph Magnes,[3] and Thomas R. Pieber[1,3,4]

[1]Division of Endocrinology and Diabetology, Department of Internal Medicine, Medical University of Graz, Graz, Austria
[2]Division of Rheumatology and Immunology, Department of Internal Medicine, Medical University of Graz, Graz, Austria
[3]Joanneum Research Forschungsgesellschaft mbH HEALTH – Institute for Biomedicine and Health Sciences, Graz, Austria
[4]Center for Medical Research, Clinical Trial Unit, Medical University of Graz, Graz, Austria

Correspondence: Thomas R. Pieber, MD, Division of Endocrinology and Diabetology, Department of Internal Medicine; Medical University of Graz, Auenbruggerplatz 15, 8036 Graz, Austria. Email: endo@medunigraz.at.

Abstract

Context: The effect of liraglutide in C-peptide–positive (C-pos) type 1 diabetes (T1DI) patients during hypoglycemia remains unclear.

Objective: To investigate the effect of a 12-week liraglutide treatment on the body glucose fluxes during a hypoglycemic clamp in C-pos T1D patients and its impact on the alpha- and beta-cell responses during hypoglycemia.

Design: This was a randomized, double-blind, crossover study. Each C-pos T1D patient was allocated to the treatment sequence liraglutide/placebo or placebo/liraglutide with daily injections for 12 weeks adjunct to insulin treatment, separated by a 4-week washout period.

Setting and Participants: Fourteen T1D patients with fasting C-peptide ≥ 0.1 nmol/L.

Intervention(s): All patients underwent a hyperinsulinemic-stepwise-hypoglycemic clamp with isotope tracer (plasma glucose (PG) plateaus: 5.5, 3.5, 2.5, and 3.9 mmol/L) after a 3-month liraglutide (1.2 mg) or placebo treatment.

Main Outcome Measure(s): The responses of endogenous glucose production (EGP) and rate of peripheral glucose disposal (Rd) were similar for liraglutide and placebo treatment during the clamp.

Results: The numbers of hypoglycemic events were similar in both groups. At the clamp, mean glucagon levels were significantly lower at PG plateau 5.5 mmol/L in the liraglutide than in the placebo group but showed similar responses to hypoglycemia in both groups. Mean C-peptide levels were significantly higher at PG-plateaus 5.5 and 3.5 mmol/L after liraglutide treatment, but this effect was not reflected in EGP and Rd. Hemoglobin A1c and body weight were lower, and a trend for reduced insulin was seen after liraglutide treatment.

Conclusions: The results indicate that 3 months of liraglutide treatment does not promote or prolong hypoglycemia in C-pos T1D patients.

Key Words: type 1 diabetes, hypoglycemia, liraglutide, beta-cell function, GLP-1 analogue, clinical trial

Abbreviations: AE, adverse events; AUC, area under the curve; $AUC_{C\text{-}peptide\ 0\text{-}240'}$ area under the curve of C-peptide levels from 0 to 240 minutes; AUC_{EGP}, area under the curve of endogenous glucose production; AUC_{GIR}, area under the curve of glucose infusion rate; $AUC_{glucose\ 0\text{-}240'}$ area under the curve of glucose levels from 0 to 240 minutes; $AUC_{insulin\ 0\text{-}240'}$ area under the curve of insulin from 0 to 240 minutes; AUC_{Rd}, area under the curve of peripheral glucose disposal; $AUC_{insulin\ 0\text{-}240'}$ area under the curve of insulin levels from 0 to 240 minutes; BMI, body mass index; C-pos, C-peptide positive; EGP, endogenous glucose production; GIR, glucose infusion rate; GLP-1, glucagon-like peptide 1; HbA1c, glycosylated hemoglobin A1c; MMTT, mixed meal tolerance test; N, number of patients; NA, not applicable; NEFA, nonesterified fatty acids; PG, plasma glucose; Rd, rate of peripheral glucose disposal; SEM, SE of the mean; SW, Shapiro-Wilk test.

Liraglutide is a glucagon-like peptide-1 (GLP-1) receptor agonist approved for the treatment of type 2 diabetes mellitus (T2D) in combination with insulin or other oral glucose-lowering agents (1). It stimulates insulin and inhibits glucagon secretion in a glucose-dependent manner, and it positively influences metabolic control due to weight loss, decreased glycosylated hemoglobin A1c (HbA1c) and improved insulin sensitivity in T2D patients (2-4). Liraglutide treatment has also reduced the cardiovascular risk in patients with T2D (5, 6). Driven by these beneficial effects in T2D patients, several studies have investigated the impact of liraglutide treatment in type 1 diabetes (T1D) patients (7-15), and beneficial effects on metabolic control have been observed in some (8, 16-18), but not all, studies (19-21).

The 2 largest long-term studies, a 52-week (ADJUNCT ONE study) (8) and a 26-week study (ADJUNCT TWO study) (17), have tested the effect of liraglutide in T1D. Both studies observed improved metabolic control (measured by the HbA1c level), reduced insulin requirement, and reduced body weight with liraglutide treatment compared to placebo. Interestingly, a subgroup analysis in the ADJUNCT TWO study revealed that the glucose-lowering effects after liraglutide treatment

Received: 9 August 2021. **Editorial Decision:** 12 June 2022. **Corrected and Typeset:** 20 July 2022

[2] Copy of "Effect of Liraglutide Treatment on Whole-body Glucose Fluxes in C-peptide-Positive Type 1 Diabetes During Hypoglycemia". Zenz S, Regittnig W, Boulgaropoulos B, Augustin T, Brunner M, Korsatko S, et al. J Clin Endocrinol Metab 2022;107:E3583–93. https://doi.org/10.1210/clinem/dgac369 [132]. No modifications were made. Copyright 2022 by Oxford University Press on behalf of the Endocrine Society. Reprinted with permission.

were more pronounced in C-peptide–positive (C-pos) T1D patients than in C-peptide–negative (C-neg) patients (17). However, in both trials an increased number of hypoglycemic events with liraglutide treatment compared to placebo have been observed (8, 17). Furthermore, in C-neg patients, a liraglutide dose-finding study over 4 weeks showed a reduced glucose infusion rate (GIR) during a standardized hypoglycemic clamp (7), suggesting either an increased endogenous glucose production (EGP) or a decreased rate of peripheral glucose disposal (Rd). Nonetheless, how liraglutide affects EGP and Rd responses during hypoglycemia was not assessed in this trial. Therefore, the aim of the presented study was to assess the whole-body glucose fluxes (EGP and Rd) under hypoglycemic conditions in C-pos T1D patients. For this purpose, we performed a hyperinsulinemic-stepwise-hypoglycemic clamp using a stable isotope tracer technique. Furthermore, because the EGP and Rd fluxes mainly depend on glucagon and insulin concentrations, we also assessed counterregulatory hormonal responses during the clamp.

Materials and Methods

Patients

We screened T1D patients with a residual beta-cell function, defined with history of acute hyperglycemia and ketonuria at T1D diagnosis, positive results for at least 1 of 4 specific islet antibodies (glutamic acid decarboxylase, protein tyrosine phosphatase, zinc transporter 8, islet cell antibodies), and with a cutoff level for fasting C-peptide of ≥0.1 nmol/L at screening visit. All patients were treated with daily insulin injections or continuous subcutaneous insulin infusion. We included patients between 18 and 64 years (both inclusive), with a body mass index (BMI) of 19.0 to 28.0 kg/m² (both inclusive) and an HbA1c ≤ 80 mmol/mol (≤9.5%).

Exclusion criteria were the use of a GLP-1 receptor agonist within 3 months before study start, a screening calcitonin > 50 ng/L, a family or personal history of multiple endocrine neoplasia type 2, a medullary thyroid cancer or a history of nonfamilial medullary thyroid carcinoma, and a history of chronic or idiopathic acute pancreatitis. Further exclusion criteria were the occurrence of a severe hypoglycemic event within 1 month prior to screening and hypoglycemia unawareness. For recruitment, we screened T1D patients with a fasting C-peptide ≥ 0.1 nmol/L who gave written informed consent for participation in clinical trials. The recruitment lasted 9 months, and finally 14 T1D patients with fasting C-peptide ≥ 0.1 nmol/L (C-pos) were enrolled and randomized to the study.

Clinical Study

We performed a randomized, single-center, double-blind, placebo-controlled, 2-period crossover study. Each patient was allocated to 1 of the 2 treatment sequences (liraglutide/placebo or placebo/liraglutide) adjunct to intensive insulin treatment for 12 weeks. A washout period of 4 weeks between the 2 periods was carried out (Fig. 1A). Starting dose of liraglutide was 0.3 mg, and the dose was increased weekly by 0.3 mg to reach a dose of 1.2 mg after 4 weeks. This final dose was then kept stable for 8 weeks. Liraglutide/placebo was administered once daily by subcutaneous injection at 10:00 pm. Insulin treatment was adjusted depending on the treatment dose and the patient's demand. Patients had to document the daily study product administration and each hypoglycemic event during the study. Written informed consent was obtained from all patients before any study-related activities were started. The clinical study was approved by the local ethics committee of the Medical University of Graz, Austria (26-070 ex 13/14) and performed in accordance with the guidelines for good clinical practice (22) and the Declaration of Helsinki (23). The study was registered at Clinicaltrials.gov (NCT02408705). Mixed meal tolerances tests (MMTTs) were performed at the beginning and the end of each treatment period (Fig. 1A). For all study days, the patients were advised to inject long-acting insulin no later than 10:00 pm 2 days before start of the MMTT, intermediate-acting insulin or neutral protamine hagedorn insulin no later than 10:00 pm 1 day before MMTT start and short-acting insulin no later than 6 hours before MMTT start. Further, no hypoglycemic event was allowed to take place in the night prior to MMTT start, and a low-carbohydrate diet and strenuous exercise later than 3 days prior to MMTT start were avoided.

Stepwise Hypoglycemic Glucose Clamp With Stable Isotope Tracer Technique

At the end of each treatment period, all patients underwent a hypoglycemic clamp. As described previously (24), a stable isotope tracer technique (25, 26) was used to determine the rates of EGP and Rd during the hypoglycemic clamp. After the MMTT (which was performed prior to the clamp) had been completed, patients stayed at the Clinical Trials Unit to manage glycinemic control during the day until the hypoglycemic glucose clamp was initiated (Fig. 1B). Last food intake was allowed at 3:00 pm. Only short-acting insulin injection was used to correct hyperglycemia, but not later than 6:00 pm. To avoid hypoglycemic events [plasma glucose (PG) ≤ 3.9 mmol/L], slight intake of rapidly absorbable carbohydrates was allowed. Blood samples for the determination of glucagon and C-peptide concentrations were drawn at 9:45 pm, and the last liraglutide/placebo administration was performed at 10:00 pm. To maintain normoglycemia overnight, patients received either a variable human soluble insulin infusion [40 U Actrapid (100 U/mL), NovoNordisk, Copenhagen, Denmark, in 99.6 mL NaCl (154 mmol/L)] until 8:00 am or a glucose infusion (10%, Fresenius Kabi, Graz, Austria), until 5:00 am. No variable glucose infusion was allowed 2.5 hours prior the first PG plateau 5.5 mmol/L (low insulin). PG was measured every 5 to 30 minutes depending on the PG level (PG target of 5.5 mmol/L ± 30%) to avoid nocturnal hypoglycemia (PG ≤ 3.9 mmol/L). At 5:00 am, a bolus (9.6 mg/kg/min) of stable tracer [6,6-$^2H_2$]-glucose solution (100 g/L, Euriso-Top, Saint-Aubin Cedex, France) was given intravenously over 1 minute and thereafter at a constant rate of 0.08 mg/kg/min until the end of the clamp. At 7:30 am, after equilibrium conditions for tracer glucose and stable euglycemia (PG 5.5 mmol/L) had been achieved (24), assessments at ambient insulin concentrations (low insulin, baseline duration: 30 minutes) were performed. At this PG plateau, a PG level deviation of ± 10% was allowed and the variable insulin infusion was fixed during the whole PG plateau. At 8:00 am, for induction of hypoglycemia, the insulin infusion was increased to a fixed rate of 1.5 mU/kg/min, and a variable glucose infusion with 4 mg [6,6-$^2H_2$]-glucose/mL was administered intravenously to keep the PG level stable at 5.5 mmol/L. After 90 minutes, the first PG plateau

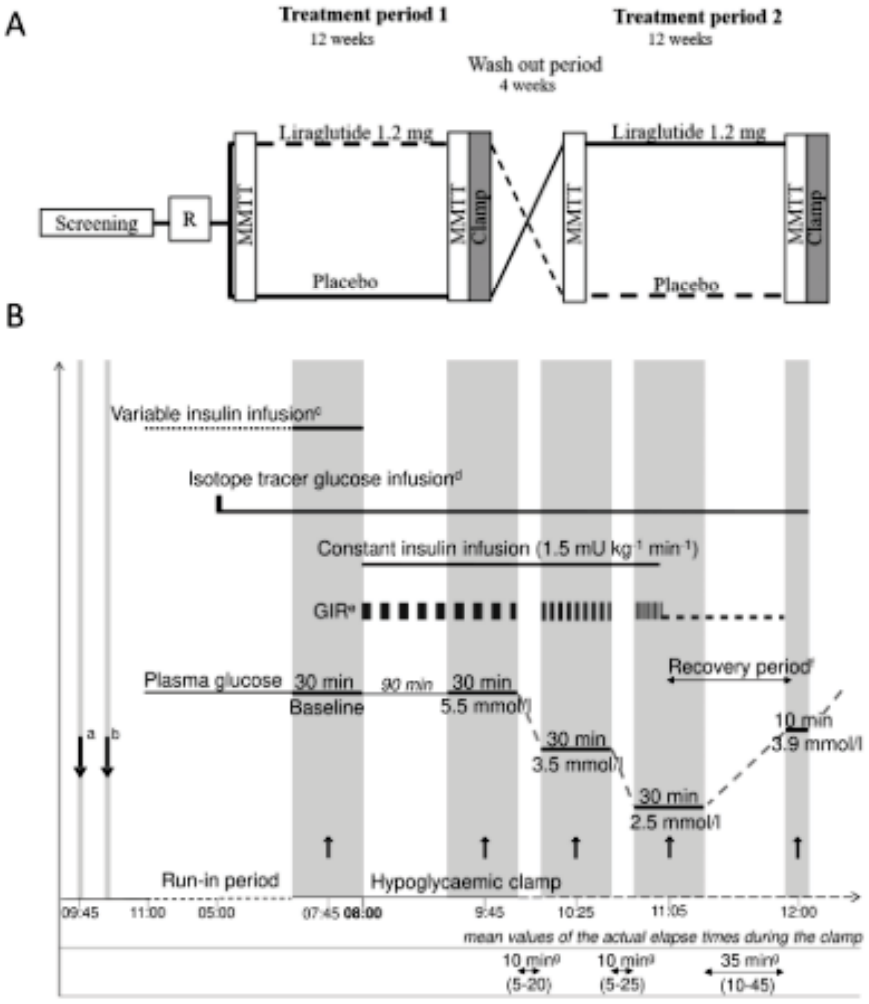

Figure 1. Study design (A) and hypoglycemic clamp design (B). The hypoglycemic clamp was performed after 12 weeks of once-daily treatment with liraglutide or placebo adjunct to insulin in C-peptide–positive T1D patients. Dose was stepwise increased from 0.3 to 0.6 to 0.9 to 1.2 mg over 4 weeks. Abbreviations: R, randomization, MMTT, mixed meal tolerance test, Clamp, hyperinsulinemic hypoglycemic clamp. †Hypoglycemic response assessments includes blood samples to assess [6,6-$^2H_2$]-glucose concentrations, counterregulatory hormone levels (glucagon, norepinephrine, epinephrine, cortisol, growth hormones), C-peptide, insulin, glycerol, lactate, nonesterified fatty acids, vital signs, hypoglycemic awareness and hypoglycemic symptoms tests. [a]Last blood sampling to measure glucagon and C-peptide concentrations before the hypoglycemic clamp was started. [b]Last liraglutide/placebo administration subcutaneously at 10:00 pm. [c]Variable insulin or glucose infusion to maintain normoglycemia overnight. No glucose infusion was allowed 2.5 hours prior to the plasma glucose (PG) plateau 5.5 mmol/L (low insulin). Insulin infusion rate was kept constant during the PG plateau 5.5 mmol/L (low insulin). [d]A bolus of 9.6 mg kg^{-1} min^{-1} of [6,6-$^2H_2$]-glucose was given for 1 minute, and a constant rate of 0.08 mg kg^{-1} min^{-1} until the end of the clamp. [e]Glucose infusion rate was with [6,6-$^2H_2$]-glucose (4 mg/mL). [f]Recovery period, defined as the time at PG plateau 2.5 mmol/L (15 minutes) after the constant insulin infusion had been stopped to reach PG plateau 3.9 mmol/L. Desired maximum time to reach plateau 3.9 mmol/L was 45 minutes. [g]"Free fall of glucose" interval in time (minutes): median (minimum-maximum).

(PG 5.5 mmol/L, high insulin) was started and kept for 30 minutes. Then, the glucose infusion was stopped, and the PG level was allowed to drop to 3.5 mmol/L, and then kept for 30 minutes at 3.5 mmol/L with a variable tracer glucose infusion. After this plateau, glucose was again stopped, and PG levels were allowed to drop to the nadir of 2.5 mmol/L. With tracer glucose infusion, nadir levels were kept at 2.5 mmol/L for 30 minutes. Fifteen minutes after having reached nadir glucose levels, the fixed insulin infusion was stopped, and the glucose infusion was tapered off to allow spontaneous recovery from hypoglycemia. If PG had not recovered 45 minutes after stop of the insulin infusion, a constant glucose infusion of 5.5 mg/kg/min was initiated to reach the last PG plateau of 3.9 mmol/L (PG plateau held for 10 minutes). The recovery period was defined as the time at PG plateau 2.5 mmol/L from termination of insulin infusion to reach PG plateau 3.9 mmol/L. Patients continued fasting (only water consumption was allowed) and stayed in a supine or

semisupine position throughout the hypoglycemic clamp. At PG plateaus 5.5, 3.5, and 2.5 mmol/L, blood sampling was performed at 0, 10, 20, and 30 minutes to assess [6,6-$^2H_2$]-glucose concentrations for the calculation of EGP and for the Rd, the levels of counterregulatory hormones glucagon, norepinephrine, epinephrine, cortisol, growth hormones, and the concentrations of insulin, C-peptide, glycerol, lactate, and nonesterified fatty acids (NEFA) were assessed. At PG plateau 3.9 mmol/L, blood samples were taken at 0 and 10 minutes.

Mixed Meal Tolerance Test

To assess the residual beta-cell function of our patients, MMTTs were performed at the beginning and the end of each treatment period (Fig. 1A) (27). After an overnight fast, each patient arrived at the Clinical Trials Unit in the morning and stayed in supine or semisupine position during the MMTT. Eight hours prior to start of the MMTT, the patients were allowed to drink 200 mL of water, but no water intake was permitted 2 hours before until 1 hour after start of the MMTT. A hand vein was cannulated for sampling arterialized venous blood, and the arm remained wrapped in a heating blanket throughout the MMTT. At 8:00 am, each patient drank a standardized liquid meal (4.8 mL Nutricia Fortimel complete per kg body weight; maximum volume: 288 mL) within 5 minutes. To evaluate the gastric emptying (GE) during the MMTT, the paracetamol absorption technique was used (28-30), which involved the enrichment of the meal with 4 mL/kg body weight of an aqueous solution of paracetamol (5 mg/mL). Blood samples were taken regularly until 240 minutes after MMTT start, and glucose, insulin, glucagon, C-peptide, and paracetamol concentrations were determined in those samples.

Blood Sample Analyses

Blood samples for glucagon measurements were collected in P800 EDTA tubes (BD™, Becton Dickinson, Franklin Lakes, NJ, USA) as described in Zenz et al (24), and they were analyzed with a solid phase 2-site enzyme immunoassay (Mercodia Glucagon ELISA, Sweden; RRID:AB_2737304). The intra-assay coefficient of variation (CV) was <5.1%. Glucose and [6,6-$^2H_2$]-glucose were collected in sodium-fluoride tubes, and analytes were quantified using gas chromatography-mass spectrometry (7890a GC, 7000b MS, Agilent Technologies, Santa Clara, CA, USA) (24, 31, 32). The tracer-to-tracee ratio was calculated using the peak areas of the glucose and [6,6-$^2H_2$]-glucose (24) to calculate the EGP and the Rd. To determine C-peptide concentrations, serum tubes were used and measured with a 2-site sandwich immunoassay (ADVIA Centaur, Siemens Healthcare Diagnostics, UK; RRID:AB_2909501; CV < 4.0%). The lower limit of quantification was ≤0.017 nmol/L. A radioimmunoassay (DRG Instruments GmbH, Germany; RRID:AB_2909502) was used to determine norepinephrine (CV ≤ 9.3%) and epinephrine concentrations (CV ≤ 10.1%). Cortisol, human growth hormones, and insulin concentrations were determined with immunoassays (ADVIA Centaur, Siemens Healthcare Diagnostics, UK; cortisol: RRID:AB_2893154; CV ≤ 3.3%; human growth hormones: RRID:AB_2909498; CV ≤ 3.4%; insulin: RRID:AB_2909499; CV ≤ 3.3%). NEFA and glycerol were measured photometrically (DiaSys, Holzheim, Germany; NEFA: CV ≤ 1.1%; glycerol: CV ≤ 1.3%) on an Olympus AU640 (Beckman Coulter, Atlanta, Georgia, USA). PG and lactate concentrations were measured at bedside with a glucose analyzer (Super GL; Dr. Müller Gerätebau GmbH, Freital, Germany; PG: CV < 2%, lactate: CV < 2.5%). Serum tubes were used for paracetamol and measured with an enzyme immunoassay (Roche Diagnostics, Basel, Switzerland, RRID:AB_2909506; CV ≤ 2.5%) on a Cobas 6000 (Roche Diagnostics). HbA1c was measured with HPLC HA 8180 Oskar (Menarini Diagnostics, Vienna, Austria; CV ≤ 0.5%).

Statistical Methods

All data are presented as mean ± SD, if not otherwise stated. The level of significance was set to alpha < 0.05 (2-sided). Based on results from a previously performed study (7), the primary endpoint was defined as the sum of the areas under the EGP curves (AUC_{EGP}) observed during the PG plateau phases 5.5 (high insulin), 3.5, 2.5, and 3.9 mmol/L. The secondary endpoints included the average EGP, Rd, PG, tracer-to-tracee ratio, C-peptide, insulin, counterregulatory hormones (glucagon, norepinephrine, epinephrine, cortisol, and growth hormone), glycerol, and lactate as well as the NEFA values observed during the individual PG plateaus of the clamp. Furthermore, additional endpoints were the sum of the areas under the GIR curves (AUC_{GIR}) observed during the PG plateau phases of the clamp and the area under the PG, C-peptide, insulin, and glucagon curves observed during the MMTT. In addition, to determine GE efficiency following meal ingestion, the area under the paracetamol curve as well as the time to reach the maximum paracetamol concentration observed during the MMTT were calculated (28-30).

All AUCs were calculated using the trapezoidal method. The outcome parameters were analyzed by means of a mixed effects model, unless otherwise indicated. The mixed effect model used the treatment sequence and period as fixed effects and subject as the random effect.

For sample size calculation we assumed a minimal clinically relevant difference of 25% between treatment with liraglutide and treatment with placebo. Further, we postulated an SD of 0.223 mg/kg/min (which corresponds to a CV of 100%); thus, a 2-tailed 1-sample *t*-test with 5% level of significance and a power of 80% would require a total of 10 patients. To provide for a drop-out rate < 30%, the number of patients had to be increased to 15 patients. No corrections for multiple testing were performed. Data analysis was done with SAS 9.2.

Results

Fourteen T1D patients with fasting C-peptide ≥ 0.1 nmol/L (C-pos; 7 female, 7 male) were enrolled into the study (Table 1). At screening visit, the patients' mean age was 33.6 ± 12.1 years, the mean diabetes duration was 3.4 ± 2 years, and the mean fasting C-peptide value was 0.23 ± 0.12 nmol/L. The patients used daily insulin injections (n = 12) or continuous subcutaneous insulin infusion (n = 2) with total daily basal insulin dose of 8 ± 7 IU [minimum-maximum (min-max) 0-28] and a total daily bolus insulin of 13 ± 6 IU (min-max 6-24). All patients completed the trial. Patients of both sequence groups (liraglutide/placebo, placebo/liraglutide) had comparable weight, BMI, fasting C-peptide levels, HbA1c values, and total mean daily insulin requirement [liraglutide: 14.9 IU ± 8.1 (min-max 2-31); placebo: 18.3 ± 12.1 IU (min-max 4-50)]. According to the MMTT/hypoglycemic exclusion criteria, no

Table 1. Clinical outcome in C-peptide–positive T1D patients at baseline and after a 12-week treatment with 1.2 mg liraglutide and placebo

		Baseline	After a 3-month treatment		Differences between baseline and 3-month treatment	
	n	Median (min, max)	Median (min, max)	*P*-value[b]	Median (min, max)	*P*-value
Body weight, kg				**<0.001**		**0.001**
Liraglutide	14	70.7 (53.1, 95.3)	68.6 (51.3, 91.3)		1.95 (0.1, 6.0)	
Placebo	14	71.1 (50.9, 94.7)	71.1 (50.9, 95.8)		−0.95 (−4.3, 1.3)	
BMI, kg/m^2				**<0.001**		**0.001**
Liraglutide	14	23.0 (19.0, 27.0)	22.1 (18.2, 25.8)		0.73 (0.0, 1.8)	
Placebo	14	22.8 (18.9, 26.8)	23.4 (20.0, 27.1)		−0.28 (−1.6, 0.5)	
Fasting C-peptide, nmol/L[a]				0.851		0.541
Liraglutide	14	0.19 (0.05, 0.58)	0.20 (0.05, 0.64)		0.02 (−0.16, 0.06)	
Placebo	14	0.21 (0.10, 0.55)	0.21 (0.06, 0.62)		0.01 (−0.19, 0.11)	
Fasting glucagon, pmol/L[a]				**0.048**		0.594
Liraglutide	14	7.9 (4.0, 33.9)	7.42 (2.6, 29.0)		0.14 (−5.2, 10.1)	
Placebo	14	7.6 (3.6, 22.8)	9.75 (2.8, 40.0)		−0.36 (−20.0, 14.1)	
Fasting PG, mmol/L				0.407		0.594
Liraglutide	14	8.1 (5.1, 12.8)	7.3 (4.6, 14.7)		0.86 (−5.2, 6.2)	
Placebo	14	9.6 (5.9, 15.1)	9.0 (5.0, 11.2)		0.71 (−2.5, 6.0)	
HbA1c, mmol/mol				**<0.001**		**0.003**
Liraglutide	14	52.3 (39, 69)	48.0 (32, 66)		3.5 (−3.0, 9.0)	
Placebo	14	50.0 (37, 73)	54.5 (40, 76)		−3.0 (−10.0, 2.0)	
HbA1c, %				**<0.001**		**0.003**
Liraglutide	14	6.9 (5.7, 8.5)	6.5 (5.1, 8.2)		0.3 (−0.3, 0.8)	
Placebo	14	6.7 (5.5, 8.8)	7.1 (5.8, 9.1)		−0.27 (−0.9, 0.2)	
Total daily insulin, IU				0.064		0.198
Liraglutide	14	17.6 (2.7, 48.7)	12.5 (2, 31)		5.1 (−7.6, 12.3)	
Placebo	14	16.8 (4, 43.3)	15.2 (4, 50)		1.4 (−12, 9)	

Level of significance is $P < 0.05$ (bolded in table 1).
Abbreviations : max, maximum; min, minimum.
[a]For assessment of fasting C-peptide and glucagon concentrations, blood samples were collected prior to mixed meal tolerance test start.
[b]Comparisons between 3-month liraglutide treatment and 3-month placebo treatment using the Wilcoxon signed rank test.

patient had had a hypoglycemic event the night prior to the MMTT or was on low-carbohydrate diet or did a strenuous exercise later than 3 days prior to the MMTT start.

At the end of the run-in period and during the first PG plateau phase (5.5 mmol/L; low insulin), patients treated with liraglutide had significantly lower insulin infusion rates compared with those treated with placebo [liraglutide: 0.095 mU/kg/min; placebo: 0.180 mU/kg/min; $P < 0.001$].

Hyperinsulinemic, Stepwise Hypoglycemic Clamp

Endogenous glucose production, rate of peripheral glucose disposal, and glucagon and C-peptide levels

The mean AUC_{EGP} and AUC_{Rd} values were comparable in both treatment groups ($P = 0.247$ and $P = 0.963$, respectively). Mean EGP and Rd rates observed during the individual PG plateau phases did not differ between the 2 treatment groups ($P > 0.131$) (Fig. 2A and 2B). When the high-dose insulin infusion was commenced, EGP rates decreased and Rd rates increased in both treatment groups (mean EGP: liraglutide: $P < 0.001$, placebo: $P < 0.001$; mean Rd: liraglutide: $P < 0.001$, placebo: $P < 0.001$) (Fig. 2A and 2B).

Mean glucagon levels of the 2 treatment groups were similar at ambient conditions but differed at PG plateau 5.5 mmol/L after the high-does insulin infusion had started. At this plateau, the liraglutide treatment group showed significantly lower mean glucagon levels than the placebo treatment group ($P = 0.001$) (Fig. 3A).

Mean C-peptide levels were significantly higher in the liraglutide than in the placebo treatment group at PG plateaus 5.5 (high insulin) and 3.5 mmol/L (PG plateau 5.5 mmol/L: $P = 0.033$; PG plateau 3.5 mmol/L: $P = 0.011$) (Fig. 3B).

Plasma glucose levels, tracer-to-tracee ratio, glucose infusion rate, and insulin levels

After induction of hypoglycemia, the mean PG values were comparable in both treatment groups. The mean tracer-to-tracee ratio remained stable within each PG plateau and did also not differ between liraglutide and placebo treatment during the hypoglycemic clamp. The AUC_{GIR} values and the mean insulin concentrations were comparable between both treatment groups during the hypoglycemic clamp. Further, no statistically significant differences of AUC_{GIR} and insulin levels

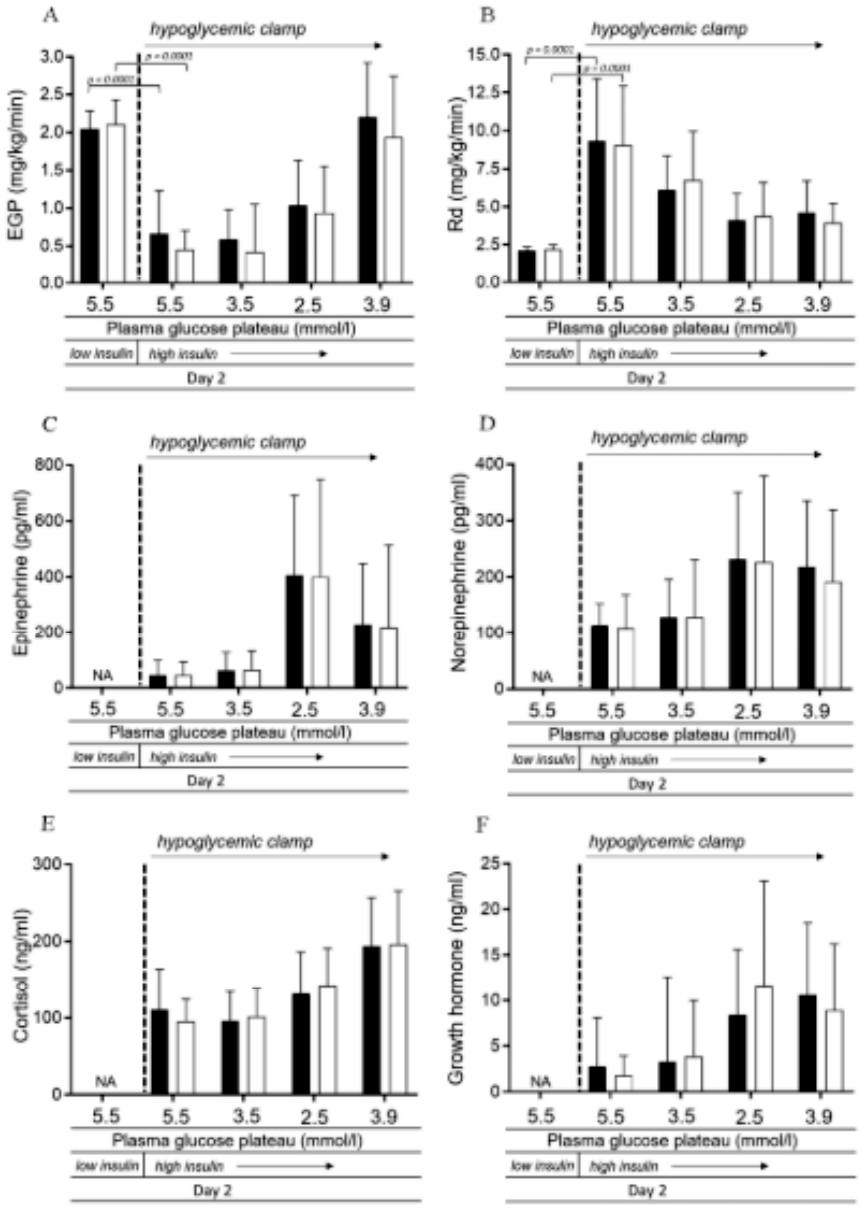

Figure 2. Mean endogenous glucose production (EGPI (A), rate of peripheral glucose disposal (Rd) (B), epinephrine (C), norepinephrine (D), cortisol (E,) and growth hormone concentrations (F) during a stepwise hypoglycemic glucose clamp with stable isotope tracer technique at baseline (5.5 mmol/L, low insulin) and at hypoglycemic clamp plateaus (high insulin) in the liraglutide (black bar) and the placebo (white bar) treatment group. Data are presented as mean ± SE of the mean. Level of significance is $P < 0.05$. Abbreviation: NA, no samples were collected at this plateau.

between the 2 treatment groups were observed during the recovery period [Supplemental Figure 2 (33)].

Other counterregulatory hormones and analyses

The mean concentrations of epinephrine (Fig. 2C), norepinephrine (Fig. 2D), cortisol (Fig. 2E), growth hormones (Fig. 2F), glycerol, lactate, and NEFA were comparable in the 2 treatment groups at all PG plateaus [Supplemental Tables 1-4 (34)]. Patients treated with liraglutide had similar hypoglycemic symptom scores and hypoglycemic awareness as placebo-treated patients at all PG plateaus during the hypoglycemic clamp.

Mixed Meal Tolerance Test and Gastric Emptying

AUC values for glucose from 0 to 240 minutes ($AUC_{glucose0\text{-}240}$) as well as the time to reach the maximum glucose concentrations did not differ between the liraglutide and the placebo group after 12 weeks of treatment (Fig. 4A and 4B). Similarly, the AUC values for C-peptide (Fig. 4C and 4D) and insulin concentrations (Fig. 4E and 4F) from 0 to 240 minutes ($AUC_{C\text{-}peptide0\text{-}240}$ and $AUC_{insulin0\text{-}240}$, respectively) were comparable between the 2 treatment groups after 12 weeks of treatment. The AUC values for glucagon were similar between the treatment groups. At the 180-minute time point, C-pos T1D patients had significantly lower glucagon levels after 3 months of liraglutide treatment compared to the baseline within the treatment group ($P = 0.021$) and compared to placebo after 3 months of treatment ($P = 0.006$) [Supplemental Figure 1 (35)]. The AUC values for paracetamol did not show any difference between the 2 treatment groups, nor did the time to reach maximum of paracetamol concentrations (Fig. 4G and 4H).

Clinical Outcomes

Lower glucagon levels were observed in the morning before the MMTT was started after the 12-week treatment in the liraglutide than in the placebo group ($P = 0.048$). Patients treated with liraglutide had significantly lower body weight, BMI, and HbA1c than patients treated with placebo (Table 1). The liraglutide treatment group showed a trend for reduced insulin requirement. During the study, 274 hypoglycemic episodes were observed, and no difference was seen between both treatment groups (liraglutide group n = 129; placebo group n = 145).

Adverse Events

Sixty-four adverse events (AEs) occurred during the clinical study, of which 55 (85.94%) were mild and 9 (14.06%) were moderate. No severe AE and 13 AEs with gastrointestinal symptoms (primary nausea, vomiting, diarrhea and obstipation) were seen.

Discussion

This study investigated for the first time the effect of a 12-week liraglutide treatment adjunct to insulin in T1D patients with fasting C-peptide ≥ 0.1 nmol/L (C-pos) during insulin-induced hypoglycemia. We observed that liraglutide treatment did not alter the EGP or the Rd responses to hypoglycemia. Also, the number of hypoglycemic events was similar in the 12-week liraglutide treatment group compared to the placebo group. These results indicate that 3 months of liraglutide treatment does not promote or prolong hypoglycemia in C-pos T1D patients.

Overall glucagon levels were similar under fasting conditions after 12 weeks of liraglutide treatment compared to placebo. Interestingly, after starting high-dose insulin infusion and maintaining euglycemias (plateau PG 5.5 mmol/L, high insulin), glucagon was suppressed to a larger extent in the liraglutide group than in the placebo group. An overall suppression of glucagon levels under hyperinsulinemic euglycemic conditions is known to occur (36), but why liraglutide treatment showed greater suppression of glucagon levels than placebo treatment remains unclear. However, during hypoglycemia (plateau PG 3.5 and 2.5 mmol/L), the glucagon secretion was comparable in both groups, indicating similar responsiveness of alpha-cells toward hypoglycemia. Liraglutide-treated patients also had statistically significantly higher C-peptide levels in the early phase of hypoglycemia induction (plateau PG 5.5 and 3.5 mmol/L), which may suggest that suppression of endogenous insulin secretion appears to be less effective. However, these effects were not reflected in the glucose fluxes, as we found no differences in the EGP or Rd responses to hypoglycemia between the 2 treatment groups.

No significant impact on the orally stimulated C-peptide secretion was observed with liraglutide compared to placebo treatment (Fig. 4). Furthermore, no differences were seen in overall glucagon, PG, and insulin levels between both groups. In addition, paracetamol concentrations were similar in both groups, which indicates that 3 months of liraglutide treatment has no effect on the GE rate in C-pos T1D patients.

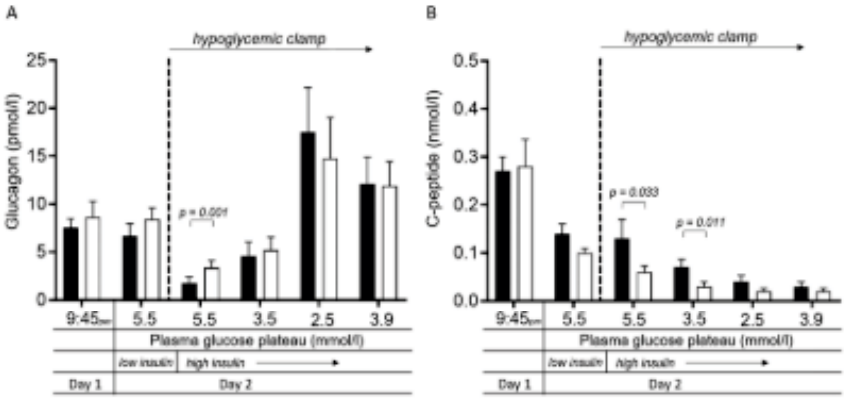

Figure 3. Mean plasma glucagon (A) and C-peptide concentrations (B) during a stepwise hypoglycemic glucose clamp with stable isotope tracer technique at day 1, at baseline (5.5 mmol/L, low insulin, day 2) and at each hypoglycemic clamp plateau (high insulin) in the liraglutide (black bar) and placebo (white bar) treatment group. Data are presented as mean ± SE of the mean. Level of significance is $P < 0.05$.

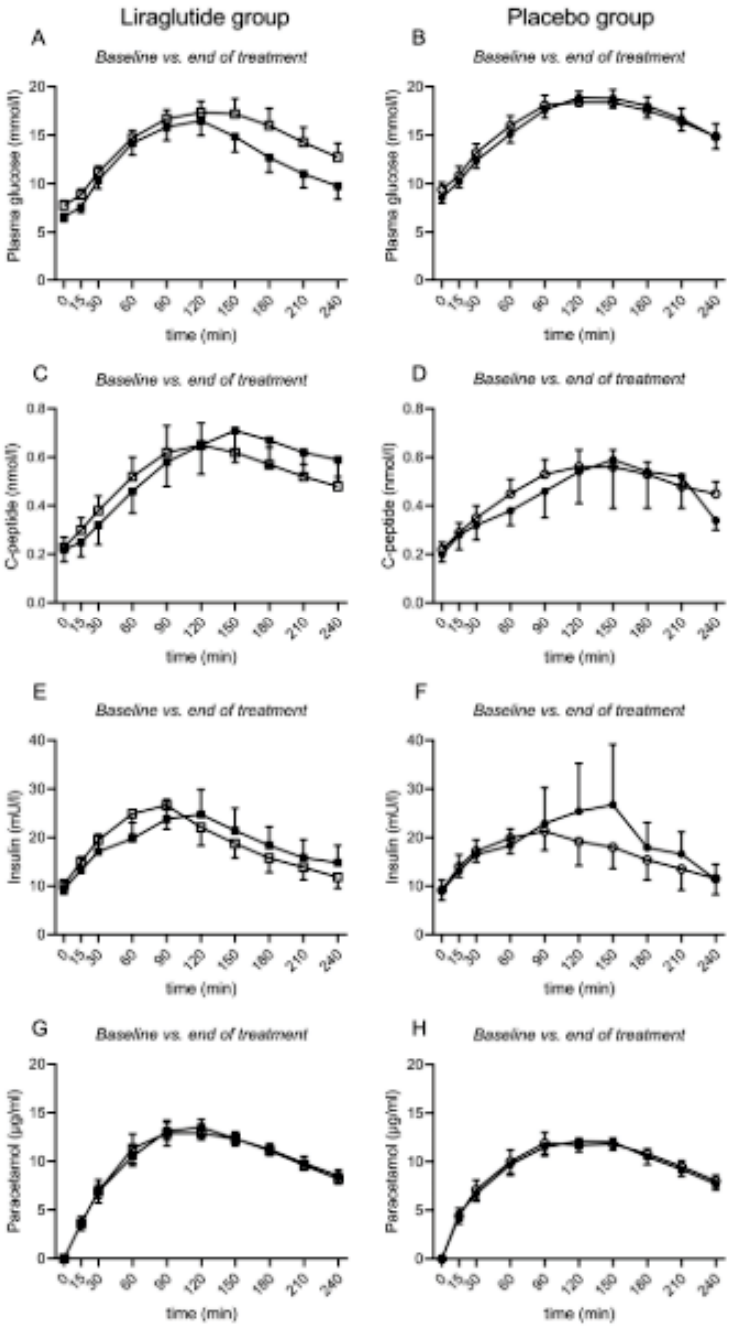

Figure 4. Plasma glucose (A and B), C-peptide (C and D), insulin (E and F), and paracetamol concentrations (G and H) observed during a mixed meal tolerance test (MMTT) for liraglutide (square in A, C, E, and G) and placebo treatment (circle in B, D, F, and H) at baseline (open symbol) and by the end of the 12-week treatment period (filled symbol). Data are presented as mean ± SE of the mean.

In line with results from previous studies in T1D patients treated with liraglutide (8, 17), we observed a positive impact on body weight and HbA1c of the liraglutide treatment. This is also reflected by a trend for reduced insulin requirements and supports the positive metabolic effect of liraglutide in C-pos T1D. However, we did not observe an improvement in insulin sensitivity with liraglutide treatment, which might be due to the limited weight loss in our C-pos patients.

In 2015, we have performed a dose-finding study with hypoglycemia in C-neg T1D patients after 4 weeks of liraglutide treatment of either 1.2 or 1.8 mg per day (7). In this group of T1D patients, we observed a significantly reduced GIR during hypoglycemia, which can be explained by either decreased insulin sensitivity or increased EGP. These findings cannot be confirmed by this study in C-pos T1D patients. We did not find differences between liraglutide treatment or placebo for both EGP and the Rd. One reason for the divergent results could be the different study populations. The C-neg patients T1D in the previous study had a longer diabetes duration (mean 21.4 years) and also a poorer metabolic control (HbA1c, BMI) than our C-pos T1D patients (24).

During the hypoglycemic clamp, mean insulin concentrations and concentrations of the counterregulatory hormones norepinephrine, epinephrine, cortisol, and human growth hormone were comparable between both treatment groups, supporting the results of our study that liraglutide does not prolong hypoglycemia in C-pos patients. Further, we observed the same frequency of hypoglycemic events between both groups, which is in contrast to results from other larger trials (8, 17) and might be explained by the careful insulin adjustment in our rather small study. In addition, our study population experienced relatively recent onset of T1D and may have some residual glucose metabolism regulation that could also limit the risk for hypoglycemic events.

It is known that the glucagon response shortly after diagnosis of T1D is associated with the level of C-peptide secretion, underlining that an insulin-glucagon interaction in islets is crucial for hypoglycemia defense (24, 36). Our data demonstrate that a 3-month treatment period with a GLP-1 receptor agonist does not change the overall response to hypoglycemia in C-pos T1D patients with mean diabetes duration of 3.4 years. If a longer treatment period (eg, 2 to 3 years) has an impact on the natural course of the disease needs to be evaluated in future studies.

As shown by Von Herrath et al (37), a combination therapy of an anti-interleukin-21 antibody and liraglutide might have a positive impact on the beta-cell function over 54 weeks in recently diagnosed T1D.

Drug adherence of the patients was high during our study. Only mild gastrointestinal side effects were observed, and no dropouts due to severe AE occurred. This high tolerance of the liraglutide treatment could be attributable to a lower starting dose (from 0.3 mg to 0.6 mg) and a slower titration (from 0.6 mg to 1.2 mg) of liraglutide than used in other studies (8, 17, 21).

Notably, the glucagon concentrations described in both treatment groups were lower compared to those found in earlier studies (7, 38, 39). This can be explained by the fact that in this study we used a highly specific double-sandwich glucagon assay which is able to measure intact glucagon only (36), whereas commonly used unspecific assays detect splice-products and inactivated fragments of glucagon in addition to intact glucagon (40).

A limitation of our study is the small sample size to detect changes in C-peptide secretion. Patients in our study were C-pos, but had a disease duration of up to 3 years. For such a mechanistic trial, it is very difficult to include patients early after diagnoses. Thus, our attempt was to investigate the effect of liraglutide in C-pos T1D patients which automatically leads to a risk of recruitment of late onset autoimmune diabetes in adult patients. However, to the best of our knowledge, there is no clear distinction between T1D and late onset autoimmune diabetes in adult patients, at least not at the level of alpha- and beta-cell function. Our findings may not be applicable to newly manifested T1D patients or C-pos T1D patients with a longer diabetes duration. Another limitation of this study is the relatively short duration of liraglutide treatment (12 weeks), which does not allow us to draw conclusions about the long-term efficacy of liraglutide in C-pos T1D patients. Liraglutide's possible long-term effects on sustainable metabolic improvements and its potential additive benefits when used early in the course of T1D as well as its possible effect to "burn out" the beta-cells sooner in C-pos T1D patients need to be further investigated. Also, our results should be interpreted with caution as a pure liquid meal test is not fully representative of the real-life conditions of T1D patients.

In conclusion, a 12-week treatment with liraglutide adjunct to insulin does not increase or prolong hypoglycemia compared to placebo in C-pos T1D patients. However, the clinical relevance of our findings remains to be evaluated in a lager study population of C-pos T1D patients.

Acknowledgments

The authors thank Ekardt Eva (Division of Endocrinology and Diabetology, Medical University of Graz, Austria) for support in organizing the study, Kojzar Harald (Division of Endocrinology and Diabetology, Medical University of Graz, Austria) for help during the recruitment phase, Urschitz Martina and Wolf Michael (Division of Endocrinology and Diabetology, Medical University of Graz, Austria) for performing the hypoglycemic clamp, Lipp Robert (Division of Endocrinology and Diabetology, Medical University of Graz, Austria) for coordinating the laboratory blood samples, Berghofer Andrea and Bischof Sarah (Division of Endocrinology and Diabetology, Medical University of Graz, Austria) for data monitoring, Tschapeller Bernd and Krainer Christian (Joanneum Research GmbH, HEALTH—Institute for Biomedicine and Health Sciences, Austria) for data management, and the patients for their participation in the study. We especially thank Fröhlich-Reiterer Elke (Division of General Paediatric, Medical University of Graz, Austria), Krebs Michael (Division of Endocrinology and Metabolism, Medical University of Vienna, Austria), Stepan Vinzenz (Department of Internal Medicine, Hospital Elisabethinen, Austria), and Schütz-Fuhrmann Ingrid (Department of Internal Medicine, Hospital Hietzing, Austria) for help with patient recruitment and Petersen H. Astrid for help with development of the study protocol. We further thank Eberl Anita and Kollmann Denise for sample analysis.

Funding

The study was supported by an unrestricted research grant from Novo Nordisk A/S.

Author Contributions

S.Z., W.R., and T.R.P. initiated and designed the study. S.Z. conducted the clinical study, and S.Z., M.B., S.H.N., R.R., C.M., S.K., and J.M. contributed to the data acquisition and collection. The statistical analysis was performed by T.A. and S.Z. S.Z., W.R., B.B., and T.P. interpreted the data

and wrote the first draft of the manuscript. All authors revised the manuscript. T.R.P. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Clinical Trial Information

Registered at Clinicaltrials.gov: NCT02408705.

Disclosures

T.R.P. is an advisory board member/advisory panel of Novo Nordisk A/S, Arecor, Astra Zeneca, Boehringer Ingelheim, Convatec, Eli Lilly, and Sanofi; received research support from Novo Nordisk A/S and Astra Zeneca; and is an employee of CSO CBmed—Center for Biomarker Research in Medicine (a publicly owned research company). The remaining authors have no conflict of interest to disclose.

Data Availability

All data sets generated during and/or analyzed during the current study are not publicly available, but they are available from the corresponding author on reasonable request. Supplementary materials, including 2 figures and 4 tables, are deposited on FigShare (33-35) and are listed in the References.

References

1. Gentilella R, Pechtner V, Corcos A, Consoli A. Glucagon-like peptide-1 receptor agonists in type 2 diabetes treatment: are they all the same? *Diabetes Metab Res Rev*. 2019;35(1):e3070. Doi: 10.1002/dmrr.3070
2. Marso SP, Daniels GH, Brown-Frandsen K, *et al*. Liraglutide and cardiovascular outcomes in type 2 diabetes. *N Engl J Med*. 2016;375(4):311-322. Doi: 10.1056/NEJMoa1603827
3. Mann JFE, Ørsted DD, Brown-Frandsen K, *et al*. Liraglutide and renal outcomes in type 2 diabetes. *N Engl J Med*. 2017;377(9):839-848. Doi: 10.1056/NEJMoa1616011
4. DiMeglio LA, Evans-Molina C, Oram RA. Type 1 diabetes. *Lancet*. 2018;391(10138):2449-2462. Doi: 10.1016/S0140-6736(18)31320-5
5. Verma S, Bhatt DL, Bain SC, *et al*. Effect of liraglutide on cardiovascular events in patients with type 2 diabetes mellitus and polyvascular disease results of the LEADER trial. *Circulation*. 2018;137(20):2179-2183. Doi: 10.1161/CIRCULATIONAHA.118.033898
6. Nauck MA, Tornøe K, Rasmussen S, Treppendahl MB, Marso SP. Cardiovascular outcomes in patients who experienced a myocardial infarction while treated with liraglutide versus placebo in the LEADER trial. *Diabetes Vasc Dis Res*. 2018;15(5):465-468. Doi: 10.1177/1479164118783935
7. Pieber TR, Deller S, Korsatko S, *et al*. Counter-regulatory hormone responses to hypoglycaemia in people with type 1 diabetes after 4 weeks of treatment with liraglutide adjunct to insulin: a randomized, placebo-controlled, double-blind, crossover trial. *Diabetes Obes Metab*. 2015;17(8):742-750. Doi: 10.1111/dom.12473
8. Mathieu C, Zinman B, Hemmingsson JU, *et al*. Efficacy and safety of liraglutide added to insulin treatment in type 1 diabetes: the adjunct one treat-to-target randomized trial. *Diabetes Care*. 2016;39(10):1702-1710. Doi: 10.2337/dc16-0691
9. Frandsen CS, Dejgaard TF, Andersen HU, *et al*. Liraglutide as adjunct to insulin treatment in type 1 diabetes does not interfere with glycaemic recovery or gastric emptying rate during hypoglycaemia: a randomized, placebo-controlled, double-blind, parallel-group study. *Diabetes Obes Metab*. 2017;19(6):773-782. Doi: 10.1111/dom.12830
10. Kuhadiya ND, Dhindsa S, Ghanim H, *et al*. Addition of liraglutide to insulin in patients with type 1 diabetes: a randomized placebo-controlled clinical trial of 12 weeks. *Diabetes Care*. 2016;39(6):1027-1035. Doi: 10.2337/dc15-1136
11. Garg M, Ghanim H, Kuhadiya ND, *et al*. Liraglutide acutely suppresses glucagon, lipolysis and ketogenesis in type 1 diabetes. *Diabetes Obes Metab*. 2017;19(9):1306-1311. Doi: 10.1111/dom.12944
12. Harris KB, Boland CL. Adjunctive role of glucagon-like peptide-1 receptor agonists in the management of type 1 diabetes mellitus. *Pharmacotherapy*. 2016;36(9):1011-1020. Doi: 10.1002/phar.1804
13. Varanasi A, Bellini N, Rawal D, *et al*. Liraglutide as additional treatment for type 1 diabetes. *Eur J Endocrinol*. 2011;165(1):77-84. Doi: 10.1530/EJE-11-0330
14. Dubé M-C, D'Amours M, Weisnagel SJ. Beyond glycaemic control: a cross-over, double-blinded, 24-week intervention with liraglutide in type 1 diabetes. *Diabetes Obes Metab*. 2018;20(1):178-184. Doi: 10.1111/dom.13063
15. Mader JK, Jensen L, Ingwersen SH, Christiansen E, Heller S, Pieber TR. Pharmacokinetic properties of liraglutide as adjunct to insulin in subjects with type 1 diabetes mellitus. *Clin Pharmacokinet*. 2016;55(11):1457-1463. Doi: 10.1007/s40262-016-0413-4
16. Kielgast U, Krarup T, Holst JJ, Madsbad S. Four weeks of treatment with liraglutide reduces insulin dose without loss of glycemic control in type 1 diabetic patients with and without residual beta-cell function. *Diabetes Care*. 2011;34(7):1463-1468. Doi: 10.2337/dc11-0096
17. Ahren B, Hirsch IB, Pieber TR, *et al*. Efficacy and safety of liraglutide added to capped insulin treatment in subjects with type 1 diabetes: the adjunct two randomized trial. *Diabetes Care*. 2016;39(10):1693-1701. Doi: 10.2337/dc16-0690
18. Kuhadiya ND, Malik R, Bellini NJ, *et al*. Liraglutide as additional treatment to insulin in obese patients with type 1 diabetes mellitus. *Endocr Pract*. 2013;19(6):963-967. Doi: 10.4158/EP13065.OR
19. Dejgaard TF, Johansen NB, Frandsen CS, *et al*. Effects of liraglutide on cardiovascular risk factors in patients with type 1 diabetes. *Diabetes Obes Metab*. 2017;19(5):734-738. Doi: 10.1111/dom.12841
20. Dejgaard TF, Frandsen CS, Hansen TS, *et al*. Efficacy and safety of liraglutide for overweight adult patients with type 1 diabetes and insufficient glycaemic control (Lira-1): a randomised, double-blind, placebo-controlled trial. *Lancet Diabetes Endocrinol*. 2016;4(3):221-232. Doi: 10.1016/S2213-8587(15)00436-2
21. Frandsen CS, Dejgaard TF, Holst JJ, Andersen HU, Thorsteinsson B, Madsbad S. Twelve-week treatment with liraglutide as add-on to insulin in normal-weight patients with poorly controlled type 1 diabetes: a randomized, placebo- controlled, double-blind parallel study. *Diabetes Care*. 2015;38(12):2250-2257. Doi: 10.2337/dc15-1037
22. International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use (ICH). *J Clin Pharmacol*. 1994;37(5):401-404. DOI: 10.1111/j.1365-2125.1994.tb05705.x
23. World Medical Association Inc. Declaration of Helsinki: ethical principles for medical research involving human subjects. *J Indian Med Assoc*. 2009;107(6):403-405.
24. Zenz S, Mader JK, Regittnig W, *et al*. Impact of C-peptide status on the response of glucagon and endogenous glucose production to induced hypoglycemia in T1DM. *J Clin Endocrinol Metab*. 2018;103(4):1408-1417. Doi: 10.1210/jc.2017-01836
25. Steele R, Wall JS, Bodo RC de, Altszuler N. Measurement of size and turnover rate of body glucose pool by the isotope dilution method. *Am J Physiol*. 1956;187(1):15-24. Doi: 10.1152/ajplegacy.1956.187.1.15

26. Powrie JK, Smith GD, Hennessy TR, Shojaee-Moradie F, Kelly JM, Sönksen PH JR. Incomplete suppression of hepatic glucose production in non-insulin dependent diabetes mellitus measured with [6,6-2H2]-glucose enriched glucose infusion during hyperinsulinaemic euglycaemic clamps. *Eur J Clin Invest*. 1992;22(4):244-253. Doi: 10.1111/j.1365-2362.1992.tb01458.x
27. Greenbaum CJ, Mandrup-Poulsen T, McGee PF, *et al*. Mixed-meal tolerance test versus glucagon stimulation test for the assessment of beta-cell function in therapeutic trials in type 1 diabetes. *Diabetes Care*. 2008;31(10):1966-1971. Doi: 10.2337/dc07-2451
28. Medhus AW, Lofthus CM, Bredesen J, Husebye E. Gastric emptying: the validity of the paracetamol absorption test adjusted for individual pharmacokinetics. *Neurogastroenterol Motil*. 2001;13(3):179-185. Doi: 10.1046/j.1365-2982.2001.00249.x
29. Heading RC, Nimmo J, Prescott LF, Tothill P. The dependence of paracetamol absorption on the rate of gastric emptying. *Br J Pharmacol*. 1973;47(2):415-421. Doi: 10.1111/j.1476-5381.1973.tb08339.x
30. Willems M, Quartero AO, Numans ME. How useful is paracetamol absorption as a marker of gastric emptying? A systematic literature study. *Dig Dis Sci*. 2001;46(10):2256-2262. Doi: 10.1023/a:1011935603893
31. Plank J, Bodenlenz M, Sinner F, *et al*. A double-blind, randomized, dose-response study investigating the pharmacodynamic and pharmacokinetic properties of the long-acting insulin analog detemir. *Diabetes Care*. 2005;28(5):1107-1112. Doi: 10.2337/diacare.28.5.1107
32. Hachey DL, Parsons WR, McKay S, Haymond MW. Quantitation of monosaccharide isotopic enrichment in physiologic fluids by electron ionization or negative chemical ionization GC/MS using di-O-isopropylidene derivatives. *Anal Chem*. 1999;71(20):4734-4739. Doi: 10.1021/ac990724x
33. Zenz S, Regittnig W, Boulgaropoulos B, *et al*. Supplemental data (Figure S2) for: Effect of liraglutide treatment on whole-body glucose fluxes in C-peptide–positive type 1 diabetes during hypoglycemia. Deposited March 9, 2022. *FigShare*. Doi: 10.6084/m9.figshare.19322861
34. Zenz S, Regittnig W, Boulgaropoulos B, *et al*. Supplemental data (Tables S1-S4) for: Effect of liraglutide treatment on whole-body glucose fluxes in C-peptide–positive type 1 diabetes during hypoglycemia. Deposited March 9, 2022. *FigShare*. Doi: 10.6084/m9.figshare.19322996
35. Zenz S, Regittnig W, Boulgaropoulos B, *et al*. Supplemental data (Figure S1) for: Effect of liraglutide treatment on whole-body glucose fluxes in C-peptide–positive type 1 diabetes during hypoglycemia. Deposited March 9, 2022. *FigShare*. Doi: 10.6084/m9.figshare.19322972
36. Rickels MR, Evans-Molina C, Bahnson HT, *et al*. High residual C-peptide likely contributes to glycemic control in type 1 diabetes. *J Clin Invest*. 2020;130(4):1850-1862. Doi: 10.1172/JCI134057
37. von Herrath M, Bain SC, Bode B, *et al*. Anti-interleukin-21 antibody and liraglutide for the preservation of β-cell function in adults with recent-onset type 1 diabetes: a randomised, double-blind, placebo-controlled, phase 2 trial. *Lancet Diabetes Endocrinol*. 2021;9(4):212-224. Doi: 10.1016/S2213-8587(21)00019-X
38. Hoffman RP, Arslanian S, Drash AL, Becker DJ. Impaired counterregulatory hormone responses to hypoglycemia in children and adolescents with new onset IDDM. *J Pediatr Endocrinol*. 1994;7(3):235-244. Doi: 10.1515/JPEM.1994.7.3.235
39. Briscoe VJ, Ertl AC, Tate DB, Davis SN. Effects of the selective serotonin reuptake inhibitor fluoxetine on counterregulatory responses to hypoglycemia in individuals with type 1 diabetes. *Diabetes*. 2008;57(12):33152453-33153322. Doi: 10.2337/db08-1000
40. Wewer Albrechtsen NJ, Hartmann B, Veedfald S, *et al*. Hyperglucagonaemia analysed by glucagon sandwich ELISA: nonspecific interference or truly elevated levels? *Diabetologia*. 2014;57(9):1919-1926. Doi: 10.1007/s00125-014-3283-z

6.3. Scientific Work Related To Thesis

6.3.1. Scientific Awards

Abstractpreis 2015 der Österreichischen Diabetes Gesellschaft

Zenz S; Regittnig W; Urschitz M; Brunner M; Korsatko S; Raml R; Narath S; Magnes C; Tschapeller B; Augustin T; Pieber T. R. Endogene Glukoseproduktion während einer induzierten Hypoglykämie bei neu diagnostiziertem und langjährigem Typ 1 Diabetes. 43. Jahrestagung der Österreichischen Diabetesgesellschaft (ÖDG). 2015; November 19-21.: Salzburg, Österreich.

Winner of the Helmsley Charitable Trust Abstract Award in Type 1 Diabetes

Mader J.K ; **Zenz S**; Baumann P; Brunner M; Puffin A; Rumpler M; Moser O; Hajnsek M; Sourij H; Pieber T.R. Glucagon response is similar in people with long versus short standing diabetes mellitus type 1. 98th Annual Meeting of the Endocrine Society (ENDO) 2016; April 1-4, Boston, USA.

2017 Young Investigator Travel Grant Award - 77th Diabetes Association Scientific Sessions

Zenz S; Regittnig W; Wolf M; Brunner M; Ekardt E; Raml R; Rupp S; Magnes C; Augustin T; Pieber TR. Effect of GLP-1 receptor agonists on pancreatic islet function in patients with C peptide positive type 1 diabetes mellitus. 77th ADA Scientific Sessions; June 9 - 13, 2017; San Diego, USA. 2017.

6.3.2. Original Research Articles

Zenz, S; Mader, JK; Regittnig, W; Brunner, M; Korsatko, S; Boulgaropoulos, B; Magnes, C; Raml, R; Narath, SH; Eller, P; Augustin, T; Pieber, TR Impact of C-Peptide Status on the Response of Glucagon and Endogenous Glucose Production to Induced Hypoglycemia in T1DM. J Clin Endocrinol Metab. 2018; 103(4):1408-1417. Doi: 10.1210/jc.2017-01836

Zenz, S; Regittnig, W; Boulgaropoulos, B; Augustin, T; Brunner, M; Korsatko, S; Münzker, J; Narath, SH; Raml, R; Magnes, C; Pieber, TREffect of Liraglutide Treatment on Whole-body Glucose Fluxes in C-peptide-Positive Type 1 Diabetes During Hypoglycemia. J Clin Endocrinol Metab. 2022; 107(9):e3583-e3593. Doi: 10.1210/clinem/dgac369.

Cited in: DiMeglio LA, Evans-Molina C, Oram RA. Type 1 diabetes. *Lancet* (London, England) 2018;391:2449–62. https://doi.org/10.1016/S0140-6736(18)31320-5.

6.3.3. Presentations / Posters / Abstracts

Zenz S; Regittnig W; Urschitz M; Brunner M; Korsatko S; Raml R; Narath S; Magnes C; Tschapeller B; Augustin T; Pieber T. R. Endogenous Glucose Production During Hypoglycemia In Patients With Newly Diagnosed And Longstanding Type 1 Diabetes. 75th Scientific Sessions, American Diabetes Association (ADA) 2015; June 5-9.; Boston, USA.

Zenz S; Regittnig W; Urschitz M; Brunner M; Korsatko S; Raml R; Narath S; Magnes C; Tschapeller B; Augustin T; Pieber T. R. Endogene Glukoseproduktion während einer induzierten Hypoglykämie bei neu diagnostiziertem und langjährigem Typ 1 Diabetes. 43. Jahrestagung der Österreichischen Diabetesgesellschaft (ÖDG). 2015; November 19-21.: Salzburg, Österreich.

Zenz, S; Regittnig, W; Urschitz, M; Brunner, M; Korsatko, S; Raml, R; Narath, S; Magnes, C; Tschapeller, B; Augustin, T; Pieber, TR. Endogenous Glucose Production during an induced Hypoglycemia in newly diagnosed and long term Type 1 Diabetes. WIEN KLIN WOCHENSCHR. 2015; 127: S136-S136.

Mader J.K ; **Zenz S;** Baumann P; Brunner M; Puffin A; Rumpler M; Moser O; Hajnsek M; Sourij H; Pieber T.R. Glucagon response is similar in people with long versus short standing diabetes mellitus type 1. 98th Annual Meeting of the Endocrine Society (ENDO) 2016; April 1-4, Boston, USA.

Zenz S; Baumann P; Brunner M; Puffing A; Rumpler M; Wolf M; Hajnsek M; Sourij H; Pieber T.R; Mader J.K. Glucagon response in C-peptide positive vs. C-peptide negative patients with type 1 diabetes under hypoglycemic clamp vs. real-life setting. 76th Scientific Sessions, American Diabetes Association (ADA) 2016; June 10-14.; New Orleans, USA.

Zenz S; Baumann P; Brunner M; Puffing A; Rumpler M; Wolf M; Hajnsek M; Sourij H; Pieber T.R; Mader J.K. Glucagon Response in C Peptide Positive vs. C Peptide Negative Patients with Type 1 Diabetes under Hypoglycaemic Clamp vs. RealLife Setting. 52th Annual Meeting of the European Association for the Study of Diabetes (EASD) 2016; September 12-16, München, Deutschland.

Zenz, S; Baumann, P; Brunner, M; Puffing, A; Rumpler, M; Wolf, M; Hajnsek, M; Sourij, H; Pieber, TR; Mader, JK. Comparison of the Glucagon Response with C-peptide positive vs. C peptide negative Female Patients with Type 1 Diabetes mellitus in a hypoglycaemic Clamp vs. Real-Life Experiment. WIEN KLIN WOCHENSCHR. 2016; 128: S413-S413.

Mader J.K; **Zenz S;** Baumann P; Brunner M; Puffin A; Rumpler M; Moser O; Hajnsek M; Sourij H; Pieber T.R. Glucagon response is similar in people with long versus short standing diabetes mellitus type 1. 52th Annual Meeting of the European Association for the Study of Diabetes (EASD) 2016; September 12-16, München, Deutschland.

Zenz S; Baumann P; Brunner M; Puffing A; Rumpler M; Wolf M; Hajnsek M; Sourij H; Pieber T.R; Mader J.K. Glukagon Antwort in C-peptid positiven vs. C-peptid negativen PatientInnen mit Typ 1 Diabetes in einem hypoglykämischen Clamp vs. Real Life Experiment. 44. Jahrestagung der Österreichischen Diabetesgesellschaft (ÖDG). 2016; November 17-19.: Salzburg, Österreich.

Zenz S; Mader J.K; Regittnig W; Brunner M; Korsatko S; Boulgaropoulos B; Magnes C; Raml R; Narath SH; Eller P; Augustin T; Pieber TR. Glucagon Response and Endogenous Glucose Production during Hypoglycaemia in C-peptide Positive and C-peptide Negative Patients with Type 1 Diabetes Mellitus. Doc Day 2025. MedUniGraz-Campus. Februar 13. Graz, Österreich.