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During Incretin-Based Therapy

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Abstract

Incretin-based therapy is associated with low risk of hypoglycemia in type 2 diabetes mellitus (T2DM) possibly due to its glucose-dependency in stimulating insulin secretion and a sustained glucagon response during hypoglycemia. This is of special relevance when it is combined with insulin or given to elderly patients since both insulin therapy and high age are associated with impaired glucagon counter-regulation. Incretin-based therapy may also be used as add-on to insulin therapy in type 1 diabetes mellitus (T1DM), where there is also an impaired glucagon counter-regulation. The studies in this thesis evaluated the glucagon and the other hormonal counter-regulations to insulin induced hypoglycemia during incretin therapy in four different patient populations. These populations were all particularly vulnerable patients with increased risk for hypoglycemia: T1DM, insulin-treated T2DM and elderly subjects with T2DM. Both dipeptidyl peptidase-4 (DPP-4) inhibition and glucagon-like peptide-1 receptor agonism (GLP-1 RA) were examined. In all, the four studies examined vildagliptin (DPP-4 inhibition) + insulin in T1DM (study 1), vildagliptin + insulin (±oral medication) in T2DM (study 2), lixisenatide (GLP-1 RA) + basal insulin + metformin in T2DM (study 3) and sitagliptin (DPP-4 inhibition) + metformin in elderly subjects with T2DM (study 4).

All studies were single-center, double-blind, randomized, placebo (PBO)-controlled crossover studies involving 18-29 subjects. Mean age was 30 (T1DM), 55, 59, and 74 (T2DM) years, respectively, in the four studies, and diabetes duration 9-14 years, mean baseline hemoglobin A1c (HbA1c) 52-61 mmol/mol and mean body mass index (BMI) 25-33 kg/m^2. Subjects received the active study medication or PBO as add-on therapy for 4 weeks (6 weeks in study 3) in random order with a four-week washout in-between. After each treatment period, the subjects underwent a hyperinsulinemic hypoglycemic clamp with glucose at 2.5 mmol/l (study 1), 2.6 mmol/l (study 2), 3.5 and 2.8 mmol/l (study 3) and 3.5 and 3.1 mmol/l (study 4); clamped glucose levels were preserved for 30 min during which the samples for counter-regulatory hormones including glucagon were collected for analysis.

Results showed that at 3.1 mmol/l and below, the glucagon and the other hormonal responses were similar during incretin therapy as during PBO, whereas at 3.5 mmol/l (study 3 and 4), the glucagon and some of the other hormonal responses were lower during incretin therapy than during PBO.

We conclude that the glucagon and the other hormonal counter-regulatory responses to hypoglycemia are preserved when incretin therapy is used in these particularly vulnerable patients. Furthermore, the results suggest that at glucose levels between 3.5 and 3.1 mmol/l inhibition of glucagon secretion vanishes during incretin therapy in these patient groups. It is suggested that this is a mechanism behind the low risk of hypoglycemia associated with incretin therapy.

Key words

Incretin, DPP-4, GLP-1, Glucagon, Hypoglycemia, Diabetes

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MADE IN SWEDEN

Have more than you show, speak less than you know

怀璧慎显，博识谨言。

Shakespeare, 莎士比亚

Contents

Abstract

Incretin-based therapy is associated with low risk of hypoglycemia in T2DM possibly due to its glucose-dependency in stimulating insulin secretion and a sustained glucagon response during hypoglycemia. This is of special relevance when it is combined with insulin or given to elderly patients since both insulin therapy and high age are associated with impaired glucagon counter-regulation. Incretin-based therapy may also be used as add-on to insulin therapy in T1DM, where there is also an impaired glucagon counter-regulation. The studies in this thesis evaluated the glucagon and the other hormonal counter-regulations to insulin induced hypoglycemia during incretin therapy in four different patient populations. These populations were all particularly vulnerable patients with increased risk for hypoglycaemia: T1DM, insulin-treated T2DM and elderly subjects with T2DM. Both DPP-4 inhibition and GLP-1RA were examined. In all, the four studies examined vildagliptin (DPP-4 inhibition) + insulin in T1DM (study 1), vildagliptin + insulin (±oral medication) in T2DM (study 2), lixisenatide (GLP-1 RA) + basal insulin + metformin in T2DM (study 3) and sitagliptin (DPP-4 inhibition) + metformin in elderly subjects with T2DM (study 4).

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List of papers

This thesis is based on the following four papers:

Study 1

J Farngren, M Persson, A Schweizer, JE Foley, B Ahrén: Vildagliptin reduces glucagon during hyperglycemia and sustains glucagon counter-regulation during hypoglycemia in type 1 diabetes. J Clin Endocrinol Metab 97:3799-3806 (2012)

Study 2

J Farngren, M Persson, A Schweizer, JE Foley, B Ahrén: Glucagon dynamics during hypoglycaemia and food-re-challenge following treatment with vildagliptin in insulin-treated patients with type 2 diabetes. Diabet Obes Metab 16:812-818 (2014)

Study 3

J Farngren, M Persson, B Ahrén: Effect of the GLP-1 receptor agonist lixisenatide on counter-regulatory responses to hypoglycemia in subjects with insulin-treated type 2 diabetes. Diabetes Care 39:242-249 (2016)

Study 4

J Farngren, M Persson, B Ahrén: Effects on the glucagon response to hypoglycaemia during DPP-4 inhibition in elderly subjects with type 2 diabetes: A randomized, placebo-controlled study. Diabet Obes Metab 20:1911-1920 (2018)

Abbreviation

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AE	Adverse event
AUC	Area under the curve
BMI	Body mass index
CGM	Continues glucose monitoring
DPP-4	Dipeptidyl peptidase 4
EDTA	Ethylene diamine tetra acetate
EGP	Endogenous glucose production
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
FDA	Food and Drug Administration
FPG	Fasting plasma glucose
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide-1
GLP-2	Glucagon-like peptide-2
GLP-1RA	Glucagon-like peptide-1 receptor agonist (agonism)
HbA1c	Glycated hemoglobin
IAH	Impaired awareness of hypoglycemia
IAPP	Islet amyloid polypeptide
NPH	Neutral protamine hagedorn
PBO	Placebo
PP	Pancreatic polypeptide
PPG	Postprandial plasma glucose
RIA	Radioimmunoassay
SPSS	Statistical package for social sciences
SGLT2	Sodium-glucose co-transporter-2
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus

Populärvetenskaplig Sammanfattning

Målet med behandling av diabetes är att åstadkomma en blodglukosnivå så nära den friska normala nivån som möjligt. Hos personer med typ 1 diabetes görs detta med insulin, medan för personer med typ 2 diabetes finns det idag ett omfattande läkemedelsutbud att välja bland. En risk med den höga målsättningen är att patienten riskerar att bli för låg i sitt blodglukos varvid man drabbas av ett blodglukosfall, s.k. hypoglykemi. Detta är för patienten oftast obehagligt och tvingar patienten att avbryta det man håller på med. Den kan även skapa psykisk oklarhet, yrsel, medvetslöshet och till och med vara dödlig. Dessa symptom och risker som uppkommer i samband med hypoglykemi kan oroa patienterna och hindra dem från att eftersträva sina behandlingsmål.

Normalt fasteblodglukos är 4,2 – 6,0 mmol/l. Det normala förloppet vid sjunkande blodglukos hos en frisk individ är att vid en nivå på ≈3,6 mmol/l stängs den egna (endogena) insulin-insöndringen av. Om blodglukos fortsätter att sjunka ytterligare så finns hormoner som frisätts med en effekt att öka blodglukos. Bland dessa hormoner finns i första hand glukagon från bukspottkörteln men även stresshormoner (adrenalin, noradrenalin, kortisol).

Hos personer med diabetes är dessa hormonella motreaktioner försämrade. De med typ 1 och långvarig typ 2 diabetes kan inte styra den egna insulininsöndringen för att anpassa sig till sjunkande blodglukosnivå. Samtidigt försämras även deras förmåga att öka glukagon och stresshormonet adrenalin efter längre tids sjukdom. Denna förmåga försämras även med stigande ålder. Äldre har dessutom ofta många andra sjukdomar som ökar risken för hypoglykemi, som t.ex. dålig njurfunktion och demens. Dessa faktorer spelar in och gör att äldre också har en ökad risk för hypoglykemi.

För ett par decennier sedan fanns enbart insulin och insulinfrisättande sulfaliknande tabletter att behandla typ 2 diabetes med. De senaste tjugofem årens forskning har gett oss ett antal nya läkemedelsprinciper att använda mot diabetes.

Inkretinbaserade läkemedel togs fram efter upptäckten av inkretineffekten. Med denna avses den ökade insulinutsöndring man får via peroralt glukosintag jämfört med intravenöst glukosintag trots att glukosnivåerna är samma vid båda tillfällena. Orsaken till detta är att oralt intag av glukos/mat stimulerar tarmhormonerna GLP-1 och GIP till insöndring vilka i sin tur stimulerar upp till 75% mer insulinsekretion från β-celler. GLP-1 hämmar också insöndringen av glukagon vilket leder till lägre nivåer av blodglukos efter matintag.

Hos personer med diabetes har GIP förlorat denna effekt och GLP-1 har försämrad effekt, men GLP-1 infusion med högre koncentration kan ändå återställa en hel del av inkretinets effekt. Ett problem är dock att GLP-1 bryts ner på ett par minuter i

cirkulationen av ett enzym som kallas DPP-4. Forskningen inom detta område har resulterat i två metoder att öka GLP-1 nivåer i cirkulationen. Vid den ena metoden har man ändrat den naturliga GLP-1 molekylens struktur så att den inte bryts ner snabbt, en så kallad GLP-1 RA (Receptor Analog). Den andra metoden är att blockera DPP-4 enzymet (DPP4 hämmare) så att kroppens egna naturliga GLP-1 stannar längre i cirkulationen och därmed får en högre koncentration.

Inkretinbaserade läkemedel är nu rekommenderade att kombineras med metformin och de kan även användas i kombination med insulin för personer med typ 2 diabetes och eventuellt även för de med typ 1 diabetes (ännu inte godkänd indikation). Användningen bland äldre har också blivit vanligare.

Frågeställning blir då om inkretinläkemedel innebär en ökad risk för hypoglykemi för dessa patientgrupper med försämrad hormonell motreaktion mot hypoglykemi med tanke på dess insulinstimulerande och glukagonhämmande verkan vid höga blodglukosnivåer.

Vi har därför utfört fyra studier på dessa patientgrupper för att testa den hormonella motreaktionen mot hypoglykemi jämfört med placebo med hjälp av ett så kallat hypoglykemiskt klamptest.

Den första studien var på insulinbehandlade typ 1 diabetiker. Vi gav dem DPP-4 hämmare (vildagliptin) i kombination med deras insulin. Två andra grupper insulinbehandlade patienter med typ 2 diabetes fick vildagliptin respektive en GLP-1 RA (lixisenatid) som tillägg i den andra och tredje studien. I den fjärde studien användes en annan DPP-4 hämmare (sitagliptin) som tillägg till metformin till äldre patienter med typ 2 diabetes. Metformin är förstahandsläkemedel hos typ 2 diabetiker och därför är denna kombination vanlig.

Behandlingen pågick i 4 - 6 veckor och därefter utfördes klamptest med hjälp av insulininfusioner så att blodglukosnivån sänktes till förutbestämda nivåer: 2,5 mmol/l i studie 1 och 2,6 mmol/l i studie 2. Två klampnivåer hölls i studie 3 och 4: först 3,5 mmol/l i båda studierna och sedan 2.8 mmol/l i studie 3 och 3,1 mmol/l i studie 4. Varje klampnivå hölls i 30 minuter så att blodprov för olika hormoner kunde tas för att studera deras motreaktion mot hypoglykemi.

Studieresultaten visar att glukagonets motreaktion är bevarad vid en blodglukosnivå på 3,1 mmol/l eller lägre. Det samma gäller övriga hormonella motreaktioner också. Detta trots att inkretinbaserade läkemedel hämmar insöndringen av glukagon vid högt blodglukosnivå.

Studiens slutsats är således att i flera olika patientgrupper med ökad risk för hypoglykemi sänker inte inkretinbaserade läkemedel glukagon vid en blodglukosnivå mellan 3,1 och 3,5 mmol/l. Istället börjar glukagonnivåerna stiga som hos placebobehandlade patienter.

Detta är en av förklaringarna till den låga risken för hypoglykemi vid inkretinterapi även hos dessa patientgrupper med försämrad hormonell motreaktion mot hypoglykemi.

Ouverture

Hypoglycemia is a feared and dangerous AE during glucose-lowering management of diabetes[1] and it is associated both with acute symptoms, which may be life threatening, and with serious long-term complications such as cardiovascular diseases[2]. Therefore, it is of great importance to avoid hypoglycemia in diabetes. In fact, avoidance of hypoglycemia is regarded as a requirement for a successful glucose-lowering therapy of the disease[3].

The different glucose-lowering options that exist today have different risks for hypoglycemia and, in general, the more recently developed therapy groups have a lower risk. Incretin-based therapy, which consists of DPP-4 inhibitors and GLP-1 RAs, belongs to the newly developed therapeutic groups and it has been documented to exhibit a low risk for hypoglycemia[4].

The work included in this thesis aimed at understanding the mechanism of the low risk for hypoglycemia with incretin therapy in diabetes with focus to explore the counter-regulatory mechanisms, in particular glucagon counter-regulation. Special interest was to explore counter-regulation during incretin treatment in patients with increased risk for developing hypoglycemia, i.e., patients with T1DM, insulin treated T2DM and elderly patients with T2DM.

Introduction

Hypoglycemia and its consequences

Definition of hypoglycemia There are different definitions of hypoglycemia. American Diabetes Association and Endocrine Society workgroups have defined hypoglycemia as "all episodes of abnormally low plasma glucose that expose the individual to potential harm" which limits long-term treatment intensification and optimization[5]. Abnormally low glucose was defined as <3.9 mmol/l which approximates the lower limit of the normal post-absorptive plasma glucose concentration. 3.9 mmol/l also approximates the glycemic threshold for activation of glucose counter-regulatory systems in nondiabetic individuals and is the upper limit of plasma glucose level reported to reduce counter-regulatory responses to subsequent hypoglycemia[5]. However, in many clinical studies, a cut-off level of 3.1 mmol/l is commonly used for definition of hypoglycemia[6]. There is also a clinical subdivision of hypoglycemia in grade 1, in which the patient is able to manage the symptoms alone, and in grade 2, in which the patient, due to cognitive symptoms, is unable to manage it alone and needs assistance by another person[7].

Acute symptoms of hypoglycemia Hypoglycemic symptoms are classified as *neurogenic* and *neuroglycopenic*. *Neurogenic symptoms* result from the physiologic changes triggered by hypoglycemia at <3.5 mmol/l and include both adrenergic and cholinergic symptoms such as palpitations, anxiety, hunger, sweating, and paresthesia respectively. In contrast, *neuroglycopenic symptoms* are the direct result of low glucose levels (usually ≤ 3.0 mmol/l) in the brain which include cognitive impairments, psychomotor abnormalities, and in, severe cases, coma[6, 7]. It should be emphasized, however, that the hypoglycemia symptoms vary between individuals and for any given individual over the time course of his/her disease. For some individuals, the hypoglycemia symptoms are not triggered until glucose levels are very low, often occurring after cognitive function is impaired. This is referred to as IAH[8].

Consequences of hypoglycemia An important consequence of hypoglycemia, particularly repeated hypoglycemia, is reduced quality of life. A common denominator is fear of a new hypoglycemic episode and this increased fear may in turn result in the deterioration of glycemic control due to a decreased desire of the patient for tight glycemic control, poorer adherence to diet therapy, and

compromised compliance for taking medication. All of this may result in deterioration of glycemic control, and hypoglycemia is therefore a risk factor for later hyperglycemia-related complications of diabetes[7].

Weight gain is another consequence to hypoglycemia and caused by increased eating in self-defense against hypoglycemia. In turn, weight gain has negative impact on health, such as increased insulin resistance, worsening of metabolic and hemodynamic changes associated with the metabolic syndrome, and deterioration of glycemia. Another serious long-term complication to hypoglycemia is increased risk for cardiovascular diseases, which has been demonstrated in long-term follow-up studies. It was thus found that severe hypoglycemia is associated with a significantly higher risk of major macrovascular events, major microvascular events, death from cardiovascular or any other causes and it may also result in dementia[7].

Hypoglycemia impairs physiological and behavioral defenses against subsequent hypoglycemia and thereby is often recurrent. The serious acute and long-term effects of repeated hypoglycemia are presented in Table 1[3, 7].

Table 1:
The consequences of hypoglycemia[3, 7]

Acute Consequences	Chronic Consequences
Tachycardia, shakiness, sweating, anxiety, irritability, and hunger • Because of sympathoadrenal activation, which is a part of the glucose counter-regulatory response Coma, seizures, headache, and cognitive dysfunction • Because of glucose levels are below the threshold for sufficient cerebral supply	Atherosclerosis, myocardial infarction and sudden death • Because of repeated sympathoadrenal activation, increased thrombotic tendency, endothelial dysfunction, inflammation due to cytokine release Increased body weight • Because of repeated defensive eating Loss of self-confidence, reduced quality of life, maintenance of higher than desirable plasma glucose levels • Because of fear for relapse of hypoglycemia

Treatment of hypoglycemia To treat hypoglycemia, providers should continue to counsel patients with fast-acting carbohydrates at the hypoglycemia alert value of 3.9 mmol/l or less. The acute glycemic response correlates better with the glucose content of food than with the carbohydrate content of food. The use of glucagon is indicated for the treatment of hypoglycemia in people unable or unwilling to consume carbohydrates by mouth[9].

Cost of hypoglycemia For both the patients, the health care system, and the society at large, hypoglycemia carries an excessive cost. The cost includes its effects on quality of life, reduced treatment satisfaction and reluctance to achieve

recommended glycemic targets. The latter will increase risks of long-term complications which have massive cost implications[7,10].

The counter-regulatory system

When blood glucose falls in a nondiabetic adult, the secretion of counter-regulatory hormones and the onset of cognitive, physiological, and symptomatic changes occur at reproducible blood glucose thresholds within a defined hierarchy (Fig. 1)[11]. These processes include inhibition of the glucose utilization in muscle and fat cells and stimulation of glucose release from the liver[6]. An initial response, the inhibition of insulin secretion from the β-cells occurs when glucose levels fall below ∼4.6 mmol/l until glucose level of ∼3.6 mmol/l[11, 12]. At lower glucose levels (<3.8 mmol/l), a series of counter-regulatory responses aiming at stimulating EGP is initiated. This includes glucagon secretion from the islet α-cells, activation of the autonomic nervous system, release of adrenaline and cortisol from the adrenals and release of growth hormone from the hypophysis[11-13]. Once the threshold for activation of an autonomic input is reached, the magnitude of activation usually increases as glucose falls further. In the marked hypoglycemic range, it is therefore likely that all three autonomic inputs, i.e. sympathetic, parasympathetic and adrenals, mediate the large glucagon response, perhaps in redundant fashion[12]. Changes in growth hormone and cortisol are lower in the hierarchy of physiologic responses to hypoglycemia than changes in insulin, glucagon, and adrenaline. Neither is critical for the correction or prevention of hypoglycemia[6]. Subjective recognition of the symptoms of hypoglycemia is fundamental to effective self-management and to prevent progression in its severity[11]. Diabetic patients have a similar hormonal counter regulatory hierarchy, however, both high age and insulin treatment are associated with impaired hormonal reaction[11].

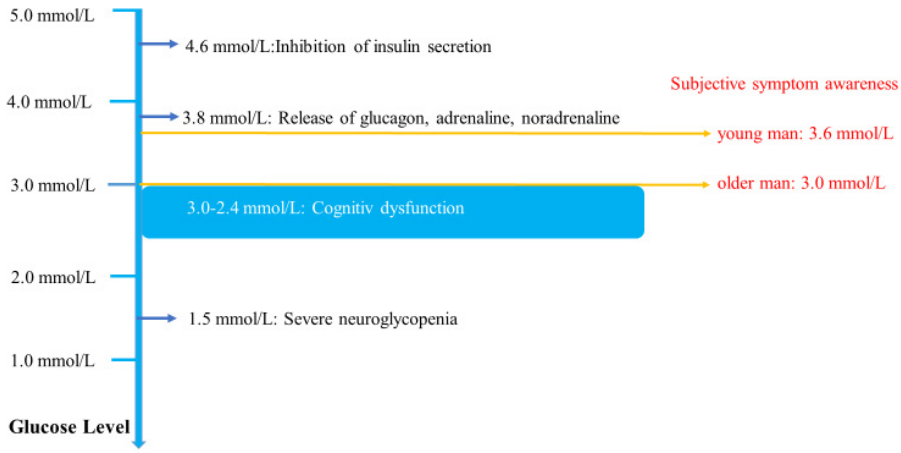


Figure 1:
Glycemic thresholds for secretion of counter-regulatory hormones and onset of physiological, symptomatic, and cognitive changes in response to hypoglycemia in the nondiabetic human[11]

Glucagon

A main counter-regulatory factor for restoring glucose levels during hypoglycemia is glucagon. In fact, a normal restoration of glucose requires a normal glucagon response[13]. Glucagon is a 29-amino acid peptide hormone, which is cleaved by prohormone convertase 2 from the proglucagon molecule in pancreatic islet α-cells. From these cells, the hormone is secreted into the hepatic portal vein and acts on G protein-coupled receptors in the liver to stimulate glucose production. By itself, glucagon largely stimulates hepatic glycogenolysis. However, in concert with other glucose counter-regulatory hormones, such as adrenaline, it mobilizes gluconeogenic precursors (lactate, amino acids, and glycerol) to the liver and stimulates hepatic gluconeogenesis. Glucagon increases also the synthesis and activity of enzymes favoring gluconeogenic and glycogenolytic pathways over their respective counterparts: glycolysis and glycogenesis. It has also a wide range of actions outside its role in glucose homeostasis, reflected by the expression of glucagon receptors in numerous tissues such as the kidneys, gastrointestinal tract, adrenal glands, heart, and brain[14, 15].

The secretion of glucagon is a function of an interplay between many stimulatory and inhibitory factors. Dominant physiological stimulators include hypoglycemia, certain amino acids, GLP-2, adrenaline, and the autonomic nervous system, while

hyperglycemia, IAPP, insulin, somatostatin, and GLP-1 all inhibit glucagon secretion[14]. The incretin hormone GIP stimulates glucagon secretion during hypoglycemia[16]. The reciprocal regulation of glucagon secretion by insulin stands high in the hierarchy of those mechanisms. In a normal circumstance, a decreasing plasma glucose concentration inhibits β-cell insulin secretion which results an increasing α-cell glucagon secretion during hypoglycemia. On the other hand, after a meal ingestion, an increasing plasma glucose concentration, possibly together with other stimuli, stimulates β-cell insulin secretion which results a inhibition in α-cell glucagon secretion[15].

Risks of hypoglycemia

The glucagon counter-regulation to hypoglycemia is a key mechanism for preventing and defending hypoglycemia. In fact, in diabetic patients, without functioning β-cells, blood insulin levels are only a function of the clearance of exogenous insulin and circulating glucagon levels do not increase appropriately when plasma glucose levels are sub-physiologic. Therefore, the inhibition of β-cell secretion and disinhibition of α-cell secretion are affected[6, 15]. This is maybe the reason that the duration of T1DM is a major risk factor for hypoglycemia, whereas in insulin-treated T2DM the duration of insulin therapy is the prime determinant of this risk[2]. In the absence of these mechanisms, patients with T1DM or advanced T2DM depend on the adrenaline secretory response which is also often attenuated and thus the risk of severe hypoglycemia increases considerably. Attenuation of the sympathoadrenal response is believed also to cause the clinical syndrome of IAH[6].

Furthermore, there are other risk factors which make some patients particularly vulnerable to hypoglycemia. These risk factors include high age, general frailty, multimorbidity, poor nutrition, history of prior hypoglycemia including impaired hypoglycemia awareness and the risk is also higher in T1DM than in T2DM. The chronic health conditions most often implicated in increasing hypoglycemia risk are kidney disease, cardiovascular disease, heart failure, depression and cognitive impairment. Older patients have often several of these risk factors at the same time and their symptoms of hypoglycemia are sometimes less obvious and atypical, such as vague neurologic symptoms that may be misinterpreted as neurologic diseases[2, 17, 18]. When using glucose-lowering therapy in patients with these risk factors for hypoglycemia, it is of particular value to use a type of therapy with a low risk for hypoglycemia.

Hypoglycemia during glucose-lowering therapy

An important risk factor for hypoglycemia is the use of glucose-lowering therapies. However, there are differences in the risk of hypoglycemia both among and within the various classes of therapies. Insulin administration and insulin secretagogues result in increased rate of hypoglycemia. For example, severe hypoglycemia estimated per 100 person-years has been shown to be at a rate of ~2.0 with insulin or insulin secretagogues while the equivalent rates for other glucose-lowering agents (including metformin, thiazolidinedione, DPP-4 inhibitor, GLP-1 RA) and no glucose-lowering agents are at 0.2 and 0.5 respectively[6, 18, 19].

The use of insulin is associated with the highest rates of hypoglycemia. Numerous studies have demonstrated that long-acting basal insulin analogs, such as insulins detemir and glargine, are associated with a reduction in the incidence of hypoglycemia compared to NPH insulins[6]. Among the oral antidiabetic agents, insulin secretagogues, such as sulfonylureas and glinides, have the highest risk of hypoglycemia. Agents that do not stimulate insulin secretion or stimulate insulin secretion in a glucose-dependent manner: metformin, thiazolidinediones, SGLT2 inhibitors and incretin therapy (both DPP-4 inhibitors and GLP-1 RAs) have a low risk of hypoglycemia[20, 21]. Fig. 2 shows results of two meta-analysis regarding risk for hypoglycemia with different therapies as monotherapy or as add on to metformin[20, 21] which is a common clinical combination considering that metformin is still the most common first line medication[22].

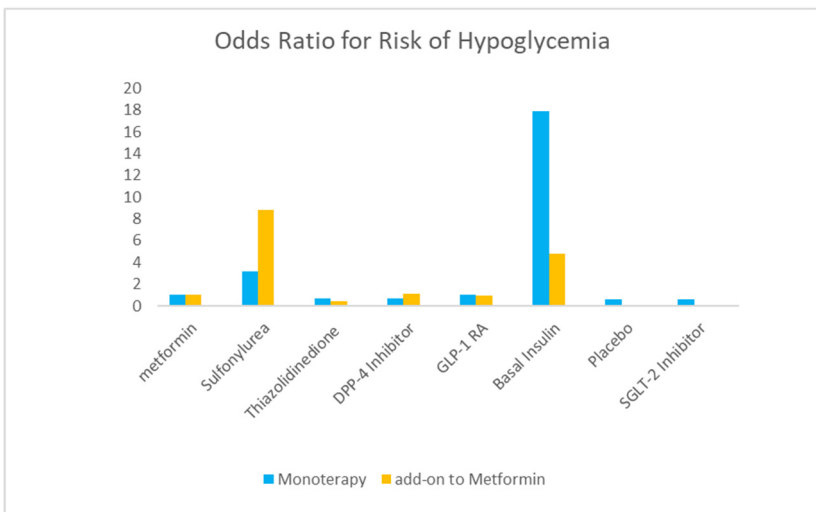


Figure 2:
Mean odd ratios for hypoglycemia of the various therapies versus the risk with metformin as monotherapy or as add-on to metformin in subjects with T2DM[20, 21]

When incretin therapies are combined with insulin secretagogues or insulin, however, the risk of hypoglycemia approaches the high rates seen with insulin or secretagogues alone[6, 23]. Fig. 3 shows results of nine phase III trials where different DPP-4 inhibitors (5 trials) or GLP-1 RAs (4 trials) added on to a variety of insulin regimens and hypoglycemia was experienced by 8–27% and 18-28% of patients, respectively (vs 7–24% and 12-29% in PBO arms)[24-32]. The rates of hypoglycemia were not increased but were on the same level as insulin treated patients.

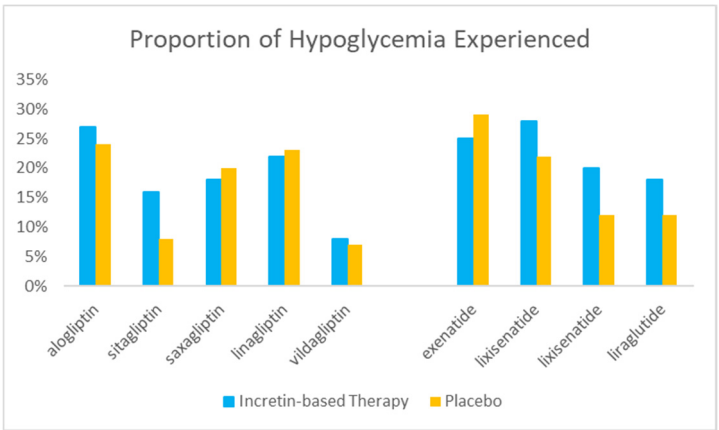


Figure 3:
DPP-4 inhibitors or GLP-1 RAs added-on to a variety of insulin regimens in Phase III trials with the mean treatment period between 24 to 30 weeks and the number of study subjects between 213 to 1261[24-32], *There are two studies with lixisenatide as active study medication.

Incretin therapy

Incretin hormones In healthy subjects, oral ingestion facilitates the disposal of absorbed glucose through the stimulation of insulin secretion from the endocrine pancreas. The observation that enteral nutrition provided a more potent insulinotropic stimulus compared with isoglycemic intravenous challenge led to the development of the incretin concept[33]. The gut hormones GIP and GLP-1 are secreted from the intestinal K- and L-cells, respectively, within minutes of nutrient ingestion and together explain most, if not all, of the incretin effect in humans[34]. The incretin effect is estimated to account for approximately 25%–75% of the total insulin secreted after oral glucose administration (i.e., the combined action of hyperglycemia and incretin hormones) depending on the glucose load[35]. In T2DM, GIP no longer stimulates glucose-dependent insulin secretion, even at supraphysiological plasma levels[36]. GLP-1, on the other hand, is still

insulinotropic and this has led to the development of compounds that activate the GLP-1 receptor with a view to improving insulin secretion[36]. However, the half-life of native GLP-1 in the circulation is less than 2 minutes owing to rapid renal clearance and inactivation by the DPP-4. Consequently, more than half of the GLP-1 that enters the portal circulation already has been inactivated before entry into the systemic circulation[34].

There are two classes of incretin therapies: DPP-4 inhibitors, which prevent the proteolytic breakdown and inactivation of GLP-1, and GLP-1RAs, which provide supraphysiological concentrations of ligands that stimulate the GLP-1 receptors. There are five GLP-1RAs currently available in Europe for the treatment of T2DM (Table 2). Individual differences between the different GLP-1 RAs in structure, pharmacokinetic and clinical effects exist, resulting in GLP-1RAs being classified as short- or long-acting[37, 38]. There are five DPP-4 inhibitors available in Europe (Table 2) with similar efficacy and safety for T2DM treatment, either as monotherapy or combination therapy[37, 39].

DPP-4 inhibitors reduce HbA1c without weight gain, and GLP-1 RAs reduce HbA1c with weight reduction. Incretin therapy is safe with very few AEs and an additional value of the therapy is a very low risk for hypoglycemia[40].

As more than half of the native GLP-1 that enters the portal circulation already has been inactivated before entry into the systemic circulation[34], a possibility is that the actions of GLP-1 are transmitted via sensory neurons in the intestine and the liver expressing the GLP-1 receptor. Also, when studying incretin-based therapy, it is important to distinguish between measurements of the intact hormone (responsible for endocrine actions) or the sum of the intact hormone and its metabolites, reflecting the total L-cell secretion and therefore also the possible neural actions[41].

Table 2:
Available incretin therapy in Europe

DPP-4 Inhibitors	GLP-1 RAs
sitagliptin	exenatide
vildagliptin	lixisenatide
linagliptin	liraglutide*
saxagliptin	dulaglutide*
alogliptin	semaglutide*

*Long acting GLP-1 RAs

Clinical studies with incretin therapy All DPP-4 inhibitors achieve glycemic efficacy in a similar fashion and elevate GLP-1 levels by 1.5- to 3-fold (i.e. near the physiological range). Once DPP-4 is maximally inhibited, HbA1c reductions are similar across this class (weighted mean differences of ~8 mmol/mol as monotherapy)[37].

Compared with PBO, GLP-1RAs reduce HbA1c levels by ~10 mmol/mol which is dependent on the choice of agent, dose, baseline HbA1c and ongoing treatment. The short acting GLP-1 RAs predominantly reduce PPG levels. By contrast, the long acting GLP-1 RAs produce reductions in both FPG and PPG as a result of suppression of fasting glucagon and increase in fasting insulin levels. Significantly greater HbA1c changes are therefore observed when compared with short acting GLP-1 RAs in head-to-head trials[37, 42].

GLP-1 RAs yield greater reduction in HbA1c and weight as compared to DPP-4 inhibitors, with increased incidence of gastrointestinal symptoms but not hypoglycemia. Replacing a DPP-4 inhibitor with GLP-1 RA provides additional benefits in glycemic control and weight loss[43]. Recent studies on cardiovascular outcomes have shown that GLP-1RAs liraglutide and semaglutide have a cardio-protective effect as reflected in significantly diminished cardiovascular events in patients with T2DM and cardiovascular risk[44]. Thus, these agents may be preferred for use in select populations with high cardiovascular risk[45].

In general, the tolerability of DPP-4 inhibitors is excellent and the number of AEs reported in clinical trials with DPP-4 inhibitors were similar in patients receiving the drugs compared with those receiving PBO. For GLP-1 RAs the most commonly observed AE is transient nausea[37].

There was controversy about whether incretin therapy is associated with pancreatitis and pancreatic cancer[46, 47]. After an extensive review by the FDA and EMA in 2014, it was concluded that studies so far did not show a causal link between incretin-based therapies and either pancreatitis or pancreatic cancer[48]. Observational studies and post-marketing surveillance provide real world evidence of safety and effectiveness of these agents and have provided reassurance that signals for pancreatitis and pancreatic cancer seen in clinical trials are not of major concern in large patient populations[4].

Incretin therapy in clinical guidelines Current international T2DM treatment guidelines place both GLP-1 RAs and DPP-4 inhibitors as second-line or third-line agents after metformin and they can also be used in combination with insulin with complementary and additive effects on glycemic control to achieve HbA1c targets[22, 49, 50]. In patients with established atherosclerotic cardiovascular disease, GLP-1 RA is recommended prior to insulin because of the benefits to reduce major adverse cardiovascular events and cardiovascular mortality. In addition, recent evidence supports the utility of GLP-1 RAs prior to insulin in patients not reaching glycemic targets with oral agent regimens since the efficacy of the two treatments was similar and GLP-1 RAs had a lower risk of hypoglycemia and beneficial effects on body weight compared with insulin[4, 22, 51]. There has also been some interest in the use of incretin therapies in the management of T1DM[52], especially for those with residual β-cell function[53]. However, these

agents are not approved for patients with T1DM according to the treatment guidelines[22].

Incretin therapy and counter-regulation to hypoglycemia

Since incretin therapy is associated with a low risk of hypoglycemia, its effect on counter-regulatory responses to hypoglycemia has been studied[54-56]. In particular, the effect of incretin therapy on the glucagon counter-regulation has been of interest, since incretin therapy is known to inhibit glucagon secretion[37] and glucagon is required for a good glucose counter-regulation to hypoglycemia[6, 13].

In healthy subjects, GLP-1 RAs have shown preserved or enhanced glucagon counter-regulation during hypoglycemia and they did not impair overall autonomic hypoglycemia counter-regulation[54, 55]. In drug naïve patients with T2DM, even a 38% higher glucagon increment after the DPP-4 inhibitor vildagliptin was reported, as compared with PBO, during hypoglycemia which was induced immediately after a meal. It was supposed that the DPP-4 inhibitor improved the ability of both α- and β-cells to sense and respond appropriately to hypoglycemia. In the same study, a preserved counter-regulatory response in adrenaline, noradrenaline, cortisol and the marker of the autonomic nervous system, PP were observed[56]. The most likely explanation behind this is the glucose-dependency of the action of the incretin-based therapy, such that the effect of lowering glucagon during hyperglycemia vanishes once glucose levels are lowered below a threshold. There is also a possibility that GIP may contribute to maintain stimulated glucagon secretion during DPP-4 inhibition, considering that GIP, the levels of which are increased after DPP-4 inhibitor, has been shown to stimulate glucagon secretion during hypoglycemia[57] and an intact GIP receptor is required for a normal glucagon response[58].

These studies on glucagon counter-regulation was performed in healthy subjects and in subjects with drug-naïve T2DM. The studies did not, therefore, explore whether incretin therapy does affect glucagon counter-regulation in particularly vulnerable subjects at increased risk for hypoglycemia.

The rationale for the present study

Study populations When incretin therapy is becoming more widely used, it is used in a variety of different patient groups. It becomes therefore of clinical importance to study the counter-regulation to hypoglycemia also in vulnerable patients who have increased risk of developing hypoglycemia, such as insulin treated patients with T2DM, patients with T1DM and elderly patients with T2DM. These vulnerable patients have a trend for a low glucagon response to hypoglycemia[11]. The compromised defences in established T1DM and advanced T2DM (those with absolute deficiency of endogenous insulin) include loss of a decrease in insulin, loss of an increase in glucagon, both of which are probably the result of β-cell failure, and attenuation of an increase in adrenaline as plasma glucose concentrations fall[3]. With increasing age, the symptoms of hypoglycemia may become less intense and the symptom profile is modified, and the magnitude of the glucagon is lower in the elderly group during mild hypoglycemia[11]. This is evident from a study showing that the generation of hypoglycemic symptoms and development of cognitive dysfunction for elderly men occur at almost the same blood glucose level[11]. This contrasted with younger men who have an interval of around 1 mmol/l in between these symptoms (Fig. 1). Counter-regulation to hypoglycemia also differs in older people with diabetes who had lower glucagon and growth hormone responses but higher adrenaline and cortisol responses during hypoglycemia. This altered counter-regulation may interfere with their ability against hypoglycemia[59]. Factors that place this population at risk include also decreased clearance of medication, impaired cognition, and inability to recognize signs and symptoms of hypoglycemia[60].

Scientific question The previous studies on the effects of incretin therapy on counter-regulatory responses to hypoglycemia have been undertaken in healthy subjects and in drug-naïve T2DM patients[54-56], i.e., in subjects having low risk for hypoglycemia by themselves. Whether the compromised counter-regulatory responses in vulnerable patients impair the potential of incretin therapy to sustain glucagon counter-regulation was therefore not known. This also relates to the important question to define the glucose level at which the inhibition of glucagon secretion vanishes during incretin therapy in various patient groups. The main scientific question in this thesis was therefore to answer if the counter-regulatory glucagon response to hypoglycemia is sustained during incretin therapy when this therapy is used in particularly vulnerable patients. To approach this scientific question, the effect of incretin therapy on the glucagon response to hypoglycemia was established in insulin treated patients with T2DM, in patients with T1DM and in elderly patients with T2DM.

Methodological considerations To study the glucagon response to hypoglycemia, the hyperinsulinemic hypoglycemia clamp technique was used in the

studies. This method is a development of the clamp technique developed by Defronzo et al. in 1979. They described the glucose clamp techniques which are two methods for quantifying insulin secretion or resistance. The *hyperglycemic clamp* requires maintaining a high blood glucose level by perfusion or infusion with glucose to quantify how fast β-cells respond to glucose. The *hyperinsulinemic euglycemic clamp* requires maintaining a high insulin level and a constant glucose level, achieved by perfusion or infusion with insulin together with glucose to quantify how sensitive the tissue is to insulin[61].

The *hyperinsulinemic hypoglycemic clamp* used in our studies is a modification of this glucose clamp technique designed to assess counter-regulatory hormone responses under standardized conditions of experimental hypoglycemia[62]. Insulin is infused to lower glucose and the resulting circulating glucose is measured bedside every 5th minute. A concomitant variable glucose infusion allows circulating glucose levels to be maintained at a desired level for 30 minutes. During this clamp period, blood is sampled for measurements of the counter-regulatory hormones, such as glucagon, adrenaline, noradrenaline, cortisol and PP.

Hypoglycemic clamps have previously been extensively used in studies of the pathophysiology of iatrogenic hypoglycemia[63-65]. They are also used to study the effects on glucose counter-regulation of glucose-lowering drugs that on theoretical grounds might impair the physiological response to hypoglycemia, e.g. by restraining increases in hepatic glucose production[66]. The hypoglycemic clamp permits the onset of secretion and the magnitude of counter-regulatory hormone responses to be quantified under standardized and reproducible conditions with a high degree of precision. Such precision is not possible using a bolus of insulin which is an alternative method for studies on hypoglycemia. Insulin injection technique causes also a less standardized and predictable reductions in blood glucose[67].

In the morning before the start of the hyperinsulinemic hypoglycemic clamp in study 1, 2 and 4 where DPP-4 inhibitors were used as the active study medication, a standardised breakfast was served before the clamp test was initiated. The reason for this was to ensure a raise in the concentrations of incretin hormones at the time of the clamp.

In all studies, we utilized a crossover design because each patient serves as his/her own control and paired t-test can be used, at the same time fewer subjects were needed for statistics. During the hypoglycemic clamp, we sampled the counter-regulatory hormones: glucagon, adrenaline, noradrenaline and cortisol. PP was also sampled because PP is released into the circulation during vagal, cholinergic activation, and the circulating levels of PP are therefore considered to reflect the degree of vagal activity[68].

Study medication Vildagliptin (in study 1, 2) and sitagliptin (in study 4) were used as the active study medication because they belong to two of the most used DPP-4 inhibitors clinically.

Sitagliptin and vildagliptin mimic the dipeptide structure of DPP-4 substrates and both are competitive reversible inhibitors with similar efficacy in lowering HbA1c levels. They are orally available and are rapidly absorbed with significant inhibition of plasma DPP-4 activity within 5 min followed by DPP-4 inhibition > 80% 24 h and 12 h post-dose of sitagliptin and vildagliptin, respectively. Sitagliptin is dosed once per day and vildagliptin twice per day with appropriate dose adjustment in patients with moderate or severe renal insufficiency as kidneys are the predominant route of elimination. Vildagliptin is not recommended for patients with mild/moderate hepatic insufficiency and liver function tests should be performed periodically when it is prescribed[69].

Lixisenatide is a once-daily short acting GLP-1 RA for the treatment of adults with T2DM. It shares some structural elements with exendin-4 with a half-life of ∼3 h and can be used in individuals with mild-to-moderate renal impairment without dose adjustment. Compared with the longer-acting GLP-1 RA, lixisenatide reduce PPG primarily due to the greater inhibition of gastric motility with either weight loss or no weight gain. The most frequent AE are gastrointestinal and transient in nature[70].

Aims of the thesis

The primary aim of this thesis was to answer the following two questions:

1. Is the glucagon counter-regulatory response to hypoglycemia preserved when incretin therapy is used in particularly vulnerable patients?
2. Are the counter-regulatory responses to hypoglycemia for adrenaline, noradrenaline, cortisol and PP preserved when incretin therapy is used in particularly vulnerable patients?

The study design also allowed a secondary aim, to answer the question at which glucose level vanishes the inhibition of glucagon secretion during incretin therapy in these various patient groups.

Subjects and methods

Study design and study population

Inclusion and exclusion criteria The study population comprised of male and female patients (non-fertile or of child-bearing potential using a medically approved birth control method) who had ability to complete the studies. They were aged >18 years and had antibody negative T2DM (except study 1 where only T1DM patients were recruited). Patients had HbA1c: between 48-68 mmol/mol in study 1 and 2, between 53-86 mmol/mol in study 3 and between 42-67 mmol/mol in study 4. Furthermore, included patients had, as glucose-lowering therapy, insulin (study 1), insulin ± oral medication (study 2), basal insulin + metformin (study 3) and metformin (study 4). Exclusion criteria were T1DM (except study 1); pregnancy or lactation; a history of recent (< 2 weeks) recurrent or severe hypoglycemia; acute infection which may affect the blood glucose control during the 4 weeks preceding the study; history of liver disease; donation of one unit blood (500 ml or more) within 2 weeks; treatment with growth hormone or an oral steroid during the 2 months preceding the study and thereafter during the whole study period. (please see each respective article for more details of inclusion and exclusion criteria)

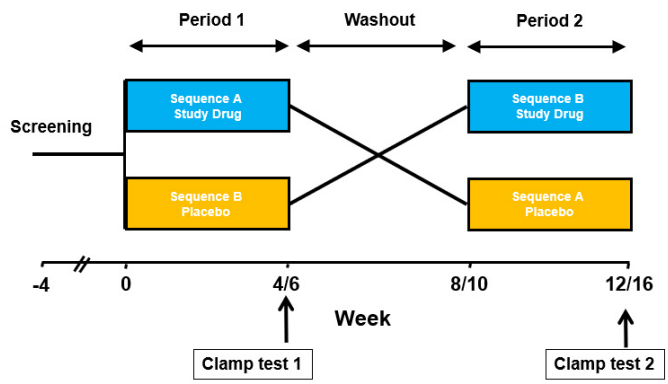


Figure 4:
The study design

Study design The studies were single-center, double-blind, randomized, PBO-controlled crossover studies. They were undertaken at the Clinical Research Unit, Medicine Department, Skåne University Hospital, Malmö, Sweden. Figure 4 illustrates the design of the studies. After the screening visit, eligible patients were randomized to the active study medication or PBO for 4 weeks (6 weeks in study 3 with the dose titration during the first 2 weeks) in addition to their background medication. Randomization were undertaken by the hospital pharmacy. At day 28 (day 42 for study 3), a clamp procedure was undertaken (see below), after which blinded study medication was discontinued for a 4-week washout period. This was followed by a second equally long treatment period with the alternative treatment, which ended with the second clamp test. Thus, all patients received the active study medication and PBO for an equal long treatment period in a random crossover design.

Sample size Sample size was planned to provide a power of >80% to detect a 10% difference in the primary endpoint (increase in glucagon levels after 30 minutes of hypoglycemic clamp) in a paired t test, as in the crossover design, based on the previous study that determined the glucagon response to insulin-induced hypoglycemia with a clamp technique in T2DM[56]. Four additional patients were recruited in each study to allow for discontinuation or other drop outs.

Clamp Procedure

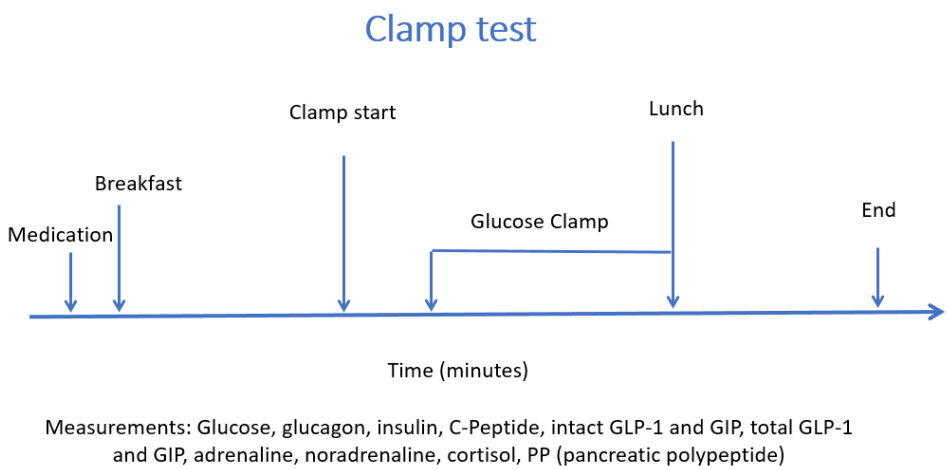


Figure 5:
The clamp test procedures

General description of the clamp technique After an overnight fast, a baseline (fasting) blood sample was taken (time, −20 minutes). Background (except insulin) and blinded study medications were then administered. In studies with DPP-4 inhibitors, a standardized breakfast was served (time, 0) and was to be consumed within 15 minutes. This aimed to stimulate the incretin hormone secretion and allowed the DPP-4 inhibitor to prevent their inactivation. In study 3, no breakfast was served because GLP-1 RA lixisenatide was studied and reached supraphysiological level before the clamp procedure. At 120 minutes (60 minutes in study 3), a hyperinsulinemic hypoglycemic clamp was initiated. Like in a prior study[56], patients received a primed infusion of insulin (Actrapid^{R}; Novo Nordisk A/S, Bagsvaerd, Denmark), with the rate depending on fasting blood glucose concentration. Four minutes after the initiation of insulin infusion, glucose infusion was initiated (200 mg/ml) at a variable rate, depending on the body surface area. After the primed infusion, the insulin and glucose infusion rates were adjusted to allow a moderate decrease in glucose level. This procedure assures a similar reduction in glucose, being independent of fasting glucose, which is of importance for standardizing the clamp procedure to allow the glucagon response to occur at the same time in each patient. Blood glucose was monitored at the bedside every 5 minutes (2.5 minutes when glucose approaching predetermined level) using the glucose dehydrogenase technique with a HemoCue^{R} device (HemoCue AB, Ängelholm, Sweden). At the end of the clamp test, the insulin infusion was discontinued, and a lunch was served. Each patient received the same lunch meal on both test days.

Modifications of the clamp technique in the different studies In study 3 and 4, the primed infusion rate was higher because of the insulin resistance in the study populations. The insulin infusion schedule results in different insulin levels in study 4 between the two treatment arms during the beginning of the clamp test, possibly because of the different postprandial glucose level, which might influence counter-regulatory results. However, it has been shown previously that differences in insulin levels do not affect the counter-regulatory response of glucagon or other counter-regulatory factors during the hyperinsulinemic hypoglycemic clamp procedure, even at a 6-fold difference in insulin levels[71].

The lowest glucose target during the hypoglycemic clamp was 2.5 mmol/l and should be maintained at least 30 minutes which was expected to stimulate all the counter-regulatory hormone responses and at the same time, with the limited hypoglycemic period, minimize the possible acute complication of hypoglycemia to the patients. In study 3 and 4, we performed a two-step hyperinsulinemic hypoglycemic clamp to explore the counter-regulatory hormone responses in more details. In study 3, the glucose clamp levels were 3.5 and 2.8 mmol/l respectively. In study 4, the glucose clamp levels were 3.5 and 3.1 mmol/l, i.e., the lower value was slightly higher than in study 3, because of the vulnerability of this elderly study

population. The acceptance of the higher glucose clamp levels in the last two studies was also because of the insulin resistance accompanied with the T2DM patients, thereby the glucose clamp level can be reached without delay and at a similar time point after the start of the clamp procedure in both test days.

Laboratory measurements

Blood sampling and methods for adrenaline, noradrenaline and cortisol
During the clamp studies, blood samples for assay of glucose, glucagon, insulin, C-peptide and PP were drawn in tubes containing EDTA (7.4 mmol/l) and were immediately centrifuged at 4 ^{0}C and plasma was frozen at −20 ^{0}C until analysis. For determination of GLP-1 and GIP, blood samples were collected into tubes containing EDTA with the addition of the DPP-4 inhibitor diprotin A (0.1 mmol/l) (Bachem, Bubendorf, Switzerland). Samples for the determination of noradrenaline and adrenaline levels were obtained in ice-chilled sodium-heparin tubes, and concentrations were determined by high-performance liquid chromatography. Samples for the determination of cortisol were taken in sodium-lithium-heparin tubes, and cortisol was determined using the Access Immunoassay Systems^{R} (Beckman Coulter Diagnostics, Fullerton, CA, USA). Cortisol, adrenaline, noradrenaline, HbA1c, fasting plasma glucose and safety laboratory assessments were measured by the Department of Clinical Chemistry (Skåne University Hospital, Malmö, Sweden). All other samples were measured at the Biomedical Center, Lund University according to standardized and validated procedures and good laboratory practice.

- **Glucagon** concentrations were analyzed with a double antibody RIA (Merck Millipore, Billerica, MA, USA) in study 1, 2 and 4 with a detection limit of 6.2 pmol/l. During RIA, antibody is mixed with known quantities of radioactive antigen, then unlabeled antigen is added to compete for antibody binding sites and displace the radioactive variant. Thereby the unlabeled antigen can be measured by measuring the amount of radioactive labeled antigen displaced. This assay utilized ^{125}I-labeled human glucagon and a glucagon antiserum to determine the level of glucagon in the samples.

  In study 3, an ELISA (Mercodia, Uppsala, Sweden) was used with a detection limit of 1 pmol/l. During ELISA, antigens from the sample are attached to antibodies on a surface. Then, another specific antibody is added, so it can bind to the antigen. This antibody is linked to an enzyme which, in the final step, react to a substrate and produces a detectable signal. Thereby the antigens can be measured.

- **Glucose** was analyzed using the glucose oxidase method (except at bedside when the glucose dehydrogenase method was used). Glucose oxidase catalyzes the oxidation of Beta D-glucose present in the plasma with the formation of hydrogen peroxide (H_2O_2). The H_2O_2, in the presence of peroxide, is involved in the oxidation of ABTS where the produced oxygen after chemical reactions converted to a colored compound and measured calorimetrically.
- **Insulin** was determined with ELISA (Mercodia, Uppsala, Sweden) with a detection limit of 6.9 pmol/l where two monoclonal antibodies are directed against separate antigenic determinants on the inulin molecule. The bound conjugate is detected by a reaction which give a colorimetric endpoint that can be read spectrophotometrically.
- **C-Peptide** was measured using double antibody RIA (Merck Millipore, Billerica, MA, USA) with a detection limit of 0.02 nmol/l. This C-Peptide assay utilized ^{125}I-labeled Human C-Peptide and a Human C-Peptide antiserum to determine the level of C-Peptide in the samples.
- **PP** were analysed with ELISA (Merck Millipore, Billerica, MA, USA) with a detection limit of 12.3 pg/ml and the assay is specific for human PP.
- **Total GIP** was determined with ELISA (Merck Millipore, Billerica, MA, USA) and the limit of sensitivity of this assay is 0.84 pmol/l. The assay is C-terminally directed and cross-reacts to 100% with GIP (1-42) and GIP (3-42).
- **Total GLP-1** was determined with ELISA (Merck Millipore, Billerica, MA, USA) and the limit of sensitivity of this assay is 1.5 pmol/l. The assay is C-terminally directed and cross-reacts to 100% with GLP-1 (7-36) and GLP-1 (9-36).
- **Intact GIP** was analysed with ELISA (Dermeditect Diagnostics, Kiel-Weilsee, Germany) in study 2 and (Crystal Chem Downers Grove, IL, USA) study 4. The assays are N-terminally directed and have a limit of sensitivity is 0.24 pmol/l and 0.78 pmol/l, respectively.
- **Intact GLP-1** were analysed with ELISA (Merck Millipore, Billerica, MA, USA). The assay is N-terminally directed and has a sensitivity of 2 pmol/l.

Data analysis and statistics

All data analyses were performed using IBM SPSS statistics. Means ± SE are shown unless otherwise noted. After breakfast (in studies 1, 2 and 4) and lunch meals (in studies 1,2 and 4) and during the hypoglycemic clamp procedure (all studies), the changes in the concentrations of all analytes were calculated as incremental values or AUC using the trapezoid rule in the completer's population. The completer´s population was defined as all randomized patients who received at least one dose of study medication and had a valid assessment of the primary variable at the end of each treatment period. Between-treatment differences in measured variables were estimated with a paired t test. There was no difference depending on the order sequence of treatment in any of the studies. Therefore, in the statistical analysis, all sequences with the active study medication and PBO, respectively, were analyzed together. A p-value of $<.05$ was considered significant. Changes in HbA1c and body weight were also assessed during the two treatment periods.

Adverse events

AEs were sought by nondirective questioning at each visit and were also recorded when mentioned by the patient during or between visits or during physical examination, laboratory testing or other assessments. Hypoglycemia was defined as either symptomatic hypoglycemia, confirmed hypoglycemia (blood glucose concentration ≤ 3.1 mmol/l) or a hypoglycemia episode that required assistance for glucose control.

Ethics, good laboratory and good clinical practices

The studies were undertaken according to Good Laboratory Practice and Good Clinical Practice in accordance with the Declaration of Helsinki. The protocols were approved by the Ethic Committee in Lund, Sweden, the Swedish Medical Product Agency, and registered at ClinicalTrials.gov (clinical trial reg. no. NCT01147276, NCT01219400, NCT02020629 and NCT02256189 for studies 1-4, respectively) and https://www.clinicaltrialsregister.eu databases (EudraCT 2010-019528-29, EudraCT 2010-020088-19, EudraCT 2012-004959-36 and EudraCT 2014-002685-70 for studies 1-4, respectively). All subjects gave written consent to participate before the study, and the studies were monitored by external monitors.

Results

Following is the summary of results of the four studies. More details of results can be found in the respective articles.

Patient disposition, demographic data and baseline data

The participating patients had clinical follow-up by the local health centers or the university hospital in Malmö/Lund and were recruited according in line with the respective study protocols. Table 3 summarizes the patient disposition and the demographic and baseline characteristics of the completer's population. In total, 152 patients were screened for the four studies, 112 patients were randomized, and 103 patients completed the studies and therefore comprised the completer´s population. The reasons for not completing after randomization in the studies were (1) non-adherence to study medication before first clamp study in two patients, (2) hospitalization because of cerebral vascular disease in two patients, (3) having abdominal pain during the first treatment period in two patients (one had active study medication and the other had PBO), (4) blood pressure fall and nausea during the first hypoglycemic clamp test in one patient, (5) change in regular medication after performing the first study test in one patient, and (6) personal reasons in one patient.

Table 3:
The demographic and baseline characteristics of the completer's population

	Study 1	Study 2	Study 3	Study 4
Study population	T1DM Insulin-treated	T2DM Insulin-treated	T2DM Insulin-treated	T2DM Elderly
Active study medication	vildagliptin	vildagliptin	lixisenatide	sitagliptin
Nr of screened patients	36	48	21	47
Nr of randomized patients	29	33	19	31
Nr of completed patients	28	29	18	28
Age (years)	30 ± 5	59 ± 6	55 ± 12	74 ± 6
Male	21 (75%)	16 (55%)	11 (61%)	17 (61%)
BMI (kg/m^2)	24.8 ± 3.3	30.7 ± 4.6	33.0 ± 5.0	30.2 ± 4.7
Hypertension treatment	0	20 (69%)	13 (72%)	17 (61%)
Hyperlipidemia treatment	2 (7.1%)	18 (62%)	14 (78%)	12 (43%)
Cardiovascular diseases	0	4 (14%)	3 (17%)	0
Cerebralvascular disease	0	1 (3%)	2 (11%)	1 (4%)
Creatinine (umol/l)	73 ±11	71 ±18	74 ±22	77 ±15
HbA1c (mmol/mol)	58 ± 6	60 ± 8	61 ± 3	52 ± 7
FPG (mmol/l)	10.5 ± 4.3	8.6 ± 2.1	9.7 ± 0.6	8.5 ± 1.6
Disease duration (years)	11.0 ± 4.4	14.0 ± 8.0	11.7 ± 7.6	9.2 ± 6.6
Metformin treatment (g/day)		1.9 ± 0.4*	2.1 ± 0.7	1.6 ± 0.7
Daily insulin dose Long-acting (U) Short-acting (U)	 30 ± 9 31±12	 34 ± 16 21 ± 13	 39 ± 22 -	 -

Mean ± SD or n (%); Cardiovascular diseases: history of myocardial infarction, percutaneous coronary Intervention or coronary artery bypass grafting; Cerebralvascular disease: history of transient ischemic attack or stroke; *: 66% patients treated with metformin.

Fasting and post-breakfast glucose, glucagon and incretin hormones

Table 4 shows the fasting and post-breakfast levels of glucose and glucagon. In all the studies, after treatment with the active study medication, fasting glucose was lower but there were no significant differences in the fasting glucagon. In studies 1, 2 and 4, a breakfast meal immediately before the clamp period was served. After breakfast, glucagon levels increased less with incretin therapy than with PBO which resulted in lower 120-min AUC after incretin therapy. Also, glucose levels increased less with incretin therapy than with PBO, except in the study 1 in T1DM patients where the increase of glucose was not significantly different compared to treatment after PBO.

All these changes, after breakfast, were associated with significant increases in intact GLP-1 (P≤0.03 in study 1, 2 and 4) and GIP (P<0.001 in study 2 and 4). In study 4, total GLP-1 and GIP were also analyzed which showed a non-significant different total GLP-1 after breakfast, but the post-breakfast total GIP was lower (P=0.044).

Table 4:
The fasting and post-breakfast levels of glucose, glucagon and post-breakfast Incretin hormones. Active study medication in red, PBO in blue.

	Study 1 vildagliptin, T1DM	Study 2 vildagliptin, T2DM	Study 3 lixisenatide, T2DM	Study 4 sitagliptin, T2DM
Fasting				
Glucose (mmol/l)	8.7±0.8 9.4±0.8 (P=0.012)	8.0±0.4 8.7±0.3 (P=0.046)	8.3±0.6 9.3±0.7 (P=0.023)	7.4±0.3 8.2±0.3 (P=0.003)
Glucagon (pmol/l)	19.6±1.5 19.4±1.4 (NS)	16.2±1.2 17.3±1.4 (NS)	10.7±1.3 9.8±1.2 (NS)	26.5±1.9 26.9±1.8 (NS)
AUC after Breakfast *				
Glucose (mol/lmin)	1.2±0.1 1.4±0.1 (NS)	1.2±0.1 1.5±0.1 (P<0.001)	-	0.2±0.02 0.3±0.03 (P=0.001)
Glucagon (nmol/lmin)	2.4±0.2 2.6±0.2 (P=0.022)	2.0±0.2 2.2±0.2 (P=0.016)	-	0.3±0.1 0.7±0.1 (P=0.02)
Intact GIP (nmol/lmin)	-	6.9±0.6 3.7±0.4 (P <0.001)	-	9.1±0.9 4.4±0.5 (P <0.001)
Intact GLP-1 (nmol/lmin)	0.3±0.10 0.1±0.03 (P=0.034)	1.7±0.2 0.7±0.1 (P<0.001)	-	1.7±0.2 0.6±0.1 (P <0.001)
Total GIP (nmol/lmin)	-	-	-	3.3±0.3 4.3±0.4 (P =0.044)
Total GLP-1 (nmol/lmin)	-	-	-	1.8±0.2 2.0±0.3 (NS)

Data show the mean ± s.e.m. for the completer's population. P-values indicate the significant level of random difference between treatment as obtained by paired *t*-test. *AUC in study 4 and intact GLP-1 in study 1 were presented as suprabasal AUC, otherwise total AUC.

Clamp results

Glucose

Blood glucose levels were well matched on the different predetermined hypoglycemic levels between the active study medication and PBO treated groups during the hypoglycemic clamp within each study. There was no difference in the amount of glucose infused between the study groups within each study during the

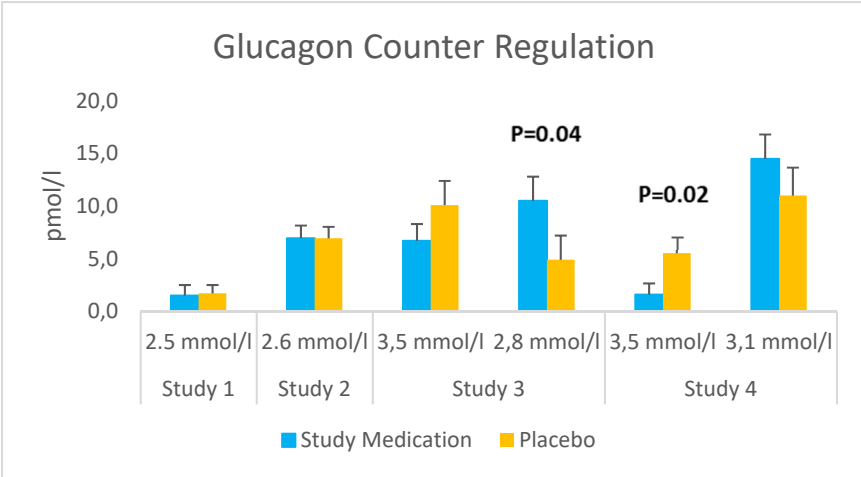
clamp period in studies 1 to 4. In studies 1, 2 and 3, total amount of infused insulin did not differ between the groups, whereas in study 4, slightly more insulin was infused in the PBO treated group compared with the active study medication treated group.

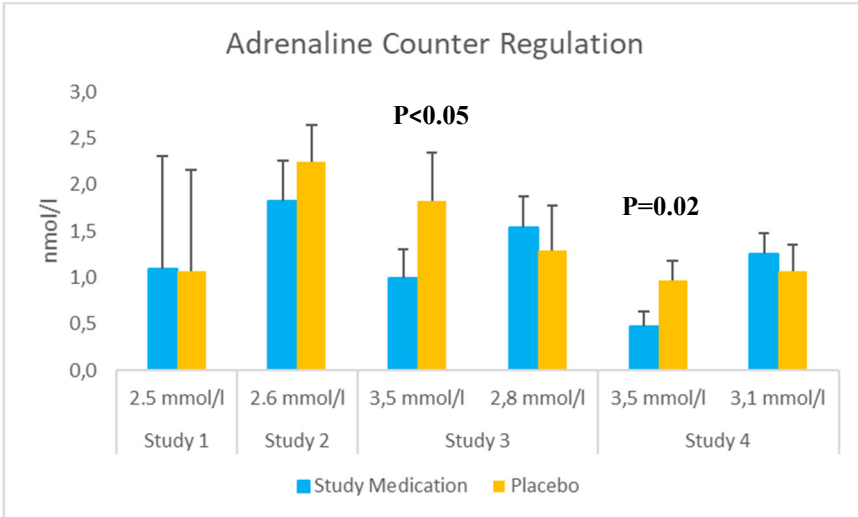
Glucagon

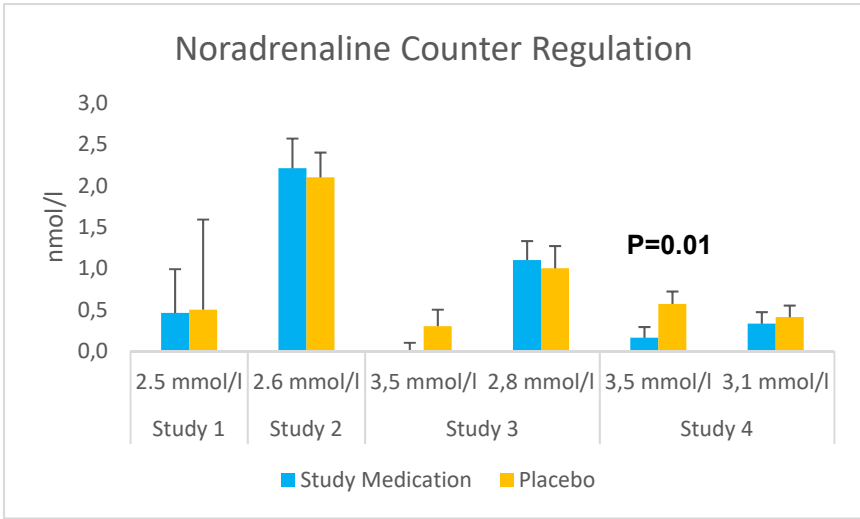
As shown in Fig. 6, in study 1 and 2, when the one-step hypoglycemic technique was performed, the glucose was clamped at 2.5 mmol/l and 2.6 mmol/l respectively, glucagon levels increased without significant differences between the treatment groups within each study. In study 3 and 4, the two-step hypoglycemic technique was performed. Glucose was first clamped at 3.5 mmol/l and then at 2.8 and 3.1 mmol/l, respectively. During the first clamp step, in study 3, the increase of glucagon was not different between PBO and active study medication, but the glucagon level was lower ($P=0.045$ in the end of the first clamp step) in the active study medication treated group. Correspondingly, in study 4, both the increase and the level of glucagon in the end of the first clamp step were less during sitagliptin than during PBO ($P\leq0.02$). However, during the second clamp step, glucagon increased more in the active study medication treated groups which resulted in no significant differences of the glucagon levels compare with that of PBO in the end of the clamp period in both two studies.

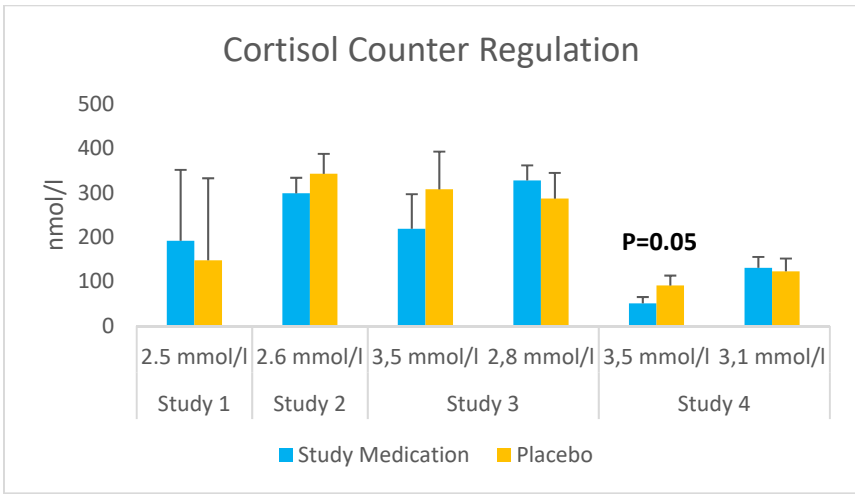
Adrenaline, noradrenaline, cortisol and PP

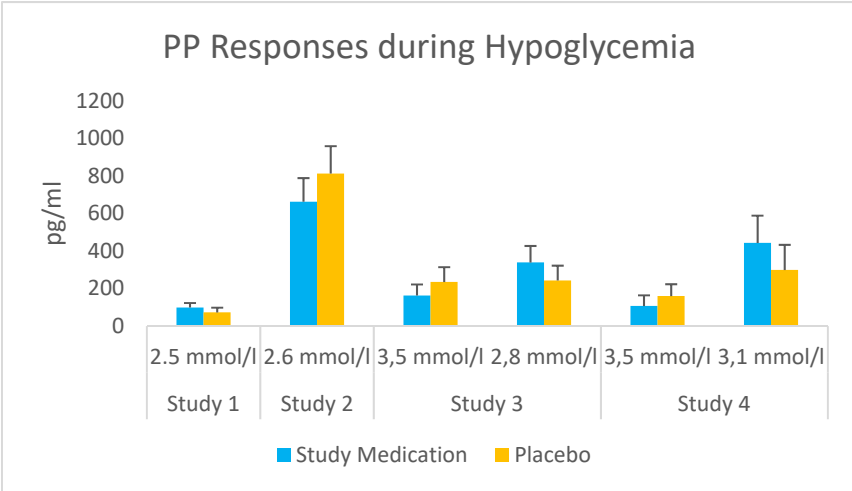
As shown in Fig. 6, in study 1 and 2, during the clamp test, the counter regulatory increases in adrenaline, noradrenaline, cortisol and PP did not differ significantly between the treatments within each study. In study 3, when the two-step hypoglycemic technique was performed, and glucose was first clamped at 3.5 mmol/l, the increase of adrenaline was impaired during incretin therapy compared to PBO. Also, in study 4, at this first glucose clamp level, the increases of adrenaline, adrenaline and cortisol were impaired by incretin therapy. However, in both study 3 and 4, when the glucose was lowered further and clamped at 2.8 and 3.1 mmol/l, respectively, all these counter-regulatory hormones increased with no significant differences and reached same levels in the end of the clamp period.


Glucagon Counter Regulation
pmol/l
20,0
15,0
10,0
5,0
0,0
P=0.04
P=0.02
2.5 mmol/l
2.6 mmol/l
3,5 mmol/l
2,8 mmol/l
3,5 mmol/l
3,1 mmol/l
Study 1
Study 2
Study 3
Study 4
Study Medication
Placebo


Adrenaline Counter Regulation
nmol/l
3,0
2,5
2,0
1,5
1,0
0,5
0,0
P<0.05
P=0.02
2.5 mmol/l
2.6 mmol/l
3,5 mmol/l
2,8 mmol/l
3,5 mmol/l
3,1 mmol/l
Study 1
Study 2
Study 3
Study 4
Study Medication
Placebo


Noradrenaline Counter Regulation
nmol/l
3,0
2,5
2,0
1,5
1,0
0,5
0,0
P=0.01
2.5 mmol/l
2.6 mmol/l
3,5 mmol/l
2,8 mmol/l
3,5 mmol/l
3,1 mmol/l
Study 1
Study 2
Study 3
Study 4
Study Medication
Placebo


Cortisol Counter Regulation
nmol/l
500
400
300
200
100
0
P=0.05
2.5 mmol/l
2.6 mmol/l
3,5 mmol/l
2,8 mmol/l
3,5 mmol/l
3,1 mmol/l
Study 1
Study 2
Study 3
Study 4
Study Medication
Placebo



Figurer 6:
Glucagon and other counter-regulatory hormonal responses during the hypoglycemic clamp

GIP and GLP-1

In study 1, intact GLP-1 was measured, in study 2 intact GIP and GLP-1 were measured and in study 4 both the total and intact GIP and GLP-1 were measured. All the measures of intact GIP and GLP-1 were significantly elevated during the hypoglycemic clamp period after the active study medication compared to PBO ($P \leq 0.03$). In contrast, total GIP and GLP-1 measured in study 4 during hypoglycemia were significantly lower during incretin therapy ($P \leq 0.03$).

Glucose, glucagon and incretin hormones after lunch

Following lunch ingestion after the hypoglycemic clamp procedure, glucose levels were analyzed in study 1, 2 and 4 and were significantly lower ($P<0.05$ in study 1 and 4) or with a trend to be lower ($P=0.12$ in study 2) after the active study medication. At the same time, the increases of glucagon, which was measured in study 2, 3 and 4, were also significantly lower during incretin therapy ($P<0.04$).

During the period after lunch, intact GIP and GLP-1 were measured in study 2 and 4 and they were significantly higher after the active study medication ($P \leq 0.02$). However, total GIP and GLP-1, which were analysed in study 4, displayed lower AUC after incretin therapy ($P \leq 0.01$).

HbA1c, body weight, BMI and background medication

HbA1c

Although this study was not designed to assess the clinical efficacy, after the treatment period with the active study medication, mean HbA1c was reduced by incretin therapy compared with PBO treatment by 3.4±1.0 mmol/mol, P=0.002; 3.6±1.0 mmol/mol, P<0.001; 4.8±1.0 mmol/mol, P = 0.042; 2.1±0.6 mmol/mol, P=0.001 respectively in studies 1-4.

Body weight and BMI

The body weight was reduced (-1.1±0.4 kg, P = 0.04) after lixisenatide treatment compared with PBO treatment in study 3, but there was no change in BMI when it was calculated in study 4 after sitagliptin treatment.

Background medication

There were no changes in background oral agents during the study period in any of the studies. In study 1 to 3, the patients had insulin as background treatment and after the treatment by active study medication, the dose of insulin had a non-significant trend of reduction in study 2 and a significant reduction in study 3 (-2.6±3.9 Units, P=0.02), but it was not changed in study 1, i.e., in the study population with T1DM.

Adverse events

Generally, the active study medications were well tolerated. A total of 34 AEs were reported in study 1 during both treatment periods (53% of AEs during active study medication treatment period), the correspondent data in study 2 to 4 were 37 AEs (57%), 29 AEs (56%) and 23 AEs (35%). The only AE that was reported by more than three patients during both treatment periods in each study was the common cold in study 1,2 and 4. In study 3 with lixisenatide as active study medication, nausea was reported in seven patients during lixisenatide treatment and in two patients during PBO treatment. Nausea was the most common AE for lixisenatide as was expected from this class of drugs[72]. The overall AE profile during treatment with the active study medication was otherwise like that during the PBO in all the 4 studies including mild hypoglycemia. No severe hypoglycemia (i.e. needing third party assistance) or other serious AEs, including pancreatitis or pancreatic cancer, were reported in the studies, except the hospitalization of one patient because of stroke during PBO treatment in study 2, one patient because of TIA during washout period (PBO/sitagliptin sequence) in study 4. Please see each respective article for more details.

Discussion

Main novelty

The studies show that at 3.1 mmol/l glucose level and below, the glucagon counter response to hypoglycemia is sustained during incretin therapy and also other counter-regulatory hormones respond as PBO treated patients in all study populations. Furthermore, the glucose threshold for glucagon counter-regulation is between 3.5 and 3.1 mmol/l during the incretin therapy. This sustained glucagon counter-regulation to hypoglycemia may contribute to the low risk of hypoglycemia during incretin-based therapy even in susceptible and fragile patient groups.

Glucagon counter-regulation

The main finding of the studies is that for insulin treated patients with either T1DM or T2DM, after adding on DPP-4 inhibitor vildagliptin, the glucagon counter-regulation to hypoglycemia was sustained at glucose level of ~2.5 mmol/l (studies 1 and 2). Furthermore, for metformin treated elderly patients with T2DM, after adding the DPP-4 inhibitor sitagliptin, glucagon was sustained even on higher glucose level, i.e., 3.1 mmol/l (study 4) which is similar to another study for T1DM patients[73]. After adding the GLP-1RA lixisenatide to long acting insulin for patients with T2DM (study 3), this sustainment was above 2.8 mmol/l glucose level (Fig. 6).

If the glucose-dependent mode of action of incretin-based therapy is preserved when it is used in these vulnerable patients as antihyperglycemic medication, there should be a hypoglycemic threshold during development of hypoglycemia where the glucagon reduction vanishes. We performed two-step hyperinsulinemic hypoglycemic clamp in study 3 and 4. At the higher glucose clamp level, i.e. 3.5 mmol/l, the glucagon was still lower during incretin therapy, which indicates that the responses were impaired in both studies. Thus, the results show that in the vulnerable patients, a glucose level of ~3.5 mmol/l is close to the glycemic threshold for the reduction of glucagon during incretin-based therapy considering the trend of

higher glucagon levels when glucose was clamped at 2.8 mmol/l and 3.1 mmol/l, respectively, in both studies (Fig. 6).

Our conclusion is in accordance with two recent studies where GLP-1 RAs were added to patients with T2DM or T1DM and glucagon were sustained at 3.3 and 3.5 mmol/l glucose levels, respectively[74, 75]. One observation is that one of the study population with T2DM had only metformin as background medication, i.e. without insulin[74]. Therefore, the slight variance of the thresholds shown in these different studies including our studies may depend on different study populations, study designs or active study medications.

It has been demonstrated that both diabetes with insulin deficiency and high age imply counter-regulatory abnormalities[3, 11]. For example, in healthy subjects, one earlier study showed that the glucose threshold during native GLP-1 treatment seems to be slightly higher and was between 3.7 and 4.2 mmol/l[54]. However, based on the results of these studies, the general conclusion is that the glucose threshold for glucagon counter-regulation against hypoglycemia in these vulnerable patients is between 3.1 and 3.5 mmol/l.

The reason for the sustained glucagon response to hypoglycemia during incretin therapy might be explained by the enhanced glucose sensitivity in α-cells, thereby promoting and not inhibiting the direct effect of hypoglycemia to stimulate glucagon secretion[76]. It might also be explained by the preserved autonomic activation of glucagon secretion, which is an important counter-regulatory mechanism during hypoglycemia[12, 77]. In case of DPP-4 inhibition, the concentration of GIP is increased by DPP-4 inhibition[78] and GIP is known to stimulate glucagon secretion during hypoglycemia[57]. Furthermore, considering the several fold higher glucagon responses during the development of hypoglycemia for patients with T2DM compared with them with T1DM in our studies (Fig. 6), the increased β-cell sensitivity in T2DM patients to reduce intra-islet insulin during hypoglycemia and thereby to increase the α-cell glucagon secretion may play an important role for the glucagon counter-regulation[15]. Therefore, T1DM with residual β cells may be an especially suitable patient group with incretin-based therapy as add on.

Adrenaline, noradrenaline, cortisol and PP

In the studies, we measured also adrenaline, noradrenaline, cortisol and PP responses during hypoglycemia, as these are known to increase during hypoglycemia[6, 12, 13, 77]. PP is released into the circulation in association with vagal, cholinergic activation, and the circulating levels of PP are therefore considered to reflect the degree of vagal activity[68]. We found that the responses of adrenaline (in both study 3 and 4), noradrenaline and cortisol (in study 4) were

significantly impaired during reduction of glucose to 3.5 mmol/l after lixisenatide or sitagliptin treatment as compared to PBO. In contrast, when further reducing glucose to 2.8 mmol/l (study 3) or 3.1 mmol/l (study 4), the responses of all these hormones were not impaired (Fig. 6). Overall, both studies therefore suggest a lower counter-regulatory response of these other hormones to mild hypoglycemia during incretin therapy, whereas the responses to more severe hypoglycemia, i.e. ~3.1 mmol/l, are sustained. The sustained counter-regulation of autonomic responses, i.e., adrenaline and noradrenaline, above this glucose level ensure the awareness of hypoglycemia[11].

During the progressive recruitment of the counter-regulatory hormones as the glucose levels fall, the inhibition of the islet β-cell results in disinhibition of the islet α-cell but only until the glucose level falls to ~3.6 mmol/l, thereafter insulin does not decease any more[79]. Instead at lower glucose levels, it should be the autonomic responses that mediate the glucagon counter-regulation. The implication is that the glucagon secretion seen when glucose levels fall less than 3.6 mmol/l is due to different, likely autonomic, mechanisms[12]. If so, the impaired adrenaline (study 3 and 4) and noradrenaline (study 4) as rapid-acting hormones[13] may have contributed to the impaired glucagon response on this glucose level, i.e. 3.5 mmol/l in both studies. However, in the other two recent studies, such association was not seen where glucagon responded similarly to 2.5 mmol/l glucose clamp level, even though the other hormones (adrenaline, noradrenaline and cortisol) were impaired[80, 81]. In one of the two studies[80], as the author mentioned, the reason may be because of the prior hypoglycemic clamp performed only 2 weeks before, i.e. not because of the active study medication. The relationship between glucagon and the other hormones during counter-regulation and their association with hypoglycemia needs therefore to be study further.

The clinical anti-hyperglycemic efficacy

Although this study was not designed to assess the clinical efficacy of adding incretin-based therapy to the vulnerable patient groups, it is worth noting that after only 4 weeks DPP-4 inhibition, HbA1c levels were reduced significantly (study 1, 2, 4) without increasing the rate of hypoglycemic episodes and a trend of insulin dose reduction was found in study 2, which is similar to what have been demonstrated as vildagliptin or other DPP-4 inhibitors added on insulin treatment in T2DM[24-28, 82-84]. In study 3, after 6 weeks treatment with the GLP-1 RA lixisenatide, HbA1c and body weight were reduced with a slight reduction of insulin dose as shown in other studies[70].

In studies 1, 2 and 4 with the DPP-4 inhibitors as the active study medication, breakfast and lunch were served before and after the hypoglycemic clamp test. Despite that fasting glucagon was not different compared with the PBO groups, we confirm in these studies the well-known effect that DPP-4 inhibition reduces prandial glucagon levels in patients with T1DM or T2DM, which was first demonstrated in 2004[85, 86]. The lower glucagon could reduce inappropriate hepatic glucose production which in turn reduces hyperglycemia. This reduction is most probably mediated by GLP-1[87]. It is not clear whether this is because of enhanced glucose sensitivity in the α-cells or a direct action of GLP-1 on glucagon secretion, which may be questioned because of only a very low GLP-1 receptor expression on alpha cells[88]. It may also be mediated by an indirect action through stimulation of somatostatin secretion, which inhibits glucagon secretion[89]. In case of T2DM, an indirect effect by stimulating β-cell secretion which in turn inhibits glucagon secretion through insulin or other products from the β-cells could also be the mechanism[90]. We also showed in study 4 that insulin and C-peptide levels after meal ingestion did not differ between sitagliptin and PBO, despite the difference in glycemia. The pattern of lower postprandial glucose and similar insulin and C-peptide levels suggests an enhancement of glucose stimulated insulin secretion after meal ingestion by DPP-4 inhibition, which is supported by the higher insulinogenic index after sitagliptin treatment. This confirms previous reports[85, 91, 92]. In study 3, glucagon levels were reduced immediately after the administration of GLP-1 RA lixisenatide, similarly as shown in the previous studies where glucagon secretion was inhibited by lixisenatide[93].

The incretin hormones

We also measured incretin hormone levels in study 1, 2 and 4 when DPP-4 inhibitors were used as the active study medication to further explore the mechanisms underlying this therapy. We thereby found that both intact GLP-1 (study 1, 2, 4) and intact GIP (study 2, 4) were maintained at higher levels by vildagliptin and sitagliptin both before and after meal ingestion (table 4). This confirms previous reports of increased intact incretin hormones after meal ingestion in drug-naive patients with T2DM[78] and it is important for improved hyperglycemia[85]. In our studies, intact GLP-1 and GIP were found to be higher even during the hypoglycemic clamp period which needs to be studied more as GLP-1 has been shown to suppress glucose production independent of islet hormones[94, 95].

Intact GLP-1 is extremely rapidly metabolized and inactivated by the enzyme DPP-4 even before the hormone has left the gut, raising the possibility that the actions of GLP-1 are transmitted via sensory neurons in the intestine and the liver expressing the GLP-1 receptor. Because of this, it is important to distinguish between

measurements of the intact hormone (responsible for endocrine actions) or the sum of the intact hormone and its metabolites, reflecting the total L-cell secretion and therefore also the possible neural actions[41].

The secretion of GLP-1 has been demonstrated previously to be reduced by DPP-4 inhibition, which is explained by a feedback inhibition by intact GLP-1 on GLP-1 secretion[96, 97]. We confirm this feedback in study 4 as the concentrations of total GLP-1 were lower after sitagliptin than after PBO during the clamp procedure and after the lunch meal, with a trend also after the breakfast meal. We also found that total GIP levels were lower after sitagliptin treatment than after PBO administration which would suggest that there is also a feedback inhibition by intact GIP on GIP secretion. This confirms previous reports about the effect of sitagliptin on total GIP levels after meal ingestion in healthy subjects and in patients with T2DM[91, 92]. Also, two other studies have shown a trend like this, although of lesser magnitude than feedback inhibition on GLP-1 secretion[96, 97]. These results are at variance with another previous study[80] and further studies are therefore warranted.

Strengths and limitations

One strength of our study is the similar study design across the different individual studies which allows us to compare the glucagon counter-regulation during hypoglycemic clamp in different study populations. For example, this design allowed us to demonstrate several fold higher glucagon secretions in patients with T2DM than in them with T1DM. The two-level hypoglycemic clamp in study 3 and 4 also gave suggestion of the glucose threshold for the glucagon counter-regulation which may be between 3.1 and 3.5 mmol/l. In these four studies, glucose was, however, clamped on different level in each individual study which makes it difficult to demonstrate more exactly the glucose threshold for glucagon counter-regulation and therefore more detailed studies with similar glucose clamp levels are needed.

Another strength is the crossover study design, which has several advantages over a parallel group design. First, the influence of confounding covariates is reduced because each patient serves as his or her own control. Second, optimal crossover designs are statistically efficient and so require fewer subjects than do non-crossover designs.

A limitation of the studies is that the treatment periods were only 4 or 6 weeks. The glucagon response, and other counter-regulatory hormonal responses, to hypoglycemia after longer treatment with incretin-based therapy remains therefore to be studied. Furthermore, a potential limitation of the study design with the DPP-4 inhibitors as the active study medication is the requirement of a breakfast meal

immediately before the clamp period, which, on one hand, is necessary to raise the concentrations of incretin hormones but, on the other hand, may have influenced the PPG level and the amount of insulin needed during the clamp test. Thus, during the clamp procedure at 3.5 mmol/l in study 4 there was a significantly higher plasma insulin level during PBO administration than that during sitagliptin treatment. However, it has previously been shown that differences in insulin levels during clamp studies do not affect glucagon responses to hypoglycemia, even within a 6-fold difference[71]. Furthermore, the hormonal responses to hypoglycemia were determined after 30-min persistent and stable hypoglycemia, which is different from the clinical situation when there is an actual increase in circulating glucose levels by the counter-regulation. However, the clamp technique, by creating a stable glucose level, is superior to quantify the glucagon and the other hormonal counter-regulations[67].

Clinical lessons and future studies

When blood glucose falls in a nondiabetic adult, the secretion of counter-regulatory hormones and the onset of cognitive, physiological, and symptomatic changes occur at reproducible blood glucose thresholds within a defined hierarchy (Fig. 1)[11]. Subjective recognition of the symptoms of hypoglycemia is fundamental for effective self-management and to prevent progression in severity of the hypoglycemia. If we don't consider the known influence of prior hyperglycemia on the thresholds for these activations, symptoms are most likely generated below the glucose threshold for glucagon counter-regulation which is close to 3.5 mmol/l shown in our studies. This applies also to the elderly where symptoms and cognitive dysfunction occurred almost simultaneously at ~3.0 mmol/l[11]. Our studies, thereby, provide a mechanism behind the low risk of hypoglycemia when incretin-based therapy is used in these vulnerable patients. The sustainment of glucagon counter-regulation during hypoglycemia by DPP-4 inhibition is a differentiation from the sulfonylureas, because the glucagon counter-regulation to hypoglycemia were impaired both by tolbutamide and glibenclamide[98, 99]. This would explain the increased risk for hypoglycemia in patients treated with sulfonylureas in comparison with DPP-4 inhibitors[100-102].

In our studies, different incretin-based medications were used, and the study populations are heterogenic: In study 1 with T1DM, the glucagon response during hypoglycemia was clearly less compared with other studies with T2DM (study 2, 3 and 4). This is also shown in two other studies[73, 75]. In study 4, the elderly population had clearly higher fasting glucagon. Thus, the possible different thresholds between different types of diabetes, between DPP-4 inhibitors and GLP-1 RAs as a class or as individual medication, between insulin treated young and

elderly populations need to be studied further. Study with glucose clamp level between 3.1 mmol/l and 3.5 mmol/l for vulnerable populations should be performed because the glucose threshold for glucagon counter-regulation seems located between these glucose levels and it is between these glucose levels the autonomic symptoms emerge as a warning signal to the patient and thereafter is the neuroglycopenic symptoms with the potential influence on the cognitive function[11].

To confirm the sustained glucagon counter-regulation after long period treatment with incretin therapy, CGM may be performed. Considering the anti-hyperglycemic effect and the glucose threshold for the initiation of glucagon counter-regulation between 3.1. and 3.5 mmol/l demonstrated by incretin-based therapy, the hypothesis is that the glucose variation should be less and this is confirmed in a post-hoc analysis of study 2, i.e. already after the four weeks treatment[103]. CGM can also provide information of the actual change of circulating glucose by the counter-regulatory hormones as in a clinical situation.

According the recent treatment guidelines, GLP-1RAs and SGLT-2 inhibitors are both recommended to newly diagnosed patients and can be used in combination[22]. Both are associated with low risk of hypoglycemia[20, 21]. However, inhibition of SGLT2 has been shown to stimulate EGP and the increase in EGP was accompanied with a small decrease in plasma insulin concentration and a large increase in plasma glucagon concentration[104, 105]. On the other side, it is well documented that the primary mechanism by which incretin therapy lower the plasma glucose is by suppressing glucagon secretion and inhibiting EGP[37]. Therefore, the glucagon counter-regulation during hypoglycemia needs to be studied when this more common combination is used clinically.

Summary, conclusions and future outlooks

In clinical practice, to improve hyperglycemia, incretin-based therapy has been suggested as add on to insulin in patients with T1DM by reducing glucagon[86] and is also recommended as add on to insulin, especially in long-acting insulin treated patients with T2DM for the beneficial effects on low risk of hypoglycemia and weight reduction[106]. We demonstrated these clinical effects in our studies which including elderly with metformin treatment. At the same time, our studies provide a mechanic basis behind the low risk of hypoglycemia.

In summary, below glucose level of 3.1 mmol/l, both the glucagon and other counter-regulatory hormonal responses are not impaired and above this glucose level the subjective recognition of the symptoms of hypoglycemia is possibly not impaired and this glucose level is well above the level before the cognitive function

is affected. The glucose threshold for glucagon counter-regulation against hypoglycemia is between 3.1 and 3.5 mmol/l, most likely close to 3.5 mmol/l, for vulnerable diabetic patients during incretin-based therapy. This is probably the mechanism behind the low risk of hypoglycemia during incretin-based therapy also in susceptible and fragile patient groups.

It is important to avoid hypoglycemia which is often associated with antihyperglycemic therapy. Perhaps, in future, new agents should be studied in regard to their impact on the counter-regulatory responses before they come into the clinical utility. Especially, the glucagon counter-regulation on the glucose level of 3.5 and 3.1 mmol/l need to be examined because sustained hormonal counter-regulation on these glucose levels imply the preserved warning symptoms and lowest glucose level before the cognitive impairment, respectively.

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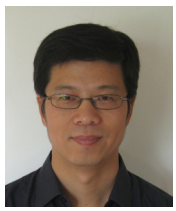
Glucagon Counter-Regulation to Hypoglycemia During Incretin-Based Therapy



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The studies were conducted from 2011 through 2019:

Study 1

J Farngren, M Persson, A Schweizer, JE Foley, B Ahrén: Vildagliptin reduces glucagon during hyperglycemia and sustains glucagon counter-regulation during hypoglycemia in type 1 diabetes. J Clin Endocrinol Metab 97:3799-3806 (2012)

Study 2

J Farngren, M Persson, A Schweizer, JE Foley, B Ahrén: Glucagon dynamics during hypoglycaemia and food-re-challenge following treatment with vildagliptin in insulin-treated patients with type 2 diabetes. Diabet Obes Metab 16:812-818 (2014)

Study 3

J Farngren, M Persson, B Ahrén: Effect of the GLP-1 receptor agonist lixisenatide on counter-regulatory responses to hypoglycemia in subjects with insulin-treated type 2 diabetes. Diabetes Care 39:242-249 (2016)

Study 4

J Farngren, M Persson, B Ahrén: Effects on the glucagon response to hypoglycaemia during DPP-4 inhibition in elderly subjects with type 2 diabetes: A randomized, placebo-controlled study. Diabet Obes Metab 20:1911-1920 (2018)

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