DISSERTATION

Glucagon responses and regulation in people with or without Type 2 Diabetes (T2DM)

Glucagonreaktionen und -regulierung bei Menschen mit oder ohne Typ-2-Diabetes (T2DM)

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List of abbreviations

Amino acid	AA
Branched-chain amino acid	BCAA
Clinical investigation day	CID
Dipeptidyl peptidase 4 inhibitor	DPP-4i
Glucose-dependent insulinotropic	GIP
Glucagon-like peptide-1	GLP-1
γ-Aminobutyric acid	GABA
Glucagon receptor	GCGR
Isomaltulose	ISO
Mixed meal test	MMT
Non-alcoholic fatty liver disease	NAFLD
Prohormone convertase 2	PC2
Saccharose/sucrose	SAC
Very low calorie diet	VLCD

Abstract

Background: Fasting hyperglucagonemia increases glucose production and induces hyperglycemia in Type 2 Diabetes (T2DM). The suppression of glucagon upon oral glucose intake is known to be impaired in people with T2DM which may be due to mitochondrial and K_{ATP} channel dysfunction. Amino acids (AA) increase glucagon secretion, which regulates AA-degradation in the urea cycle, but their contribution to postprandial hyperglucagonemia in T2DM remains unclear. Furthermore, exogenously applied GIP stimulates while GLP-1 inhibits glucagon secretion in humans. However, their roles in mixed meals are in debate.

Methods: Three clinical trials were included in the thesis. In Study 1, 13 subjects with T2DM and 15 healthy controls consumed high-protein mixed meal tests (MMT) plus 50g SAC or ISO randomly which is known to elicit opposite profiles of GIP and GLP-1 secretion. In Study 2, 9 controls and 8 patients with T2DM received different doses of 5, 10 and 30g of oral pure whey and casein which was dissolved in 250 ml water to investigate glucagon responses to different proteins as well as the differences in subjects with and without T2DM. Study 3 recruited 36 overweight /obese (BMI 27-45 kg/m²) patients with orally-treated T2DM. All patients conducted very low-calorie diet (VLCD) by shakes as replacements for 3 months to achieve 15kg weight loss. Clinical investigation days (CID) happened at baseline (V1), after 1 week (V2) and 3 months (V3) intervention along with medical and nutritional consultation. Anthropometry and MMT was performed on each CID, blood samples at different time-points were taken before and after the intervention. Incretin hormones (GIP and GLP-1), glucagon, C-peptide, and insulin were determined.

Results: In Study 1 participants with T2DM showed excessive glucagon responses within 15 min and lasting over 3h, while the controls showed small initial and delayed greater glucagon responses to MMTs. In Study 2 both whey and casein consumption increased glucagon release dose-dependently and there was no significant shift between controls and T2DM patients. The AUC glucagon to different dosage of whey and casein was moderately increased in patients with T2DM. In Study 3, the levels of glucose, insulin and glucagon decreased significantly (all $p< 0.001$) after 3-month of VLCD intervention. Meanwhile, insulin sensitivity and the other metabolic profiles improved remarkably.

Conclusions: The rapid hypersecretion of glucagon after MMTs in T2DM patients is unaffected by endogenous GIP and GLP-1 compared to controls. Protein intake contributes to postprandial hyperglucagonemia in T2DM with an increase in the absolute response. The defective suppression of glucagon by glucose combined with hypersecretion to protein is required for the exaggerated response. The reduction of glucagon might play an essential role in diabetes remission after 15kg weight loss through VLCD intervention.

Zusammenfassung

Hintergrund: Die Nüchtern-Hyperglukagonämie erhöht die Glukose-Produktion und induziert Hyperglykämie bei Diabetes Typ 2 (T2DM). Die Hemmung von Glukagon nach der oralen Glukoseaufnahme ist bei Menschen mit T2DM bekanntermaßen beeinträchtigt, was auf eine Dysfunktion von Mitochondrien und K_{ATP}-Kanälen zurückgeführt werden kann. Aminosäuren (AA) erhöhen die Glukagonsekretion, wodurch der AA-Abbau im Harnstoffzyklus reguliert wird. Dennoch bleibt ihr Beitrag zur postprandialen Hyperglukagonämie bei T2DM unklar. Darüber hinaus stimuliert exogen appliziertes GIP die Glukagonsekretion, während GLP-1 die Sekretion beim Menschen hemmt. Dennoch ist ihre Rolle bei gemischten Mahlzeiten umstritten.

Methoden: In dieser Arbeit wurden drei klinische Studien berücksichtigt.In der ersten Studie nahmen 13 Probanden mit T2DM und 15 gesunde Kontrollpersonen eine gemischte proteinreiche Testmahlzeit (MMT) plus 50 g SAC oder ISO (randomisiert) zu sich, die bekanntermaßen entgegengesetzte Profile der GIP- und GLP-1-Sekretion auslösen. In der zweiten Studie erhielten 9 Kontrollpersonen und 8 Probanden mit T2DM verschiedene orale Dosen von 5, 10 oder 30 g reiner Molke und Kasein, um die Glukagonreaktionen auf verschiedene Proteindosen und die Unterschiede zwischen Personen mit und ohne T2DM zu untersuchen. Die dritte Studie rekrutierte 36 übergewichtige/adipöse (BMI 27-45 kg/m2) Probanden mit oral behandeltem T2DM. Alle Probanden führten eine VLCD durch, indem sie 3 Monate lang Shakes als Ersatz konsumierten, um einen Gewichtsverlust von 15 kg zu erreichen. Klinische Untersuchungstage (CID) fanden bei Baseline (V1), nach 1 Woche (V2) und 3 Monaten (V3) Intervention zusammen mit medizinischer und ernährungswissenschaftlicher Beratung statt. Bei jedem CID wurden eine Anthropometrie und ein MMT durchgeführt.

Bestimmt wurden Inkretine (GIP und GLP-1), Glukagon, C-Peptid und Insulin. Glukagon, C-Peptid und Insulin wurden bestimmt.

Ergebnisse: Teilnehmer mit T2DM zeigten übermäßige Glukagon-Reaktionen innerhalb von 15 Minuten, die über 3 Stunden anhielten, während die Kontrollgruppe anfänglich kleine und verzögerte, größere Glukagon-Reaktionen auf MMTs zeigte. Molkenprotein und Casein erhöhten die Glukagon-Freisetzung dosisabhängig und es gab keinen signifikanten Unterschied zwischen den Kontrollen und T2DM. Die AUC Glukagon für Molkenprotein und Casein war bei Patienten mit T2DM mäßig erhöht. Nach 3 Monaten VLCD nahmen die Werte für Glukose, Insulin und Glukagon signifikant ab (alle $p < 0{,}001$). Gleichzeitig verbesserten sich die Insulinsensitivität sowie die anderen Stoffwechselprofile bemerkenswert.

Fazit: Die schnelle Hypersekretion von Glukagon nach gemischten Mahlzeiten bei T2DM-Patienten wird im Vergleich zu Kontrollen durch endogenes GIP und GLP-1 nicht beeinflusst. Proteinzufuhr trägt zur postprandialen Hyperglukagonämie bei T2DM bei und erhöht die absolute Reaktion. Die mangelhafte Suppression von Glukagon durch Glukose in Kombination mit einer Hypersekretion von Protein ist für die starke Reaktion erforderlich. Die Reduktion von Glukagon nach einer 15 kg-Gewichtsabnahme durch eine VLCD-Intervention könnte eine wesentliche Rolle bei der Diabetes-Remission spielen.

1 Introduction

1.1 Regulation of glucagon in type 2 diabetes (T2DM)

Glucagon is a 29-amino acid pancreatic hormone which regulates glucose metabolism to maintain glucose homeostasis during fasting by stimulation of gluconeogenesis and glycogenolysis, and additionally inhibits glycogenesis and glycolysis, as well as induces ketone production through multiple mechanisms (1-3). Fasting and postprandial hyperglucagonemia was proposed to play a critical role in hyperglycemia and to represent an initial step in the pathogenesis in type 2 diabetes (T2DM) in the early 1970s (4, 5). This was confirmed with glucagon receptor (GCGR) antagonists which effectively reduced fasting glucose but also increased hepatic transaminases and caused dyslipidemia (6-8). In recent years, hyperglucagonemia was advocated as an early driver of hyperglycemia and as an initial step in the pathogenesis of T2DM (9), and both deficient insulin secretion by β-cells as well as postprandial hyperglucagonemia are thought to contribute to hyperglycemia in patients with T2DM, although the underlying mechanisms are still unclear. By contrast, several studies confirmed that glucagon also exerts metabolically beneficial effects by stimulating insulin release, increasing energy expenditure, and reducing appetite (10-12). Glucagon has been also proved to have hypolipidemic effects, causing decreases in triglycerides and cholesterol, and increases in free fatty acid oxidation (13).

The secretion and inhibition of glucagon are regulated by multiple substances with both intrinsic and paracrine mechanisms (Fig.1). However, the mechanisms differ in healthy subjects and patients with T2DM. It's widely accepted that glucagon secretion is inhibited by oral glucose intake in healthy individuals while this suppression is impaired in patients with diabetes, which may aggravate glucose control in particular postprandial glucose (14). Glucagon suppression is affected with multiple mechanisms, such as α-cell membrane depolarization, which determines closing of Na^+ and Ca^{2+} channels (15).

1.2 Interactions between incretins (GLP-1 and GIP) and glucagon

The incretion hormones glucose-dependent insulinotropic (GIP) and glucagon-like peptide-1 (GLP-1) are best known for their potent insulinotropic effects and both incretin hormones are known to modulate glucagon secretion via α-cells (16). Evidence from previous studies demonstrated that GLP-1 is a potent inhibitor of pancreatic glucagon

secretion, while GIP increases glucagon secretion, both in a glucose-dependent manner (17, 18). In healthy subjects, GLP-1 inhibits glucagon release during hyperglycemia, while GIP acts during hypoglycemia and in the euglycemic state (17, 19-21). However, the incretin effects are impaired in T2DM. Regarding glucagon secretion via α-cells in healthy subjects, exogenous administration of GIP stimulates, while GLP-1 inhibits glucagon release during hypoglycaemia and euglycemia but ceases to have an effect in hyperglycemia. However, administration of GIP stimulates glucagon release independently of glucose levels in T2DM (19, 22). Moreover, glucagon release from α-cells was shown to stimulate insulin release by acting on glucagon and GLP-1 receptors and was required for intact insulin secretion in mice. However, other specific studies investigating the role of glucagon in β-cell function examined isolated islets from mice with genetic deletion of glucagon receptors, showing that glucose-stimulated insulin secretion was impaired (23, 24). In addition, insulin secretion was overstimulated in mice with β-cell overexpression of the glucagon receptor (25).

Our group previously reported the responses of GLP-1, GIP, insulin/C-peptide and glucagon to 50 g of SAC or ISO in patients with T2DM and controls, which also showed that basal levels of glucagon were markedly higher in T2DM patients than healthy controls (26). Meanwhile, T2DM patients showed a moderate increase of glucagon release after 50 g SAC followed by a suppression while healthy controls showed an inhibition starting after 30 min without previous increase. Meanwhile, the glucagon response to ISO was more delayed and the initial increase as well as the subsequent suppression were less pronounced (26, 27).

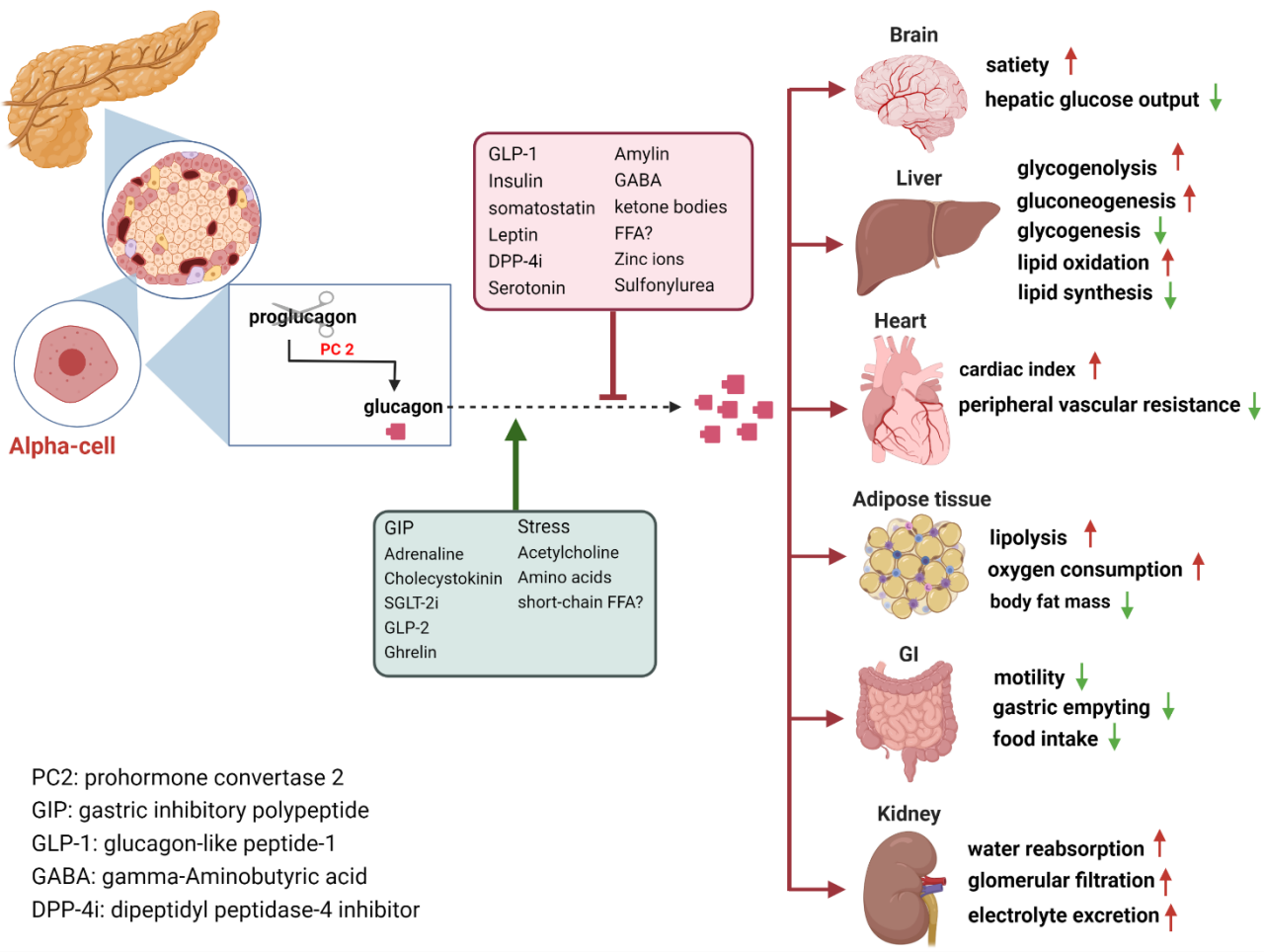


Figure 1. Glucagon receptors expressed in different sites with multiple functions and regulation of glucagon secretion. This graph was created with *biorender.com*

1.3 Effects of sugars (isomaltulose and sucrose) and protein on glucagon

Glucagon secretion is inhibited in response to oral glucose intake to lower blood glucose in healthy individuals while paradoxical hyperglucagonemia after a glucose load exacerbating hyperglycemia was reported in patients with T2DM (28-30) and patients with gestational diabetes (31). Glucose may inhibit glucagon via insulin release from β-cells by acting on neighboring α-cells or through a GABA, serotonin and somatostatin mediated intra-islet ultrashort loop feedback. Moreover, islets studies on animals suggested that hyperglycaemia dysregulates glucagon secretion by impairing mitochondrial function and thereby the function of K_{ATP}-channels in α- and β-cells (15, 32). Among different sugars, the disaccharide isomaltulose (ISO) is characterized by a slow, yet full hydrolysis in the small intestine which contributes to its low-glycemic properties (26). Saccharose (SAC) has a 1,2-glycosidic bond which is rapidly cleaved by intestinal α-glucosidase enzymes while ISO with an α-1,6 linkage is more resistant toward enzymatic breakdown. SAC and

ISO are known to be differentially stimulated by GIP and GLP-1 secretion which also effects glucagon release (27, 33).

While the suppression of glucagon after ingestion of carbohydrates has been extensively investigated, the powerful stimulation of glucagon by amino acids (AA) has received much less attention (34-38). At present it's already established that AA or proteins induced a strong stimulation of glucagon which in turn regulated AA degradation, which is well known as liver - α cell feedback (39-41). Most circulating AAs have been shown to potently stimulate both glucagon and insulin secretion in animals and humans, although not all AAs are identical in their glucagonotropic effects. As early as the 1970s, Unger et al had found that alanine infusion induced an increase in glucagon secretion accompanied with very little stimulatory effect on insulin secretion in dogs, lysine contributed to a lesser extent to α-cell secretion, while branched-chain amino acids (BCAA) had no effects on glucagon secretion but elicit a significant insulin response (36, 42-44). In the following decades, clinical studies often utilized AAs, although the effects of individual AA on glucagon secretion remain in controversy. Holst et al. suggested regarding the interactions between glucagon and AA, that defective glucagon signaling to the liver results in hyperaminoacidemia, which further stimulates the secretion of glucagon, possibly resulting in hyperplasia of α cells (45). Additionally, which AAs are capable of stimulating glucagon secretion directly from pancreatic α-cells or via increasing glucagon signaling remains mysterious.

On the other hand, Cappozi and coworkers demonstrated that glucagon-induced insulin release contributes to decrease blood glucose concentrations particularly in mixed meals through α-β cell crosstalk (46). The insulinotropic effect of glucagon and its positive effects in metabolism are now being increasingly recognized which is supported by the positive effects of glucagon agonists in combination with incretin agonism in recently designed peptide polyagonists (11, 46, 47). Remarkably, despite the stimulation of glucagon, the acute or prolonged intake of protein in combination with carbohydrates improves rather than deteriorates glucose metabolism in T2DM patients (48, 49). Prandial increases in endogenous glucagon may therefore provide metabolic benefits (50) ; therefore, we are aiming to investigate glucagon responses to mixed meals and pure protein in order to explore beneficial diets as novel therapy strategy for T2DM patients.

In the presented project, we first used the differential effects of SAC and ISO to study differing GIP or GLP-1 responses in combination with AA-induced elevated glucagon stimulation, evaluating the role of protein-induced hyper-responsiveness of glucagon in the setting of a mixed meal. In this initial trial, we assessed whether an interaction between incretin hormone secretion and glucagon levels can be seen or if there are other, possibly more important factors regulating glucagon secretion. Secondly, in order to explain the role of protein in the overshooting response to a mixed meal, we additionally assessed the acute dose-dependent glucagon responses to whey and casein. Finally, we investigated whether the decrease of glucagon drives diabetes remission in T2DM patients after weight loss by hypocaloric dietary intervention.

2 Methods

2.1 Ethics of clinical trials

This dissertation consisted of three different clinical trials (Study 1, Study 2 and Study 3) investigating different aspects of glucagon responses to sugar-augmented or non-augmented mixed meal tests (MMTs) and pure protein (whey and casein). The protocols and all amendments of those three randomized double-blind clinical trials were approved by the Ethics Committee of the Charité Universitätsmedizin Berlin and conducted in accordance with the Declaration of Helsinki, and registered at Clinical Trials.gov (No. NCT02219295, NCT04564391 and NCT05295160). It was carried out in the Department of Clinical Nutrition at the German Institute of Nutrition (DIfE) and Charité Universitätsmedizin Berlin. All the participants provided written informed consent prior to the study.

2.2 Study designs and participants

The eligibility of all potential participants was assessed by our study doctor before the beginning of the study. For this purpose, blood samples and urine samples were taken to detect clinical parameters including liver function, kidney function and so on. Furthermore, blood pressure, anthropometry as well as body composition were measured. An oral glucose tolerance test (OGTT) was also carried out in the metabolically healthy subjects to evaluate glucose metabolism. Patients with insulin therapy or other medications which might affect glucose metabolism; malignant diseases; serious cardiovascular disease, heart attack or stroke less than 6 months ago; serious diabetic complications and psychic disorders were excluded from all studies.

For all three trials, all participants were asked to pause their anti-diabetic medications shortly before the clinical investigation days. The overviews of the study designs are presented in Figure 2A-2C.

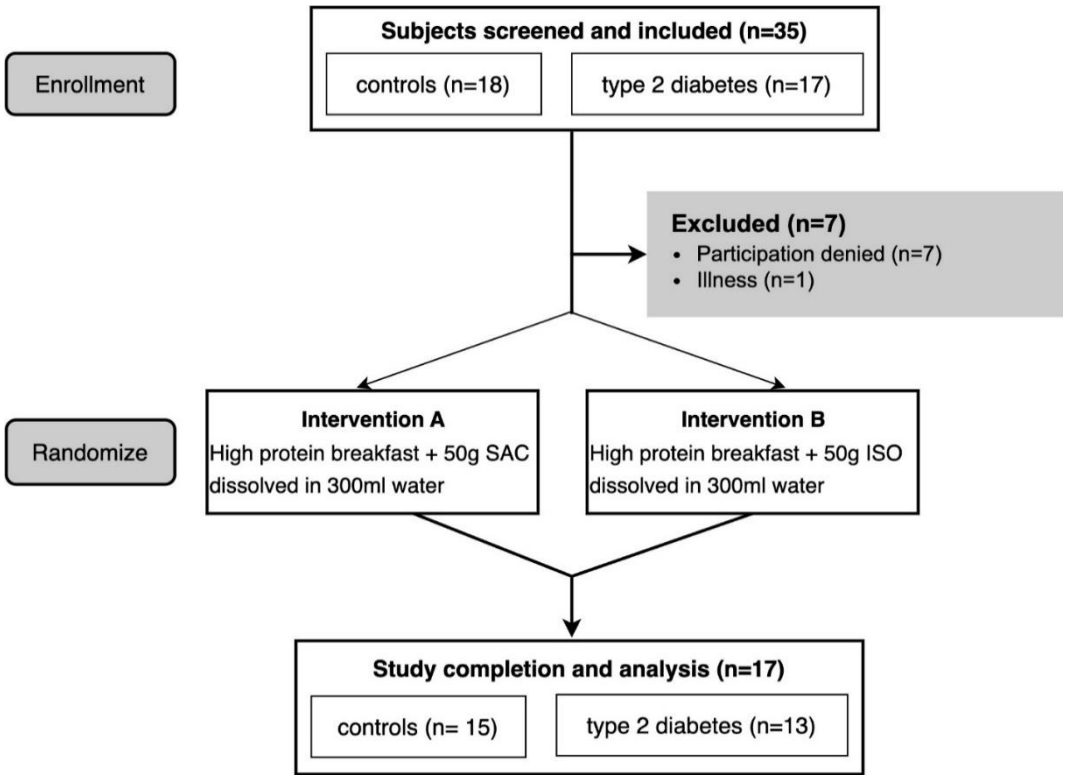
In Study 1, 15 healthy obese volunteers and 13 patients with T2DM were recruited and underwent MMTs containing 26.9 g protein from curd (20% whey, 80% casein) and an additive of either 50 g ISO (Isomaltulose/Palatinose™, BENEO, GmbH, Mannheim, Germany) or SAC (saccharose/sucrose, Südzucker, Mannheim, Germany) in the form of a

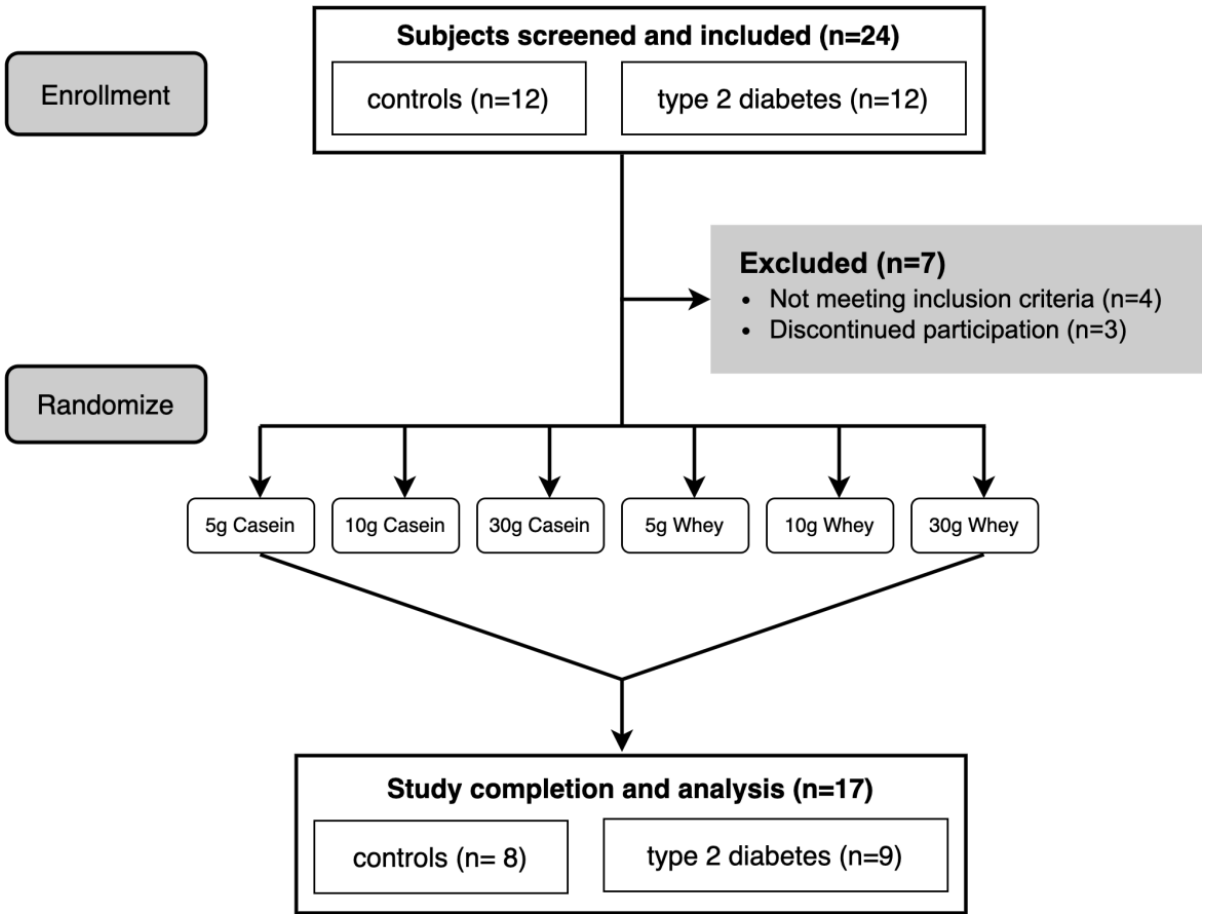
citrus drink (500 ml) in randomized order containing 466kcal total (Table 1). Both groups were examined on two non-consecutive intervention days.

In Study 2, 8 control patient with Metabolic Syndrome (MS), but normoglycemia and 9 orally-treated T2DM patients completed protein tests on six non-consecutive interventions days, with different dosages (5g, 10g, 30g) of pure whey and casein which was dissolved in 250ml water to compare glucagon responses to pure proteins.

In Study 3, 39 overweight / obese (BMI 27 - 45 kg/m²) non-insulin-dependent patients with T2DM were included. All participants agreed to conduct VLCD intervention (600 kcal/day for females and 800 kcal/day for males, respectively) to achieve 15kg weight loss by meal replacement formula diet which consisted of a portion of non-starchy vegetables and 3 or 4 formula diet shakes (OPTIFAST® or HEPAFAST®) every day. No sugar substitutes or alcohol was allowed during the intervention. Clinical investigation days were conducted at baseline (V1), after 1-week (V2) and after 12-weeks of VLCD intervention or after 15kg weight loss within 12-week (V3). For each visit anthropometry, body composition and MMTs were determined along with medical and nutritional consultation.

All consumables and technical equipments, assays and softwares which were used in 3 studies are listed in Table 2.


Enrollment
Subjects screened and included (n=35)
controls (n=18)
type 2 diabetes (n=17)
Excluded (n=7)
• Participation denied (n=7)
• Illness (n=1)
Randomize
Intervention A
High protein breakfast + 50g SAC dissolved in 300ml water
Intervention B
High protein breakfast + 50g ISO dissolved in 300ml water
Study completion and analysis (n=17)
controls (n= 15)
type 2 diabetes (n=13)


Enrollment
Subjects screened and included (n=24)
controls (n=12)
type 2 diabetes (n=12)
Excluded (n=7)
• Not meeting inclusion criteria (n=4)
• Discontinued participation (n=3)
Randomize
5g Casein
10g Casein
30g Casein
5g Whey
10g Whey
30g Whey
Study completion and analysis (n=17)
controls (n= 8)
type 2 diabetes (n=9)

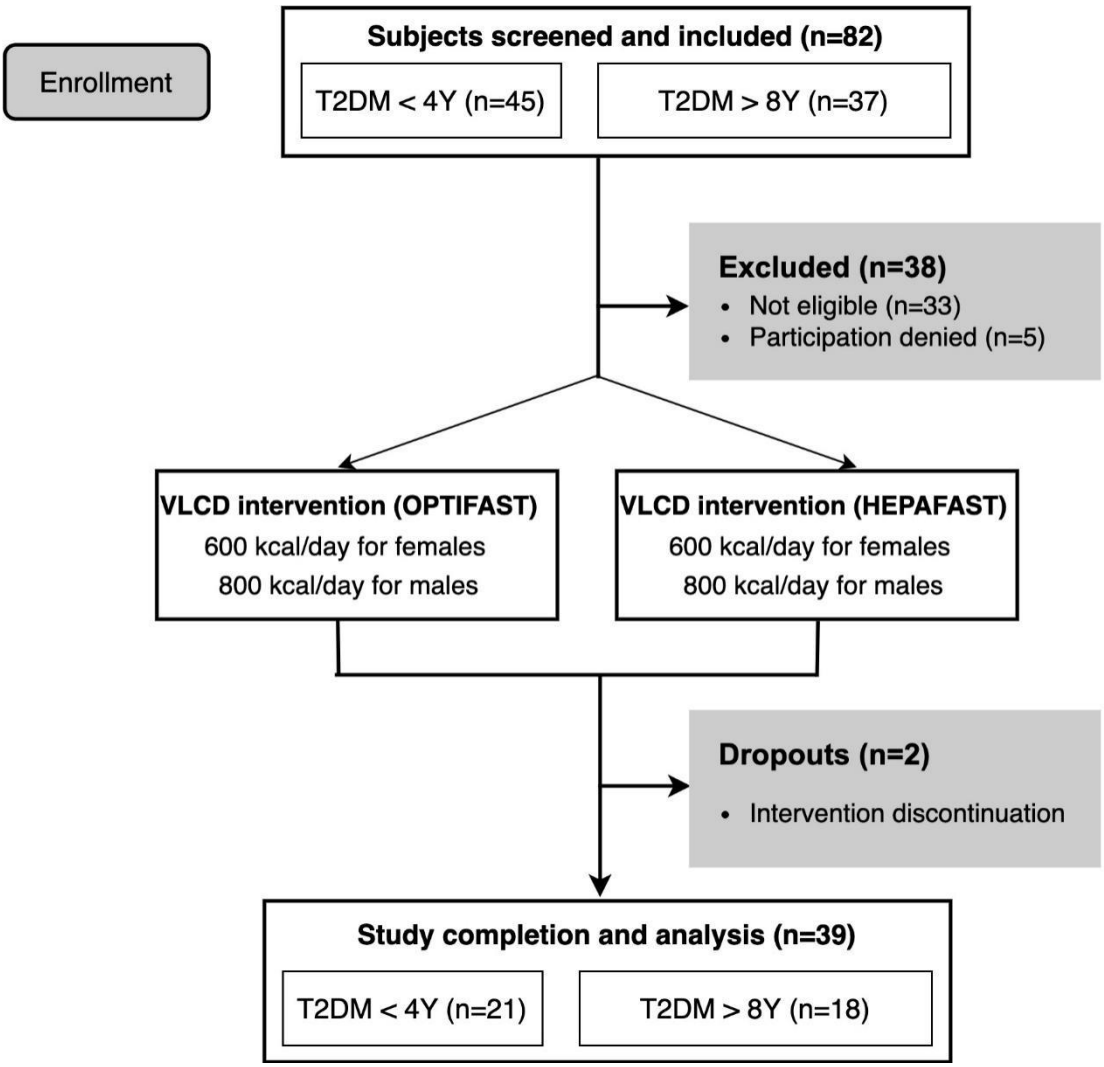


Figure 2: The overview design of Study 1 (A), Study 2 (B), and Study 3 (C)

Table 1: The composition of Mixed Meal test of Study 1

weight (g)	meal composition	energy (kcal)	carbohydrate (g)	fat (g)	protein (g)	fibre (g)
250	curd, 20%	240.0	9.0	11.0	26.3	0
25	Raspberries	11.0	1.0	0	0.3	1.2
10	coffee cream, 10% fat	12.0	0	1.0	0.3	0
300	Water	0	0	0	0.0	0
50	SAC or ISO	203.0	50.0	0	0.0	0
In total		466.0	60.0	12.0	26.9	1.2

2.3 Biomarkers measurements

On each clinical investigation day, a catheter was placed in a forearm vein wrapped in a heating pad for sampling of arterialized blood. Blood samples were taken before the start of MMTs (Study 1 and Study 3) or pure proteins (Study 2) and then 15, 30, 60, 90, 120 and 180 minutes thereafter. Immediately after the blood collection, serum samples were clotting for 10 min at room temperature while plasma samples were collected in pre-chilled EDTA or/and DPP-4 inhibitors (2.5 mM, Merck Millipore) tubes and centrifuged immediately for 10 min at 4°C. Supernatant was stored at -80°C until further analysis. Glucose concentrations and clinical routine parameters were measured in serum using ABX Pentra 400 (HORIBA). Total as well as percentage fat mass and fat free mass were determined using Air Displacement Plethysmography (BOD POD, Body Composition System; Life Measurement Instruments, Concord, CA). Plasma GLP-1 and GIP were detected by an electrochemiluminescent method (Meso Scale Discovery, Gaithersburg, MD); insulin, C-peptide and glucagon were measured by ELISA immunoassay (Mercodia, Uppsala, Sweden). The sensitivity and specificity have been reported before (51).

2.4 Calculations and Statistical analysis

The distribution of variables was evaluated by Shapiro–Wilk test. For the analysis of the difference of glucose, insulin, C-peptide, glucagon, GIP, GLP-1 between different time-points, the repeated measures ANOVA (rmANOVA) was performed using the Greenhouse-Geisser correction if sphericity was not given. Comparisons between the interventions were performed using paired t-test or Wilcoxon test depending on distribution. To compare the patient groups either or student's unpaired t-test or Mann-Whitney-U-test was used; to compare the changes before and after intervention (V1 and V3) paired-t-test or Wilcoxon signed-rank test was performed depending on the distribution of data. Areas under the curve (AUC) and incremental areas under the curve (iAUC) of glucose, insulin, C-peptide, glucagon, GIP, GLP-1 were calculated by SPSS using the trapezoid rule. Results are described as Mean ± SD in tables, statistical significance is defined as $p < 0.05$. All statistical calculations were performed using SPSS 28.0 SPSS Inc, Chicago, IL, USA). The graphs were generated by GraphPad prism 9 (California, USA) and R Studio 4.2 (Germany).

Table 2: List of consumables and technical equipment, assays and software

Consumables and appliances	Manufacturer with address
Analysis Systems / Devices	
ABX Pentra 400	Horiba Diagnostics, Montpellier, France
MESO QuickPlex SQ 120	Meso Scale Diagnostics LLC, Rockville, Maryland
Measuring instruments Anthropometry	
Anthropometer GPM Model 100	Seritex, NJ, USA
Bioimpedance Analyzer Quantum /S	Akern Srl Florence, Italy
BOD POD (Body Composition System)	Life Measurement Instruments, Concord, CA
Assays and specific chemicals	
Active GLP-1, Total Human GIP Multiplex Assay Kit	Meso Scale Diagnostics LLC, Rockville, Maryland
DPP 4-Inhibitor	Merk-Millipore, St. Charles, MO, USA
Mercodia C-Peptid ELISA Kit	Mercodia AB, Uppsala, Sweden
Mercodia Glucagon ELISA Kit	Mercodia AB, Uppsala, Sweden
Mercodia Insulin ELISA Kit	Mercodia AB, Uppsala, Sweden
Bulk Pure Whey Protein	BULK, Germany
Bulk Micellar Casein	BULK, Germany
Softwares	
Discovery WorkBench 4.0	Meso Scale Diagnostics LLC, Rockville, Maryland
PRODI® (Version 6.5)	Nutri-Science GmbH, Hausach, Germany
SPSS 28.0	IBM (Chicago, USA)
R Studio	R Studio (PBC, USA)

Results

The participant characteristics of Study 1 and Study 2, including anthropometric measures and routine blood parameters, are presented in table 3. In Study 1, 15 controls (age 62 ± 9 years) and 13 subjects with T2DM (age 65 ± 8 years) completed the study; in Study 2, 9 controls (age 67 ± 9 years) and 8 subjects with T2DM (age 55 ± 9 years) conducted all protein tests. All participants were middle age, moderate obese, and HbA1c was significantly higher in T2DM patients than controls for both studies.

Table 3: Characteristics of the participants in Study 1 and Study 2

Parameters	Study 1		Study 2	
	healthy	type 2 diabetes	healthy	type 2 diabetes
N	15	13	8	9
female: male	6/9	3/10	4/4	2/7
Age (years)	62 ± 9	65 ± 8	55 ± 9	67 ± 9
BMI (kg/m²)	31.7 ± 4.3	30.7±4.4	32.6 ± 6.3	30.7 ± 4.9
WHR	0.97± 0.12	1.03±0.16	0.96 ± 0.06	0.99 ± 0.09
ALT (U/L)	23 ± 7	31 ± 14	25 ± 9	32 ± 18
GGT (U/L)	24 ± 14	37 ± 17	50 ± 50	44 ± 39
Total cholesterol (mg/dl)	209 ± 43	205 ± 43	213 ± 45	154 ± 33
Triglycerides (mg/dl)	142 ± 62	168 ± 89	120 ± 38	159 ± 74
HDL-cholesterol (mg/dl)	50 ± 12	62 ± 8	55 ± 11.7	47.2 ± 11.9
LDL-cholesterol (mg/dl)	132 ± 35	124 ± 35	145 ± 41	86 ± 28
HbA1c (%)	5.7±0.3	6.9±0.9	5.4 ± 0.2	6.7 ± 0.7

Data are described as Mean ± SD. BMI: body mass index; WHR: waist-to-hip-ratio; ALT: alanine transaminase; HDL: high-density lipoprotein; LDL: low-density lipoprotein; GGT: gamma-glutamyl transferase; HbA1c: glycated hemoglobin A1c.

3.1 Study 1

3.1.1 Glucose, insulin and C-peptide responses to MMTs

The concentration of glucose increased much more strongly in T2DM patients compared to controls at all time-points both after SAC ($p< 0.001$) and ISO ($p < 0.001$) ingestion (Fig. 3A). AUC glucose (*180min) was remarkably higher in T2DM than controls after both SAC and ISO consumption (Fig. 3B). By contrast, there were no significant differences when comparing ISO and SAC consumption for both groups (3A).

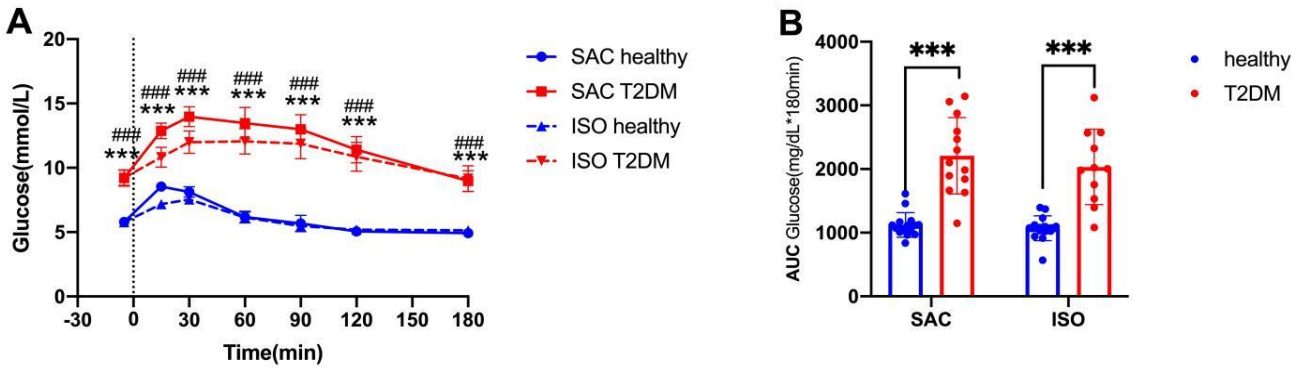


Figure 3: Glucose responses at different time-points (A) and AUC glucose (B) to the mixed meal tests (MMTs) in healthy subjects (blue) and T2DM patients (red). Values were described as Mean ± SEM, significant difference to SAC and to ISO between two groups was indicated as * and $^{\#}$, respectively. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; $^{\#}p < 0.05$, $^{\# \#}p < 0.01$, $^{\# \# \#}p < 0.001$.

3.1.2 Insulin and C-peptide responses to MMTs

Both ISO and SAC induced a significant stimulation of insulin secretion but it was prolonged by 30 and 60 minutes with ISO compared to SAC in healthy and T2DM subjects, respectively. Thus, in T2DM patients, the insulin levels were slightly delayed compared to controls ($SAC_{insulin}$ ($p = 0.006$), $ISO_{insulin}$ ($p = 0.001$)) (Fig. 4A). However, AUC insulin did not differ significantly between healthy controls and T2DM patients or between sugars ($p>0.05$) (Fig. 4B).

C-peptide curves showed an approximate direct proportionate with regard to the postprandial concentration-time course of insulin within both controls and T2DM subjects ($SAC_{C\text{-}peptide}$ ($p = 0.012$), $ISO_{C\text{-}peptide}$ ($p = 0.001$)) (Fig. 4C). Meanwhile, as we have expected, the whole responses of C-peptide to both SAC ($p=0.016$) and ISO ($p=0.027$)

consumption are significantly higher in T2DM patients compared to healthy controls, which was confirmed with calculation of AUC (*180min) C-peptide (Fig. 4D).

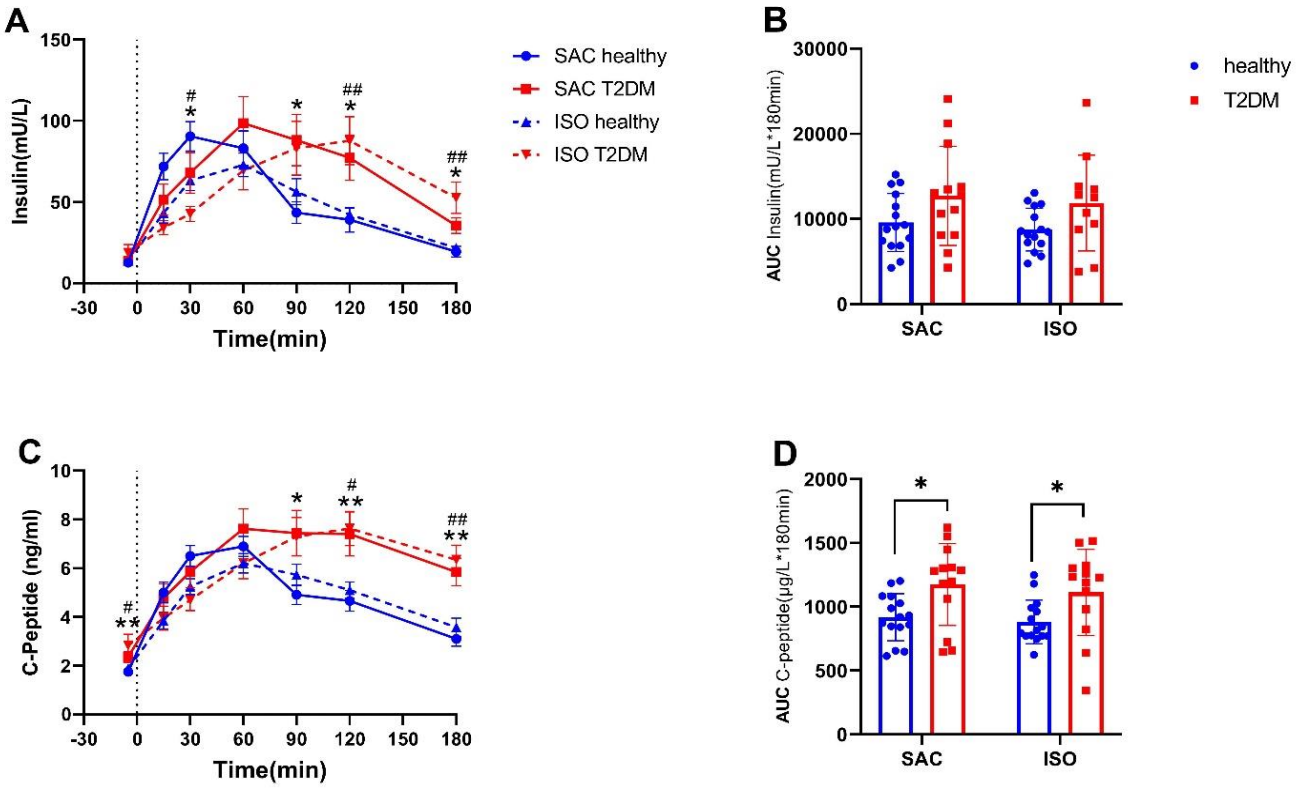


Figure 4: Insulin (A) and C-peptide (C) responses at different time-points and AUC insulin (B) and AUC C-peptide (D) to the mixed meal tests (MMTs) in healthy subjects (blue) and T2DM patients (red). Values were described as Mean ± SEM, significant difference to SAC and to ISO between two groups was indicated as * and #, respectively. *p < 0.05, **p < 0.01, ***p < 0.001; #p < 0.05, ##p < 0.01, ###p < 0.001.

3.1.3 GIP and GLP-1 responses to MMTs

There was no significant difference between the postprandial concentrations of GIP (SAC p = 0.692 and ISO p = 0.693) or GLP-1 (SAC p = 0.315 and ISO p = 0.592) between controls and patients with T2DM after administration of both disaccharides (Fig.5A-5C). Results for the comparison of disaccharides within each group are shown in supplemental materials. In healthy subjects, SAC induced a significantly higher rise in GIP than ISO at 15 min (p = 0.041), 30 min (p = 0.006) and 60 min (p = 0.004) (Fig. 5A). In T2DM patients, this was observed with significant differences also after 15min (p = 0.002), 30min (p = 0.008) and 60 min (p = 0.027) (Fig. 5A). ISO caused a greater increase in GLP-1 than SAC both in healthy subjects and in subjects with type 2 diabetes (Fig.5B and 5D). However, there was no significant difference of the overall responses (AUC) of both GIP and

GLP-1 to ISO vs. SAC within healthy controls and T2DM subjects, and between the two cohort groups (Fig. 5B and 5D).

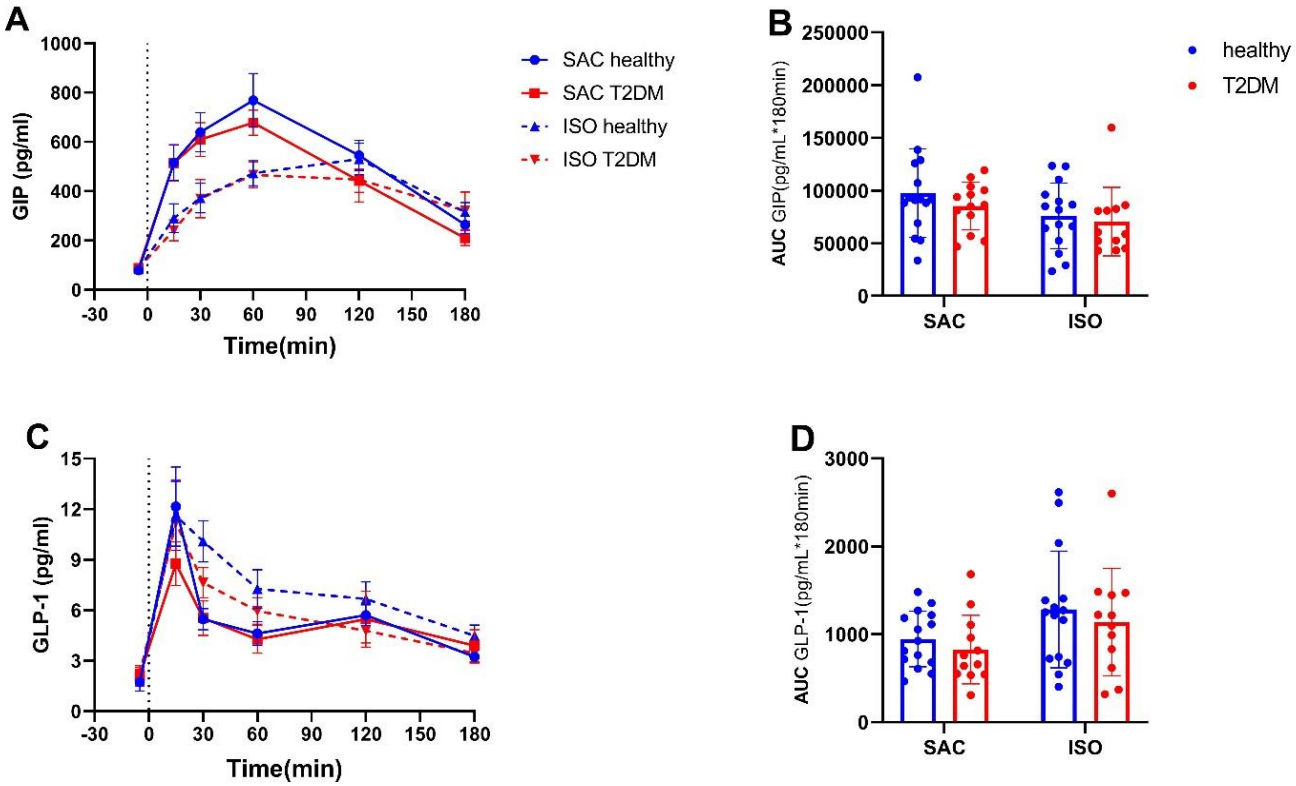


Figure 5: GIP (A) and GLP-1 (C) responses at different time-points, AUC GIP (B), AUC GLP-1 (D) to the mixed meal tests (MMTs) in healthy subjects (blue), and T2DM patients (red). Values were described as Mean ± SEM.

3.1.4 Glucagon response to MMTs

In healthy subjects, we observed a biphasic increase in glucagon secretion in the MMT with a small rise after 15 min and a larger increase starting after 60 min with a maximum after 120 min (Fig.6A). By contrast, subjects with T2DM showed a very rapid rise in glucagon to maximal levels already after 15 min and maintained these elevated levels until 120 min after the meal resulting in a significant difference between the curves over time after the MMT in subjects with T2DM compared to controls, both after the ingestion of SAC ($p<0.05$) and ISO ($p<0.05$) (Fig. 6A). However, the whole response which was evaluated by AUC (*180min) to both SAC and ISO did not show significant differences between two groups (Fig. 6B)

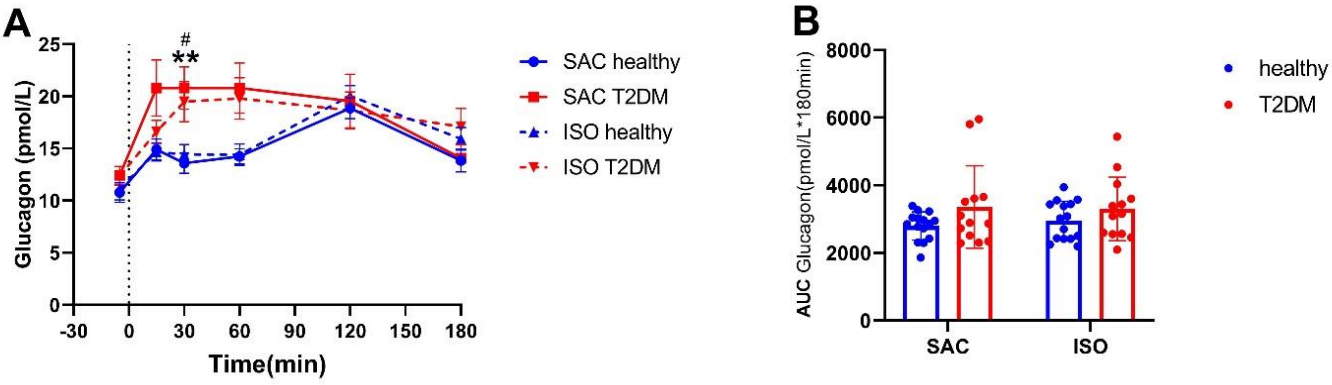


Figure 6. Glucagon responses at different time-points (A) and AUC glucagon (B) to the mixed meal tests (MMTs) in healthy subjects (blue) and T2DM patients (red). Values were described as Mean ± SEM, significant difference to SAC and to ISO between two groups was indicated as * and #, respectively; *p < 0.05; #p < 0.05.

3.2 Study 2

3.2.1 Glucose responses to 5g, 10g and 30g of casein and whey

Both casein and whey with different dosages induced remarkably higher glucose response points in subjects with T2DM compared to controls through all time due to significantly higher baseline in T2DM patients (Fig. 7A-7F), which are also confirmed with the whole response which was calculated by AUC (*180min) glucose (Fig.10A).

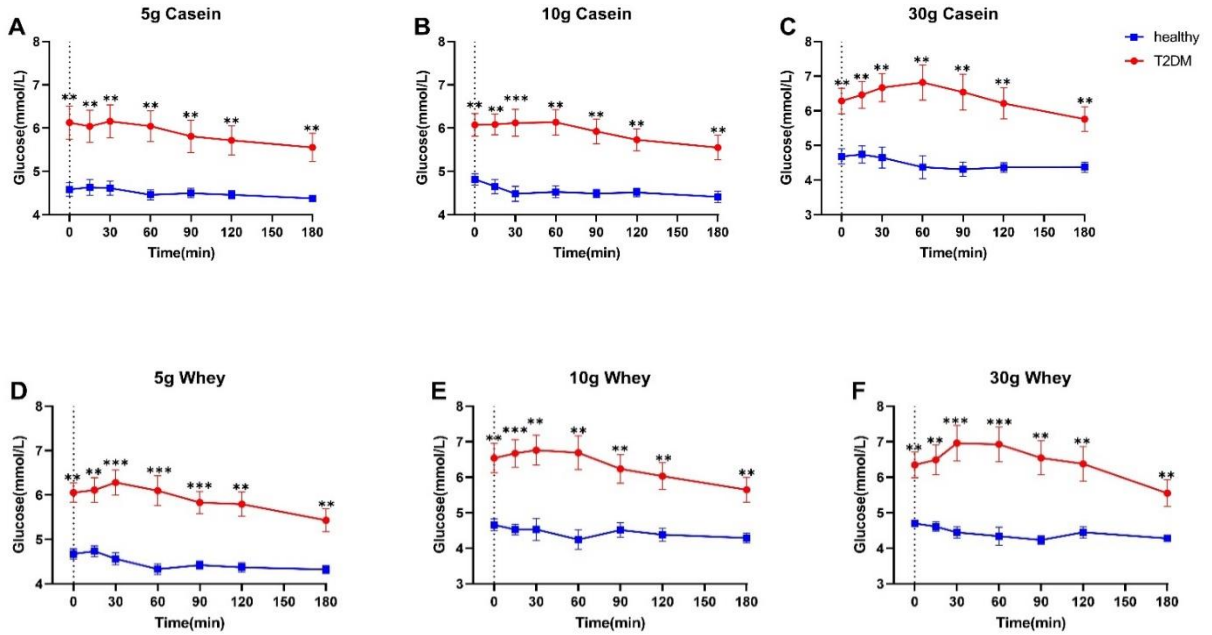


Figure 7: Glucose responses to 5, 10 and 30 g casein (A-C) or whey protein (D-F) in controls (blue) and patients with T2DM (red) in protein tests. Values are described as Mean± SEM, *p < 0.05, **p < 0.01, ***p < 0.001.

3.2.2 Insulin responses to 5g, 10g, 30g of casein and whey

Fasting insulin did not show differences between two groups. For postprandial insulin concentrations, postprandial responses after different dosages of whey and casein at the various time points did not differ significantly either (Fig. 8A-8F).

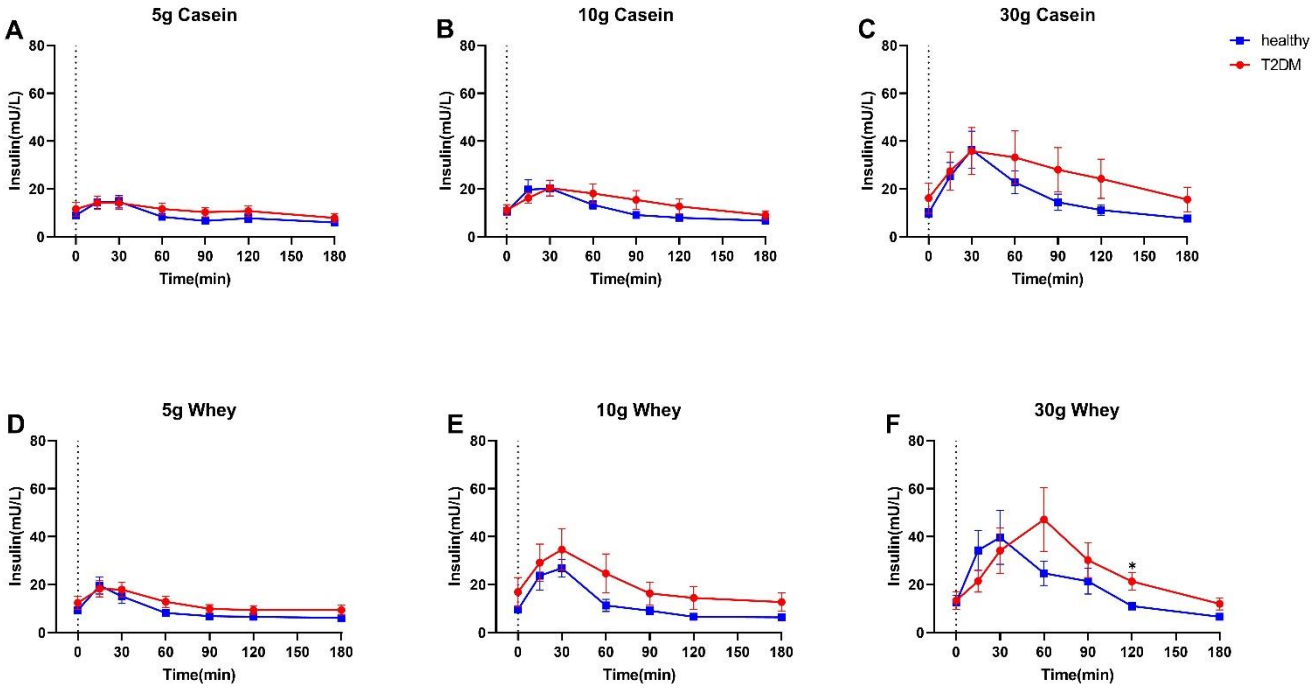


Figure 8: Insulin responses to 5, 10 and 30 g casein (A-C) or whey protein (D-F) in controls (blue) and patients with T2DM (red) in protein tests. Values are described as Mean ± SEM.

3.2.3 Glucagon responses to 5g, 10g, 30g of casein and whey

Both whey and casein with all different dosages (5g, 10g and 30g) induced hyper-secretion of glucagon in subjects with T2DM compared to healthy controls. Meanwhile, both groups showed dose-dependent increases in glucagon (Fig. 9A-9F). The whole response of glucagon to 30g casein and 10g whey calculated with AUC was significantly higher in T2DM compared to controls, but not in the other four test conditions, even though they showed the same trend (Fig. 10C).

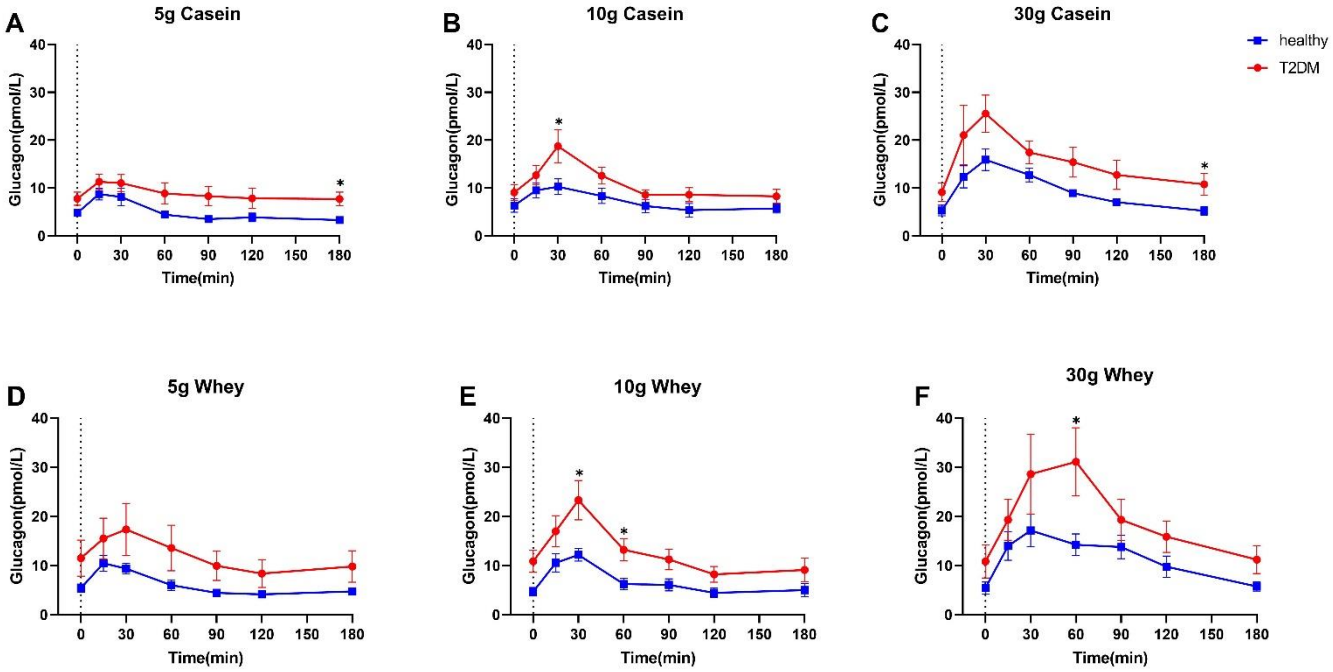


Figure 9: Glucagon responses to 5, 10 and 30 g casein (A-C) or whey protein (D-F) in controls (blue) and patients with T2DM (red) in protein tests. Values are described as Mean ± SEM, *p<0.05.

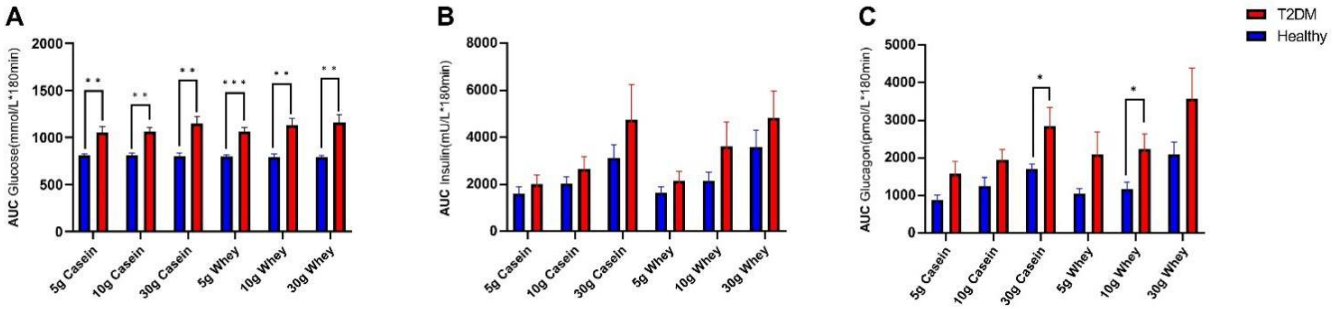


Figure 10: AUC (*180min) glucose (A), insulin (B) and glucagon (C) to 5g, 10g and 30 g whey or casein protein in controls (blue) and patients with T2DM (red) in protein tests. Values are described as Mean± SEM, *p < 0.05, **p < 0.01, ***p < 0.001.

3.3 Study 3

The characteristics and clinical parameters of participants before and after intervention in Study 3 is described in Table 4. In total 44 overweight / obese patients (age 55 ± 11 years) with T2DM were recruited and 39 patients were analyzed. After the 3-months hypocaloric dietary intervention, we observed significant improvements of all parameters related to glucose and fat metabolism (including lipid metabolism profiles and liver enzymes) as expected (Table 4). Glucose, insulin and glucagon levels decreased significantly through all time-points resulting in markedly improved fasting and postprandial insulin sensitivity

and a reversion to non-diabetic fasting glucose levels in the participants, and HbA1c also decreased significantly after 3-month intervention (Table 4). Obviously, the improvement of insulin sensitivity and the reduction of hyperglucagonemia led to a normalization of glucose levels and greatly reduced insulin requirements.

Table 4: Characteristics and parameters of participants in Study 3 at baseline (V1) and after 3-month intervention (V3)

Parameters	V1	V3	p value
N (Male: Female)	36 (17/19)		/
Age (year)	55 ± 11		/
BMI (kg/m²)	34.9 ± 4.8	29.8 ± 4.6	<0.001***
WHR	1.02 ± 0.08	0.96 ± 0.09	<0.01**
HbA1c (%)	7.0 ± 0.9	5.9 ± 0.4	<0.001***
AST (U/L)	31.1 ± 11.8	26.4 ± 9.7	<0.05*
ALT (U/L)	39 ± 17	26 ± 11	<0.001***
Total cholesterol (mg/dl)	195 ± 38	156 ± 28	<0.001***
Triglyceride (mg/dl)	189 ± 130	114 ± 62	<0.001***
HDL-cholesterol (mg/dl)	52 ± 15	45 ± 10	<0.001***
LDL-cholesterol (mg/dl)	120 ± 34	95 ± 25	<0.001***
HOMA-IR	4.7 ± 3.9	1.6 ± 0.8	<0.001***
Matsuda	3.6 ± 1.9	7.6 ± 3.8	<0.001***

Data are described as Mean ± SD. BMI: body mass index; WHR: waist-to-hip-ratio; AST: aspartate aminotransferase; ALT: alanine transaminase; HDL: high-density lipoprotein; LDL: low-density lipoprotein; HbA1c: glycated hemoglobin A1c; HOMA-IR: homeostatic model assessment for insulin resistance. *p < 0.05, **p < 0.01, ***p < 0.001.

The whole responses of glucose, insulin, glucagon which were calculated with AUC showed remarkable reductions after 3-month intervention compared with baseline (Fig. 12).

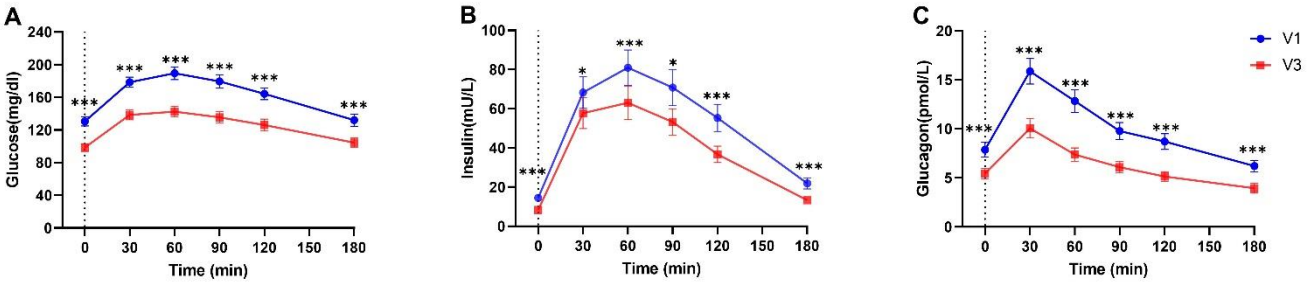


Figure 11. Glucose (A), insulin (B) and glucagon (C) responses to MMTs before (V1) and after 3-month (V3) intervention. Values are means ± SEMs, *p < 0.05, **p < 0.01, ***p < 0.001.

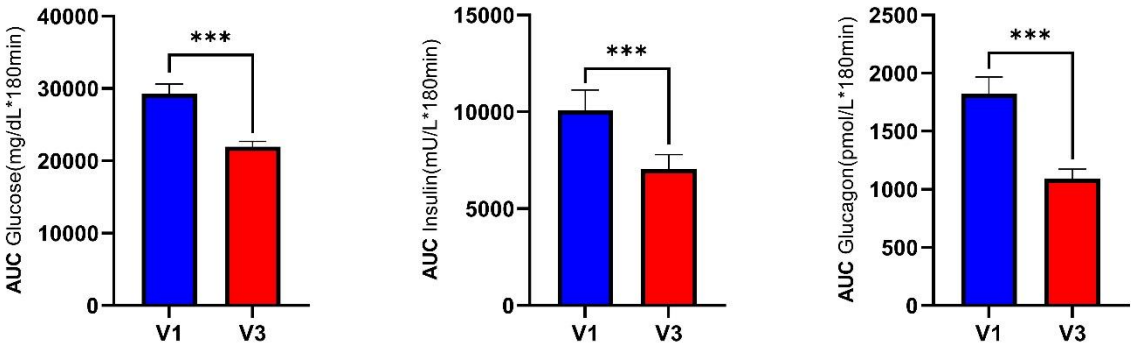


Figure 12. AUC (*180min) glucose (A), insulin (B) and glucagon (C) responses to MMTs before (V1) and after 3-month (V3) intervention. Values are described as Mean ± SEM, ***p < 0.001.

4. Discussion

4.1 Short summary of results

Whether the increased glucagon release is deleterious or beneficial is still in debate and its role in metabolism as an important diabetogenic hormone versus a protective hormone - improving β-cell function and reducing fatty liver is highly controversial. Our primary and novel finding is an accelerated and excessive glucagon secretion upon intake of high protein mixed meals in patients with T2DM compared to controls which is not evident with the single components. We compared the glucagon responses to mixed meal with different sugars (SAC or ISO) in Study 1 and pure proteins (whey and casein) with different dosage alone in Study 2, which revealed modestly impaired suppression of glucagon after SAC or ISO and modest glucagon increases in responses to the proteins. The rather excessive responses in the MMTs markedly exceed the responses to the single components and therefore indicate a potentiated response to the combined components. We further addressed the role of endogenous GIP and GLP-1 by using the 1,2- and 1,6-linked glucose-fructose dimers SAC and ISO in the mixed meals, which induce opposite profiles of the incretins but did not affect glucagon responses.

On the other hand, previous studies had shown that diabetes remission was achieved in over 80% of participants by dietary, hypocaloric intervention. Thus, in Study 3 we investigated glucagon change in patients with T2DM by 3-month VLCD to achieve 15kg weight loss and we found that diabetes remission is associated with a highly significant reduction in fasting and postprandial glucagon release which has not been addressed before.

4.2 Interpretation of results

The stimulation of glucagon release by individual AA was previously characterized in dogs and humans (36, 37, 52, 53). In study 1, we observed an excessive and more rapid secretion of glucagon to MMTs in T2DM patients, which differed markedly from that of healthy subjects. In T2DM, glucagon levels rose maximally within 15 – 30 min and showed a monophasic persistent increase compared to a much slower increase and a biphasic course in the healthy group. The overshooting and biphasic response glucagon response to MMTs might be attributable to protein-curd, which consisted of 20% of whey and 80% of casein. Whey protein is a soluble protein, which is easily digestible and

causes a rapid increase in AA levels (54), while casein is precipitated by gastric acid and resorbed more slowly which induced slowly rise of AA levels (55).The smaller first peak would be induced by the rapid uptake of whey protein combined with the suppression of glucagon release by glucose. The second one, larger increase would then be caused by the greater amount of casein. The lack of inhibition of glucagon by sugar combined with the stimulation by the protein would not quantitatively explain this response in our studies. The response may be related to the hyperglycemia-induced alteration of K_{ATP}-channel function as suggested recently (32) which would be enhanced by an interaction of protein and glucose. However, an additive dysregulation of intra-islet somatostatin, GABA or serotonergic mechanisms proposed to mediate the inhibition of glucagon in intact islets cannot be excluded (56-58).

In Study 2, glucagon responses to different dosage of pure proteins showed modestly greater increases to 10 or 30 g of whey and casein in patients with T2DM vs controls. Although responses were enhanced in T2DM patients, this was observed with 10 and 30 g of protein and the enhancement was modest and borderline significant.

Meanwhile, we investigated the potential roles of incretins for the enhanced glucagon response seen in T2DM. We combined 30g of protein with either SAC or ISO to elicit opposite incretin responses under physiological conditions. It is well established that subjects with T2DM show a diminished suppression of glucagon after an OGTT and it was suggested that GIP contributes to this phenomenon, raising the question whether this explains the exaggerated glucagon responses observed here (34, 59). By the administration of the different disaccharides, we induced varying responses of incretins to establish whether a difference in incretin levels would also cause a difference in postprandial glucagon secretion. While a significant difference in GIP and GLP-1 levels was obtained, we could not see a significant variation in glucagon levels neither in healthy nor in T2DM participants.

El and Campbell proposed that the effect of GIP on glucagon is altered in T2DM. There is no stimulation of glucagon during hyperglycemia in healthy subjects while in T2DM, infusion of GIP continued to stimulate glucagon regardless of glucose levels (19). However, this effect does not explain the meal-induced hypersecretion of glucagon since we did not observe differences between SAC vs ISO despite different incretin levels. However, the greater GIP release with SAC as compared to ISO did not result in greater

glucagon responses from our study. We propose that there is probably no dose-related glucagon response to GIP in humans. Either basal stimulation by GIP is sufficient for the glucagon response or GIP is not involved in glucagon release in healthy humans. Moreover, there is no indication for a role of GIP in the exaggerated protein response in T2DM.

In Study 3, we found that in patients with T2DM who achieved 15kg weight loss by 3-month VLCD intervention, diabetes remission was achieved. This was determined by weight loss induced improvements in insulin sensitivity, insulin secretion and a reduction of hyperglucagonemia. The reduction of glucagon levels may be sufficient to reduce insulin requirements in the presence of improved insulin sensitivity and may therefore not require changes of β-cell function. Our investigation highlights that diabetes remission by weight loss may be associated with substantial changes of glucagon secretion in individuals with elevated baseline levels.

4.3 Embedding the results into the current state of research

While glucagon has traditionally been considered an obstacle to diabetes management, and the dysregulation of glucagon signaling is a well-known factor in the development of T2DM, recent research has shown that it may also have potential therapeutic benefits. For instance, GLP-1/GIP receptor dual agonists and GLP-1/GIP/glucagon receptor triagonists have been shown to have potent glucose-lowering effects, reduce body weight, and improve cardiovascular risk factors in people with T2DM. These medications may have potential in the treatment of obesity and non-alcoholic fatty liver disease (NAFLD). However, they are still in the early stages of development and more research is needed to fully understand their safety and efficacy.

Meanwhile, there is a growing interest in the potential role of high protein diets in reducing liver fat, which is a major risk factor for the development of NAFLD. While the mechanisms underlying the effects of higher protein diets on liver fat are not fully understood, recent research suggests that glucagon may play a key role. Glucagon is established to stimulate the breakdown of liver glycogen and promotes the production of glucose by the liver. In addition, glucagon has been shown to increase the oxidation of fatty acids in the liver and promote the breakdown of triglycerides, which may contribute to the reduction of liver fat seen with high protein diets.

Our previous studies have found that high protein diet remarkably reduced liver fat content and improve liver function, glucose was improved after knockout of glucagon receptor (GCGR) in mice as well treat humans with acute and prolonged pharmacological receptor antagonisms (GRAs), although accompanied with hepatic side effects as increase in transaminases, liver fat accumulation and dyslipidemia, and the underlying mechanisms are not well understood. While it is not clear whether the effects of higher protein diets on liver fat are specifically induced by glucagon, there is evidence to suggest that glucagon may be involved. For example, a recent study found that the administration of glucagon increased the rate of fatty acid oxidation in the liver, which is consistent with the proposed mechanism of action of higher protein diets. In our study, we observed that patients showed hyper-response to protein intake compared to healthy controls. Further studies are needed to better understand the underlying mechanisms and to determine the optimal dietary strategies for the prevention and treatment of NAFLD and T2DM.

4.4 Strengths and weaknesses of the study(s)

1. Limitations of the study apply to relatively small number of participants tested and the age group. 28 subjects in Study 1 and 17 subjects in Study 2, which were divided into two groups – healthy controls and T2DM. There were methodological limits to the statistical evaluation due to relatively small numbers; therefore, the results may show weaknesses or less significant difference with regard to reliability.
2. It was not a consecutive cross-over design with the combination of Study 1 and Study 2 as one study was based on the previous one. Furthermore, due to the COVID-19 pandemic, it was almost impossible to achieve that each participant completed 9 clinical visits in total, so the results we concluded might not be robust.
3. There was only one study group with VLCD intervention in Study 3, thus there are might be some confounding factors on diabetes remission (our primary study objective) could not be concluded in the case of weight loss in only one experimental group. Meanwhile, the only comparisons could only be performed before and after intervention, not during the intervention.
4. Another limitation is the partially low heterogeneity of the study groups – control and T2DM. In order to correct for interpersonal variability, subjects with unremarkable carbohydrate metabolism were matched to the T2DM population with respect to defined parameters, including BMI. While the T2DM patients showed a very

good glycemic control and only weakly pronounced concomitant diseases, the subjects of the control already showed first signs of the development of a disturbed carbohydrate metabolism (insulin resistance). As a result, some of the effects presented may have been overestimated or underestimated.

5. We did not determine the circulating concentrations of AAs in all studies in particular Study 1 and 2, therefore, we could not investigate AA kinetics and comparisons after MMT or pure proteins. More detailed studies are indeed required in the future, for example, looking for glucagon responses to intravenous injections of alanine in humans.

4.5 Implications for practice and/or future research

First of all, our Study 1 observed a monophasic persistent increase in glucagon levels in T2DM patients compared to a slower and biphasic response in healthy individuals, and further investigation of the mechanisms underlying the exaggerated glucagon response to meals in T2DM patients is needed. Previous study proposed an additive dysregulation of intra-islet somatostatin, GABA, or serotonergic mechanisms to mediate the inhibition of glucagon in intact islets. Therefore, future research could investigate the specific role of these mechanisms in regulating glucagon release in patients with T2DM. Meanwhile, this study combined 30 g of protein with either SAC or ISO to elicit opposite incretin responses under physiological conditions and found that although there was a significant difference in GIP and GLP-1 levels, there was no significant variation in glucagon levels in either healthy or T2DM participants. Future research could further explore the relationship between incretin hormones and glucagon re-lease and their contribution to the exaggerated glucagon response seen in T2DM patients.

The Study 2 showed that T2DM patients had modestly greater increases in glucagon levels in response to 10 or 30 g of whey and casein compared to healthy individuals. However, the enhancement was modest and borderline significant. Future research could explore the impact of different proportions of whey and casein (20%: 80%; 40%: 60%; 50%: 50%) as well as other types of proteins on glucagon release in T2DM patients.

The Study 3 found that most patients with T2DM who achieved 15 kg weight loss through a 3-month VLCD intervention achieved diabetes remission, which was deter-mined by weight loss induced improvements in insulin sensitivity, insulin secretion, and a reduction

of hyperglucagonemia. Therefore, future research could further investigate the impact of weight loss on glucagon secretion and diabetes remission in patients with T2DM.

5. Conclusions

Glucagon has received much attention recently and its role in metabolism as an important diabetogenic hormone versus a protective hormone - improving β-cell function and reducing liver fat - is highly controversial. In the Study 1, we demonstrated that the rapid hypersecretion of glucagon after mixed meals in patients with T2DM compared to controls is unaffected by endogenous incretins. The defective suppression of glucagon by glucose combined with hypersecretion to protein is required for the exaggerated response.

In the second study, glucagon responses to different dosages of pure proteins showed modestly greater increases in T2DM patients compared to healthy controls, but the enhancement was modest and borderline significant. The study also investigated the potential role of incretins in the enhanced glucagon response in T2DM but did not observe significant differences in glucagon levels despite different incretin levels.

In the third study, patients with T2DM who achieved a 15 kg weight loss through a 3-month VLCD intervention showed diabetes remission and a reduction in glucagon levels. The reduction in glucagon levels may be sufficient to reduce insulin requirements with improved insulin sensitivity and may not require changes in β-cell function. The study highlights that diabetes remission by weight loss may be associated with substantial changes in glucagon secretion in individuals with elevated baseline levels. The results suggest that elevated glucagon secretion in T2DM may represent a rescue mechanism attempting to reestablish metabolic balance, and future trials may exploit the differences in the effects of different types of protein on glucagon release to assess the beneficial or deleterious effects on T2DM.

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Statutory Declaration

“I, Jiudan Zhang, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic [Glucagon responses and regulation in people with or without Type 2 Diabetes (T2DM)]; [Glucagonreaktionen und regulierung bei Menschen mit oder ohne Typ-2-Diabetes (T2DM)], independently and without the support of third parties, and that I used no other sources and aids than those stated.

All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons. All tables and graphs in the thesis as well as the publications were generated by myself independently.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; http://www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me.”

Date Signature

Declaration of your own contribution to the publications

I, Jiudan Zhang, was born in Anhui China, contributed the following publications:

Jiudan Zhang, Olga Pivovarova-Ramich, Stefan Kabisch, Mariya Markova, Silke Hornemann, Stephanie Sucher, Sascha Rohn, Jürgen Machann, Andreas F.H Pfeiffer, High Protein Diets Improve Liver Fat and Insulin Sensitivity by Prandial but Not Fasting Glucagon Secretion in Type 2 Diabetes. Front Nutr. 2022 May 19;9:808346. doi: 10.3389/fnut.2022.808346. eCollection 2022.

Database remediation:

Study data were collected by study physicians, study nurses and directly by the patients. Data were then transferred directly into the electronic database. I independently performed the structuring, expansion and error correction of these databases.

Evaluation and Analysis:

I independently performed the statistical analysis. It was performed with SPSS software packages for Windows (IBM, version 28.0, Chicago, IL, USA), R Software and R Studio (Germany). All graphs were generated by Graphpad Prism 9 (Graphpad California, USA). For this purpose, the distribution of all data was checked. Possible outliners were analyzed and, when it was necessary, excluded from the analysis. For further statistical analysis, the normal distribution and variance homogeneity of the metric data were first analyzed (Kolmogorov-Smirnov test for normality, Levene's test for variance homogeneity). For normally distributed data, comparisons within and between control group and T2DM group was analyzed with Student t-tests (Paired or Unpaired). Non-normally distributed variables were transformed to achieve normal distribution or analyzed by nonparametric tests (Wilcoxon or Mann-Whitney U test). For the analysis of the difference between time courses (0-180 min) a repeated measures ANOVA (rmANOVA) was performed using the Greenhouse-Geisser correction if sphericity was not given. A model with one between-subject factor and two within-subject factors was used. Pearson correlation or Spearman correlation analysis was used for normal distributed and non-normally distributed variables, respectively to determine the correlations between different hormone responses to MMT and pure Whey or Casein. Areas under the curve (AUC) and incremental areas under the curve (iAUC) were calculated by SPSS using the trapezoid rule. Statistical significance is defined as $p < 0.05$.

Manuscript Writing:

I independently undertook the writing of the article. Continuous further development took place through the supervision and criticism of my supervisors: Dr.Stefan Kabisch, and Prof. Andreas F.H Pfeiffer. The concept and the protocol of the study was initiated by Prof.Pfeiffer. All data from the patients were collected with Dr.Kabisch, study nurses and myself. All data was statistically evaluated and visualized (all tables and graphs) by me independently.

Data analysis and critical interpretation were written in the first draft of the manuscript by me and revised by all other authors. Up to the final version, it required repeated updates of the primary data set and renewed statistical evaluations.

[Jiudan Zhang] contributed the following to the below listed publications:

Publication 1: [Jiudan Zhang, Olga Pivovarova-Ramich, Stefan Kabisch, Mariya Markova, Silke Hornemann, Stephanie Sucher, Sascha Rohn, Jürgen Machann, Andreas F H Pfeiffer], [High Protein Diets Improve Liver Fat and Insulin Sensitivity by Prandial but Not Fasting Glucagon Secretion in Type 2 Diabetes], [Frontiers Nutrition], [2022]

Contribution: Jiudan Zhang performed the statistical analysis of this completed project, generated all the figures and tables for this publication based on my statistical evaluation independently, and wrote the manuscript.

Signature, date and stamp of first supervising university professor / lecturer

Signature of doctoral candidate

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High Protein Diets Improve Liver Fatand Insulin Sensitivity by Prandial butNot Fasting Glucagon Secretion in Type 2 Diabetes

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Glucagon (GCGN) plays a key role in glucose and amino acid (AA) metabolism by increasing hepatic glucose output. AA strongly stimulate GCGN secretion which regulates hepatic AA degradation by ureagenesis. Although increased fasting GCGN levels cause hyperglycemia GCGN has beneficial actions by stimulating hepatic lipolysis and improving insulin sensitivity through alanine induced activation of AMPK. Indeed, stimulating prandial GCGN secretion by isocaloric high protein diets (HPDs) strongly reduces intrahepatic lipids (IHLs) and improves glucose metabolism in type 2 diabetes mellitus (T2DM). Therefore, the role of GCGN and circulating AAs in metabolic improvements in 31 patients with T2DM consuming HPD was investigated. Six weeks HPD strongly coordinated GCGN and AA levels with IHL and insulin sensitivity as shown by significant correlations compared to baseline. Reduction of IHL during the intervention by 42% significantly improved insulin sensitivity [homeostatic model assessment for insulin resistance (HOMA-IR) or hyperinsulinemic euglycemic clamps] but not fasting GCGN or AA levels. By contrast, GCGN secretion in mixed meal tolerance tests (MMTTs) decreased depending on IHL reduction together with a selective reduction of GCGN-regulated alanine levels indicating greater GCGN sensitivity. HPD aligned glucose metabolism with GCGN actions. Meal stimulated, but not fasting GCGN, was related to reduced liver fat and improved insulin sensitivity. This supports the concept of GCGN-induced hepatic lipolysis and alanine- and ureagenesis-induced activation of AMPK by HPD.

Keywords: glucagon, insulin sensitivity, liver fat content, alanine, type 2 diabetes, non-alcoholic fatty liver disease (NAFLD), high protein diet

INTRODUCTION

Glucagon (GCGN) increases glucose production in the liver, stimulates insulin release from beta cells and contributes to maintaining normal levels of glucose in a close interplay with insulin in healthy subjects (1, 2). Hyperglucagonemia was proposed as an early driver of hyperglycemia and as an initial step in the pathogenesis of type 2 diabetes mellitus (T2DM) (3, 4) although the causes of hyperglucagonemia remain controversial (5). Insulin resistance of the alpha-cell was proposed to impair the inhibition of glucagon secretion by insulin and may thereby increase GCGN levels (3). Glucagon release is directly and acutely stimulated by amino acids (AA)
(6) and drives their hepatic degradation in the urea cycle (7, 8), which generates a liver-alpha-cell feedback loop. Non-alcoholic fatty liver disease (NAFLD) is a frequent consequence of obesity and associated with increased levels of AA (9) which was proposed to result from fatty liver-induced hepatic resistance to the GCGN-induced degradation of AA. The ensuing hyper-aminoacidemia may in turn stimulate GCGN-release and induce fasting and postprandial hyperglucagonemia in obesity and diabetes mellitus. The increase of fasting GCGN is thought to increase glucose production and to induce hyperinsulinemia which will further aggravate NAFLD and insulin resistance (10). The product of GCGN and alanine was recently proposed as an indicator of hepatic GCGN resistance and was associated with hepatic fat content (11). Fatty liver is closely linked to insulin resistance and increased levels of AAs, such that the overlap and interdependence of both phenomena make it difficult to separate the causes.

Although GCGN antagonists reduced blood glucose levels in T2DM patients they increased hepatic transaminases, induced fatty liver and dyslipidemia (5, 12–14). This raised awareness of the positive actions of GCGN such as the induction of lipolysis and lipid oxidation, inhibition of appetite and increase in energy expenditure (5, 15, 16). Moreover, recent work unraveled an important role of intra-islet GCGN release from alpha cells in maintaining beta cell responses (5, 17, 18). This work was backed by the development of GCGN agonists in peptide polyagonists combining GCGN, GLP-1, and/or GIP to treat T2DM (5). As AAs are potent inducers of GCGN secretion, high protein diets (HPDs) might be used to increase GCGN release and thereby profit from its benefits (16). Indeed, we recently tested HPDs without restriction of calorie intake in patients with T2DM and observed improvements of insulin sensitivity, hepatic fat content, circulating fatty acids, uric acid, and markers of inflammation and redox metabolism (19–23).

This raises the question, whether (a) fatty liver is quantitatively linked to fasting glucagon secretion and hepatic GCGN resistance in T2DM as reflected by elevated fasting AAand the GCGN–alanine index and (b), whether a reduction of liver fat would improve the hepatic GCGN resistance in people with T2DM as might be expected if NAFLDis a primary cause of hyperglucagonemia. As NAFLD is also closely linked to insulin resistance, the reduction of liver fat should improve alpha-cell insulin sensitivity and may thereby reduce fasting and postprandial GCGN release. Because alpha-cell-GCGN-stimulated insulin secretion is largely mediated by GLP-1 receptors, GCGN-resistance might not alter the response to protein- and AA intake-induced insulin secretion in mixed meal tolerance tests (MMTTs).

A second aspect arises from potential beneficial effectsof GCGN in obesity and T2DM: GCGN specifically drives intrahepatic lipolysis and lipid oxidation through a recently discovered inositol trisphosphate-receptor-1 (INSP3-R1) dependent signal pathway and thereby is a powerful stimulus to reduce liver fat (24). Preclinical studies moreover suggest a centrally mediated inhibition of hepatic lipogenesis by GCGN (16). Indeed, isocaloric HPDs which strongly stimulate GCGN release, have been used to reduce liver fat in patients with T2DM by over 40% which most likely was mediated by the increase in GCGN-induced hepatic lipolysis (19, 20). This raises the question whether GCGN resistance of the liver would impair the action of GCGN and thereby serve as a marker of the prospective effectiveness of HPD for the reduction of liver fat in people with NASH/NAFLD.

This analysis was performed to assess the interplay of intrahepatic lipids (IHLs) with plasma levels of GCGN and hepatic GCGN-resistance in study participants with T2DM before and after extensive loss of liver fat achieved by the intake of HPDs (30%E of protein) for 6 weeks. We assessed whether thereis (a) a correlation of IHL with insulin sensitivity and GCGN resistance determined by the GCGN–alanine index at baseline and after the intervention, (b) whether an extensive reduction of IHL by isocaloric HPD affects insulin or GCGN sensitivity, (c) whether GCGN sensitivity at baseline determines the effect of theHPD on loss of IHL, and whether (d) GCGN sensitivity affects the secretion of insulin induced by a mixed meal, i.e., whether the ultra-short loop feedback between alpha- and beta-cells changes.

MATERIALS AND METHODS

The analysis is based on the "LeguAN" intervention trialin subjects (18–80 years) with T2DM, which was registeredat ClinicalTrials.gov (NCT02402985). Participants with orally treated T2DM, matched for age, sex, body mass index (BMI), glycated hemoglobin A1c (HbA1c), and anti-diabetic medications, were randomized using computer algorithm to 6 weeks of isocaloric diets which contained 30% of energyintake (%E) as protein, 40%E as carbohydrates, and 30%E asfat (20). All participants received individually adapted dietary instructions and meal plans by an experienced dietician and Master in Nutrition (SS) and were partially supplied with foods during the 6 weeks. The overall composition of SAFA (10%E), MUFA (10%E), and PUFA (10%E) was kept similar as much aspossible and dietary intake was calculated with the computer program PRODI as described in detail in the supplements ofrefs (19, 20). The study participants completed MMTTs before and at the end of the study which consisted of breakfast (MMTT1) and lunch (MMTT2) with detailed profiles of insulin,GCGN, glucose, and AA over 360 min. The original study compared plant vs. animal protein rich diets which showed similar improvements of IHL, insulin sensitivity, fasting glucose,

HbA1c, visceral adipose tissue (VAT), inflammatory, liver, and redox markers ref (19–23). The groups were therefore combined in the current analysis. The separation into two groups with changes of liver fat above vs. below the median comprised animal/plant protein of 7/8 in the higher and 9/7 in the lower liver fat change groups. Changes of protein intakes, blood urea nitrogen (BUN) and urinary nitrogen excretion relative to changes in IHL, GCGN, and homeostatic model assessment for insulin resistance (HOMA-IR) are shown in **Supplementary Figures 2–4**. The free fatty acid (FFA) in serum showed a decrease of all saturated fatty acids (C14–C22), no change of linoleic acid and a small increase of alpha-linoleic acid as reported previously (20). All subjects signed informed consent prior to participation. A total of 31 subjects were included who performed proton magnetic resonance spectroscopy (^{1}H-MRS) of the liver and MRI for VAT on a 1.5 T whole body imager (Magnetom Avanto, Siemens Healthcare, Erlangen, Germany) at baseline and after 6-weeks of high-protein dietary intervention (19–21). Body composition (fat mass and lean mass) was determinedby Air Displacement Plethysmography (BOD POD, COSMED, Italy). Routine parameters were measured in serum using ABX Pentra 400 (Horiba, Japan). Insulin and glucagon in serum samples were measures by ELISA (Mercodia, Sweden). Plasma AA levels were determined by liquid chromatography tandem mass spectrometry analysis.

Calculations

Index of whole-body insulin resistance (HOMA-IR) was calculated as: fasting insulin (mU/L)× fasting glucose in (mmol/L)/22.5 (25). Matsuda index was calculated according to Matsuda and DeFronzo (26).

The GCGN–alanine index and the GCGN–AA-index were calculated as fasting glucagon × fasting alanine or other AA, respectively, according to the previous publication (12). The glucose disposal rate (*M*-value) was calculated from the infusion rate of exogenous glucose during steady state of the hyperinsulinemic euglycemic clamp (HEC) as previously described.

Statistical Analysis

For statistical analysis, all variables are described as mean ± SD. Normal distribution was evaluated by Shapiro–Wilk-test. According to the normal or non-normal distribution, statistical comparison of variables at baseline and after 6-weeks high protein intervention between two groups was performed by independent *t*-test or Mann–Whitney *U*-test; Paired *t*-testor Wilcoxon signed rank test was used within groups. The repeated measures ANOVA was used to analyze differences at different time-points.

For correlation analysis, non-normally distributed data (GCGN–AA index, IHL, and HOMA-IR) were logarithmically transformed to approximate a linear distribution. Spearman'snon-parametric rank or Pearson correlations were conducted depending on the normality of data distribution. Areas under the curve (AUC) and incremental areas under the curve (iAUC) were calculated by GraphPad prism 8 (CA, United States) using the trapezoid rule.

A p-value < 0.05 was considered statistically significant. All statistical calculations were performed using SPSS 26.0 (IBM, United States). All graphs were generated by GraphPad prism 8 (CA, United States).

RESULTS

We studied 31 study participants with orally treated T2DM whose characteristics are shown in **Table 1**. The intrahepatic lipidcontent (IHL) was 15.4 9.8% determined by ^{1}H-MRS and correlated highly with insulin sensitivity measured as HOMA-IR($\rho = 0.554$, $p = 0.001$) (**Figure 1A**) and with fasting GCGN levels ($\rho = 0.454$, $p = 0.012$) (**Figure 1B**). VAT, determined by MRI,

TABLE 1 | Parameters at baseline (Week 0) and after the HPD intervention of all study participants (Week 6).

Parameter (n = 31)	Week 0	Week 6	p-Value
Age (years)	64.6 ± 6.0		
Gender (male/female)	19 m/12 f		
Liver fat content (MR-S; %)	15.4 ± 9.8	8.8 ± 8.1	<0.001***
Body weight (kg)	89.4 ± 14.2	87.4 ± 14.0	<0.001***
BMI (kg/m^2)	30.6 ± 3.7	29.9 ± 3.5	<0.001***
Waist circumference (cm)	102.9 ± 10.9	100.6 ± 10.7	<0.01**
Fasting glucose (mmol/L)	9.6 ± 1.5	8.8 ± 1.5	<0.001***
Fasting insulin (mU/L)	8.4 ± 4.7	7.9 ± 5.4	0.16
Fasting glucagon (pmol/L)	8.2 ± 3.5	8.4 ± 3.7	0.63
Fasting C-P (µg/L)	1.9 ± 0.8	1.9 ± 0.9	0.40
Insulin/glucagon ratio	1.1 ± 0.72	0.89 ± 0.42	0.056
C-P/glucagon ratio	0.27 ± 0.17	0.23 ± 0.08	0.23
iAUC glucagon (pmol/L)	992.1 ± 577.4	829.3 ± 502.3	0.313
HbA1c	6.8 ± 0.70	6.4 ± 0.69	<0.001***
HOMA-IR	3.5 ± 1.9	3.1 ± 2.0	<0.05*
Matsuda index	4.5 ± 3.1	5.0 ± 2.9	<0.05*
M-value	4.9 ± 2.1	5.5 ± 1.9	<0.01**
AST (U/L)	25.2 ± 8.7	21.8 ± 6.1	<0.01**
ALT (U/L)	28.2 ± 9.9	26.5 ± 8.4	0.13
AST/ALT ratio	0.87 ± 0.21	0.84 ± 0.19	0.54
GGT (U/L)	44.1 ± 26.2	30.8 ± 15.9	<0.001***
TG (mmol/L)	1.7 ± 0.59	1.6 ± 0.66	0.22
TC (mmol/L)	5.3 ± 0.97	4.62 ± 0.95	<0.01**
LDL-C (mmol/L)	3.4 ± 0.89	2.9 ± 0.85	<0.01**
HDL-C (mmol/L)	1.1 ± 0.26	0.96 ± 0.17	<0.01**
CREA (µmol/L)	81.3 ± 16.2	77.5 ± 16.7	<0.05*
BUN (mmol/L)	6.0 ± 0.95	7.8 ± 1.8	<0.001***
eGFR (mL/min/1.73 m^2)	78.6 ± 15.2	82.6 ± 15.2	<0.05*
Urine urea (mmol/24 h)	403.0 ± 134.2	564.0 ± 200.2	<0.001***
VAT (L)	6.0 ± 2.1	5.8 ± 1.9	<0.01**
Fat mass (%)	35.8 ± 7.3	33.9 ± 7.0	<0.05*
Lean mass (%)	64.0 ± 7.3	66.2 ± 7.0	<0.05*

*BMI, body mass index; C-P, C-peptide; iAUC, incremental area under curve; HbA1c, glycated hemoglobin A1c; HOMA-IR, homeostatic model assessment for insulin resistance; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transferase; TG, triglycerides; TC, total cholesterol; CREA, creatinine; BUN, blood urea nitrogen; eGFR, estimated glomerular filtration rate; VAT, visceral adipose tissue. *p < 0.05; **p < 0.01; ***p < 0.001.*

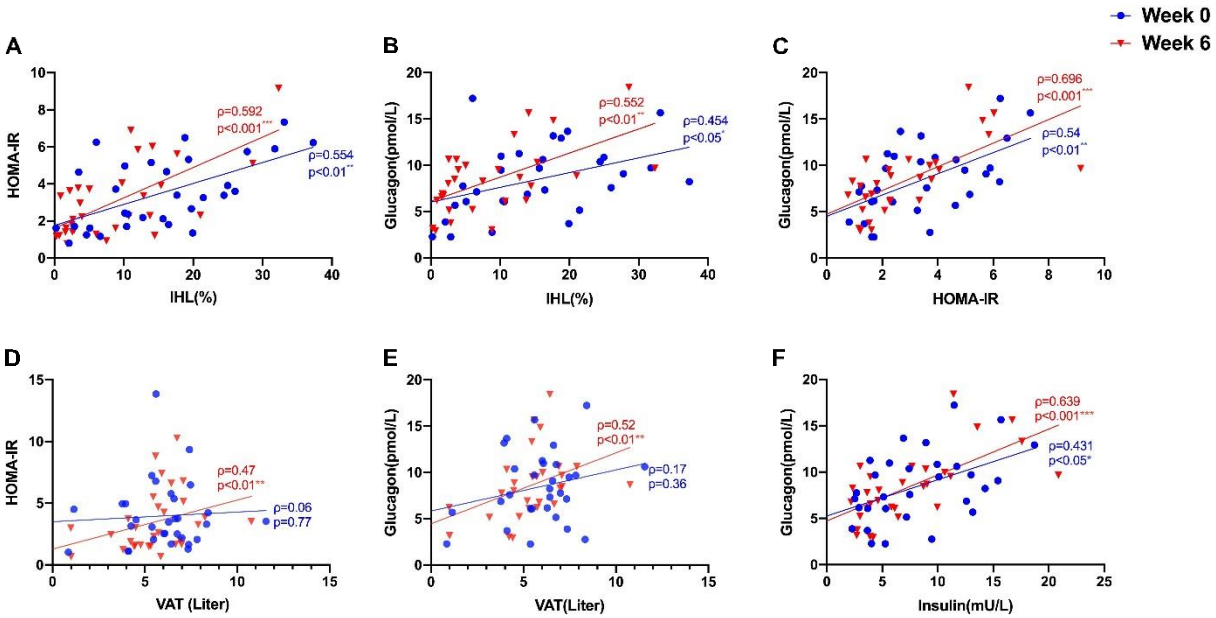

FIGURE 1 | Correlations between (A) IHL (%) and insulin sensitivity (HOMA-IR); (B) IHL (%) and GCGN; (C) HOMA-IR and GCGN; (D) visceral adipose tissue (VAT) and HOMA-IR; (E) VAT and GCGN; (F) correlations between fasting insulin and GCGN before (Week 0, blue) and after high protein intake intervention in the entire study group (Week 6, red). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

did not correlate with GCGN (ρ = 0.17, $p = 0.36$) (**Figure 1E**). The intervention resulted in markedly reduced liver fat content by 6.6%, slightly but significantly reduced VAT and significant improvements of HbA1c, fasting glucose, and insulin sensitivity (HOMA-IR, Matsuda index, and *M*-value) (**Table 1**) (19, 20). The levels of fasting GCGN did not change significantly (**Table 1**).

Correlation of Glucagon, Glucagon–Alanine Index, and Insulin Sensitivity With Intrahepatic Lipid andVisceral Adipose Tissue

Glucagon levels correlated with IHL and insulin sensitivity before and after the intervention (**Figures 1B,C**) and with VAT after the intervention (ρ = 0.52, $p = 0.004$) (**Figure 1D**). In order to assess hepatic GCGN sensitivity, we calculated the GCGN–alanine index as proposed (12) which correlated modestly with IHL at baseline (ρ = 0.369, $p < 0.05$). Insulin sensitivity calculated by HOMA-IR correlated trendwise and non-significantly with the GCGN–alanine index at baseline (ρ = 0.352, $p = 0.057$) (**Figure 2A**). Remarkably, the correlations of the GCGN–alanine index became highly significant upon the high protein intake for6 weeks for IHL (ρ = 0.652, $p < 0.001$) (**Figure 2B**) and for insulinsensitivity (ρ = 0.644, $p < 0.001$) (**Figure 2A**). Similarly, increasedcorrelations were observed between GCGN–alanine index and BCAA, glutamine, or histidine as well as between total AAs andwith IHL or HOMA-IR (**Supplementary Table 3**). The intakeof the high-protein diet thus greatly increased the alignment of GCGN and AA as reflected by their increasing correlation with liver fat and insulin sensitivity.

Improvements of Insulin Sensitivity Upon Reduction of Liver Fat Are Dissociated From Changes of the Glucagon–Alanine Index

Glucagon is likely a key player in the protein-induced reduction of liver fat by high protein intake (24). The reductions of liver fat in our study showed large differences between individuals. We therefore hypothesized that these differences might be related to hepatic GCGN resistance resulting in impaired GCGN-induced hepatic lipolysis and induction of ureagenesis.

We therefore analyzed the participants according to changes above or below the median of liver fat change. This resulted in a significant difference of liver fat reduction between the groups although baseline levels of IHL did not differ significantly (**Table 2**). The lesser liver fat reduction group shifted from 17.4 to 12.7% IHL and thus maintained a high liver fat content even after the relative reduction by 27%. The greater liver fat reduction group decreased IHL by 65% from 13.3 to 4.6 ±3.8% and thus –in average – below the defined threshold of fatty liver of 5.56% IHL. The modest reduction of weight and waist circumference was around 2 kg and 2 cm, respectively, identical in both groupsas were modest reductions of visceral and total adipose tissue and modest increases in muscle mass (**Table 2**).

Fasting glucose decreased significantly in both groups while fasting insulin decreased significantly in the greater liver fat reduction group only. Fasting GCGN did not change significantly in either group. Insulin sensitivity expressed by HOMA-IR, Matsuda index, or *M*-value improved significantly in the group with greater IHL reduction but not in the lesser IHL-reduction group resulting in a significant difference between the groups.

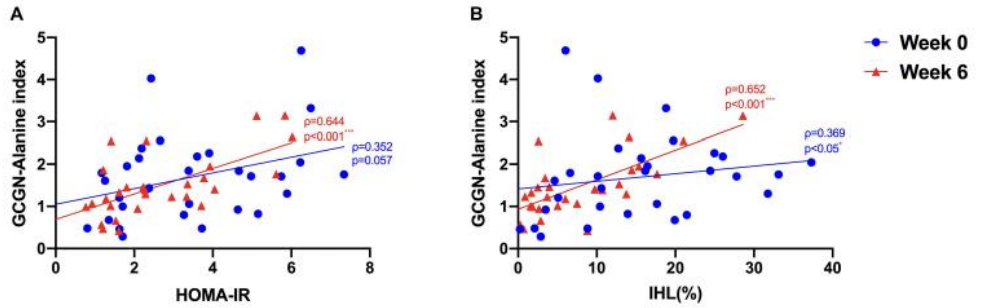

FIGURE 2 | Correlations between **(A)** GCGN–alanine index and HOMA-IR; **(B)** GCGN–alanine index and IHL (%) before (Week 0, blue) and after the intervention in the entire study group (Week 6, red). *$p < 0.05$; ***$p < 0.001$.

TABLE 2 | Parameters at baseline (Week 0) and after HPD intervention (Week 6) of study participants with lower (below median) and higher (above median) reduction of intrahepatic lipid content (IHL).

Parameter	Lower liver fat reduction (n = 16) below median			Higher liver fat reduction (n = 15) above median			$p$week 6 vs week 0
	Week 0	Week 6	p	Week 0	Week 6	p	
Age (years)	63.0 ± 5.7			66.3 ± 6.0			
Gender (male/female)	8 m/8 f			11 m/4 f			
Liver fat content (MR-S; %)	17.4 ± 10.7	12.7 ± 9.2	<0.001***	13.3 ± 8.6	4.6 ± 3.8	<0.001***	<0.05*
Body weight (kg)	89.0 ± 14.0	86.7 ± 13.6	<0.001***	89.6 ± 15.6	86.8 ± 15.4	<0.001***	0.96
BMI (kg/m^2)	31.0 ± 4.1	30.2 ± 4.0	<0.001***	30.2 ± 3.3	29.5 ± 3.1	<0.001***	0.96
Waist circumference (cm)	102.5 ± 10.4	100.7 ± 10.3	0.07	103.2 ± 11.8	100.6 ± 11.5	<0.01**	0.54
Fasting glucose (mmol/L)	9.3 ± 1.0	8.8 ± 1.1	<0.05*	10.0 ± 1.8	8.9 ± 1.8	<0.01**	0.12
Fasting insulin (mU/L)	8.4 ± 4.9	8.9 ± 6.4	0.28	8.3 ± 4.6	6.9 ± 4.1	<0.05*	<0.05*
Fasting glucagon (pmol/L)	8.2 ± 3.2	9.2 ± 4.0	0.24	8.7 ± 4.5	7.6 ± 3.4	0.51	0.18
Fasting C-P (ug/L)	1.9 ± 0.9	1.9 ± 1.0	0.59	1.8 ± 0.8	1.7 ± 0.8	0.07	0.11
AUC insulin (MMT1)	8915.3 ± 6880.0	9039.2 ± 7201.4	0.75	10163.1 ± 6425.7	8503.3 ± 4852.5	<0.05*	<0.05*
AUC insulin (MMT2)	6322.0 ± 4262.6	5923.3 ± 3508.7	0.14	6062.9 ± 4109.5	4926.7 ± 2844.5	0.06	0.35
AUC glucagon (MMT1)	2917.4 ± 869.5	3051.3 ± 1018.9	0.35	2925.1 ± 1004.8	2672.3 ± 1046.9	<0.05*	0.08
AUC glucagon (MMT2)	2988.1 ± 829.9	2755.9 ± 831.8	0.08	2651.7 ± 1089.3	2439.4 ± 1181.6	0.08	0.98
HbA1c	6.7 ± 0.54	6.3 ± 0.47	<0.01**	7.0 ± 0.81	6.6 ± 0.84	<0.05*	0.80
HOMA-IR	3.4 ± 1.9	3.4 ± 2.4	0.77	3.6 ± 2.0	2.6 ± 1.5	<0.01**	<0.05*
Matsuda index	4.8 ± 3.7	4.7 ± 2.9	0.72	4.2 ± 2.5	5.4 ± 3.0	<0.01**	<0.05*
M-value	5.0 ± 2.4	5.3 ± 2.0	0.28	4.8 ± 1.8	5.8 ± 1.7	<0.01**	0.11
AST (U/L)	26.4 ± 9.7	21.8 ± 5.8	<0.05*	24.0 ± 7.7	21.7 ± 6.5	0.16	0.34
ALT (U/L)	29.9 ± 12.7	27.8 ± 9.4	0.15	26.4 ± 5.5	25.1 ± 7.2	0.48	0.42
AST/ALT ratio	0.88 ± 0.24	0.82 ± 0.16	0.61	0.87 ± 0.18	0.86 ± 0.22	0.81	0.67
GGT (U/L)	48.4 ± 23.6	36.0 ± 17.9	<0.001***	39.5 ± 28.7	25.2 ± 11.5	<0.05*	0.81
TG (mmol/L)	1.7 ± 0.54	1.8 ± 0.74	0.33	1.7 ± 0.66	1.4 ± 0.52	<0.05*	<0.05*
TC (mmol/L)	5.2 ± 0.88	4.8 ± 1.0	<0.01**	5.4 ± 1.1	4.5 ± 0.88	<0.001***	<0.05*
LDL-c (mmol/L)	3.3 ± 0.86	3.0 ± 0.91	<0.05*	3.5 ± 0.94	2.9 ± 0.82	<0.01**	0.58
HDL-c (mmol/L)	1.1 ± 0.27	0.95 ± 0.14	<0.01**	1.2 ± 0.27	0.96 ± 0.21	<0.001***	0.18
Creatinine (μmol/L)	82.6 ± 17.5	79.9 ± 18.5	0.41	79.9 ± 15.2	74.9 ± 14.7	<0.05*	0.49
BUN (mmol/L)	6.0 ± 1.0	7.8 ± 1.7	<0.01**	5.9 ± 0.94	7.8 ± 1.9	<0.01**	0.38
eGFR (mL/min/1.73 m^2)	77.1 ± 16.1	80.2 ± 15.9	0.38	80.3 ± 14.6	85.1 ± 14.6	<0.05*	0.61
Urine urea (mmol/day)	377.6 ± 79.3	507.6 ± 158.5	<0.01**	430.4 ± 175.0	624.7 ± 227.9	<0.01**	<0.05*
VAT (L)	6.0 ± 2.2	5.6 ± 2.1	<0.01**	5.9 ± 2.1	5.5 ± 1.9	0.12	0.92
Fat mass (%)	36.4 ± 9.0	34.8 ± 8.9	<0.01**	35.2 ± 4.9	32.6 ± 3.8	0.11	0.52
Lean mass (%)	63.6 ± 9.0	65.2 ± 8.9	<0.01**	64.8 ± 4.9	67.4 ± 3.8	0.11	0.52

*BMI, body mass index; C-P, C-peptide; HbA1c, glycated hemoglobin A1c; HOMA-IR, homeostatic model assessment for insulin resistance; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transferase; TG, triglycerides; TC, total cholesterol; CREA, creatinine; BUN, blood urea nitrogen; eGFR, estimated glomerular filtration rate. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.*

By contrast, the GCGN resistance indices calculated for alanine or AA did neither change significantly within, nor differ between the groups (**Supplementary Tables 1, 2**, **Figure 3**). However, the reduction of liver fat showed a borderline correlation with the change of GCGN ($\rho = 0.344$, $p = 0.077$) but not with the change of the GCGN–alanine index.

Notably, the correlations of the GCGN–AA indices with IHL and insulin sensitivity became highly significant for virtually all AA from baseline to follow-up, indicating a close alignment of GCGN-regulated AA-metabolism with IHL and insulin sensitivity (**Supplementary Table 2**). Thus, the reduction of liver fat is linked to a reduction of insulin resistance but not of GCGN resistance estimated by the GCGN–alanine index even upon extensive reductions of liver fat. However, the role of the GCGN–AA-hepatic axis appears to become enhanced which we interpret to reflect beneficial actions of GCGN.

Does Glucagon Resistance Impair the High Protein Diet-Induced Loss of Liver Fat?

We then asked whether hepatic GCGN resistance may relate to impaired degradation of IHL by GCGN in response to HPD and therefore compared participants above with those belowthe median of the GCGN–alanine index regarding responses of IHL to high-protein diet. Indeed, the GCGN–alanine index in the upper half was associated with higher liver fat comparedto the lower half both at baseline (20.9 ± 9.2 vs. 11.9 ± 9.4%; $p < 0.05$) and after 6 weeks (11.4 ± 7.3 vs. 4.1 ± 3.9%; $p < 0.01$). However, the absolute magnitude of liver fat reduction did not differ between the groups (6.8 ± 5.3 vs. 6.6 ± 5.1%; $p > 0.05$) and we did not find an indication that a higher GCGN–alanine index impairs the HPD-induced reduction of liver fat (**Table 3** and **Supplementary Figure 1**).

Does Glucagon Play a Role for Circulating Free Fatty Acids?

We previously reported that HPDs reduced circulating saturated FFAs which associated with the changes in IHL (19). In view of the regulation of hepatic lipid metabolism by GCGNwe assessed associations between circulating GCGN and FFA.

TABLE 4 | Correlations between GCGN and FFA and DNL-index before (Week 0) and after (Week 6) HPD intervention.

Parameters ($n = 31$)	Week 0	Week 6
C14:0	$\rho = 0.253$ $p = 0.186$	$\rho = 0.417$ $p < 0.05$*
C15.0	$\rho = -0.071$ $p = 0.713$	$\rho = 0.094$ $p = 0.626$
C17.0	$\rho = 0.315$ $p = 0.096$	$\rho = 0.272$ $p = 0.153$
C16:0	$\rho = 0.388$ $p < 0.05$*	$\rho = 0.524$ $p < 0.01$**
C18.0	$\rho = 0.522$ $p < 0.01$**	$\rho = 0.489$ $p < 0.01$**
DNLindex = 16:0/18:2n6	$\rho = 0.531$ $p < 0.01$**	$\rho = 0.456$ $p < 0.05$*

*C14:0: myristic acid; C16:0: palmitic acid; C18:0: stearic acid; C15:0: pentadecanoic acid; C17:0: heptadecanoic acid; DNLindex: de novo lipogenesis index. *p < 0.05; **p < 0.01.*

Indeed, GCGN correlated significantly with palmitic and stearic acid and the *de novo* lipogenesis index, both before and after the intervention supporting a role of GCGN in the regulation of lipogenesis. This would be expected due to the AMPK induced inhibition of ACC (**Table 4** and **Supplementary Table 7**). In agreement, there was no correlation with odd numbered FFA or unsaturated FFA or indices of desaturase or elongase activities (**Supplementary Table 7**).

Assessment of Beta-Cell Stimulation by High Protein Diet – Does the Glucagon Response Play a Role?

Capozzi and coworkers recently proposed that GCGN-induced insulin secretion contributes to lowering of blood glucose concentrations particularly in mixed meals (17, 18). Wewondered whether changes of AAs and GCGN responses to protein challenges occurred in response to the reductions ofliver fat by HPD. Fasting levels of AA did not change in response to the intervention. Fasting levels of GCGN and insulin were highly correlated ($\rho = 0.431$, $p < 0.05$), and the

TABLE 3 | Parameters at baseline (Week 0) and after HPD intervention (Week 6) between lower (below median) and higher (above median) basalGCGN–alanine index groups.

Parameters	Week 0 Glucagon–alanine index			Week 6 Glucagon–alanine index		
	Lower ($n = 16$) below median	Higher ($n = 15$) above median	p	Lower ($n = 16$) below median	Higher ($n = 15$) above median	p
Liver fat content (MRS; %)	11.9 ± 9.4	20.9 ± 9.2	<0.05*	4.1 ± 3.9	11.4 ± 7.3	<0.01**
Insulin/glucagon ratio (fasting)	1.4 ± 0.84	0.85 ± 0.48	0.052	0.89 ± 0.42	0.83 ± 0.32	0.88
Insulin/glucagon ratio (60 min)	5.2 ± 3.7	2.9 ± 1.6	0.07	3.7 ± 2.0	4.2 ± 2.8	0.84
Insulin/glucagon ratio (120 min)	4.6 ± 2.8	3.5 ± 2.2	0.18	3.8 ± 2.0	4.3 ± 2.7	0.77
Insulin/glucagon ratio (180 min)	2.6 ± 1.6	2.4 ± 1.6	0.33	2.3 ± 1.1	2.4 ± 1.3	0.95

TABLE 5 | Area under the curve-insulin and AUC-GCGN levels at baseline (Week 0) and after HPD intervention (Week 6) in MMTT 1 and MMTT 2.

Parameters	MMTT 1			MMTT2		
	Week 0	Week 6	*p*	Week 0	Week 6	*p*
AUC insulin	9482.6 ± 6410.0	8722.8 ± 5890.0	0.17	6233.7 ± 4015.9	5394.6 ± 3102.0	<0.05*
AUC glucagon	2917.4 ± 899.8	2856.6 ± 1007.4	0.51	2907.4 ± 964.2	2656.0 ± 979.8	<0.05*

*AUC, area under the curve. *p < 0.05; **p < 0.01.*

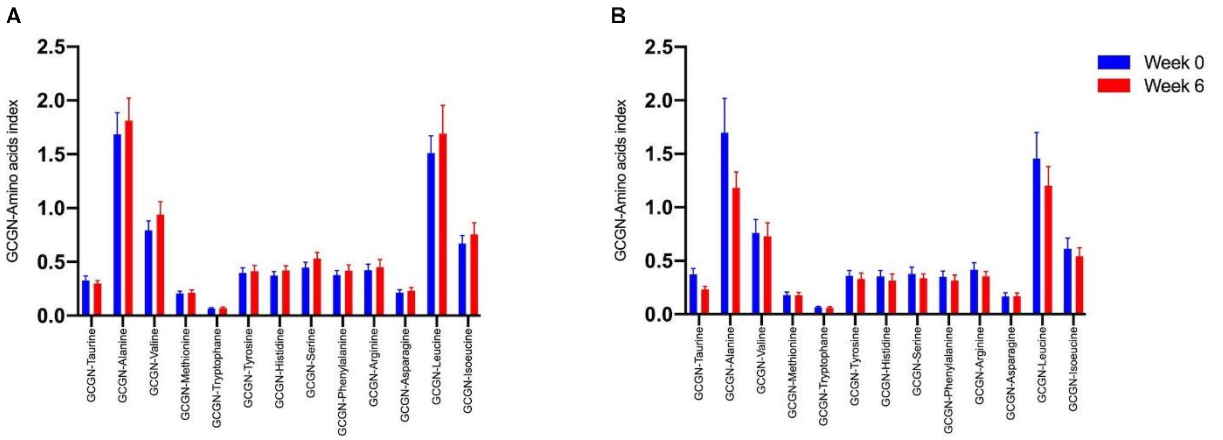

FIGURE 3 | Glucagon–alanine index between (A) lower IHL reduction groups; (B) higher IHL reduction group before (Week 0, blue) and after the intervention (Week 6, red).

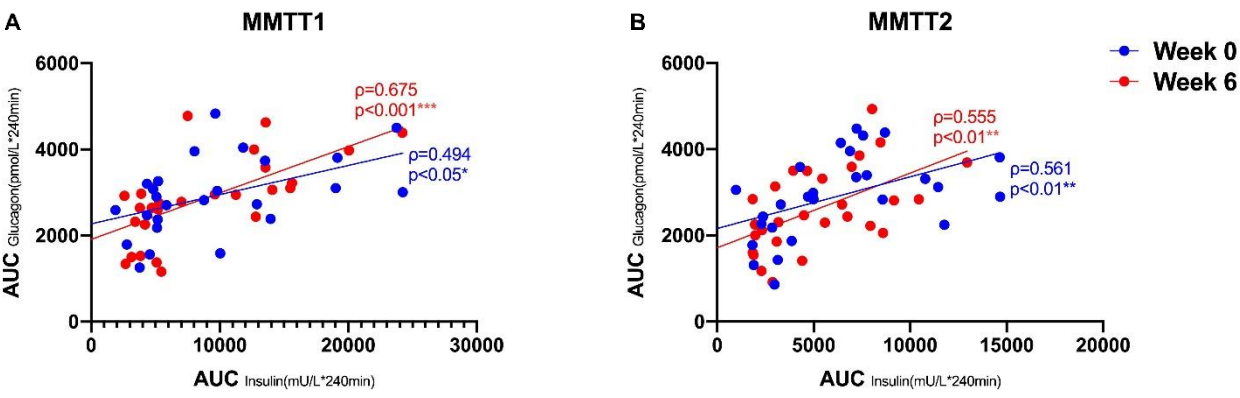

FIGURE 4 | Correlations between AUC (*240 min) insulin and AUC (*240 min) GCGN in MMTT 1 (A) and MMTT 2 (B) before (Week 0, blue) and after the intervention (Week 6, red). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

correlation increased markedly after the intervention ($\rho = 0.639$, $p < 0.001$) (**Figure 1F**).

We therefore tested whether the AA and GCGN responses to intake of 30 g protein/mixed meal were related to the liver fat content by analyzing identical successive breakfast (MMTT1) and lunch (MMTT2) before and after the intervention. The reduction of IHL resulted in reduced overall responses of insulin and GCGN in the MMTTs (**Table 5** and **Figure 4**). This was accompanied by significantly and selectively reduced increases of alanine but not of other AAs (**Figure 5**). We then performed the same calculations for the groups above and below the median with greater and lesser liver fat reduction. Indeed, the reductions of insulin-, GCGN-AUC in the meal tests were only observed in the greater liver fat reduction group while alanine-AUC was reduced in both groups (**Table 2** and **Figures 6A,B**).

The insulin/GCGN ratios were, moreover, significantly higher in participants with a fasting and postprandial GCGN–alanine index below compared to above the median at baselineindicating relatively less GCGN release (**Table 3**). Remarkably, the insulin/GCGN ratio decreased markedly from 1.42 ± 0.84 to 0.89 ± 0.42 at 0 min and also over the meal test in the lower

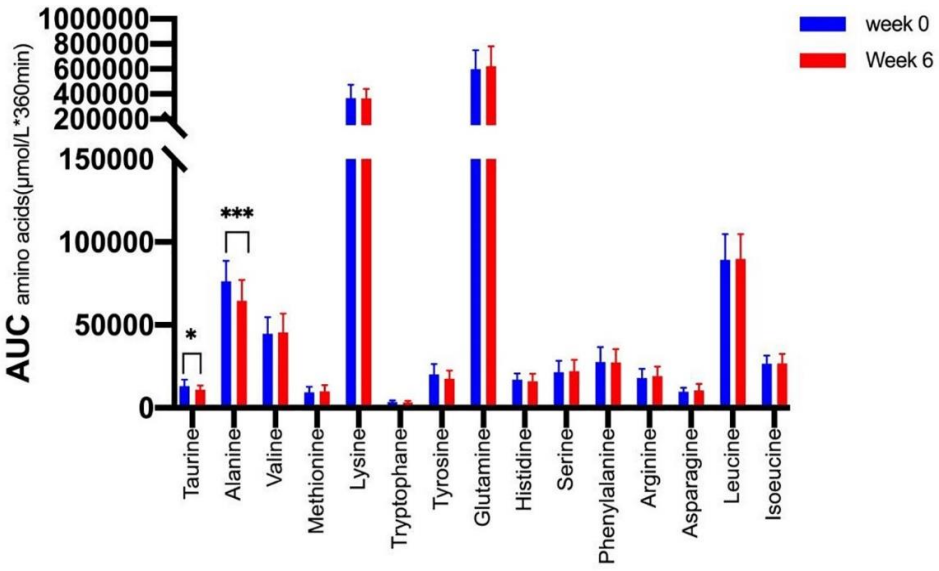

FIGURE 5 | Area under the curve (*360 min) of amino acids in the MMTTs before (Week 0, blue) and after the intervention (Week 6, red). $*p < 0.05$; $***p < 0.001$.

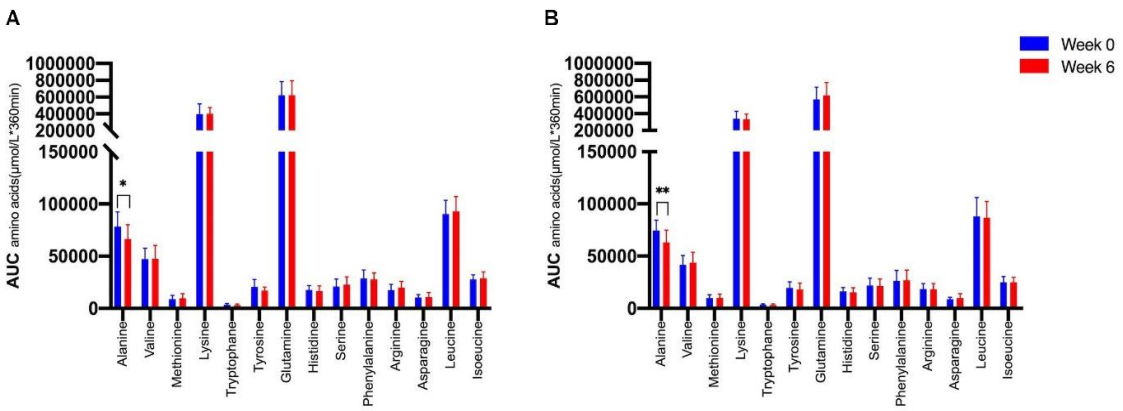

FIGURE 6 | Area under the curve (*360 min) of amino acids in the MMTTs between (A) lower IHL reduction group; (B) higher IHL reduction group before (Week 0, blue) and after the intervention (Week 6, red). $*p < 0.05$; $**p < 0.01$.

GCGN–alanine index group (with lower liver fat) indicatinga greater relative secretion of GCGN. By contrast, the higher GCGN–alanine index group showed no significant change.

The ratios did not differ significantly between the higherand lesser IHL-reduction group before or after the intervention although the insulin/GCGN-ratio also decreased numerically in the greater liver fat reduction group (**Supplementary Table 4**).

DISCUSSION

The role of GCGN in the dysregulation of glucose and lipid metabolism is debated at present (5, 10, 15). GCGN is consistently elevated in people with fatty liver and T2DM and contributes to hyperglycemia as shown with GCGN antagonists (27, 28). However, GCGN antagonists increased dyslipidemia, IHL and liver enzymes (13). Glucagon selectively induces hepatic lipolysis and enhances insulin secretion within pancreatic islets (17, 24).

Our findings confirm (A) a positive correlation of fasting GCGN with hepatic fat content and insulin sensitivity in subjects with T2DM, obesity, and fatty liver. We report (B) that extensive, but not moderate, reductions of IHL after 6 weeks HPD induce the expected improvement in insulin sensitivity but do not alter fasting levels of GCGN. However, (C) postprandial stimulation of GCGN is reduced in parallel to reductions of insulin due to the better insulin sensitivity. However, the insulin/GCGN ratio in MMTTs decreased in participants with a greater reduction of liver fat and extensive metabolic improvements. Thus, the fasting and postprandial levels of GCGN relative to insulin increased indicating that higher GCGN responses were associated with

metabolic improvements. Moreover, (D) a selective reduction of the postprandial response of alanine was observed whichmay indicate enhanced hepatic GCGN sensitivity which strongly regulates alanine metabolism (see below). Therefore, increasing GCGN by HPD indeed allows metabolic improvements which suggests that the beneficial actions of GCGN outweigh the negative role in glucose production and parallel the positive results of GCGN co-agonists.

Correlation of Glucagon or the Glucagon–Alanine Index With Intrahepatic Lipid and Insulin Sensitivity

Glucagon is potently stimulated by increases of AA and then regulates not only the hepatic degradation of AA in the urea cycle but also increases hepatic glucose production and stimulates insulin secretion (5). This increase of glucose production is physiologically compensated for by increased insulin releaseand glucose disposal in healthy subjects (29) which remains functional in people with T2DM despite of an impaired insulin response to glucose (21).

The correlation of IHL with the GCGN–alanine index was borderline significant at baseline and GCGN alone showed a higher correlation with IHL than the GCGN–alanine index which thus reflects variable alanine levels. The correlation ofthe GCGN–alanine index with insulin sensitivity (HOMA-IRor *M*-value) was non-significant at baseline while a significant correlation with GCGN was observed. The GCGN–alanine index did not improve the association of GCGN with insulin sensitivity or fatty liver as might be expected if the dysregulation of fasting alanine plays a primary role. This was also true for all other GCGN–AA indices. As the GCGN–alanine index multiplies Ala (or other AA) with GCGN one would expect a higher correlation of the index than of GCGN alone if alanine contributes to the increased GCGN levels.

Remarkably, after the HPD intervention, the correlation of GCGN–alanine index with IHL and HOMA-IR became highly significant. This also applied to other GCGN–AA indices. We interpret this to reflect a greater impact of AA in the regulation of IHL and insulin sensitivity in combination with GCGN dueto the increased protein intake. High protein intake results inthe oxidation of AAs in muscle which employs the alanine– glucose (or Cahill) cycle to shuttle the amino groups to the liver for detoxification in the urea cycle (30). GCGN was shown to directly regulate both ALT enzymes (GPT and GPT-2) at the transcriptional level (31) as the first step of AA detoxification in the hepatic urea cycle. This may reflect the primary role of GCGN in the reduction of IHL due to GCGN induced hepatic lipolysis through the INSP3R1 mediated pathway which was recently described and provides an important explanation for the effect of HPD (24). The urea cycle was moreover shown to activate AMPK due to the consumption of ATP by arginino-succinate synthase which results in AMPK-induced inhibition of hepatic acetyl-CoA carboxylase and thus of lipogenesis (32). The high correlations of GCGN with saturated FFA and the *de novo* lipogenesis index support the role of GCGN in the regulation of lipid metabolism which became apparent in human studies with GCGN antagonist-induced dyslipidemia and fatty liver. Obviously, this also applies to HPD-induced increases of GCGN. The reduction of the metabolically toxic saturated FFA, in particular palmitic acid (C16:0) by HPD likely involves a regulation of *de novo* lipogenesis by GCGN, possibly due to inhibition of ACC1 by increased AMPK activity in the liver.

Dissociation of Improvements of Fasting Insulin- and Glucagon-Sensitivity in Response to Reduced Intrahepatic Lipid

The associations of GCGN with increased fasting AA, insulin resistance and fatty liver appear to support its negative rolein the obesity and diabetes-associated metabolic dysregulation.A stimulation of GCGN by high protein intake should therefore further deteriorate metabolism (10). The alternative view interprets the increase of GCGN as a defensive response inan attempt to reset metabolism (5). Indeed, the HPD induced marked improvements of metabolism (19, 20, 22). However, an extensive reduction of IHL by 42% upon consumption of HPD for 6 weeks did not alter fasting GCGN, AA-levels, or the GCGN– alanine index, indicating that IHL is not directly related to fastingGCGN or AA levels in people with T2DM. By contrast, the reduction of liver fat resulted in a significant improvement of insulin sensitivity as shown by either HOMA-IR, Matsuda index, or *M*-value. Moreover, other markers of metabolism improved such as uric acid, CRP, and blood lipids (19, 20). Therefore,the HPD induced *meal related* increase most likely explains the metabolic improvements while fasting GCGN may be of minor importance.

The decrease in liver fat with HPDs was remarkably variable which might be related to hepatic GCGN resistance, because GCGN most likely drives the liver fat reduction by specifically enhancing hepatic lipolysis and inhibiting lipogenesis (24, 32). We therefore compared subjects above the median and below the median of liver fat reduction. The upper 50th percentile lost 27% of IHL which resulted in 12.7% IHL after the intervention while the lower 50th percentile lost 65% of liver fat whichled to 4.6% IHL on average which is below the threshold definition of fatty liver. Although all indices of insulin resistanceimproved significantly only in the greater IHL-reduction group, there was no significant difference in fasting GCGN, GCGN– alanine index, or other fasting GCGN–AA indices. There were also no significant changes in the fasting levels of AA. This shows that changes of insulin sensitivity and GCGN sensitivity as calculated by the GCGN–alanine index in response to metabolic improvements can be dissociated in T2DM mellitus. Therefore, the alpha-cell response in the fasting state appearsto be less responsive to reductions of liver fat than other metabolic parameters.

Improvements of Meal-Related Insulin and Glucagon Responses

Meal related responses of GCGN are thought to be exaggerated in T2DM although this has received little attention with regards to responses to protein intake previously. We assessed whether the reduction of IHL would alter the GCGN response to protein

intake. The same protein rich MMTTs were performed before and after the intervention such that each individual could serveas its own control. This showed a reduction of insulin and GCGN responses in the MMTTs in the presence of greater reduction of liver fat. Moreover, the levels of GCGN relativeto insulin increased supporting a contribution of GCGN to the metabolic improvements.

Altered Alanine Responses May Reflect Changes of the Glucose-Alanine Cycle

Remarkably, the plasma levels of alanine in the meal challenge tests were selectively reduced after 6 weeks of HPD, accompanied by a pronounced reduction of IHL, while the other AA and total AA did not change. The glucose-alanine cycle is well known to play a key role in glucose and AA metabolism (33). Alanine is generated by transamination from other AA used as energy substrates in muscle and transports amino groups to the liver which detoxifies the ammonium groups by delivery to the urea cycle. GCGN was shown to preferentially increase hepatic alanine uptake several-fold as compared to other AA (33). Alanine was recently shown to directly regulate mitochondrial oxidative metabolism in fasted humans (34). Mouse studies identified alanine as an intracellular activator of AMPK in hepatocytes which was dependent on ALT1 and the extraction of intermediate metabolites of the TCA-cycle (35). Alanine supplementation resulted in improved glucose metabolism of lean or obese mice. The alanine metabolic pathway was shown to be reversibly dysregulated in obese mice and humans and associated with impairments of ureagenesis (36, 37). We interpret the selective reduction of alanine in the MMTTs therefore as an indication of more effective use of alanine and improved mitochondrial oxidative function which may partially explain the improvements of glucose metabolism. As GCGN primarily regulates hepatic alanine uptake and metabolism, the reduced levels may indicate an improved prandial hepatic GCGN sensitivity. Notably, insulin resistance of protein metabolism was shown to be more pronounced in the fasting state while postprandial responses were close to normal (38). In analogy, postprandial GCGN actions may adapt preferentially to metabolic improvements.

A remarkable observation was that levels of urea were higher in subjects with greater liver fat reduction and showeda greater increase during the HPD intervention. This may indicate that there was a higher efficiency of GCGN to induce AA degradation and ureagenesis which may support the lossof IHL (32) as discussed above. In addition, GCGN was shown to specifically induce hepatic secretion of cAMP intothe bloodstream to regulate kidney function which is a further energy-expensive signaling pathway (39). The improvementsof IHL and insulin sensitivity in the entire cohort indicatea sufficiently preserved capacity of the liver to handle AA metabolism and to profit from its consequences in response to HPD. However, there appear to be subgroup-specific differencesin the capacity to respond to HPD which are not well understood at present.

An important concern regarding high protein intake is a potential impairment of renal function due to the increased delivery of urea (40). GCGN was shown to participate in the adaptation of the kidney to increased protein intake (41). However, there is no conclusive evidence that limitation of protein intake prevents the progression of renal failure in T2D in randomized prospective studies (42, 43). Nevertheless, high protein intake should be avoided in patients with renalimpairment until better evidence is available.

Limitations of the study apply to the relatively small number of patients who displayed a well-controlled non-insulin requiring diabetes and were characterized in considerable detail. The study used plant or animal protein supplements which differed in AA composition and there was a gender dysbalance in the groups above or below the median of liver fat reduction. The patients were Caucasian and of moderately advanced age. We did not study direct responses to exogenous administration of GCGN which may allow more sensitive assessment of GCGN responses. However, the high protein MMTTs reflect the real-life situation.

CONCLUSION

Although fasting levels of GCGN are positively correlated with insulin resistance and IHL, increasing prandial GCGN secretion by HPD improves IHL, insulin sensitivity, fasting glucose, and circulating free saturated fatty acids. This associates with a selective reduction of alanine in meal challenge tests whichis known to be primarily regulated by GCGN. Alanine links GCGN-stimulated glucose and AA-metabolism and might playa key role in augmenting insulin sensitivity and in inhibitionof lipogenesis through AMPK-dependent pathways. Moreover, the metabolic improvements are associated with a reduction of meal stimulated insulin and GCGN secretion but a greater GCGN relative to insulin secretion. Together these findings suggest a primary role of prandial GCGN in the HPD-induced metabolic improvements which appears to be associated with an increased GCGN sensitivity.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the University of Potsdam. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MM, OP-R, and SH conducted the experiments by dietary consultation and collected the data. SK, MM, OP-R, and SH analyzed and interpreted the experimental data. SR analyzed

the samples, designed the study, and interpreted the data. JZ performed the statistical analysis, designed the figures and tables, and wrote the manuscript. AP designed the study and wrote the manuscript. All authors read and revised the manuscript, contributed to discussion, and approved the final version of this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.808346/full#supplementary-material

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

Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Publication list

1. **J. Zhang,** O. Pivovarova-Ramich, S. Kabisch, M. Markova, S. Hornemann, S. Sucher, S. Rohn, J. Machann, A. F. H. Pfeiffer. High Protein Diets Improve Liver Fat and Insulin Sensitivity by Prandial but Not Fasting Glucagon Secretion in Type 2 Diabetes. Front Nutr. 2022 May 19;9:808346. doi: 10.3389/fnut.2022.808346. eCollection 2022. (Impact Factor 2022: 6.59)

2. **J. Zhang**, S.M Schäfer, S. Kabisch, M.Csanalosi, B. Schuppelius, M. Kemper, M. Markova, N. MT. Meyer, O. Pivovarova-Ramich, F. Keyhani-Nejad, S. Rohn, A. F. H. Pfeiffer. Implication of sugar, protein and incretins in excessive glucagon secretion in type 2 diabetes after mixed meals. Clin Nutr. 2023 Apr;42(4):467-476. doi: 10.1016/j.clnu.2023.02.011. (Impact Factor: 7.643)

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