Aus der Klinik für Endokrinologie, Stoffwechsel- und Ernährungsmedizin der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Dysfunctional Network of Type 2 Diabetes Mellitus: Influence of Diet, Genes and Brain

zur Erlangung des akademischen Grades
Doctor rerum medicinalium (Dr. rer. medic.)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

von

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Datum der Promotion: 26.06.2022

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II Abbreviations

Body weight	BW
Cardiovascular diseases	CVD
Central GIPR knock out mice	$GIPR^{CNS-/-}$
Central nervous system	CNS
Diabetes Nutrition Algorithms in Patients with Overt Diabetes Mellitus	DiNA-D
Diabetes Nutrition Algorithms in PREDIABETES	DiNA-P
Diastolic blood pressure	DBP
Flow-mediated dilation	FMD
Free fatty acids	FFA
Genome-wide association studies	GWAS
Glucose tolerance test	GTT
Glucose-dependent Insulinotropic Peptide	GIP
Glucose-dependent Insulinotropic Polypeptide Receptor	GIPR
Glycated hemoglobin	HbA1c,
Heterozygous	HET
High density cholesterol	HDL
High-fat diet	HFD
Homozygous major allele	HAM
Homozygous minor allele	HMI
Humanized GIPR control	$hGIPR^{+/+}$
Hypothalamic GIPR knock out mice	$GIPR^{HYPO-/-}$
Insulin tolerance test	ITT
Itrahepatic lipid	IHL
Knock-out	KO
Leguminosen – Anbau und Nutzung	LeguAN
Lipoprotein lipase	LPL
Low density cholesterol	LDL
Low-fat	LF
Meal Tolerance Test	MTT
Nonalcoholic fatty liver disease	NAFLD
Optimal Fibre Trial for Diabetes Prevention	OptiFiT
Semi-synthetic control diet	CD

Single nucleotide polymorphisms	SNP
Systolic blood pressure	SBP
Total abdominal adipose tissue	TAT
Total cholesterol	TCHO
Triglycerides	TAG
Type 2 diabetes mellitus	T2DM
Very-low-carbohydrate diet	VLC
Visceral adipose tissue	VAT

1. Abstract, Zusammenfassung

1.1 Abstract

Type 2 diabetes mellitus (T2DM) incidence, prevalence, and costs are rapidly increasing simultaneously with obesity, cardiovascular diseases (CVD), and nonalcoholic fatty liver disease (NAFLD), clustering a dysfunctional metabolic network. Given the complex relationship between these metabolic disorders and their potential to influence each other, T2DM research regarding nutritional management requires a multidisciplinary approach considering independent measures for NAFLD and CVD. Furthermore, the link between T2DM, NAFLD, and CVD is embedded in a complex interplay between metabolites, genes, and external factors such as diet. In this context, the Glucose-dependent Insulinotropic Polypeptide Receptor (GIPR) has emerged as a novel target for the prevention and treatment of T2DM and co-morbidities.

We firstly investigated the effect of a 3-week hypocaloric intensive low-carb vs. low-fat intervention on endothelial function, intrahepatic lipid (IHL) content, and adipose tissue accumulation in T2DM patients (n=36). On a second investigation, we examined the effect of GIPR SNP rs10423928 on glucose metabolism in a cross-sectional setting and tested its interaction with diverse iso- to hypocaloric 3 weeks diets (low-carb, low-fat) in T2DM and prediabetes patients (n=497). Lastly, we aimed to investigate whether whole brain or hypothalamic GIPR signaling is involved in the development of insulin resistance and obesity in constitutively central (CaMKIIa-Promotor, n=10) and hypothalamic (Nkx2.1-Promotor^{-}, n=12) KO mouse models, undergoing a 14-weeks low-fat or control diet.

Compared with the very-low-carbohydrate diet (VLC), the low-fat (LF) diet showed significantly greater enhancement of flow-mediated dilation (FMD). Endothelial function showed a positive correlation with protein intake and fat intake in the LF group, while it revealed a negative correlation with protein intake in the VLC diet group.

Moreover, we observed that carriers of the A allele of GIPR SNP rs10423928 had a significantly higher fasting glucose but lower 2 h glucose levels after an oral glucose challenge compared to individuals homozygous for the major allele T. A-carriers also had significantly higher Cederholm Index values. We also observed a significant SNP-diet effect on IHL reduction in prediabetic as well as in diabetic subjects.

Results of our animal study showed that after 14 weeks on a high-fat diet, mice with hypothalamic deletion of GIPR had similar weight gain progression and maintenance of insulin sensitivity compared to control diet-fed mice.

Taken all together, our data indicate that a LF diet has favorable effects on endothelial function in T2DM subjects. Prediabetic subjects carrying the A allele of GIPR SNP rs10423928 showed better glucose metabolism and insulin sensitivity. However, IHL improvements seem to underlie an SNP-diet interaction in T2DM subjects.

Additionally, our findings point out that hypothalamic KO mice (Nkx2.1-Promotor) are protected against HFD -induced weight gain and loss of insulin sensitivity suggesting central GIPR as a novel target for the treatment of T2DM and comorbidities.

1.2 Zusammenfassung

Die Inzidenz, Prävalenz und Kosten von Typ-2-Diabetes mellitus (T2DM) steigen derzeit mit Adipositas, Herz-Kreislauf-Erkrankungen (CVD) und nichtalkoholischer Fettlebererkrankung (NAFLD). Angesichts der komplexen Beziehung zwischen diesen Störungen und ihrem Potenzial sich gegenseitig zu beeinflussen, erfordert die T2DM-Forschung zum Ernährungsmanagement einen multidisziplinären Ansatz für NAFLD und CVD. Darüber hinaus ist die Verbindung zwischen T2DM, NAFLD und CVD in ein komplexes Zusammenspiel zwischen Metaboliten, Genen und externen Faktoren wie der Ernährung eingebettet. In diesem Zusammenhang hat sich der Rezeptor für das Glukoseabhängige Insulinotrope Peptid (GIPR) als neues Ziel für die Prävention und Behandlung von T2DM und Komorbiditäten herauskristallisiert.

Zunächst untersuchten wir die Auswirkung zweier diätetischer Interventionen auf die Endothelfunktion, den Gehalt an intrahepatischem Lipid (IHL) und die Ansammlung von

Fettgewebe. In einem zweiten Projekt untersuchen wir die Wirkung von GIPR SNP rs10423928 und seine Wechselwirkung mit verschiedenen Ernährungsansätzen bei Diabetes- und Prädiabetes-Patienten. Letztlich wollen wir die Beteiligung des zentralen GIPR-Signalwegs an der Entwicklung von Insulinresistenz und Adipositas in vivo untersuchen.

Im Vergleich zu einer kohlenhydratarmen Diät (VLC) zeigte eine fettarme Diät (LF) eine signifikante Verbesserung der flussvermittelten Vasodilatation. Die Endothelfunktion zeigte eine positive Korrelation mit der Protein- und Fettaufnahme in der LF-Gruppe, während sie eine negative Korrelation mit der Proteinaufnahme in der VLC-Diätgruppe aufwies.

Außerdem beobachteten wir, dass Träger des GIPR SNP rs10423928 A-Allels eine signifikant höhere Nüchternglukose zeigten, aber im 2-Stunden-oralen-Glukose-Toleranz-Test niedrigere Glukosespiegel aufwiesen. A-Träger hatten auch signifikant höhere Cederholm-Indexwerte. Ansonsten beobachteten wir auch einen signifikanten SNP-Diät-Effekt auf die IHL-Reduktion bei prädiabetischen und diabetischen Probanden.

Die Ergebnisse unserer Tierstudie zeigen, dass Mäuse mit hypothalamischer Deletion des GIPR nach 14 Wochen fettreicher Ernährung eine ähnliche Gewichtszunahme und Insulinsensitivität aufweisen wie Mäuse, die mit Kontroll-Diät (10% Fett) gefüttert wurden.

Insgesamt deuten unsere Daten darauf hin, dass eine LF-Diät die Endothelfunktion bei T2DM-Probanden günstig beeinflusst. Prädiabetische Probanden, die das A-Allel von GIPR SNP rs10423928 tragen, zeigten einen günstigeren Glukose-Stoffwechsel und bessere Insulinsensitivität. IHL-Verbesserungen weisen auf eine SNP-Diät-Interaktion hin.

Zusätzlich konnten wir zeigen, dass hypothalamische GIPR-KO-Mäuse (Nkx2.1-Promotor) vor HFD-induzierter Gewichtszunahme und Verlust der Insulinsensitivität geschützt sind, was den hypothalamischen GIPR als Target für die Behandlung des T2DM und Begleiterkrankungen hervorhebt.

2. Introduction

The development of type 2 diabetes (T2DM) in the 21st century has become one of the major challenges to human health. The affected population is still increasing, and the use of insulin to treat T2DM is projected to increase by more than 20% from 2018 to 2030 [1]. In addition, its incidence, prevalence, and costs are rapidly increasing simultaneously with obesity, cardiovascular diseases (CVD), and nonalcoholic fatty liver disease (NAFLD), embracing a dysfunctional metabolic network. Although these associations between T2DM, CVD, and NAFLD may result from a shared combination of various common risk factors, such as dyslipidemia, insulin resistance (IR), and low-grade inflammation [2, 3], growing evidence suggests that there is a causal and bidirectional influence on the nature of these comorbid conditions [4-8]. It is well established that obesity is the main trigger of this network and consequently energy restriction results in significant improvement of T2DM [9], NAFLD [10], and CVD risk factors [11]. Obesity is the result of a positive energy balance between energy intake and energy expenditure [12]. Thus, dietary and lifestyle interventions are an adequate tool for treating obesity and preventing further metabolic alterations. The most common dietary strategies are hypocaloric and entail the restriction of either carbohydrates or fat. Over the past years, a hand full of meta-analyses comparing the benefits of these diets reported conflicting results on weight loss and improvement of blood lipids, inflammation, and glucose metabolism markers [13-19]. However, these studies do not take into consideration the complex relationship between these metabolic disorders and their potential to influence each other.

Taking this together, T2DM research regarding nutritional management requires a multidisciplinary approach considering independent measures for NAFLD and CVD such as intrahepatic lipid (IHL) content and endothelial function. It is well established that vascular endothelial cells play a pivotal role in the maintenance of cardiovascular homeostasis. The endothelium owns several mechanisms to control the internal integrity and stability [20]. Hereby, nitric oxide (NO), a key vasodilator released by the endothelial cells, plays a central role in the maintenance of vascular tone and is considered a functional marker of endothelial dysfunction [20]. However, endothelial dysfunction also results from inflammation and oxidative stress-induced for instance by an up-regulation of monocyte chemoattractant protein-1 (MCP-1) and adhesion molecules [21].

With a non-invasive procedure, it is possible to quantify the ability of the brachial artery to dilate in response to shear stress. The Flow Mediated Dilation (FMD) technique has been utilized in

numerous studies to establish the effect of hypocaloric diets, bariatric surgery or/and exercise [22-28] in weight loss and management. Indeed, vascular function is influenced by low-fat (LF) and low-carbohydrate (LC) diets [28-33]. Hereby, weight loss is a key element for the restoration of vessel stability and integrity, but also the reduction of visceral fat [27, 34], liver fat [28] and glycated hemoglobin A1c (HbA1c) levels [29] contribute to an enhanced FMD. Although, hypocaloric nutritional strategies are a common tool used for T2DM management due to their quick metabolic benefits, the beneficial effect of diverse macronutrient composition of these diets is still under dispute regarding its impact on endothelial function in T2DM patients.

Furthermore, the link between T2DM, NAFLD, and CVD is embedded in a complex interplay between metabolites, genes, and external factors such as diet. In this context, the Glucose-dependent Insulinotropic Polypeptide Receptor (GIPR) has emerged as a novel target for the prevention and treatment of T2DM and co-morbidities. Glucose-dependent Insulinotropic Peptide (GIP) is secreted from K cells located in the proximal small intestine after meal ingestion and mediates a major part of the food-dependent secretion of insulin constituting the entero-insular axis [35-37]. This effect is best demonstrated when glucose is orally ingested [38] although GIP secretion is also enhanced by proteins or fats [39]. Accumulating evidence suggests that GIP is involved in the development of obesity, NAFLD, and CVD in addition to its involvement in T2DM pathophysiology. GIPR activation promotes glucose uptake and free fatty acids (FFA) re-esterification in fat cells [40] and upregulates the lipoprotein lipase (LPL) gene [41], which contributes to the fat-accumulating effect of GIP. In fact, GIPR antagonists have been proposed as a strategy to prevent and even treat T2DM and adiposity [42]. Furthermore, reducing the receptor signaling by lowering the endogenous GIP secretion was shown to be effective for treating NAFLD [43] and insulin resistance [44]. In support of this, genome-wide association studies (GWAS) have begun to illuminate the importance of GIPR single nucleotide polymorphisms (SNP) The human GIPR gene, located to chromosome 19, encompasses 14 exons with a size of circa 14 kb [45]. To the best of our knowledge, within the GIPR gene, 13 single nucleotide polymorphisms (SNP) have been described. SNP rs10423928 of GIPR is situated within a noncoding region. It is well known, that intronic gene variants can influence gene splicing, transcription, and translation, shifting gene expression [46]. This specific SNP rs10423928 consists of the exchange of thymine by an adenine base (T/A). The functional consequence of this base conversion is implicated in glucose and insulin response. The minor allele A has been repeatedly described as the risk allele in non-diabetic individuals due to its associations with

increased 2-h glucose, fasting proinsulin levels, and lower ß-cell function [47-49]. Though, the A allele was also linked to impaired glucose- and GIP-stimulated insulin secretion and a reduction in BMI, lean body mass, and waist circumference in T2DM and non-T2DM subjects [50]. In addition, carriers of the A allele variant also showed better insulin sensitivity [51]. However, intervention studies addressing the effects of GIPR polymorphisms in T2DM and their interaction with diet are missing.

Although little is known about the behavior of GIPR in humans, more specific support comes from mice studies which have shown that genetic deletion of GIPR exerts beneficial effects inhibiting the development of obesity, hepatic steatosis, and insulin resistance [52]. Mice lacking GIPR and exposed to an obesogenic environment (ovariectomy, high-fat diet, or sucrose-rich diet) do not exhibit body weight gain, liver steatosis nor an increase in visceral and subcutaneous fat mass compared to wild type (WT) mice [53, 54]. GIP signaling stimulates glucose uptake and free fatty acids (FFA) re-esterification in fat cells [40] and upregulates the lipoprotein lipase (LPL) gene [41]. All these factors contribute to the fat-accumulating effect of GIP. In fact, GIPR antagonists have been proposed as a strategy to prevent and even treat type 2 diabetes mellitus (T2DM) and adiposity [42]. An in vivo study suggested that deleting GIPR specifically in adipose tissue reduces IL-6 plasma levels which may be in part responsible for the protective effect against insulin resistance and hepatic steatosis of interrupting GIP-GIPR interactions [55].

Interestingly, the ubiquitous expression of GIPR in the central nervous system (CNS) [56] revealed the potential of this receptor to modulate energy balance-related hypothalamic circuits [57, 58]. Further investigation on the central action of GIPR and its impact on IR, adipose tissue, and liver is required.

A compelling review published in 2018 highlighted GIP and GIPR action in insulin sensitivity, adipose tissue, liver, inflammation, and hypothalamus, increasing the evidence that the interruption of GIPR signaling might have a protective effect against the development of T2DM perhaps through metabolic regulation in adipose tissue and hypothalamic responses [59]

Within this framework, the aim of this doctoral work was first to investigate the short-term effect of a low-carbohydrate, ketogenic diet vs a low-fat diet on endothelial function, IHL, and adipose tissue accumulation, as independent risk factors of CVD and NAFLD in T2DM subjects. Secondly to examine the effect of GIPR SNP rs10423928 on glucose metabolism parameters in diabetic and prediabetic subjects. Furthermore, we investigate the interaction between IHL and GIPR SNP rs10423928 within diverse nutritional approaches. The third aim of this doctoral work was to

investigate the central and hypothalamic GIPR. Through the use of novel mice models, we examined the role of central and hypothalamic GIPR signaling on the development of obesity and glucose metabolism impairment.

3. Methods

3.1 Human intervention studies

All human studies were conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of the Charité and the University of Potsdam. All subjects provided written informed consent for their participation in the respective study.

3.1.1 Effect of low-fat diet vs low-carbohydrate diet on endothelial function, intrahepatic lipid (IHL), and adipose tissue accumulation

Participants and study design

To examine and compare the effect of a hypocaloric very-low-carbohydrate (VLC) diet versus a hypocaloric low-fat (LF) diet on endothelial function, IHL, and adipose tissue accumulation, a study subgroup of the DiNA-D (Diabetes Nutrition Algorithms in Patients with Overt Diabetes Mellitus) (registered at www.ClinicalTrials.gov (NCT02459496)), in which subjects agreed to the additional assessment of the endothelial function, was analyzed. Complete details of this study are described elsewhere [60]. After the initial screening (V0) subjects were randomized on either a very VLC or LF diet. Routine clinical measurements, assessment of endothelial function and IHL content were performed twice, at baseline (V1) and post-intervention (V2) of the respective dietary intervention.

Assessment of the endothelial function

The assessment of the endothelial function was performed with the flow-mediated dilation (FMD) method, a non-invasive technique, which quantifies the ability of the brachial artery to dilate in response to shear stress [30]. Brachial artery ultrasound was performed using a highly sophisticated computerized system to identify the intima media complex by edge detection software (Brachial Analyzer version 5.10.6, Medical Imaging Applications LLC, Iowa City, IA, USA).

Assessment of intrahepatic lipids and adipose tissue content

Magnetic resonance for the determination of IHL was performed using proton magnetic resonance spectroscopy (1H-MRS) on a 1.5 T Magnetom Avanto (Siemens Healthineers, Erlangen, Germany) applying a single voxel STEAM (Stimulated Echo Acquisition Mode) technique (TE (echo time) = 10 ms, TR (repetition time) = 4 s). Abdominal adipose tissue was calculated from axial T1-weighted fast-spin echo images between the hip and shoulder with subjects lying in a prone position with outstretched arms. An automatic segmentation procedure based on fuzzy clustering and orthonormal snakes was employed to compute total abdominal adipose tissue (TAT) and visceral adipose tissue (VAT) depots [61].

Clinical parameters

Levels of HbA1c were determined in the fasting state in serum/plasma with ABX Pentra 400 (Horiba, Fukuoka, Japan). For determining the blood levels of insulin (Mercodia, Uppsala, Sweden), an enzyme-linked immunosorbent assay was used. Glucose and CRP (C-reactive protein) were determined by turbidimetric immunoprecipitation. A fasting blood sample was collected at baseline and post-intervention diet; serum lipids (total cholesterol (TCHO), high-density cholesterol (HDL), low-density cholesterol (LDL), triglycerides (TAG), and further routine parameters were measured in serum/plasma by using ABX Pentra 400 (Horiba, Fukuoka, Japan). All measurements of systolic blood pressure (SBP) and diastolic blood pressure (DBP) were performed after a previous resting time of 10 min at room temperature using a standardized blood pressure cuff system (BOSO ABI system 100, BOSO, Jungingen, Germany).

Dietary intervention

The VLC diet was aimed to be ketogenic with a carbohydrate daily intake of <40 g (60–70% fat, 5–10% carbohydrate, 20–30% protein) and included a reduction of caloric intake to 1200–1500 kcal/day.

The LF diet was characterized by an intake of less than 30% of the total energy intake (E%) of fat (<30% fat, 50% carbohydrate, 20% protein) and a calorie intake of 1000–1200 kcal/day. Study participants got a flavored meal replacement powder (MODIFAST® (OTC Siebenhandl GmbH, Ulm, Germany)). In addition, the intake of 200 g of raw or steamed vegetables was allowed.

Nutritional evaluation

All subjects were asked to prepare food intake documentation throughout the intervention (21 days). Diet records were analyzed for macronutrient content using Prodi software (Version 6.2, Nutri-Science, Hausach, Germany). A mean value of 4 days before starting the diet was calculated for the dietary intake at baseline. A mean value of 3 weeks diet (21 days) was calculated for the dietary intake at the endpoint.

Sample size calculation

The information on the sample sizes is based in part on the extensive, long-term experience in the quantification of metabolic phenotypes. The selected sample size subjects were calculated using the sensitivity power analysis of the program G * Power 3.1.9.7.

The overall sample size calculation was estimated on previous literature on NAFLD as primary outcome. A non-inferiority power calculation for FMD (SD of 0.75 %-pt.) based on a clinically relevant difference in changes of 1%-pt.[62, 63] indicates that with a 5% significance level and a power of 90 %, at least 13 observations per group are required for the detection. Given the intensity of the diet and potential technical difficulties in the acquisition of reliable FMD data before and after the diet, an additional buffer of 10 subjects covering dropouts and insufficient compliance was added, resulting in a total of 36 subjects for this analysis.

Statistical analysis

Statistical analysis was conducted using the SPSS software package for Windows (IBM, version 20.0, Chicago, IL, USA). Data were analyzed for normality by the Kolmogorov–Smirnov test. Comparison within and between diet groups was analyzed using a Student's t-test (paired and unpaired). Non-normally distributed variables were analyzed by non-parametric tests (Wilcoxon or Mann–Whitney-U-Test). Analysis of variance (ANOVA) for repeated measurements was performed for assessing diet-FMD interaction effects. A model with one between subject factor (diet groups) and two within-subject factors (V1 and V2) and adjusted for kilocalories intake change per kg body weight (BW) was used. Pearson correlation (for non-normally distributed variables Spearman correlation) analysis was performed to identify the strength of relations between macronutrients intake (g) per kg BW and FMD after dietary intervention. Statistical significance was defined as $p < 0.05$.

3.1.2 Effect of GIPR SNP rs10423928 on glucose metabolism and its interaction with diverse nutritional approaches in diabetic and prediabetic subjects

Participants and study design

Data of prediabetic subjects were obtained from two intervention trials, "Diabetes Nutrition Algorithms in PREDIABETES (DiNA-P)" registered at www.ClinicalTrials.gov as NCT 02609243 and the "Optimal Fibre Trial for Diabetes Prevention (OptiFiT)" as NCT 01681173. Complete details of DiNA-P and OptiFiT have been described elsewhere [64, 65].

In addition, data of T2DM subjects were extracted from the DiNA-D study [60], described above, and the LeguAN (Leguminosen – Anbau und Nutzung) study [66], registered at www.ClinicalTrials.gov as NCT02402985.

Dietary interventions

DINA-D study [60]

Details see above

DiNA-P [64] study and OptiFiT [65] study

Baseline data of prediabetic subjects were obtained from two intervention trials, "Diabetes Nutrition Algorithms in PREDIABETIC (DiNA-P)" registered at clinicaltrials.gov as NCT 02609243 and the "Optimal Fibre Trial for Diabetes Prevention (OptiFiT)" as NCT 01681173. Complete details of DiNAP and OptiFiT have been described elsewhere [64, 65]. Both studies were conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of the University of Potsdam. All subjects provided written informed consent for their participation in the study. In total of 424 prediabetic subjects were included (nDiNA-P = 262, nOptiFiT = 162).

DiNA-P-dietary intervention.

In brief, the VLC diet was aimed to be ketogenic with a carbohydrate daily intake of <40 g (60-70 E % fat, 5-10 E% carbohydrate, 20-30 E% protein) and includes a reduction of caloric intake to 1200-1500 kcal/d. This is identical to the design in DINA-D (described in 3.1.1).

The LF diet is characterized by a calorie intake of 1000-1200 kcal/d, and less than 30 E % of the total energy intake of fat (<30 E% fat, 50 E% carbohydrate, 20 E% protein). In contrast to DiNA-D, study subjects from DiNA-P were not provided with a meal replacement powder.

Subject of both diets (VLC and LF) visited the respective study facility twice, at baseline (W0) and after 3 weeks (W3) diet were routine clinical measurements and MRI/1H-MRS (Magnetic resonance imaging/Magnetic resonance spectroscopy) assessments were performed.

LeguAN study [66]

Isocaloric high protein diet (HP): After the initial screening the type 2 diabetes patients had an HP intake (30 E% fat, 40 E% carbohydrate, 30 E% protein) for 6-weeks (W6). Routine clinical measurements and MRI/1H-MRS assessment were performed at baseline (W0) and after 6 weeks (W6) diet.

Genotyping

Genomic DNA was isolated from buffy coat, whole blood, or serum, depending on sample availability. Genotyping was performed by ViiA™ 7 System using TaqMan™ SNP Genotyping Assay rs10423928 ID C_30103605_10 (Applied Biosystems, Thermo Fisher Scientific).

Measurement of glucose metabolism and insulin sensitivity

Glucose tolerance test – prediabetic subjects of DiNA-P and OptiFiT

The 2h glucose challenge (oGTT) was measured after a 12 h overnight fast. Blood samples were taken before the consumption of 75g glucose and at 60 and 120min to measure glucose, insulin and C-peptide. Capillary blood glucose concentrations were measured immediately using the glucose oxidase method (Super-GL glucose analyzer; Dr. Müller Gerätebau, Freital, Germany); venous serum blood samples were analyzed batch-wise after storage (Horiba ABX Pentra 400, Montpellier, France). Serum insulin and C-peptide were measured using an ELISA technique (Mercodia, Uppsala, Sweden).

Meal Tolerance Test-T2DM subjects in DiNA-D

The glycemic profile in response to the intake of a liquid formula mixed meal (BOOST® High Protein Complete Nutritional Drink Very Vanilla, Nestlé Nutrition, Vevey, Switzerland) was measured (Meal Tolerance Test (MTT)) to study the ability to regulate blood glucose in T2DM subjects. The test meal was standardized to 360 mL (378 g). The standard test meal (360 mL) contained 365 kcal, 9 g fat, 50 g carbohydrates, and 23 g protein. Serial sampling for analytes was performed -15, 0, 10, 15, 20, 30, 60, 90, 120- and 180-min post-meal.

Meal Tolerance Test-T2DM subjects in LeguAN

In this study the MTT was based on a standardized meal according to their dietary intervention. The subjects were asked to eat the meal within 15 minutes. After the meal was finished blood samples were taken right before (0 min) and then 30, 60, 90, 120, 180, and 240 min [66].

Common indices that are based on fasting parameters or describe the glycemic status under dynamic conditions were used to determine insulin sensitivity/resistance. Fasting and dynamic indices[67-69] were calculated using oGTT (prediabetic) or MTT (T2DM) data.

Measurements of adipose tissue accumulation and clinical parameters

Calculation and analysis of TAT, VAT, and IHL content and clinical parameters are described above in 3.1.1.

Sample size calculation

The information on the sample sizes is based in part on the extensive, long-term experience in the quantification of metabolic phenotypes. The selected sample size subjects were calculated using the sensitivity power analysis of the program G * Power 3.1.9.7. based on the primary outcome of IHL% described for the OptiFiTOptiFiT study described elsewhere [65].

The power calculation the DiNA-P study was based on the primary outcome of IHL with an estimated effect size; 2.1 %-pts. (SD 4.5 %-pts) and 90 % power to detect the effect. The estimated number of participants to show these effects was 98 subjects per group. In additional, a buffer of 20% covering dropouts and insufficient compliance was added, resulting in a total of 245 subjects.

For the DiNA-P study was based on IHL with an estimated effect size; 3.0 %-pts. (SD 6.5 %-pts) and 90 % power to detect the effect. The estimated number of participants to show these effects was 100 subjects per group. In additional, a buffer of 20 % covering dropouts and insufficient compliance was added, resulting in a total of 250 subjects.

The LeguAN study power calculation was powered for IHL; 6.5 %-pts. (SD 6.5 %-pts) and 80 % power to detect effect. The estimated number of participants to show these effects was 17 subjects per group. Adding an additional buffer of 15% to cover dropouts and insufficient compliance a total of 40 subjects was calculated.

Statistical analysis

Hardy-Weinberg-equilibrium for GIPR genotype was calculated by Pearson's χ^2 test to compare the genotype and allele frequencies within prediabetic and T2DM subjects using Microsoft® Excel ®, 2016. IBM SPSS for Windows (version 20.0, Chicago, USA) was used to perform the main statistical analysis. After plausibility assessment data was examined for normality by Kolmogorov–Smirnov test. Student t-test (paired and unpaired) was computed for the comparison within and between groups (Non-normally distributed variables were analyzed by non-parametric tests (Wilcoxon or Mann-Whitney-U-Test)). In addition, BW, BMI, fasting glucose, HbA1c, TAT, VAT, and IHL were analyzed by Analysis of variance (ANOVA) for repeated measurements for

assessing GIPR SNP rs10423928-diet interaction effects. A model with genotype and diet group as between-subject factors and two within-subject factors (baseline and after dietary intervention) was used for prediabetic subjects (DiNA-P) and T2DM (DiNA-D) participants. For the LeguAN study the model was set with one between-subject factor (genotype) and two within-subject factors (baseline and after dietary intervention), thus comprising both diet groups (AP and PP) into one (HP).

3.2 Animal Study

3.2.1 Effect of central GIPR on the development of high fat-induced obesity and glucose and insulin tolerance

Animals

Experimental protocols were approved by the local governmental animal ethical committee in the State of Brandenburg, Germany. (2347-36-2017) Animals were kept in accordance with the NIH guidelines for care and use of laboratory animals.

Generation of transgenic mice

In order to understand the importance of GIPR in the human central nervous system and its effect on obesity-associated insulin resistance, a "targeting strategy" that involves the generation of a humanized GIPR was selected with the aim to generate a constitutive KO of the human brain GIPRs using Cre-mediated recombination. Human exons 5-7 were flanked by loxP sites. The positive selection markers neomycin and puromycin were flanked by FRT (neomycin resistance - NeoR, intron 7) and F3 sequences (puromycin resistance - PuroR, intron 4). The human GIPR protein is expressed under the control of the endogenous mouse GIPR promoter. Replacing the mouse GIPR genomic region in exons 3-14 with its human "counterpart" led to the loss of function of the mouse GIPR gene.

The C57BL / 6 was bred in the Jackson Laboratory. GIPR-lox / lox mice were provided by Taconic Bioscience and delivered to Prof. Wolfrum (ETH Zurich). Our GIPR-lox / lox mice were purchased from the ETH Zurich.

The constitutive KO allele was mediated by Cre/lox recombination. The use of the CaMKIIa and Nkx2.1 Cre mice allows for more specific deletions of central GIPR (CaMKIIa) as well as specifically hypothalamic GIPR.

Dietary intervention

Mice were fed with a semi-synthetic control diet (CD) (10% fat, 70% carbohydrates, 20% protein) or a high-fat diet (HFD) (fat 60%, 20% carbohydrates, 20% protein) for 14 weeks (both diets of Research Diets, Inc. New Brunswick, NJ).

Body composition

Mice BW was measured weekly using an electronic scale. Body fat and lean mass were determined at indicated times using nuclear magnetic resonance spectroscopy (Mini Spect MQ10 MRS Analyser Bruker, Karlsruhe, Germany) after three weeks and after 14 weeks on the diet.

Insulin tolerance test

Insulin tolerance test (ITT) was performed once in the eighth intervention week. Insulin (0.75 units per kg of BW) was injected intraperitoneally at the age of 13 weeks. After 2 hours of fasting, immediately before insulin application, blood samples were taken from the tail-tip for glucose measurements and at 10, 30, 60 and 120 min after.

Glucose tolerance test

To analyze glucose metabolism, a glucose tolerance test (GTT) was carried out once in the ninth intervention week. In this case, 2 g of glucose per kg of BW were administered orally using a curved button cannula after 10 hours of fasting. Blood samples were taken from the tail-tip just before glucose administration, and 15, 30, 60, and 120 minutes after glucose administration.

Sample size calculation

The information on the sample sizes is based in part on the extensive, long-term experience in the quantification of metabolic phenotypes. The selected sample size of 20 animals per group was calculated using the program G * Power 3.1.9.7 with the settings recommended for animal experiments ($\alpha = 0.05$, Power (1-ß) =80 %), an effect size of 1.0 and the variance of effect size of 0,75. Since the role of central GIPR has not yet been investigated, we assume that around 50% of the GIP effect known from whole-body KOs is centrally mediated. Due to breeding complications and delays not all planned animals could be included in the current doctoral work.

Statistical analysis

Data were analyzed using ANOVA for repeated measurements and two-tailed Student's t-test for paired and unpaired samples (Non-normally distributed variables were analyzed by non-parametric tests). In order to analyze diet effects on each genotype a model with one between-subject factor (diet) and either ITT or GTT glucose levels as within-subject factor was performed. The analysis of BW changes (%) for this model included 7 time points (week 1,4,6,8,10,12,14) in order to achieve sufficient residual degrees. For the general analysis including all genotypes, a second model was employed with two between-subject factors (Genotype and diet) and either BW changes (%), ITT, or GTT glucose levels as within-subject factor (SPSS 11.5, Chicago, IL). $P < 0.05$ was considered significant.

4. Results

4.1 Effect of dietary intervention on endothelial function, intrahepatic lipid (IHL), and adipose tissue accumulation

Description of the examined T2DM subjects

A total of 36 T2DM patients completed the intervention study with additional FMD measurements. Participants of both genders took part in the analysis (21 females and 15 males). Subjects had on average 63 years of age (range: 42–76 years), were mostly overweight to mildly obese (mean BMI = 33 ± 5 kg/m^2) with a mean HbA1c level of 6.5 ± 0.8% (47 ± 8 mmol/mol). Furthermore, 64% of all participants showed a liver fat content above the cut-off value of 5.56% for the clinical diagnosis of NAFLD [70] (mean IHL = 12.9 ± 9.5 %). About 86 % were never-smokers or former smokers.

Endothelial function

Figure 1 illustrates endothelial function variations in the LF as well as in the VLC diet ANOVA showed a significant interaction effect between diet and FMD.

Adipose tissue and IHL accumulation

Participants of both, the LF and the VLC diet, significantly reduced BW by −4.1 kg and −5.2 kg, respectively after 3 weeks of dietary intervention. Correspondingly, body fat content decreased significantly in different compartments in both diet groups and IHL content decreased extensively by 35–36% relative to the initial levels (Figure 1).

Clinical parameters

HbA1c levels decreased significantly only in the VLC group. Both dietary strategies showed comparable improvements in total cholesterol and HDL although significances were slightly greater in the LF group. Further significant improvements were observed for SBP (Table 1).

Nutritional evaluation

Moreover, the intake of energy, protein and carbohydrate showed significant differences within and between diet groups. Total fat consumption showed no significant alterations in the VLC diet, while, as expected, decreased significantly in the LF group, resulting in a significant difference between diet groups. (Table 2)

Accordingly, after 3 weeks on a LF diet, FMD showed a significant positive correlation with protein (g/kg BW) and fat intake (g/kg BW). On the other hand, the VLC group showed a significant negative correlation with protein intake (g/kg BW) (Figure 2).

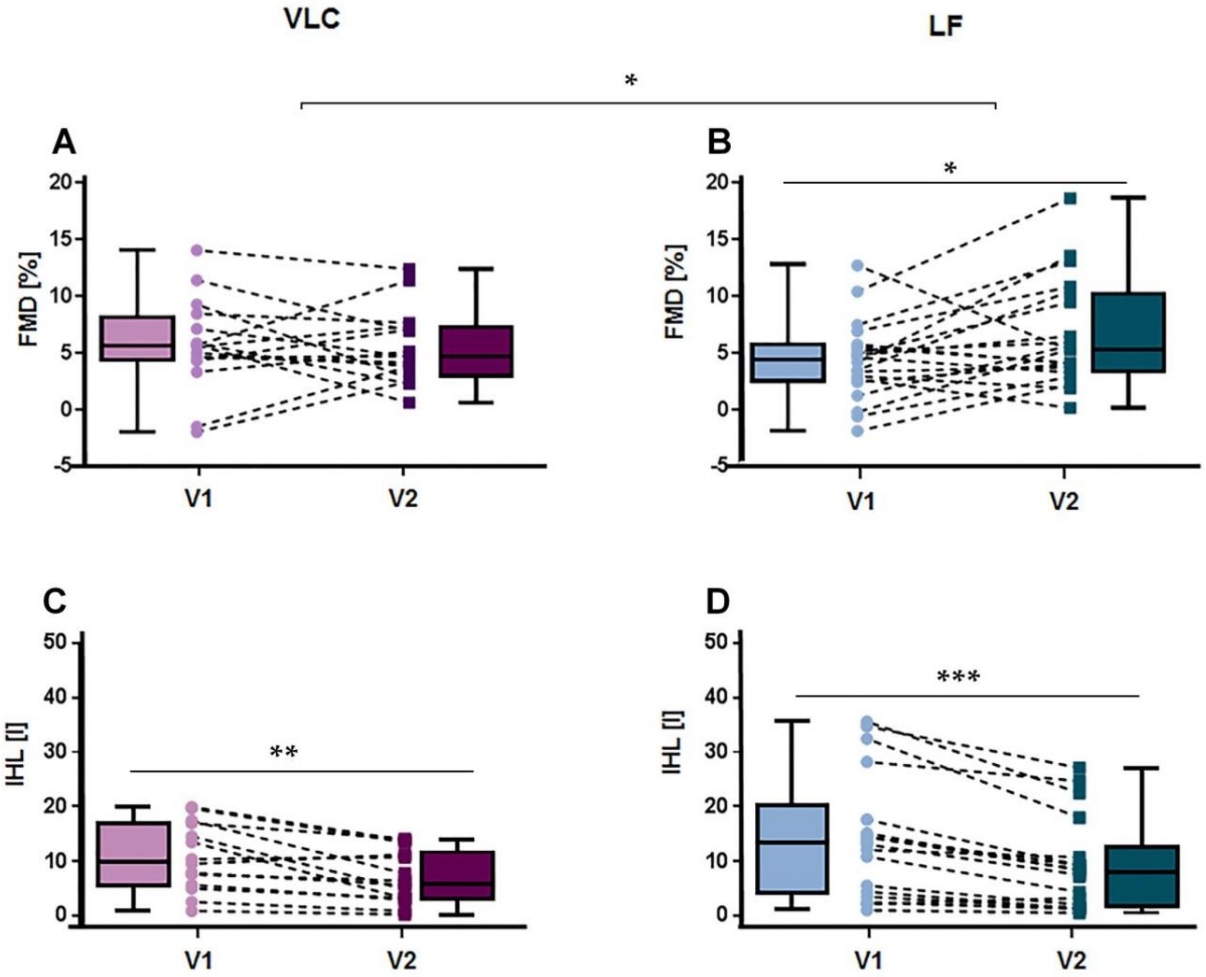

Figure 1 Acute effects on FMD and IHL after 3 weeks diet. Boxplots and individual changes in endothelial function (A) and intra hepatic lipids (C) post-intervention on the very-low-carbohydrate (VLC) diet; changes in endothelial Function (B) and intra hepatic lipids (D) after 3 weeks on the low-fat (LF) diet. FMD = flow mediated dilation, IHL = intra hepatic lipids, NVLC = 16, NLF = 20, V1 = Visit 1, V2 = Visit 2, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ [60].

Body composition and metabolism	Visit	VLC n	VLC mean	VLC ±SD	Δ	LF n	LF mean	LF ±SD	Δ	V1 vs V2 VLC p-value	V1 vs V2 LF p-value	VLC vs LF Δ p-value	VLC vs LF V1 p-value	VLC vs LF V2 p-value
Weight [kg]	V1	16	93.9	21	-4.1	20	97.6	23	-5.2	**<0.001**	**<0.001**	ns	ns	ns
	V2	16	89.8	20		20	92.5	21						
BMI [kg/m²]	V1	16	32.1	4.5	-1.3	20	32.7	4.9	-1.6	**<0.001**	**<0.001**	ns	ns	ns
	V2	16	30.8	4.3		20	31.1	4.6						
TAT [l]	V1	14	23.4	6.9	-1.7	18	22.9	5.3	-1.7	**0.001**	**<0.001**	ns	ns	ns
	V2	14	21.7	5.9		18	21.2	5.1						
VAT [l]	V1	14	5.3	1.4	-0.4	18	6.8	2.5	-0.7	**0.024**	**<0.001**	ns	**0.042**	ns
	V2	14	4.9	1		18	6.1	2.3						
IHL [%]	V1	14	10.8	6.3	-3.8	17	14.5	11	-5.2	**0.003**	**<0.001**	ns	ns	ns
	V2	14	7	4.9		17	9.3	8.4						
HbA1c [%]	V1	16	6.7	1	-0.6	20	6.2	0.6	-0.2	**<0.001**	ns	**0.001**	ns	ns
	V2	16	6.1	0.7		20	6.1	0.6						
CRP [mg/l]	V1	16	3.1	4.1	-1.3	20	3.1	2.7	-11	**0.025***	**0.008***	ns*	ns*	ns*
	V2	16	1.7	1.5		20	2	2.8						
T. CHO [mg/dl]	V1	16	200	39	-26.7	20	178	33	-31	**0.001**	**<0.001**	ns	ns	**0.042**
	V2	16	173	39		20	147	36						
HDL [mg/dl]	V1	16	56	16	-2	20	49	18	-4	ns	ns	ns	ns	ns
	V2	16	54	12		20	45	14						
LDL [mg/dl]	V1	16	127	36	-20.9	20	109	28	-26	**0.004**	**<0.001**	ns	ns	**0.035**
	V2	16	106	31		20	83	31						
TAG [mg/dl]	V1	16	133	65	-29.9	20	161	65	-35	**0.003**	**0.042**	ns	ns	ns
	V2	16	103	72		20	127	77						
SBP [mmHg]	V1	15	134	17	-9	19	134	21	-6	ns	ns	ns	ns	ns
	V2	15	126	10		19	128	19						
DBP [mmHg]	V1	15	85	11	-5	19	84	13	-6	ns	ns	ns	ns	ns
	V2	15	80	9		19	78	11						

Table 1 Anthropometric and clinical parameter. p-values of mean from t-test for analyzing differences within each group, which are presented (* = non-parametric test in the case of a missing normal distribution). BMI = body mass index, TAT = total body fat, VAT = visceral adipose tissue, IHL = intra hepatic lipids. TCHO= total cholesterol, HDL = high-density cholesterol, LDL= low-density cholesterol, TAG = triacylglycerides, CRP = C-reactive protein, HbA1c = glycated hemoglobin, SBP = systolic blood pressure in the right arm, DBP = diastolic blood pressure in the right arm, V1 = Visit 1, V2 = Visit 2 and Δ = V2 − V1. Results are shown as mean ± SD [60].

Endothelial function	Visit	n	VLC mean	±SD	Δ	n	LF mean	±SD	Δ	VLC p-value	LF p-value	visit p-value	visit x time p-value	
FMD [%]	V1	16	5.74	4.1	-0.26	20	4.32	3.5	2.27	ns	**0.024**	ns	**0.034**	
	V2	16	5.48	3.2		20	6.59	4.7						
Macronutrient intake	**Visit**	**n**	**VLC mean**	**±SD**	Δ	**n**	**LF mean**	**±SD**	Δ	VLC p-value	LF p-value	Δ p-value	V1 p-value	V2 p-value
Calories [kcal]	V1	14	2035	663	-717	15	2022	790	-1098	**<0.001**	**<0.001**	**<0.001**	ns	**0.001**
	V2	14	1318	220		15	925	61						
Protein [g/kg bw]	V1	14	0.95	0.2	0.21	15	0.94	0.2	-0.26	**0.002**	**<0.001**	**<0.001**	ns	**<0.001**
	V2	14	1.16	0.3		15	0.67	0.2						
Carbohydrate [g]	V1	14	196	69	-157	15	203	93	-85	**<0.001**	**0.006**	**0.005**	ns	**<0.001**
	V2	14	39	10		15	118	11						
Fat [%]	V1	14	40	9	14	15	38	6	-16	ns	**<0.001**	**<0.001**	ns	**<0.001**
	V2	14	54	6		15	21	2						

Table 2 FMD and macronutrient intake. Values are presented as mean (±SD), p-values of mean from t-test for analyzing differences within each group are presented. FMD was calculated using ANOVA for repeated measurements adjusted for kilocalories intake changes per kg BW. p-values of mean from t-test for analyzing difference within each group in macronutrients (and energy) are presented. p-value was based on g per kg BW. Difference significant at $p < 0.05$. FMD = flow-mediated dilation V1 = Visit 1, V2 = Visit 2 and $\Delta = V2 - V1$ [60].

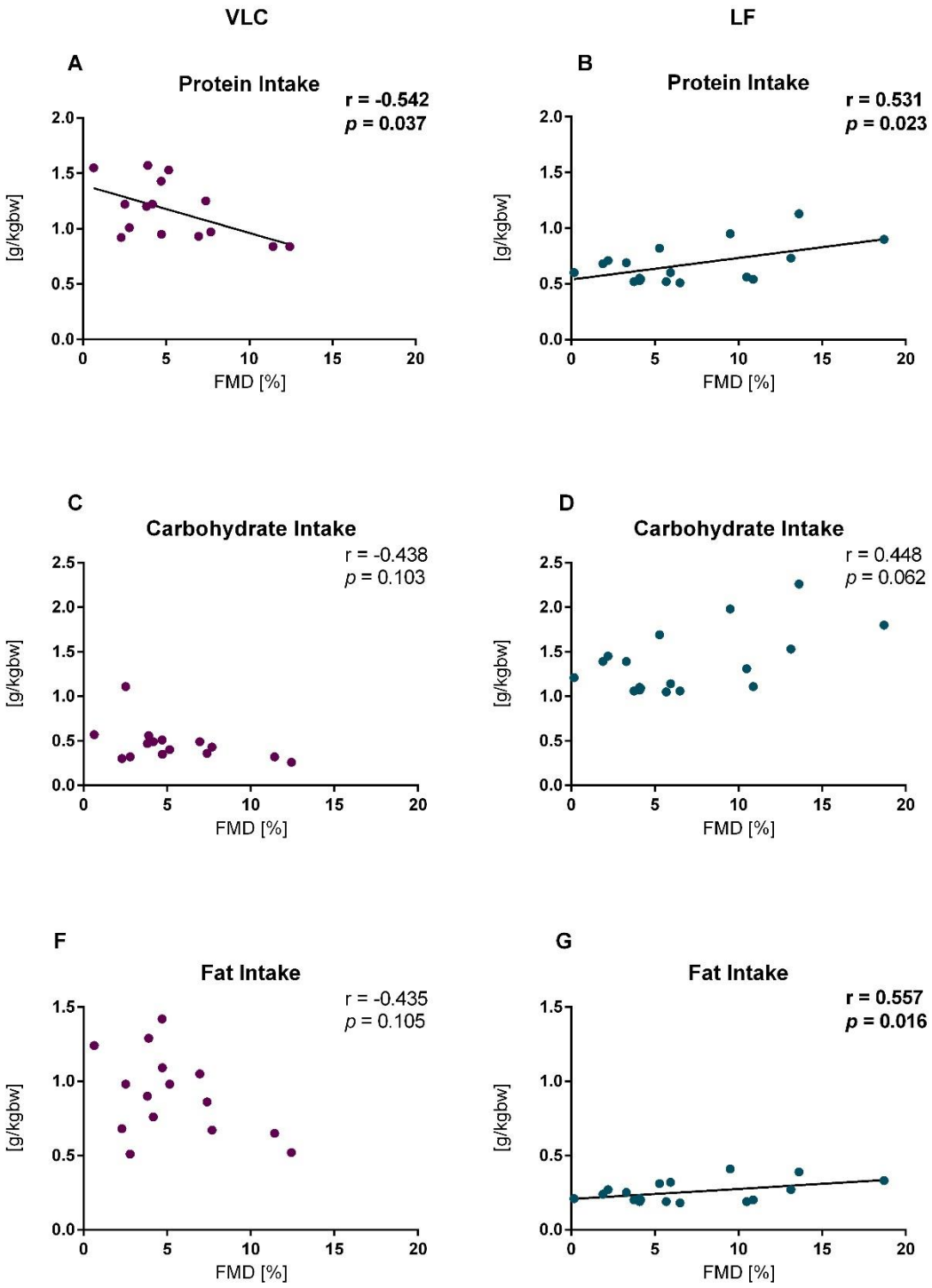

Figure 2 Correlations between flow mediated dilation (FMD) and macronutrient intake after 3 weeks diet. (A) Protein intake at Visit 2 (V2) in the very-low-carbohydrate (VLC) diet, n = 15, (B) protein intake at V2 in the low-fat (LF) diet, n = 18, (C) carbohydrate intake at V2 in the VLC diet, n = 15 (D) carbohydrate intake at V2 in the LF diet, n = 18, (E) fat intake at V2 in the VLC diet, n = 15 (F), fat intake at V2 in the LF diet, n = 18. A–F show data after macronutrient intake correction for BW at V2 [60].

4.2 Effect of GIPR SNP rs10423928 on glucose metabolism and its interaction with diverse nutritional approaches in diabetic and prediabetic subjects – Genetic Analysis

Genotype and allele frequencies of the prediabetic and T2DM cohorts are summarized in. Table 3.

rs10423928	Genotype			Allele		p-value
	HMA	HET	HMI	AF	(%)	
Prediabetic (n)	208	195	21	T	72	**0.004**
(%)	49	46	5	A	28	
T2DM (n)	35	35	3	T	72	ns
(%)	48	48	4	A	28	

Table 3 Genotypic and allelic frequencies within the cohorts. A. Values are presented as frequencies and percentages. HMA: Homozygous major allele (T/T), HET: Heterozygous (A/T). HMI: Homozygous minor (A/A). AF: Allelic frequency. P-value for HWE

Baseline glucose metabolism and insulin sensitivity

Prediabetic subjects

Baseline data of 424 prediabetic subjects (263 females, 161 males) were included for the current analysis. Participants were on average 60 years old (range: 29 - 81 y), were mostly overweight to mildly obese (mean BM I= 31.6 ± 5.6 kg/m^2) with a mean HbA1c level of 5.7 ± 0.4 %. Results from analyzed variables involving glucose metabolism and insulin sensitivity showed that A allele carriers (HET & HMI) had significantly higher levels of fasting glucose and lower 2 h glucose levels after an oral glucose challenge compared to T/T homozygous individuals. Subjects carrying the A allele also had significantly higher Cederholm Index values (Table 4 shows values presented as mean (±SD)).

T2DM subjects

A total of 73 T2DM subjects (36 females, 37 males) aged between 46-76 years were genotyped and analyzed. Individuals had a mean HbA1c level of 6.7 % and a mean BMI of 32.1 kg/m^2. We found no significant differences between rs10423928 variant carriers (HMA vs HET & HMI) regarding glucose metabolism and insulin sensitivity (Table 5).

Adipose tissue and IHL accumulation

After excluding participants, whose IHL MRI data was incomplete, no significant baseline differences between rs10423928 variant carriers (HMA vs HET &HMI) were found either in prediabetic or T2DM subjects (Table 6).

	HET & HMI			**HMA**			p-value
	n	Mean	± SD	n	Mean	± SD	
Weight [kg]	216	89	18	208	90	19	ns*
BMI [kg/m^2]	216	31	6	208	32	6	ns*
Glucose Metabolism Parameters							
Hba1c [%]	201	5.6	0.4	197	5.7	0.4	ns*
Fasting Glucose [mgdL]	216	102.0	12.3	208	99.1	11.9	**0.004***
Glucose 30 [mgdL]	213	179.1	29.2	206	177.2	25.8	ns
Glucose 60 [mgdL]	216	192.2	40.3	208	196.5	35.8	ns
Glucose 90 [mgdL]	211	171.8	41.9	206	176.9	36.3	ns
Glucose 120 [mgdL]	216	143.5	29.6	208	150.0	28.3	**0.008***
Fasting insulin [mU/l]	171	9.4	5.2	168	10.0	6.6	ns*
Insulin 60 [mU/l]	171	102.4	81.5	169	93.6	64.6	ns*
Insulin 120 [mU/l]	171	99.3	109.3	169	94.6	72.1	ns*
Fasting C-Peptid [µg/l]	168	1.6	0.8	154	1.8	1.1	ns*
C-Peptid 60 [µg/l]	168	8.0	3.8	155	7.5	3.3	ns*
C-Peptid 120 [µg/l]	168	8.7	4.1	155	8.6	4.0	ns*
Insulin Sensitivity Indices							
HOMA IR	171	2.4	1.4	168	2.5	1.8	ns*
Matsuda Index	142	6.0	3.9	105	6.6	3.8	ns*
Disposition Index	142	974.9	1130.6	105	934.6	932.8	ns*
Stumvoll Index	166	-0.6	0.2	167	-0.6	0.2	ns
Cederholm index	216	614.8	8.7	208	611.9	8.8	**<0.000***

Table 4 Baseline Glucose metabolism and insulin sensitivity within the prediabetic cohort. Values are presented as mean (±SD). p-values of mean from t-test for analyzing difference between A carriers and T/T homozygous are presented (*= non-parametric test in case of missing normal distribution). Difference significant at $p < 0.05$. HMA: Homozygous major allele (T/T), HE: Heterozygous (A/T), HMI: Homozygous minor (A/A). BMI = body mass index, HbA1c = glycated hemoglobin, HOMA IR = Homeostasis model assessment.

	HMA			HET / HMI			p-value
	n	Mean	± SD	n	Mean	± SD	
Weight [kg]	35	91.3	14.3	38	95.7	18.3	ns
BMI [kg/m^2]	35	31.8	4.3	38	32.5	4.8	ns
Glucose Metabolism Parameters							
Hba1c [%]	35	6.8	1.1	38	6.6	0.7	ns
Fasting Glucose [mg/dl]	35	133.4	47.2	36	131.9	42.8	ns*
Glucose 30 [mg/dl]	34	176.4	54.0	33	169.6	50.0	ns*
Glucose 60 [mg/dl]	35	193.1	62.2	35	181.7	53.2	ns
Glucose 90 [mg/dl]	35	168.6	65.6	35	171.7	62.7	ns
Glucose 120 [mg/dl]	34	181.2	63.4	33	180.3	63.0	ns
Fasting insulin [mU/l]	33	7.3	3.7	36	7.7	5.0	ns*
Insulin 60 [mU/l]	33	66.7	47.1	36	62.7	46.6	ns*
Insulin 120 [mU/l]	33	51.1	41.4	34	43.7	37.0	ns*
Fasting C-Peptid [µg/l]	33	1.8	0.9	36	1.9	1.0	ns*
C-Peptid 60 [µg/l]	33	3.9	2.3	36	3.6	2.4	ns*
C-Peptid 120 [µg/l]	33	5.4	2.1	35	5.1	2.6	ns*
Insulin Sensitivity Indices							
HOMA IR	33	46.6	34.0	36	44.3	30.4	ns*
Stumvoll Index	32	-0.6	0.2	32	-0.6	0.2	ns
Cederholm index	35	615.8	8.6	35	614.2	8.9	ns

Table 5 Baseline Glucose metabolism and insulin sensitivity within the T2DM cohort. Values are presented as mean (±SD), p-values of mean from t-test for analyzing difference between A-carrier s and T/T homozygous are presented (*= non-parametric test in case of missing normal distribution). Difference significant at $p < 0.05$. HMA: Homozygous major allele (T/T), HET: Heterozygous (A/T), HMI: Homozygous minor (A/A). BMI =body mass index, HbA1c = glycated hemoglobin, HOMA IR = Homeostasis model assessment

	HMA			HET / HMI			p-value
	n	Mean	± SD	n	Mean	± SD	
Prediabetic							
TAT [l]	106	20.2	7.6	153	20.7	7.1	ns*
VAT [l]	107	4.9	2.1	153	5.2	2.2	ns*
IHL [%]	107	10.2	13.6	151	9.3	7.8	ns*
T2DM							
TAT [l]	33	21.0	6.0	35	22.0	5.6	ns*
VAT [l]	33	6.3	2.3	35	5.8	2.8	ns*
IHL [%]	33	14.2	9.2	34	12.9	10.2	ns

Table 6 Baseline fat deposition within prediabetic and T2DM subjects. Values are presented as mean (±SD), p-values of mean from t-test for analyzing difference between A carriers and T/T homozygous are presented (*= non-parametric test in case of missing normal distribution). Difference significant at $p < 0.05$. HMA: Homozygous major allele (T/T), HET : Heterozygous (A/T), HMI : Homozygous minor (A/A). TAT = total body fat, VAT = visceral adipose tissue, IHL = intrahepatic lipids

GIPR SNP rs10423928-diet interaction

Prediabetic subjects

152 prediabetic subjects ((DiNA-P) nHET=84, nHMA= 68), were analyzed for the interaction between GIPR SNP rs10423928, diet, and body fat accumulation.

After excluding participants, whose IHL MRI data was missing or incomplete, a total of 89 females and 63 males with a mean age of 60.2 ± 8.7 years took part in this analysis. Participants of this study were classified as prediabetic patients and overweight with a mean HbA1c of 5.7 ± 0.4 %, an average BW of 91.2 ± 18.6 kg, and a mean IHL content of 10.8 ± 8.1% at baseline. Results of prediabetic subjects showed that after 3 weeks on a hypocaloric VLC or LF diet subjects reduced BW, BMI, total and visceral body fat depots content independently of the diet type and genotype. Fasting glucose and IHL reduction showed a significant diet-SNP rs10423928 interactions effect (Table 1). Participants carrying the A-allele showed a greater reduction of liver fat under the VLC diet, on the contrary, T/T subjects showed a greater reduction of liver fat in response to the LF diet (Figure 3).

T2DM subjects

64 T2DM subjects ((DiNA-D nHET=16, nHMA= 17, (LeguAN) nHET=17, nHMA= 14) were analyzed for the interaction between GIPR SNP rs10423928, diet and body fat accumulation.

According to ANOVA for repeated measurement, GIPR SNP rs10423928 did not significantly influence improvements in DiNA-D study (Table 7). On the other hand, GIPR SNP rs10423928 showed a significant diet x SNP influence for IHL response in 31 T2DM subjects who underwent an HP diet for 6 weeks (LeguAN study). Participants presented at baseline with a mean age of 64.5 ± 6.0 years and the mean BMI was 30.6 ± 3.7 kg/m². Most participants (80%) had an IHL content above the cut-off value of 5.56% for the clinical diagnosis of NAFLD [32].

Figure 3 displays the analysis performed within and between genotypes. Liver fat content was determined at baseline and after 6 weeks HPD. A highly significant reduction of IHL in both genotypes was observed. However, the HMA (T/T) group showed significantly greater responses to the HPD compared with HET (T/A) group, having an IHL reduction almost twice as large. No significant difference was found between genotypes for the reduction in BW, BMI, TAT and VAT (Table 7).

	HET			HMA			Time	Time x SNP	Time x Diet	Time x Dietx SNP
	n	Δ mean	±SD	n	Δ mean	±SD	p-value	p-value	p-value	p-value
Prediabetes										
Weight [Kg]	81	-3.69	2.06	66	-3.86	2.37	<0.001	ns	ns	ns
BMI [kg/m^2]	61	-1.23	0.62	52	-1.36	0.81	<0.001	ns	ns	ns
Fasting Glucose [mgd	53	-0.45	0.71	43	-0.18	0.57	<0.001	**0.038**	ns	ns
Hba1c [%]	57	-0.07	0.47	44	-0.08	0.31	0.03	ns	ns	ns
TAT [%]	81	-1.53	1.67	61	-1.78	2.67	<0.001	ns	ns	ns
VAT [%]	81	-0.45	0.59	61	-0.41	0.95	<0.001	ns	ns	ns
IHL [%]	84	-4.75	4.27	68	-4.63	4.00	<0.001	ns	ns	**0.012**
Diabetes DINA-D										
Weight [Kg]	16	-4.39	3.11	17	-5.06	2.66	**<0.001**	ns	ns	ns
BMI [kg/m^2]	16	-1.42	0.98	17	-1.68	1.12	**<0.001**	ns	ns	ns
Fasting Glucose [mgd	13	-27.65	23.67	9	-35.03	24.55	**<0.001**	ns	ns	ns
Hba1c [%]	16	-0.37	0.53	17	-0.32	0.44	**<0.001**	ns	**0.001**	ns
TAT [%]	16	-1.72	1.41	17	-2.02	2.30	**<0.001**	ns	ns	ns
VAT [%]	16	-0.39	0.50	17	-0.72	0.67	**<0.001**	ns	ns	ns
IHL [%]	16	-4.30	4.46	17	-3.99	5.02	**<0.001**	ns	ns	ns
LeguAN										
Weight [Kg]	17	-2.35	1.88	14	-1.71	1.43	**<0.001**	ns		
BMI [kg/m^2]	17	-0.79	0.61	14	-0.64	0.54	**<0.001**	ns		
Fasting Glucose [mgd	17	-0.86	1.16	14	-0.40	0.89	**0.002**	ns		
Hba1c [%]	17	-0.42	0.33	14	-0.44	0.61	**<0.001**	ns		
TAT [%]	16	-0.44	1.10	14	-0.69	0.78	**0.003**	ns		
VAT [%]	16	-0.34	0.84	14	-0.48	0.45	**0.003**	ns		
IHL [%]	17	-4.97	3.21	14	-8.72	6.08	**<0.001**	**0.036**		

Table 7 SNP interaction effect. Values are presented as mean of changes (±SD), p-values of ANOVA for repeated measurements for analyzing SNP interaction´s effect. Difference significant at $p < 0.05$. HMA: Homozygous major allele (T/T), HET: Heterozygous (A/T). BMI =body mass index, HbA1c = glycated hemoglobin, TAT = total body fat, VAT = visceral adipose tissue, IHL =intra hepatic lipids

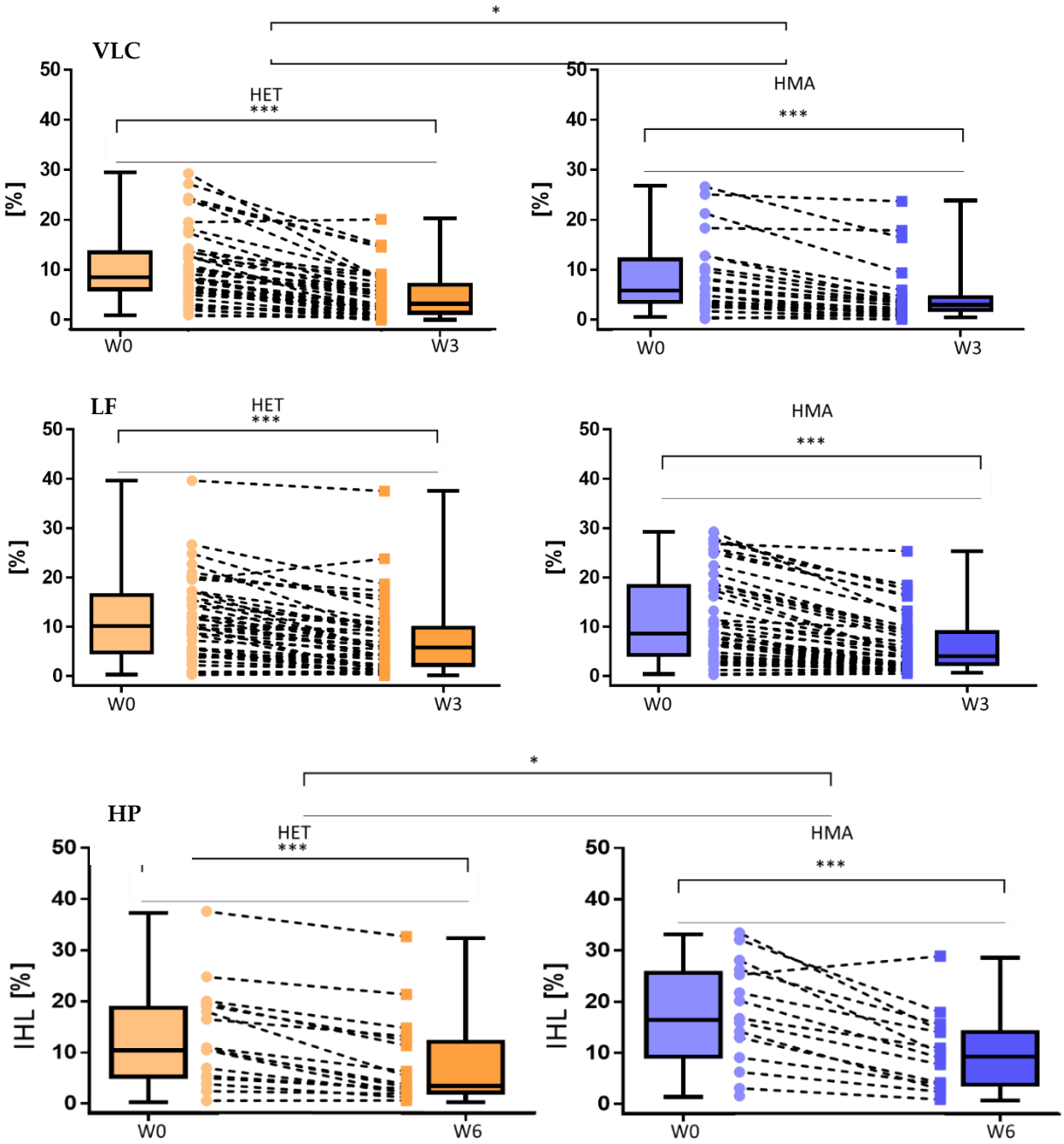

Figure 3 Effects on IHL after 3 weeks either on a VLC or LF diet in SNP rs10423928 in prediabetic subject and after 6 weeks HPD diet in SNP rs10423928 in T2DM subject. Boxplots and individual changes in intra hepatic lipids. VLC: nHET= 40, nHMA=24 LF: nHET= 44, nHMA=44 HPD: nHET= 17, nHMA=24. Results are shown as mean ± SD: P values from Student t-test (paired and unpaired) or non-parametric tests (Wilcoxon or Mann-Whitney-U-Test)∗p < .05; ∗∗p < .01; ∗∗∗p < .001

4.3 Effect of central GIPR on the development of high fat-induced obesity and glucose and insulin tolerance

A total of 34 mice were included in this analysis (n $hGIPR^{+/+}$= 12, n $hGIPR^{CNS-/-}$ =10 and n $hGIPR^{HYPO-/-}$ = 12). Genotype and sex frequencies of each diet group are summarized below in Table 8.

		$hGIPR^{+/+}$	$hGIPR^{CNS-/-}$	$hGIPR^{HYPO-/-}$
		n	**n**	**n**
CD	Female	3	2	3
	male	3	3	3
HF	Female	3	2	3
	male	3	3	3

Table 8 Genotype and gender frequencies. hGIPR+/+= control mice, hGIPRCNS-/- = central KO mice, hGIPRHYPO-/- =Hypothalamic KO mice.

Body weight and body composition

Control mice

As expected, throughout the intervention mice ($hGIPR^{+/+}$) gradually gained BW with a significant percent difference between diet groups (Figure 3A) with a significant diet effect. After 14 weeks on an HFD, control mice experienced a significant increase of FM (g), compared to the measurements on the third week (Figure 4B). This increase was significantly lower in the CD fed group. There was no significant difference in LM between diet groups (Table 9).

Deletions of central GIPR

To investigate the role of GIPR in obesity and T2DM development, we first determined the effect of its central deletion ($hGIPR^{CNS-/-}$). Figure 3A shows the effect of central KO on BW percentual changes over each intervention week. Analysis of ANOVA for repeated measurements showed that during 6–14 weeks of age the $hGIPR^{CNS-/-}$ mice increased BW percent changes with a significant difference between diet groups. After 3 and 14 weeks, FM (g) did not show significant alterations between nor within diet groups. (Figure 4B) (Table 9). CD fed mice exhibited a significant increase of LM within 11 weeks.

Deletions of hypothalamic GIPR

Furthermore, we assessed the effect of specific hypothalamic deletion ($hGIPR^{HYPO-/-}$). BW percent changes did not differ significantly between diet groups over 14 weeks according to ANOVA analysis. Figure 4C illustrates the analysis of single time points. No significant difference was detected for FM, however, LM increased significantly in both diet groups. Figure 3D displays differences in FM (g), $hGIPR^{HYPO-/-}$ exhibited significantly lower FM content compared to control mice ($p = 0.010$) after 3 weeks on a HFD diet with a mean difference of 1.4 g. (Table 9 shows values presented as mean (±SD). The second ANOVA model with two between-subject factors (Genotype and diet) showed a significant diet effect, but there was no significant genotype effect nor diet-genotype interaction.

Insulin and glucose tolerance test

Control mice

Control mice on HFD diet showed significantly decreased insulin sensitivity and glucose tolerance compared to CD-fed mice. Furthermore, glucose area under the curve (AUC) was significantly higher for the HFD group. Mice on a HFD also had significantly increased fasting glucose levels.

Deletions of central GIPR

During the ITT, a significant re-increase in plasma glucose levels in the HFD group compared to control diet group was found. (Figure 4F includes analysis of single time points). This distinction was also revealed for ITT AUC. To determine glucose tolerance, mice fasted for 12 hours, thereafter GTT was performed. Independently, no significant difference between diet groups was found. Although fasting glucose levels were significantly higher in HFD fed mice (Figure 4E) (Table 9).

Deletions of hypothalamic GIPR

$hGIPR^{HYPO-/-}$ mice showed no significant differences between diet groups for insulin sensitivity nor glucose tolerance. Hypothalamic KO mice fed with an HFD had lower glucose levels throughout ITT compared to control mice ($hGIPR^{+/+}$) fed with the same diet. Figure 4F illustrates the differences of each time point.

GTT ANOVA analysis with two between-subject factors (Genotype and diet) showed a significant effect of diet, but there was no significant genotype nor diet-genotype interaction effect.

Interestingly, for ITT, the diet had a significant effect. Nevertheless, diet-genotype interaction was not evident.

	hGIPR$^{+/+}$						hGIPR$^{CNS-/-}$						hGIPR$^{HYPO-/-}$					
	CD			HF			CD			HF			CD			HF		
Body weight	**n**	**mean**	**±SD**	**n**	**mean**	**±SD**	**n**	**mean**	**±SD**	**n**	**mean**	**±SD**	**n**	**mean**	**±SD**	**n**	**mean**	**±SD**
Baseline [g]	6	18.0	2.1	6	17.7	2.6	5	17.9	2.1	5	17.6	1.9	6	16.5	1.7	6	18.3	2.8
Week 1 [g]	6	19.5	4.0	6	19.2	3.1	5	18.8	2.6	5	18.9	3.2	6	17.6	2.3	6	19.4	3.8
Week 2 [g]	6	20.1	4.9	6	20.3	3.5	5	19.2	2.9	5	20.3	3.7	6	18.2	2.8	6	20.2	4.2
Week 3 [g]	6	21.3	4.9	6	21.5	4.0	5	19.9	3.0	5	21.6	3.9	6	19.4	3.4	6	21.6	4.4
Week 4 [g]	6	21.6	4.7	6	22.8	4.7	5	20.0	3.0	5	22.7	4.6	6	19.6	3.0	6	22.0	4.6
Week 5 [g]	6	21.1	4.7	6	23.5	5.3	5	20.9	3.5	5	23.8	4.9	6	20.6	2.6	6	22.8	4.8
Week 6 [g]	6	22.1	5.2	6	24.9	6.0	5	21.9	3.7	5	25.0	5.3	6	21.1	2.9	6	24.2	5.0
Week 7 [g]	6	22.3	4.8	6	27.0	5.8	5	22.0	3.1	5	26.0	5.9	6	21.3	3.2	6	24.0	5.6
Week 8 [g]	6	22.0	4.5	6	26.1	5.7	5	22.3	3.5	5	25.7	5.7	6	22.0	4.1	6	24.5	5.8
Week 9 [g]	6	23.7	4.4	6	25.7	6.6	5	22.9	3.6	5	26.7	5.0	6	23.2	3.1	6	25.9	5.8
Week 10 [g]	6	23.6	5.4	6	26.1	7.4	5	22.0	3.0	5	26.8	6.0	6	22.8	4.1	6	26.5	7.2
Week 11 [g]	6	24.5	5.8	6	27.7	7.4	5	22.5	3.3	5	27.8	7.6	6	22.9	4.2	6	28.2	8.5
Week 12 [g]	6	23.9	5.8	6	29.6	8.3	5	22.6	3.2	5	28.4	7.7	6	22.5	4.3	6	28.7	9.4
Week 13 [g]	6	24.2	5.9	6	30.1	8.4	5	22.6	2.7	5	27.3	6.1	6	23.0	4.2	6	28.3	8.7
Week 14 [g]	6	24.5	6.0	6	32.0	8.1	5	23.2	3.3	5	28.9	6.2	6	23.3	4.7	6	29.7	9.3
Week 1 [%]	6	7.3	9.5	6	8.6	2.8	5	5.3	3.7	5	7.3	7.2	6	6.4	5.2	6	5.4	6.0
Week 2 [%]	6	10.5	14.0	6	14.8	3.6	5	7.4	4.6	5	14.8	9.5	6	10.0	8.8	6	9.8	9.0
Week 3 [%]	6	17.1	13.2	6	21.4	5.4	5	11.3	4.7	5	22.4	9.8	6	16.9	12.0	6	17.4	8.9
Week 4 [%]	6	18.9	11.9	6	28.3	9.1	5	11.9	4.4	5	28.3	12.9	6	18.5	11.9	6	19.5	9.7
Week 5 [%]	6	16.4	12.7	6	31.9	12.0	5	16.7	6.6	5	34.5	14.8	6	24.6	9.7	6	23.7	9.9
Week 6 [%]	6	21.8	14.6	6	39.7	14.8	5	21.8	7.7	5	41.4	16.3	6	27.6	10.6	6	31.5	9.4
Week 7 [%]	6	22.8	12.5	6	51.8	18.6	5	22.8	5.8	5	46.6	19.1	6	28.8	13.1	6	30.1	12.9
Week 8 [%]	6	21.3	11.4	6	46.5	13.3	5	24.2	6.2	5	45.3	18.7	6	32.5	16.3	6	32.5	14.2
Week 9 [%]	6	30.8	9.7	6	44.1	20.7	5	27.9	7.3	5	51.4	16.4	6	40.3	10.9	6	40.4	12.8
Week 10 [%]	6	30.0	15.0	6	46.0	24.4	5	23.4	7.6	5	51.2	20.3	6	37.7	16.6	6	43.1	19.5
Week 11 [%]	6	34.4	16.1	6	54.9	21.1	5	25.7	8.9	5	56.3	28.1	6	38.1	17.3	6	51.1	25.2
Week 12 [%]	6	31.4	16.6	6	64.8	23.6	5	26.2	7.4	5	59.9	28.4	6	35.8	18.0	6	53.9	30.0
Week 13 [%]	6	33.0	17.6	6	67.7	23.5	5	26.5	6.6	5	54.3	21.5	6	39.0	16.8	6	51.6	25.8
Week 14 [%]	6	34.6	17.6	6	79.1	20.3	5	29.5	8.9	5	63.6	21.7	6	40.4	20.0	6	59.3	28.4
FM [g]																		
wk 3	6	3.9	2.1	6	3.6	1.0	5	2.6	0.4	5	3.0	0.9	6	2.4	0.6	6	2.2	0.4
wk 14	6	4.2	3.4	5	10.7	4.7	5	2.4	0.6	5	7.6	4.3	6	2.4	0.6	6	6.9	4.7
LM [g]																		
wk 3	6	15.4	3.1	6	15.6	2.8	5	15.7	3.0	5	16.3	3.1	6	15.0	2.6	6	17.3	4.5
wk 14	6	18.5	2.9	5	20.9	3.3	5	19.0	2.9	5	19.0	2.2	6	19.3	3.7	6	21.0	4.6
Glucose Tolerance [glucose in mmol/l]																		
0 min	6	7.2	0.3	6	10.3	1.6	5	7.9	1.8	5	9.8	1.3	5	7.9	1.8	5	9.8	1.3
15 min	6	6.3	1.0	6	8.8	1.3	5	6.5	0.5	5	9.3	1.9	5	6.5	0.5	5	9.3	1.9
30 min	6	5.7	1.8	6	7.9	1.5	5	5.6	1.1	5	7.0	0.8	5	5.6	1.1	5	7.0	0.8
60 min	6	5.2	2.0	6	8.1	2.0	5	5.6	1.2	5	7.2	1.3	5	5.6	1.2	5	7.2	1.3
120 in	6	6.8	1.2	6	9.6	1.4	5	6.4	0.9	5	10.2	2.3	5	6.4	0.9	5	10.2	2.3
AUC	6	727.3	158.3	6	1041.5	150.9	5	745.2	104.9	5	1004.7	139.8	5	745.2	104.9	5	1004.7	139.8
Insulin Tolerance [glucose in mmol/l]																		
0 min	6	4.4	0.2	6	5.5	0.5	5	4.4	0.5	5	6.1	1.4	5	4.4	0.5	5	6.1	1.4
15 min	5	17.2	4.3	6	19.6	2.9	5	16.8	2.3	5	16.5	1.1	5	16.8	2.3	5	16.5	1.1
30 min	5	15.7	4.2	6	24.4	2.7	5	19.7	5.0	5	22.5	3.4	5	19.7	5.0	5	22.5	3.4
60 min	5	10.2	2.2	6	18.0	4.0	5	13.3	4.1	5	16.9	4.3	5	13.3	4.1	5	16.9	4.3
120 in	5	7.9	1.9	6	8.7	1.9	5	8.4	1.6	5	9.2	1.5	5	8.4	1.6	5	9.2	1.5
AUC	5	1474.8	240.8	6	2072.0	210.2	5	1685.4	335.7	5	1907.1	268.2	5	1685.4	335.7	5	1907.1	268.2

Table 9. Detailed BW and composition over 14 weeks and ITT and GTT glucose levels Values are presented as mean (±SD), M= body fat mass, LM = lean mass. hGIPR$^{+/+}$= control mice, hGIPR$^{CNS-/-}$ = central KO mice, hGIPR$^{HYPO-/-}$ =Hypothalamic KO mice, CD: control diet, HFD: high-fat diet.

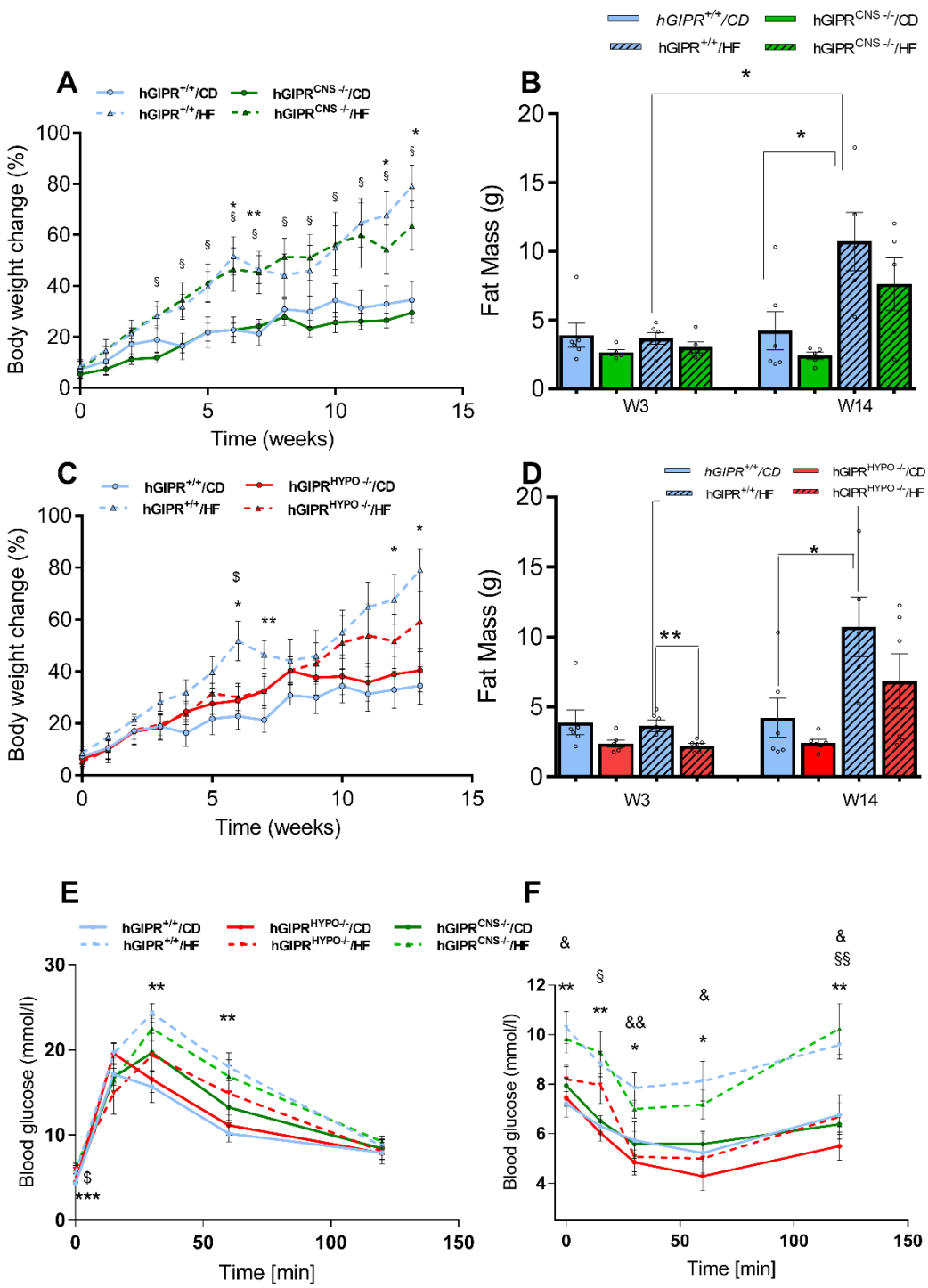

Figure 4 Body weight, body composition and Insulin and glucose tolerance test. hGIPR$^{+/+}$= control mice, hGIPR$^{CNS-/-}$ = central KO mice, hGIPR$^{HYPO-/-}$ = hypothalamic KO mice. CD: control diet, HFD: high fat diet. CD: nhGIPR$^{+/+}$ = 6, n hGIPR$^{HYPO-/-}$ =6 LF: n hGIPR$^{CNS-/-}$ = 5, HFD nhGIPR$^{+/+}$ = 6, nhGIPR$^{HYPO-/-}$ =6 LF: n hGIPR$^{CNS-/-}$ = 5. Results are presented as mean ± SEM. P values from Student t-test (paired and unpaired) or non-parametric tests. ∗p < 0.05; ∗∗p < 0.01; ∗∗∗p < 0.001. A, C: BW perceptual alterations over 14 wk. CD vs HFD = *Significance within hGIPR$^{+/+}$, § Significance within hGIPR$^{CNS-/-}$, $ Significance between hGIPR$^{+/+}$and hGIPR$^{Hypo-/-}$. B, D: Body fat mass after 3 and 14 wk. * represent all significances. E, F: Insulin and glucose tolerance test. CD vs HFD = *Significance within hGIPR$^{+/+}$, § Significance within hGIPR$^{CNS-/-}$, $^{$}$ Significance of hGIPR$^{Hypo-/-}$, $^{&}$ significance between hGIPR$^{+/+}$ and hGIPR$^{Hypo-/-}$. Results of ANOVA for repeated measurements are not illustrated.

5. Discussion

5.1 Effect of dietary intervention on endothelial function, intrahepatic lipid (IHL) and adipose tissue accumulation

Our first aim was to investigate the effect of a LF vs a VLC diet in T2DM subjects while paying particular attention to IHL accumulation and endothelial function as independent measures for comorbidities. Both dietary strategies, VLC and LF, induced a significant reduction of BW, TAT, VAT and IHL, tCHO, LDL and TAG. In addition, only the VLC diet reduced significantly HbA1c levels after three weeks. These results suggest that the VLC diet has an acute effect on risk factors, previously associated with damaged endothelial function[23, 24, 71]. Interestingly, our results suggest that the LF diet had an acute vasodilation effect compared to the VLC diet, whereby the LF diet showed a significant increase of 2.27%. In fact, this beneficial effect of LF diets has already been described in previous studies [23, 24, 34, 71].

After three intervention weeks, the VLC diet showed a negative correlation between FMD and protein intake, while the LF diet showed a positive correlation. The VLC diet nature is an increase in animal protein sources (meat, cheese), while the meal replacement powder of LF diet is rich in isolated milk protein and contains defined fats. Milk proteins are high quality proteins based on bioavailability, digestibility, amino acid requirements, and bioactive peptides. Currently, there is substantial interest in whey protein and casein to improve vascular health, which has been extensively reviewed [72]. Different research groups showed the positive effect of whey protein [73, 74] and casein [75] supplementation on FMD. Milk protein can decrease the levels of soluble adhesion molecules sVCAM-1 and sICAM-1 [72, 76], biomarkers of endothelial dysfunction. Certainly, protein consumption is often tied to fat consumption, animal-based protein consumption is associated with a higher intake of saturated fats, which are disadvantageous to cardiovascular health. However, results revealed that in the LF diet group subjects with the lowest fat consumption had the lowest FMD, while subjects with the highest intake of fat had the highest FMD. On the contrary, in the VLC group (although the correlation was not significant) subjects with the lowest fat consumption tended to have the highest FMD, and subjects with the highest intake of fat had the lowest FMD. This endothelial response can be linked to the different fatty acid composition of the diets. A study demonstrated that the exchange of saturated fatty acids (SFA) by monounsaturated fatty acids (MUFA) increased endothelium-dependent vasodilatation [77]. Essential unsaturated fatty acids, such as the n-3 FFA alpha-linolenic acid (ALA) and the n-6 FFA

linoleic acid serve as precursors to bioactive lipids of the prostanoid family with powerful endothelial activity [78], however, the supplementation of n-3 polyunsaturated fatty acids (PUFA) did not confirm an improved FMD in a study [79]. It is worth mentioning that the VLC in this study was meant to be rich in unsaturated fatty acids. Thus, we advocate the beneficial effect of the LF diet on the strategic role of proteins in modulating fat metabolism particularly modulating free fatty acids (FFA) and lipolytic activity [66]. In this context, FFA are considered as inductors of oxidative stress and inflammation in endothelial and vascular smooth muscle cells [80, 81].
Furthermore, we previously demonstrated that LF diet up-regulates GLUT3 expression and down-regulates GLUT5 expression in subcutaneous AT of T2DM patients [82]. Among the fructose transporters, GLUT5 is the only transporter specific for fructose with no affinity for glucose or galactose. A hypoxic state is common during the progression of obesity accompanied by inflammatory processes and is associated with increases in GLUT5 expression in adipocytes [83, 84]. Adipose tissue inflammation induces systemic inflammation, which leads to an atherogenic state [85].
Several studies investigating the effect of LF and VLC diets on FMD have been performed in the past years. Phillips et al. [24] and Varady et al. [23] observed a similar endothelial LF benefit after 6 weeks of diet. However, both research groups also observed a significant decrease in endothelial function after the VLC diet. Our study cannot confirm this observation, since we found no significant reduction in this diet group. In contrast to the VLC diet of the DINA-D study the high fat (VLC) diet of previously mentioned studies was characterized by a carbohydrate intake of ca. 20 g/d and by a higher intake of saturated fatty acids (SFA), which probably has adverse effects on endothelial function [52, 53].

5.2 Effect of GIPR SNP rs10423928 on glucose metabolism and its interaction with diverse nutritional approaches in diabetic and prediabetic subjects - genetic analysis

In order to treat and prevent T2DM and its co-morbidities based on dietary strategies, it is necessary to consider genetic mechanisms in this already complex interplay. Therefore, our second objective was to examine the effect of GIPR SNP rs10423928 and its interaction with diverse nutritional approaches in diabetic and prediabetic subjects. Prediabetic subjects showed a significantly higher fasting glucose but lower 2 h glucose levels after an oral glucose challenge compared to individuals homozygous for the major allele T in subjects. A-carriers also had significantly higher Cederholm Index values, indicating a higher insulin sensitivity. Accordingly,

a study, which only included T2DM subjects, showed that carriers of the A allele had a better insulin sensitivity perhaps attributable to lower osteopontin (OPN) levels, a cytokine, which is specifically abundant in adipose tissue of obese individuals. Therefore, reduced GIPR function may be a mechanism explaining the A allele protective effect. GIP stimulates OPN expression, promoting adipose tissue inflammation and insulin resistance [21]. The lack of effect in the T2DM cohort could be attributed to the medication for management of blood glucose and/or the smaller sample size. However, selection bias should be taken into consideration, since healthier subjects were omitted from the study, and/or subjects with diabetes with very high glucose levels and insulin therapy were not included.

However, the impact of GIPR SNP rs10423928 variant on glucose metabolism and insulin sensitivity is a matter of speculation. Saxena et al., [48] showed a link between GIPR A allele variant and T2DM. Another study in T2DM and non-diabetic subjects associated the A allele of GIPR rs10423928 with impaired glucose- and GIP-stimulated insulin secretion [20].

Moreover, our outcomes suggest a potential diet-gene interaction on IHL response in prediabetes and T2DM subjects.

Prediabetic participants carrying the A-allele showed greater improvement of liver fat accumulation in response to a VLC diet, while T/T subjects had a greater benefit due to the LF diet. After six weeks on a HP diet, GIPR SNP s10423928 T homozygous individuals with T2DM showed a significantly greater response compared to A-allele carriers, having an IHL reduction almost twice as large. The potential mechanisms underlying these findings are unknown, however, considering the direct effect of nutrition on endogenous GIP secretion, genetic variants in GIPR are excellent candidates of potential diet-gene interactions. Such interactions have been already described for the A variant of GIPR rs10423928 with coffee, sucrose and fiber intake [86, 87]. Epidemiological evidence suggests that dietary intake of carbohydrate and fat could potentially modulate the T2DM risk depending on the GIPR genotype. Homozygous for AA, non-diabetic subjects consuming high-fat, low-carbohydrate diets showed reduced risk. On the contrary, T/T genotype showed lower T2DM risk when consuming a high-carbohydrate/ low-fat diet [87]. To the best of our knowledge, this is the first study to date to assess the relationship of GIPR rs10423928 – diet interactions on body fat accumulation. Further studies perhaps with a larger sample size are needed in order to provide novel evidence for the development of effective dietary strategies based on the genetic background in T2DM patients.

5.3 Effect of central GIPR on the development of high fat-induced obesity and glucose and insulin tolerance

The interruption of GIPR signaling has a protective effect against the development of T2DM dysfunctional network induced by an obesogenic environment. The distribution of GIP and its receptor in the brain suggests a key function on food intake and energy homeostasis. Therefore, we aimed to investigate whether the central GIPR signaling is involved in the development of HFD-induced fat mass accumulation, as well as impaired glucose tolerance and insulin sensitivity. In order to generate specific statements about GIPR in the brain, crossing with the respective Cre mice with homozygous flanked humanized GIPR animals ($hGIPR^{+/+}$) was the method of choice. The use of the CaMKIIa and Nkx2.1 Cre mice allowed specific deletions of central GIPR ($hGIPR^{CNS-/-}$) as well as explicitly hypothalamic GIPR ($hGIPR^{HYPO-/-}$). After 14 weeks on an HFD hypothalamic KO mice appeared to be protected against HFD-induced weight gain and loss of insulin sensitivity compared to CD-fed mice. $hGIPR^{HYPO-/-}$ animals also gained less FM after 3 weeks in an HFD compared to control animals with a variation of 1.4 g.

As expected, HFD fed control animals gained weight proportionally and showed a BW gain change of 45%, compared to their respective control diet group. Central abolition of GIPR resulted in an HFD -induced increase of 34 % of BW, while hypothalamic abolition of 19 % of BW (compared to the same genotype on a CD). In fact, CD feed and HFD feed $hGIPR^{HYPO-/-}$ mice did not show significant differences in BW over 14 weeks. Furthermore, we demonstrated that GIPR deletion in the hypothalamus significantly preserved insulin sensitivity independent of dietary fat content. Comparable studies investigating the full deletion of GIPR repeatedly demonstrated the protective effect of this measure. Mice lacking GIPR exposed to an obesogenic environment (ovariectomy, high-fat diet, or sucrose-rich diet) do neither exhibit BW gain, liver steatosis, nor an increase in visceral and subcutaneous fat mass compared to wild type mice [43, 52, 88]. Since we did not obtain any major results with $hGIPR^{CNS-/-}$ mice, it is tempting to speculate that results observed by previous study groups are in part due to the interruption of hypothalamic GIPR signaling. The hypothalamus is a well-established appetite control center. Signal hormones, neuropeptides, and other messengers promote hyperphagia, hypophagia, or both. The secretin family of peptides that includes GIP is involved in a variety of brain functions, including neuro-modulation, neurogenesis, brain development, regulation of food intake, BW, and body temperature [89, 90]. Correspondingly, a study found increased mRNA of anorexigenic genes of POMC and cocaine- and amphetamine-regulated transcript (CART) in GIPR KO mice exposed to

a high glycaemic index diet. On the contrary, mice exposed to a low glycaemic index diet had a decreased expression of the orexigenic Agouti-related protein (AgRP) [54]. This suggests that hGIPR$^{HYPO-/-}$ mice could have been affected by inhibition of appetite and food intake. However, a recent in-vitro study showed that direct activation of hypothalamic GIPR+ cells potently impairs food intake [58], which would lead to increased food intake in our hGIPR$^{HYPO-/-}$ model. Another possibility of how the deletion of hypothalamic GIPR hindered weight gain and maintained insulin sensitivity despite a HFD relies on shielding of leptin signaling by the decreased hypothalamic level of Suppressor Of Cytokine Signaling 3 (SOCS3) [91]. Hypothalamic SOCS3 impairs leptin sensitivity. Most recognized biological actions of leptin are inhibition of food intake and stimulation of insulin sensitivity and metabolic rate. Studies based on larger sample size and molecular pathways analysis are required to establish the real role of hypothalamic GIPR for the development of T2DM dysfunctional network.

6. Conclusion

In conclusion, we demonstrate that both dietary strategies are efficient tools for reducing several risk factors for CVD, such as BW, TAT, VAT, IHL, blood glucose, and blood lipids in T2DM patients. However, only the LF diet provided positive effects on flow-mediated arterial dilation due to a protein-fat metabolism interaction. Our results on GIPR SNP rs10423928 analysis suggest an advantageous effect of minor allele A contributing to improved ß-cell response and a potential diet-SNP effect on IHL response. A previous work of our study group showed that prediabetic subject on the VLC had decreased blood pressure compared to the LF diet subjects, Furthermore, the LF diet had a greater reduction in LDL-cholesterol and less inflammation [92]. This was also demonstrated by several Meta-analyses [16, 93]. However, Mansoor et al., concluded: “Further investigations are needed, and may contribute to an improved understanding of the large variability in individual response to dietary intervention”. It would be of interest to investigate if GIPR SNP rs10423928 does have an impact on individual response to dietary intervention on endothelial function and further CVD risk factors. It has been previously reported that GIP induces Endothelin-1, osteopontin (OPN) [94], and other pro-inflammatory molecules [72, 76], which promote atherosclerosis. Vasodilatory nitric oxide (NO) [95] and foam cell formation [96] also seem to be involved in GIP/GIPR interaction. Interestingly, a study of GIPR SNP rs10423928 showed that A allele carriers have reduced receptor function, lower adipose tissue OPN mRNA levels and better insulin sensitivity [51] Additionally, adipocytes, and macrophages communicate

involving MCP-1, which introduces adipose tissue inflammation via GIP signaling [97]. Deletion of adipocyte GIPR signaling led to decreased expression of interleukin-6 (IL-6), a pro-inflammatory cytokine, in HFD fed mice [69] These events are closely connected to CVD since adipose inflammation induces systemic inflammation and atherogenic state [85]. Furthermore, we reported that GIPR hypothalamic signaling could be in part responsible for HFD -induced BW gain and insulin resistance. We discussed above its possible implications on protecting leptin signaling, which could contribute to endothelial function, perhaps by upregulating vascular endothelial growth factor [98] and stimulating NO release dependent vasodilation [99]. There is abundant evidence to corroborate that GIPR represents a validated therapeutic target for diet-induced T2DM and comorbidities. Further investigation should be conducted in order to answer open questions and characterize the mechanism of action of peripheral and central GIPR that will help understanding the pathophysiologic nature of this dysfunctional network.

III References

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IV Eidesstattliche Versicherung

„Ich, Renate Luzía Barbosa Yañez, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema „Dysfunctional Network of Type 2 Diabetes Mellitus: Influence of Diet, Genes and Brain“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren/innen beruhen, sind als solche in korrekter Zitierung kenntlich gemacht. Die Abschnitte zu Methodik und Resultaten werden von mir verantwortet.

Ich versichere ferner, dass ich die in Zusammenarbeit mit anderen Personen generierten Daten, Datenauswertungen und Schlussfolgerungen korrekt gekennzeichnet und meinen eigenen Beitrag sowie die Beiträge anderer Personen korrekt kenntlich gemacht habe (siehe Anteilserklärung). Texte oder Textteile, die gemeinsam mit anderen erstellt oder verwendet wurden, habe ich korrekt kenntlich gemacht.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Erstbetreuer/in, angegeben sind. Für sämtliche im Rahmen der Dissertation entstandenen Publikationen wurden die Richtlinien des ICMJE (International Committee of Medical Journal Editors; www.icmje.og) zur Autorenschaft eingehalten. Ich erkläre ferner, dass ich mich zur Einhaltung der Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis verpflichte.

Weiterhin versichere ich, dass ich diese Dissertation weder in gleicher noch in ähnlicher Form bereits an einer anderen Fakultät eingereicht habe.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§§156, 161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

Datum Unterschrift

V Anteilserklärung

Anteilserklärung an der erfolgten Publikation:

Ich Renate Luzía Barbosa-Yañez, geboren in La Paz, Bolivien, habe folgenden Anteil an der Publikation gehabt:

Renate Luzía Barbosa-Yañez, Ulrike Dambeck, Linna Li, Jürgen Machann, Stefan Kabisch and Andreas F.H. Pfeiffer, Acute Endothelial Benefits of Fat Restriction over Carbohydrate Restriction in Type 2 Diabetes Mellitus: Beyond Carbs and Fats, Nutrients 2018, Dec 2018. doi.org/10.3390/nu10121859

Geteilte Erstautorenschaft mit Ulrike Dambeck

Sanierung der Datenbank:

Auf Grund des großen Datenvolumens der DiNA-D-Studie mussten regelmäßig Probandendaten aus den Akten in die elektronische Datenbank übertragen werden. Studiendaten wurden vom Studienarzt, den weiteren StudienmitarbeiterInnen oder direkt durch die Patienten erhoben. Danach wurden die Daten direkt in die elektronische Datenbank überführt. Die Strukturierung, Erweiterung und Fehlerbereinigung dieser Datenbank nahm ich eigenständig vor. Zudem wurde für die Probanden, die für die Untersuchung der flussvermittelten Vasodilatation zugesagt hatten, eine zusätzliche Datenbank eröffnet. Durch die fertiggestellte elektronische Datenbank war es möglich, die obenstehenden Daten übersichtlich und vereinfacht über eine Eingabemaske einzugeben, bzw. für Analysen auszugeben. Ich übernahm die Kontrolle des Datenflusses und der Daten, die ins Computersystem übertragen worden sind. Dabei erfasste und korrigierte ich ungenaue, unleserliche oder unvollständige Aufzeichnungen der Daten, fehlerhafte oder unvollständige Erfassung auf elektromagnetischen Datenträgern, fehlerhafte Zuordnungen von separat oder nachträglich erhobenen Daten und nachträgliche sowie nicht vollständig dokumentierte Änderungen.

Auswertung und Analyse:

Die statistische Analyse nahm ich eigenständig vor. Sie wurde mit dem SPSS-Softwarepaket für Windows (IBM, Version 20.0, Chicago, IL, USA) durchgeführt. Dazu wurde die deskriptiven Maße (insbesondere Minimum und Maximum) der metrischen Parameter überprüft. Mögliche Ausreißer wurden analysiert und, wenn notwendig, von der Analyse ausgenommen. Für weitere statistische Analysen wurde zunächst die Normalverteilung und Varianzhomogenität der metrischen Daten analysiert (Kolmogorov-Smirnov-Test auf Normalität, Levene-Test für die Varianzhomogenität). Der Vergleich innerhalb und zwischen Ernährungsgruppen wurde mit einem Student-T-Test (gepaart und ungepaart) analysiert. Nicht-normalverteilte Variablen wurden transformiert, um Normalverteilung zu erreichen, oder durch nichtparametrische Tests (Wilcoxon- oder Mann-Whitney-U-Test) analysiert. Eine Varianzanalyse (ANOVA) für wiederholte Messungen wurde durchgeführt, um die Auswirkungen der Wechselwirkung zwischen Ernährung und der flussvermittelten Vasodilatation zu bewerten. Es wurde ein Modell mit einem Zwischensubjektfaktor und zwei Innersubjektfaktoren verwendet. Eine Analyse der Pearson-Korrelation (für nicht-normalverteilte Variablen: Spearman-Korrelation) wurde durchgeführt, um die Stärke der Beziehungen zwischen der Aufnahme von Makronährstoffen pro kg Körpergewicht und der flussvermittelten Vasodilatation nach ernährungsbedingten Eingriffen zu bestimmen.

Schreiben des Artikels:

Das Verfassen des Artikels nahm ich eigenständig vor. Ein kontinuierlicher Weiteraufbau erfolgte durch die Supervision und Kritik meines Betreuers Dr. med. Stefan Kabisch und meines Doktorvaters, Prof. Dr. med. Andreas F.H. Pfeiffer. Bis zur finalen Version bedurfte es wiederholter Aktualisierungen des Primärdatensatzes und erneuter statistischer Auswertungen.

Die Erstellung von allen Tabellen und Abbildungen für die oben genannte Publikation nahm ich eigenständig vor.

_____

Unterschrift, Datum und Stempel des/des erstbetreuenden Hochschullehrers/in

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Unterschrift des Doktoranden/der Doktorandin

Anteilserklärung für nicht publizierte Projekte

Ich Renate Luzía Barbosa-Yañez, geboren in La Paz, Bolivien, habe folgendes Manuskript eingereicht:

Renate Luzía Barbosa-Yañez, Mariya Markova, Ulrike Dambeck, Caroline Honsek, Jürgen Machann, Rita Schüler, Stefan Kabisch, Andreas F H Pfeiffer, Predictive effect of GIPR SNP rs10423928 on glucose metabolism liver fat and adiposity in prediabetic and diabetic subjects. (Zum Zeitpunkt der Abgabe der Dissertation der Publikation befand sich dieses Paper unter Begutachtung; inzwischen akzeptiert und veröffentlicht: December 2019, Peptides 125(6):170237)

Sanierung der Datenbank:

Auf Grund des großen Datenvolumens der DiNA-D und DiNA-P-Studie mussten regelmäßig Probandendaten aus den Akten in die elektronische Datenbank übertragen werden. Studiendaten wurden vom Studienarzt, den weiteren StudienmitarbeiterInnen oder direkt durch die Patienten erhoben. Danach wurden die Daten direkt in die elektronische Datenbank überführt. Durch die fertiggestellte elektronische Datenbank war es möglich, die obenstehenden Daten übersichtlich und vereinfacht über eine Eingabemaske einzugeben bzw. für Analysen auszulesen. Ich übernahm die Kontrolle des Datenflusses und der Daten, die ins Computersystem übertragen worden sind. Dabei erfasste und korrigierte ich ungenaue, unleserliche oder unvollständige Aufzeichnungen der Daten, fehlerhafte oder unvollständige Erfassung auf elektromagnetischen Datenträgern, fehlerhafte Zuordnungen von separat oder nachträglich erhobenen Daten und nachträgliche sowie nicht vollständig dokumentierte Änderungen.

Genotypisierung

Die Genotypisierung nahm ich eigenständig vor. Die genomische DNA wurde aus Buffy Coat, Vollblut oder Serum-Proben isoliert, je nach Probenverfügbarkeit. Die Genotypisierung wurde mit ViiA™ System und TaqMan™ SNP Genotyping Assay rs10423928 ID C_30103605_10 (Angewandte Biosysteme, Thermo Fisher Scientific) durchgeführt.

Die statistische Analyse nahm ich eigenständig vor. Sie wurde mit dem SPSS-Softwarepaket für Windows (IBM, Version 20.0, Chicago, IL, USA) durchgeführt. Das Hardy-Weinberg-Gleichgewicht für den GIPR-Genotyp wurde berechnet mit Pearsons $\chi 2$-Test zum Vergleich des Genotyps und der Allelfrequenzen innerhalb prädiabetischer und T2DM-Patienten mit Microsoft® Excel®, 2016. Zum Vergleich zwischen homozygoten Träger des Hauptallels T (HMA) und A-Allelträgern (heterozygot T/A (HET) und homozygot für das Nebenallel A (HMI)) wurde eine Einweg-Varianzanalyse (ANOVA) mit Genotyp (HMA vs HET, HMI) als Zwischensubjektfaktor vorgenommen. Außerdem wurden die Daten von oGTT und MTT durch ANOVA mit wiederholten Messungen analysiert.

Schreiben des Artikels:

Das Verfassen des Artikels nahm ich eigenständig vor. Ein kontinuierlicher Weiteraufbau erfolgte durch die Supervision und Kritik meines Betreuers Dr. med. Stefan Kabisch und meines Doktorvaters, Prof. Dr. med. Andreas F.H. Pfeiffer. Bis zur finalen Version bedurfte es wiederholter Aktualisierungen des Primärdatensatzes und erneuter statistischer Auswertungen.

Die Erstellung von allen Tabellen und Abbildungen nahm ich eigenständig vor. Die Erfassung des Manuskripts für eine Publikation nahm ich eigenständig vor. Das Manuskript befand sich unter Begutachtung, als diese Doktorarbeit eingereicht wurde.

_____

Unterschrift, Datum und Stempel des/der erstbetreuenden Hochschullehrers/in

_____

Unterschrift des Doktoranden/der Doktorandin

Ich Renate Luzía Barbosa-Yañez, geboren in La Paz, Bolivien, habe folgenden Anteil an diesem Forschungsprojekt gehabt:

Effect of central GIPR for the development of high fat-induced obesity and glucose and insulin tolerance

Erfassung des Genehmigungsantrags

Das Verfassen des Genehmigungsantrags nahm ich eigenständig vor. Ein kontinuierlicher Weiteraufbau erfolgte durch meinen Doktorvater, Prof. Dr. med. Andreas F.H. Pfeiffer, mit Hilfe von Prof. Dr. André Kleinridders und Dr. Anja Voigt.

Generierung von KO-Mäusen:

Die C57BL/6 wurde im Jackson Laboratory gezüchtet. GIPR-lox/lox Mäuse wurden von Taconic Bioscience zur Verfügung gestellt und an Prof. Wolfrum (ETH Zürich) ausgeliefert. Unsere GIPR-lox/lox-Mäuse wurden von der ETH Zürich bezogen. Diese wurden in der Tierhaltung des MRL mit den jeweiligen Cre-Mäusen verpaart und gezüchtet. Die Erzeugung des konstitutiven KO-Allels nahm ich mittels Cre-Rekombination eigenständig vor. Dafür war die Kreuzung mit den jeweiligen Cre-Mäusen mit homozygot flankierten GIPR-Tieren das Mittel der Wahl, um spezifische Aussagen über GIPR im Gehirn tätigen zu können. Die Verwendung der CaMKIIa- und der Nkx2.1-Cre-Mäuse ermöglicht spezifischere Deletionen zentraler GIPR (CaMKIIa) sowie speziell hypothalamischer GIPR. GIPR-lox/lox-Mäuse wurden mit den jeweiligen Cre-Mäusen verpaart. Aus der Zucht von GIPR-lox/lox und GIPR-lox/lox Cre +/- entstanden jeweils 50 % Kontrolltiere sowie 50% KOexperimentelle Tiere, denen GIPR-Lox/lox in CaMKIIa- oder Nkx2.1-exprimierenden Neuronen fehlt. Die resultierende Phänotypisierung und Genotypisierung nahm ich eigenständig vor.

Körpergewicht

Das Körpergewicht der abgesetzten Mäuse habe ich wöchentlich ab der 4. Lebenswoche bis zum Alter von 20 Lebenswochen protokolliert.

Insulintoleranztest (ITT)

Der Insulintoleranztest erfolgt in adulten Versuchstieren im Alter von 14 Wochen. Insulin (0,75 Units pro kg Körpergewicht) wurde hierfür intraperitoneal injiziert (Volumen ca. 200 – 400 µl, abhängig vom Körpergewicht). Um die Injektion durchzuführen, wurde die

Maus für wenige Augenblicke aus ihrem Heimatkäfig genommen, fachgerecht fixiert und die Injektion in das Abdomen durchgeführt. Zu den Zeitpunkten 15, 30, 60 und 120 min nach Insulingabe wurde der Blutzuckerwert der Tiere mit Blutzuckermessstreifen und -messgerät aus der Schwanzspitze genommen. Für die Blutabnahmen wurden die Mäuse für nicht länger als 1 Minute im Restrainer gehalten.

Glukosetoleranztest (GTT)

Zur Analyse des Glukosestoffwechsels wurde einmalig ein sogenannter Glukosetoleranztest durchgeführt. Dabei wurden den 16 Stunden gefasteten adulten Versuchstieren im Alter von 16 Wochen 2 g Glukose pro kg Körpergewicht oral mittels einer gebogenen Knopfkanüle appliziert (Volumen ca. 200 - 400 µl, abhängig vom Körpergewicht). Für die wiederholten Blutentnahmen unmittelbar vor der Glukosegabe (0 Minuten) sowie 15 Minuten, 30 Minuten, 60 Minuten und 120 Minuten nach der Glukosegabe wurde eine einmalige minimalinvasive Inzision an der Schwanzspitze gesetzt. Die Blutabnahmen fanden auf dem Gitterdeckel des Heimatkäfigs statt und dauerten nicht länger als 1 Minute.

Körperzusammensetzung (NMR)

Körperfett- und Magermasse der Mäuse wurden mittels NMR-Analyzer mit Nuclear Magnetic Resonance (NMR)-Messung zu den Zeitpunkten 10 und 17 Wochen nach Fütterungsbeginn ermittelt. Dabei wurde der Anteil an Muskel und Fett in den Mäusen bestimmt. Hierzu wurden die Tiere einzeln im NMR-Gerät (Bruker’s Minispec MQ10) arretiert und die Körperzusammensetzung wurde während der 1-minütigen Messung bestimmt.

Die Daten wurden unter Verwendung von ANOVA für wiederholte Messungen und zweiseitigem Student-t-Test für gepaarte und ungepaarte Proben analysiert (nicht-normalverteilte Variablen wurden durch nicht-parametrische Tests analysiert). Um die Auswirkungen der Ernährung auf jeden Genotyp zu analysieren, wurde ein Modell mit einem Faktor zwischen den Vergleichsgruppen (Diät) und entweder dem ITT- oder GTT-Glukosespiegel als innerhalb des Subjektfaktors durchgeführt. Die Analyse der BW-Veränderungen (%) für dieses Modell umfasste 7 Zeitpunkte (Woche 1,4,6,8,10,12,14), um ausreichende Restgrade zu erreichen. Für die allgemeine Analyse unter Einbeziehung aller Genotypen wurde ein zweites Modell mit zwei Zwischensubjektfaktoren (Genotyp und Ernährung) und entweder BW-Änderungen (%), ITT- oder GTT-Glukosespiegeln als Inner-Subjet-Faktor (SPSS 11.5, Chicago, IL) verwendet. $P < 0,05$ wurde als signifikant angesehen.

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Unterschrift des Doktoranden/der Doktorandin

VI Journal Summary List (ISI Web of Knowledge[SM])

Journal Data Filtered By: **Selected JCR Year: 2017** Selected Editions: SCIE,SSCI
Selected Categories: **"NUTRITION and DIETETICS"** Selected Category Scheme: WoS
Gesamtanzahl: 81 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	Annual Review of Nutrition	5,528	8.886	0.005230
2	PROGRESS IN LIPID RESEARCH	5,302	8.435	0.006750
3	Advances in Nutrition	3,937	6.853	0.012870
4	AMERICAN JOURNAL OF CLINICAL NUTRITION	58,213	6.549	0.055760
5	CRITICAL REVIEWS IN FOOD SCIENCE AND NUTRITION	10,197	6.015	0.011670
6	NUTRITION REVIEWS	7,526	5.788	0.010600
7	International Journal of Behavioral Nutrition and Physical Activity	8,371	5.548	0.019780
8	CLINICAL NUTRITION	10,558	5.496	0.016870
9	PROCEEDINGS OF THE NUTRITION SOCIETY	5,238	5.347	0.006230
10	INTERNATIONAL JOURNAL OF OBESITY	22,185	5.151	0.032040
11	FOOD CHEMISTRY	90,665	4.946	0.101120
12	NUTRITION RESEARCH REVIEWS	2,164	4.586	0.001840
13	CURRENT OPINION IN CLINICAL NUTRITION AND METABOLIC CARE	4,842	4.534	0.007130
14	EUROPEAN JOURNAL OF NUTRITION	5,669	4.423	0.011650
15	JOURNAL OF NUTRITIONAL BIOCHEMISTRY	9,815	4.414	0.014150
16	JOURNAL OF NUTRITION	38,804	4.398	0.029930
17	JOURNAL OF PARENTERAL AND ENTERAL NUTRITION	5,287	4.249	0.007990
18	Nutrients	12,031	4.196	0.032520
19	Obesity	17,578	4.042	0.037840
20	Journal of the Academy of Nutrition and Dietetics	3,687	4.021	0.014370
21	INTERNATIONAL JOURNAL OF EATING DISORDERS	8,732	3.897	0.010160
22	NUTRITION	10,167	3.734	0.013010
23	BRITISH JOURNAL OF NUTRITION	26,011	3.657	0.035400
24	Nutrition Journal	4,484	3.568	0.009540

VII Publication

nutrients

MDPI

Article

Acute Endothelial Benefits of Fat Restriction over Carbohydrate Restriction in Type 2 Diabetes Mellitus: beyond Carbs and Fats

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Nutrients 2018, 10(12), 1859; https://doi.org/10.3390/nu10121859

Received: 30 October 2018 / Revised: 26 November 2018 / Accepted: 27 November 2018 / Published: 1 December 2018

Abstract: Background: Cardiovascular diseases (CVD) are the major cause of mortality in type 2 diabetes patients (T2DM). Causes are embedded in a complex interplay between excess body fat, insulin resistance and serum lipid anomalies. Endothelial homeostasis is strongly affected by this pathogenic network. Even though metabolic changes and weight loss improve vascular endothelial function, the effect of different dietary approaches is still uncertain for type 2 diabetes patients. Objective: We aim to compare the acute effects of a hypocaloric very low carbohydrate (VLC) diet versus a hypocaloric low fat (LF) diet on flow mediated dilation (FMD), intrahepatic lipid (IHL) accumulation and visceral adipose tissue as independent risk factors of CVD in T2DM patients. Design: Thirty-six T2DM patients (age 63 ± 8 years, 60% females) were randomly assigned to the VLC diet (4-10 % of total energy intake (E)) or to the LF diet (<30 % E) for 3 weeks. Endothelial function was assessed by the flow mediated dilation (FMD) method. Adipose tissue depots and IHL were determined by magnetic resonance. Results: Both dietary strategies reduced body weight, body fat content and IHL. Unexpectedly, the LF group experienced significantly greater enhancement of FMD, compared to the VLC group. The FMD showed a positive correlation with protein intake and fat intake in the LF group, while it revealed a negative correlation with protein intake in the VLC diet group. Conclusions: Reduction of total and hepatic adiposity was shown to be successful using either the VLC or LF hypocaloric diets, however improvements in FMD may be related to the interplay of fat and protein intake.

Keywords: type 2 diabetes; endothelial function; intrahepatic lipids; low fat diet; low carbohydrate diet; protein intake

1. Introduction

The control of Type 2 diabetes mellitus (T2DM) has become a global challenge. Its incidence, prevalence and costs are rapidly increasing often in parallel with obesity, cardiovascular diseases (CVD) and nonalcoholic fatty liver disease (NAFLD), embracing a dysfunctional metabolic network. Excess body weight seems to be the main driver of this pathogenic network and probably provides the common link between T2DM, NAFLD and CVD through insulin resistance, hyperlipidemia, hypertension and low-grade inflammation [1,2]. Given that CVD is the major cause of mortality in T2DM [3], the impact of this dysfunctional network on vascular events needs to be considered. It is well established that vascular endothelial cells play a pivotal role in the maintenance of cardiovascular homeostasis. The endothelium accounts with two main mechanisms to control the internal stability: vasodilation and vasoconstriction [4]. Perhaps the most important mechanism for endothelial relaxation in arteries is the endothelium dependent synthesis of nitric oxide (NO), an effective vasodilator and a functional marker of endothelial dysfunction [4]. In fact, endothelial dysfunction has been associated with T2DM [5], NAFLD [6] and excessive fat mass [7].

With a non-invasive methodology it is possible to quantify the ability of the brachial artery to dilate in response to shear stress [8]. The Flow Mediated Dilation (FMD) technique has been applied in several clinical studies to determine the effect of weight loss (by hypocaloric diets, bariatric surgery or/and exercise) [9–13], low fat (LF) and low carbohydrate (LC) diets [14–19] on vascular function. Indeed, weight loss is a key determinant for restoration of vessel stability and integrity, but also reduction of visceral fat [20], liver fat [21] and glycated hemoglobin A1c (HbA1c) levels [22] contribute to an enhanced FMD.

Hypocaloric nutritional strategies are a common tool used for T2DM management due to its rapid metabolic benefits. However, the macronutrient composition of such low caloric diets is still under dispute regarding its impact on endothelial function in T2DM patients.

Therefore, the aim of this study was to examine and compare the effect of a hypocaloric very low carbohydrate (VLC) diet versus a hypocaloric LF diet on FMD, intrahepatic lipid (IHL) accumulation and visceral adipose tissue (VAT), as independent risk factors of CVD in T2DM patients and to evaluate the macronutrient composition of each diet and its relation to FMD at intervention completion.

2. Materials and Methods

2.1. Participants and Study Design

The DiNA-D (Diabetes Nutrition Algorithms in Patients with Overt Diabetes Mellitus) study is a randomized, parallel group, intervention study with adult T2DM patients. Following the approval of the Ethics Committee of the Charité and of the University of Potsdam, conducted in accordance with the Declaration of Helsinki, the study was registered at www.ClinicalTrials:gov (NCT02459496). The study was conducted at the clinical ward in Berlin (located at the Charité Campus Benjamin Franklin). Study participants were recruited via local advertisements. Volunteers, who met the inclusion criteria, provided a written informed consent according to GCP guidelines. After the initial screening (V0) subjects were randomized in a 1:1 ratio to either a VLC or LF diet using computer generated random numbers and stratified according to age, sex, body mass index (BMI) and waist circumference. Subject visited the study facility twice, at baseline (V1) and post-intervention (V2) of the respective dietary intervention, where routine

clinical measurements and MRI/1H-MRS (magnetic resonance imaging/magnetic resonance spectroscopy) (Siemens Healthineers, Erlangen, Germany) assessments were performed.

This paper reports the outcomes of the study subgroup with assessment of the endothelial function, over a 3 week period, conducted between October 2013 and February 2016.

2.2. Dietary Intervention

The VLC diet was aimed to be ketogenic with a carbohydrate daily intake of <40 g (60–70% fat, 5–10% carbohydrate, 20–30% protein) and includes a reduction of caloric intake to 1200–1500 kcal/d. Study participants received recipes, daily meal plans and a substitution list with common food to restrict carbohydrates and calories intake.

The LF is characterized by a calorie intake of 1000–1200 kcal/d, and less than 30% of the total energy intake (E%) of fat (<30% fat, 50% carbohydrate, 20% protein). Study participants were provided with a flavored meal replacement powder (MODIFAST® (OTC Siebenhandl GmbH, Ulm, Germany)) (supplement Table S1). In addition, the intake of 200 g of raw or steamed vegetables was allowed.

2.3. Body Weight and Body Composition

Weight and height were determined with the electronic weighing and measuring station seca 764 (Approval class III, Seca Ltd., Birmingham, England).

2.4. Liver Fat and Visceral Fat

Magnetic resonance for determination of IHL and adipose tissue compartments was performed under fasting conditions as described elsewhere [23]. Briefly, IHL were quantified by proton magnetic resonance spectroscopy (1H-MRS) on a 1.5 T Magnetom Avanto (Siemens Healthineers, Erlangen, Germany) applying a single voxel STEAM technique (TE = 10ms, TR = 4s). Abdominal adipose tissue was quantified from axial T1-weighted fast-spin echo images between hip and shoulder with subjects lying in prone position with outstrechted arms Total abdominal adipose tissue (TAT) and VAT depots were quantified by an automatic segmentation procedure based on fuzzy clustering and orthonormal snakes [24].

2.5. Clinical Parameters

Levels of HbA1c were determined in the fasting state in serum/plasma with ABX Pentra 400 (Horiba, Fukuoka, Japan). For determining the blood levels of insulin (Mercodia, Uppsala, Sweden), enzyme linked immunosorbent assay was used. Glucose and CRP (c-reactive protein) was determined by turbidimetric immunoprecipitation.

A fasting blood sample was collected at baseline and post-intervention diet; serum lipids (total cholesterol (T.CHO), high density cholesterol (HDL), low density cholesterol (LDL), triglycerides (TAG) and further routine parameters were measured in serum/plasma by using ABX Pentra 400 (Horiba, Fukuoka, Japan).

All measurements of systolic blood pressure (SBP) and diastolic blood pressure (DBP) were performed after a previous resting time of 10 min at room temperature using a standardized blood pressure cuff system (BOSO ABI system 100, BOSO, Jungingen, Germany).

2.6. Endothelial Function

All measurements were performed by an experienced member of the staff according to a standardized protocol in the morning. Furthermore, participants were asked to pause taking their antidiabetic medications 3 days prior to the measurement and to avoid the consumption of alcohol, caffeine and tobacco for 8 h before the measurement.

Brachial artery ultrasound was performed in the fasted state using a 12-MHz probe and high resolution ultrasound. Ultrasound images were video recorded and digitalized using a highly sophisticated computerized system to detect the intima media complex by edge detection software (Brachial Analyzer version 5.10.6, Medical Imaging Applications LLC, Iowa City, IA, USA).

Participants were asked to rest quietly for 10 min before the measurement started. In this time a sphygmomanometric cuff was placed around the right arm just above the medial epicondyle of the humerus and the identification of the endothelium was performed. A longitudinal image of the artery anterior and posterior vessel wall was visualized, the subject's arm was held steady and the resting baseline images of the brachial artery diameter were video-recorded for 5 s in B-Mode [8]. Thereafter the volumetric blood flow measurement was performed using the PW-Doppler mode. Arterial occlusion was then generated by inflating the sphygmomanometric cuff on the right arm to 260 mmHg for 1 min and retained for 5 min. The post occlusion dilation of the artery was video-recorded for 5 min. During the depressurization, the increase in flow velocity was also recorded and a 5 sec sequence was saved in B mode within the following 15 sec. In the time interval of 4 min after the cuff release, a video sequence was recorded every 15 s at the beginning and every 30 s during the last minute in the B-screen. Beside the initial diameter, the shear stress mediated dilatation was recorded at 14 time points. A video sequence entailed 94 single images and thus provided 94 values of the luminal diameter. For the calculation of the FMD values were averaged and from a total of 14 time points, the peak diameters were used.

2.7. Diet Compliance and Evaluation

To ensure compliance participants of both groups were given dietary counseling and support at baseline by a certified nutritionist and during the diet phase telephone follow up was carried out. Furthermore, all subjects were asked to document their food intake throughout the intervention (21 days). Diet records were analyzed for macronutrient content using Prodi software (Version 6.2, Nutri-Science, Hausach, Germany). A mean value of 4 days before starting the diet was calculated for the dietary intake at baseline. A mean value of 3 weeks diet (21 days) was calculated for the dietary intake at endpoint

2.8. Statistical Analysis

Statistical analysis was conducted using the SPSS software packagefor Windows (IBM, version 20.0, Chicago, USA). The results are expressed as mean ± SD. After plausibility assessment, extreme values of FMD were excluded from analysis: two study participants, one of each diet group, had FMD values beyond the outer borders of the boxplot analysis, and therefore were considered extreme outliers. Data was examined for normality by KolmogoroV–SmirnoV test. For comparison within and between diet groups Student t-test (paired and unpaired) was performed. Non-normally distributed variables were analyzed by non-parametric tests (Wilcoxon or Mann-Whitney-U-Test). FMD was separately analyzed by Analysis of variance (ANOVA) for repeated measurements for assessing diet-FMD interaction effects. A model with one between subject factor (diet groups) and two within subject factors (V1 and V2) was used and was adjusted for kilocalories intake changes per kg BW.

Pearson correlation analysis was performed to identify the strength of relations between macronutrients intake (g) per kg body weight (BW) and FMD after dietary intervention. Non-normally distributed variables were analyzed by Spearman correlation analysis.

Statistical significance was defined as $p < 0.05$.

3. Results

A total of 36 T2DM patients completed the intervention study (Figure 1), Participants were 22 females and 15 males, on average 63 years of age (range: 42–76 years), were mostly overweight to mildly obese (mean BMI = 33 ± 5 kg/m^2) with a mean HbA1c level of 6.5 ± 0.8% (47 ± 8 mmol/mol). Furthermore, 64% of all participants showed a liver fat content above the cutoff value of 5.56% for the clinical diagnosis of NAFLD [25] (mean IHL = 12.9 ± 9.5 %). About 86 % were nonsmokers or former smokers (Table 1).

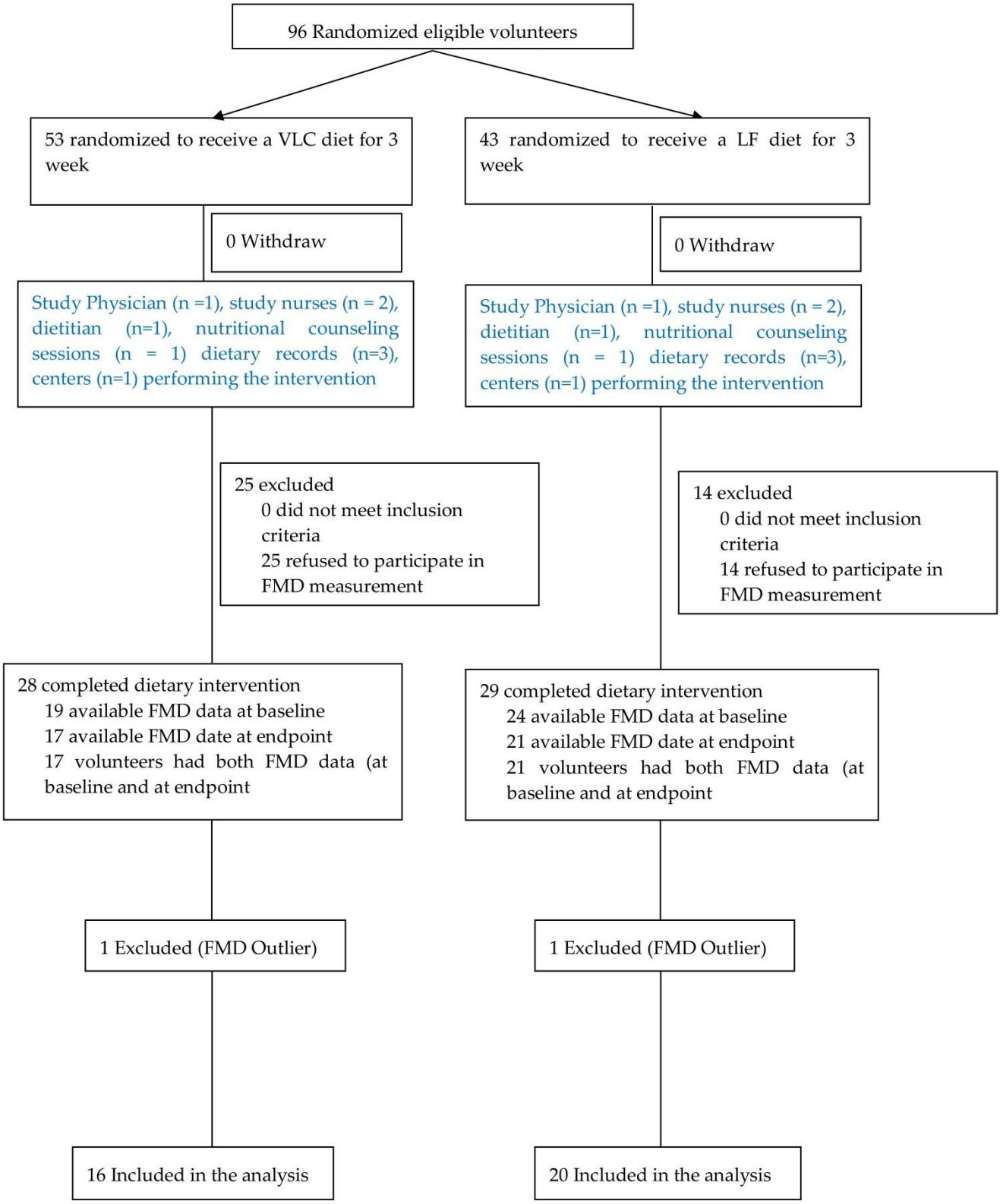

Figure 1. Flow diagram of the subjects included in this study. Adapted version of "CONSORT flow diagram for individual randomized controlled trials of nonpharmacological treatments" [32] illustrating participants inclusion to the FMD sub-group. FMD: Flow mediated dilation; VLC: very low carbohydrate diet; LF: low fat diet.

Table 1. Baseline characteristics of subjects.

General Parameters		Mean	±SD
Age [1]		63	(±8)
		N	%
Sex [2]	♀	22	60
	♂	14	40
VLC	♀	11	69
	♂	5	31
LF	♀	10	53
	♂	9	47
Lifestyle [2]		*n*	(%)
Smoking Status	Never smoker	14	39
	Smoker	5	14
	Ex-smoker	17	47
Medication [2]			
Oral antihyperglycemic agents	0	13	37.
	1	16	45
	2	4	11
Insulin	0	30	86
	1	3	9
Lipid lowering agents	0	20	57
	1	13	37
Blood pressure lowering agents	0	6	18
	1	15	46
	2	7	21
	3	4	12
	4	1	3
Bradycardic antihypertensive agents	0	19	54
	1	12	34
	2	2	6

Values are presented as mean (±SD) [1] or as frequency (%) [2]. *n* = 36. VLC: very low carbohydrate diet, LF: low fat diet.Medication: 1 = 1 agent, 2 = 2 different agents, 3 = 3 different agents, 4 = 4 different agents.

Table 2 displays results of anthropometric differences within and between diet groups. In brief, after 3 weeks dietary intervention, both, the LF and the VLC diet significantly reduced body weight by −4.1 kg and −5.2 kg, respectively ($p_{VLC} < 0.001$, $p_{LF} < 0.001$). Similarly, body fat content decreased significantly in different compartments in both diet groups (TAT ($p_{VLC} = 0.001$, $p_{LF} < 0.001$), VAT ($p_{TAT} = 0.024$, $p_{VAT} < 0.001$). and IHL content decreased extensively by 35%–36% relative to the initial levels ($p_{VLC} = 0.003$, $p_{LF} < 0.001$)) (Figure 2).

Table 2. Anthropometric differences within and between diet groups.

										V1 vs V2		**VLC vs LF**		
Anthropometry	**Visit**	**n**	**VLC Mean**	**±SD**	**Δ**	***n***	**LF Mean**	**±SD**	**Δ**	VLC ***p*-Value**	LF ***p*-Value**	Δ ***p*-Value**	V1 ***p*-Value**	V2 ***p*-Value**
Weight (kg)	V1	16	93.9	20.7	−4.1	20	97.6	22.6	−5.2	**<0.001**	**<0.001**	ns	ns	ns
	V2	16	89.8	19.7		20	92.5	20.9						
BMI (kg/m²)	V1	16	32.1	4.5	−1.3	20	32.7	4.9	−1.6	**<0.001**	**<0.001**	ns	ns	ns
	V2	16	30.8	4.3		20	31.1	4.6						
TAT [l]	V1	14	23.4	6.9	−1.7	18	22.9	5.3	−1.7	**0.001**	**<0.001**	ns	ns	ns

VAT [l]	V2	14	21.7	5.9		18	21.2	5.1							
	V1	14	5.3	1.4	−0.4	18	6.8	2.5	−0.7	**0.024**	**<0.001**	ns	**0.042**	ns
	V2	14	4.9	1		18	6.1	2.3						
IHL (%)	V1	14	10.8	6.3	−3.8	17	14.5	11.3	−5.2	**0.003**	**<0.001**	ns	ns	ns
	V2	14	7	4.9		17	9.3	8.4						

Values are presented as mean (±SD), *p*-values of mean from *t*-test for analyzing difference within each group are presented Difference significant at $p < 0.05$. BMI =body mass index, TAT = total body fat, VAT = visceral adipose tissue, IHL = intra hepatic lipids. V1 = Visit 1, V2=Visit 2. Δ = V2−V1.

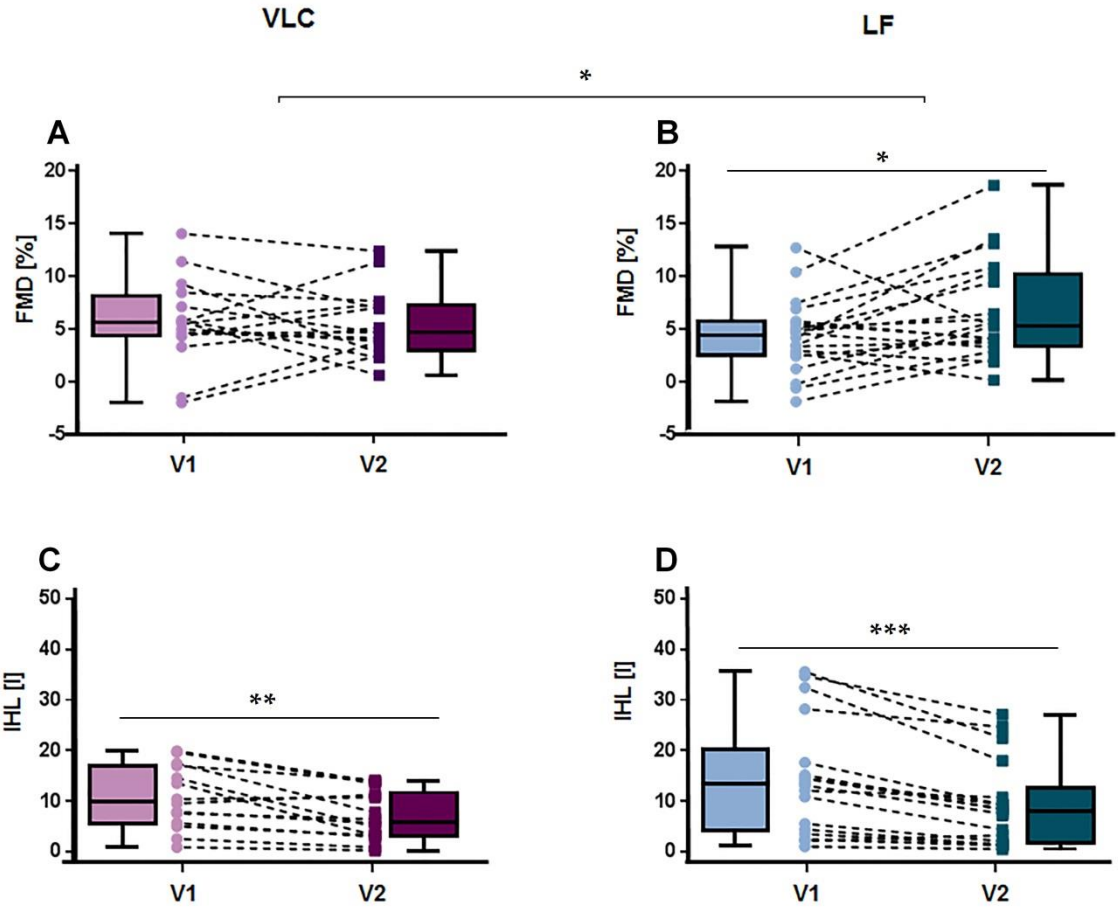

Figure 2 Acute effects on FMD and IHL after 3 weeks diet. Boxplots and individual changes in endothelial function (**A**) and intra hepatic lipids (**C**) post-intervention on the VLC diet; changes in endothelial function (**B**) and intra hepatic lipids (**D**) after 3 weeks on the LF diet. FMD = flow mediated dilation, IHL = intra hepatic lipids, $N_{VLC} = 16$, $N_{LF} = 20$, V1 = Visit 1, V2=Visit 2 $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

However, HbA1c levels decline significantly only in the VLC group ($p_{VLC} < 0.001$, $p_{LF} = 0.054$, $p_{\Delta} = 0.001$). No further significant differences were found between diet groups in any of these variables. Despite the fact that both dietary approaches showed similar improvements in total cholesterol ($p_{VLC} = 0.001$, $p_{LF} < 0.001$), LDL ($p_{VLC} = 0.004$, $p_{LF} < 0.001$) and TAG ($p_{VLC} = 0.003$, $p_{LF} = 0.042$), LDL ($p_{V2} = 0.035$) and total cholesterol ($p_{V2} = 0.042$) had significantly greater decreases in the LF compared to the VLC diet.

Further significant improvements was observed for SBP, (p_{VLC} = 0.003, p_{LF} = 0.042) (Table 3).

Table 3. Clinical parameters Differences within and between diet groups.

										V1 vs V2			VLC vs LF	
Clinical			**VLC**		**Δ**		**LF**		**Δ**	VLC	LF	Δ	V1	V2
Parameters	**Visit**	***n***	**Mean**	**±SD**		***n***	**Mean**	**±SD**		***p*-Value**	***p*-Value**	***p*-Value**	***p*-Value**	***p*-Value**
HbA1c (%)	V1	16	6.7	1	−0.6	20	6.2	0.6	−0.2	**<0.001**	ns	**0.001**	ns	ns
	V2	16	6.1	0.7		20	6.1	0.6						
CRP (mg/l)	V1	16	3.1	4.1	−1.3	20	3.1	2.7	−1.1	**0.025 ***	**0.008 ***	ns *	ns *	ns *
	V2	16	1.7	1.5		20	2	2.8						
T. CHO (mg/dl)	V1	16	200	39	−27	20	178	33	−31	**0.001**	**<0.001**	ns	ns	**0.042**
	V2	16	173	39		20	147	36						
HDL (mg/dl)	V1	16	56	16	−2	20	49	18	−4	ns	ns	ns	ns	ns
	V2	16	54	12		20	45	14						
LDL (mg/dl)	V1	16	127	36	−21	20	109	28	−26	**0.004**	**<0.001**	ns	ns	**0.035**
	V2	16	106	31		20	83	31						
TAG (mg/dl)	V1	16	133	65	−30	20	161	65	−35	**0.003**	**0.042**	ns	ns	ns
	V2	16	103	72		20	127	77						
SBP (mmHg)	V1	15	134	17	−9	19	134	21	−6	ns	ns	ns	ns	ns
	V2	15	126	10		19	128	19						
DBP (mmHg)	V1	15	85	11	−5	19	84	13	−6	ns	ns	ns	ns	ns
	V2	15	80	9		19	78	11						

Values are presented as mean (±SD), *p*-values of mean from t-test for analyzing difference within each group are presented (*= non-parametric test in case of missing normal distribution). T. CHO= total cholesterol, HDL = high-density cholesterol, LDL= low-density cholesterol, TAG = triacylglycerides, CRP = c-reactive protein, HbA1c = glycated hemoglobin, SBP = systolic blood pressure in the right arm, DBP = diastolic blood pressure in the right arm, V1 = Visit 1, V2=Visit 2. Δ = V2-V1.

FMD showed no significant differences between groups at baseline (p_{FMD} = 0.267) and did not change significantly in the VLC diet group (p_{VLC} = 0.782) post-intervention (Figure 2). However, after the LF diet FMD increased significantly (p_{LF} = 0.024) (Figure 2).

Nutritional data were adjusted for kg BW (Past studies have proposed that weight loss [10,11] are effective in improving flow mediated vasodilation. For this reason, macronutrients intake was corrected for weight (kg/BW). The intake of energy (p_{VLC} < 0.001, p_{LF} < 0.001, p_{Δ} < 0.001, p_{V2} = 0.001), protein (p_{VLC} = 0.002, p_{LF} < 0.001, p_{Δ} < 0.001, p_{V2} < 0.001) and carbohydrate (p_{VLC} < 0.001, p_{LF} = 0.006, p_{Δ} = 0.005, p_{V2} < 0.001) showed significant differences within and between diet groups (Table 4). Total fat intake showed no significant changes in the VLC diet, while, as expected, decreased significantly in the LF group (p_{LF} < 0.001), resulting in significant differences between diet groups (p_{Δ} < 0.001, p_{V2} < 0.001.

Table 4. Macronutrient intake differences within and between diet groups.

			VLC		**Δ**		**LF**		**Δ**	VLC	LF	Δ	V1	V2
Macronutrient Intake	**Visit**	***n***	**Mean**	**±SD**		***n***	**Mean**	**±SD**		***p*-Value**	***p*-Value**	***p*-Value**	***p*-Value**	***p*-Value**
Calories (kcal)	V1	14	2035	663	−717	15	2022	790	−1098	**<0.001**	**<0.001**	**<0.001**	ns	**0.001**
	V2	14	1318	220		15	925	61						
Protein (g/kg bw)	V1	14	0.95	0.15	0.21	15	0.94	0.2	−0.26	**0.002**	**<0.001**	**<0.001**	ns	**<0.001**
	V2	14	1.16	0.26		15	0.67	0.18						
Carbohydrate (g)	V1	14	196	69	−157	15	203	93	−85	**<0.001**	**0.006**	**0.005**	ns	**<0.001**

	V2	14	39	10		15	118	11						
Fat (%)	V1	14	40	9	14	15	38	6	−16	ns	**<0.001**	**<0.001**	ns	**<0.001**
	V2	14	54	6		15	21	2						

Values are presented as mean (±SD), p-values of mean from t-test for analyzing difference within each group are presented. Difference significant at $p < 0.05$. V1 = Visit 1, V2=Visit 2. Δ = V2−V1. Macronutrients (and energy) *p*-value is based on g per kg BW calculations. Results are shown as mean ± SD.

Accordingly, ANOVA model for FMD was adjusted for energy intake changes. Table 5 shows the significant interaction effect between diet and FMD was found (p_{visit} =0.267, $p_{\text{visit x diet}}$ = 0.034) (Table 5).

Table 5. Differences within diet groups and diet interaction effect on FMD.

			VLC		**Δ**		**LF**		**Δ**	VLC	LF	**Visit**	**Visit x Time**
Endothelial Function	**Visit**	***n***	**Mean**	**±SD**		***n***	**Mean**	**±SD**		***p*-Value**	***p*-Value**	***p*-Value**	***p*-Value**
FMD (%)	V1	16	5.74	4.06	−0.26	20	4.32	3.5	2.27	ns	**0.024**	ns	**0.034**
	V2	16	5.48	3.21		20	6.59	4.69					

Values are presented as mean (±SD), p-values of mean from t-test for analyzing difference within each group are presented. FMD was calculated using ANOVA for repeated measurements adjusted for kilocalories intake changes per kg BW. Difference significant at $p < 0.05$. FMD = flow mediated dilation V1 = Visit 1, V2=Visit 2. Δ = V2-V1.

After 3 weeks on a LF diet FMD showed a significant positive correlation with protein (g/kg BW) ($r = 0.531$, $p = 0.023$) and fat intake (g/kg BW) ($r = 0.557$, $p = 0.016$). On the contrary, the VLC group showed a significant negative correlation with protein intake (g/kg BW) ($r = −0.542$, $p = 0.037$) (Figure 3).

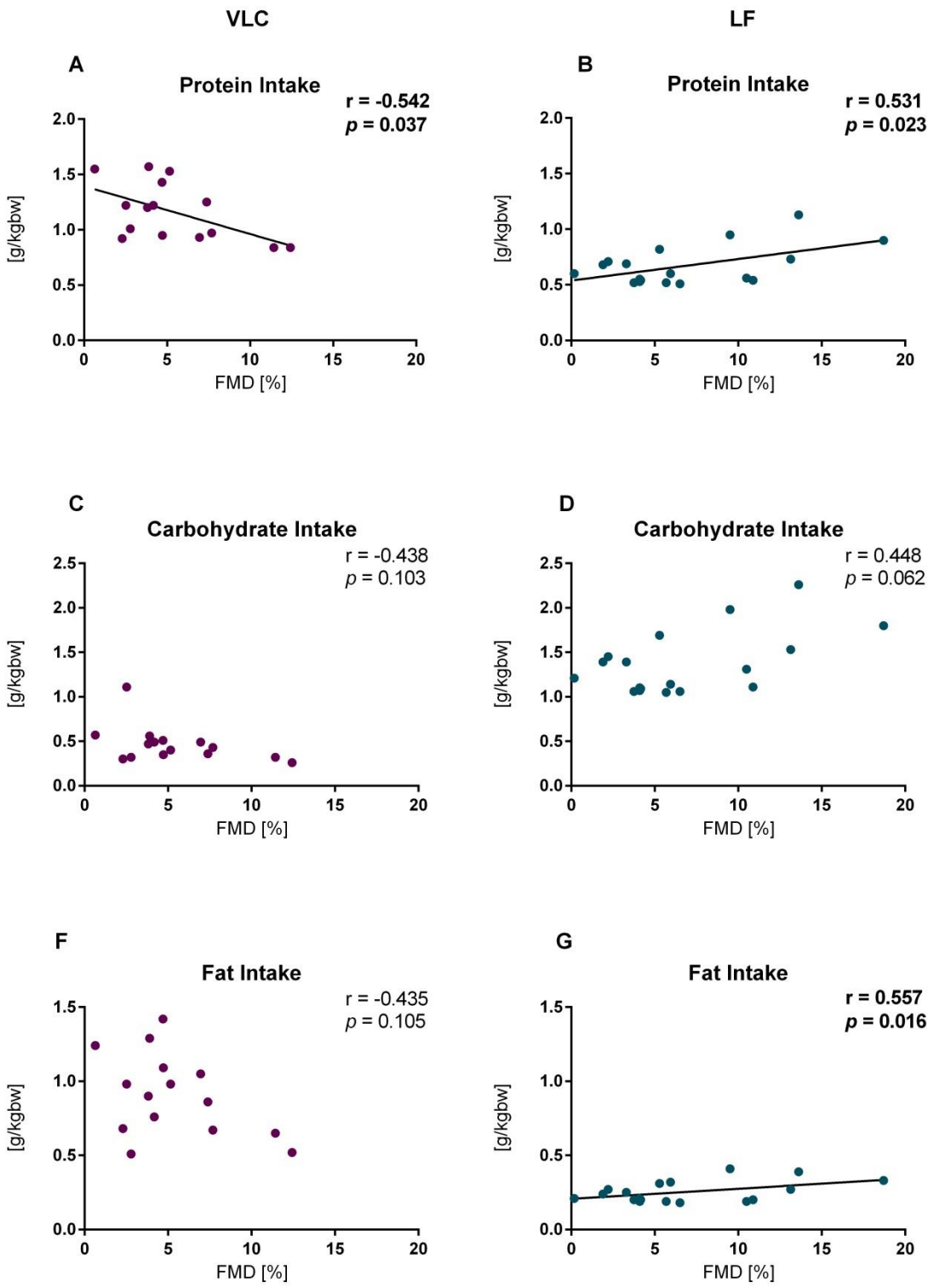

Figure 3. Correlations between FMD and macronutrient intake after 3 weeks diet. (**A**) Protein intake at V2 in the VLC diet, n = 15, (**B**) protein intake at V2 in the LF diet, *n* = 18, (**C**) carbohydrate intake at V2 in the VLC diet, *n* = 15 (**D**) carbohydrate intake at V2 in the LF diet, *n* = 18, (**E**) fat intake at V2 in the VLC diet, *n* = 15 (**F**), fat intake at V2 in the LF diet, *n* = 18. A–F show data after macronutrient intake correction for body weight at V2.

4. Discussion

In the present study we observed that both dietary strategies, VLC and LF, effectively reduced body weight, TAT, VAT and IHL. This suggests that both diets have an acute effect in this pathologic network which has been previously associated with damaged endothelial function [5–7] [20,21]. However, independently of the body fat decreasing success of both diets, subjects who participated in the LF diet experienced significantly greater enhancement of the ability of the branchial artery to dilate, compared to subjects in VLC diet. We show that the favorable endothelial nature of the LF diet mainly relates to its protein quantity and source, both correspondent to fat content and it shares in saturated and unsaturated fatty acids.

After just 3 weeks on a VLC or a LF diet subjects lost significant weight and decreased TAT, VAT, IHL, T. CHO, LDL, and TAG. In spite of the fact that only the VLC diet significantly reduced HbA1c levels and in agreement with previous studies [12,13,16,17], our outcomes suggest that the LF diet had an acute vasodilation effect compared to the VLC diet, whereby the LF diet showed a significant increase of 2.27% points.

According to the evaluation of diet records, subjects of both diet groups adhered well to the respective diets. However, we detected a significant difference in energy consumption between diet groups. This may be related to the previously mentioned weight loss difference between diet groups. Subjects on the LF diet consumed 381 Kcal less than the VLC diet group and therefore lost 1.1 kg more body weight. Consequently, macronutrient intake analysis was corrected for kg BW revealing that the VLC diet increased their protein ingestion by 0.21g/ kg BW, while the LF diet decreased it by -0.26g/kg BW.

4.1. Protein Intake

Our results demonstrate that this positive FMD effect of the LF diet is partially driven by protein. Post-intervention the VLC diet showed a negative correlation and the LF diet showed a positive correlation of FMD with protein intake (g/kg BW). Certainly, protein consumption is often tied to fat consumption. Dependent on the protein source the total fat intake and ratio between fatty acids can vary. The required carbohydrate restriction of the VLC diet increased the amount of animal protein sources (meat, cheese), while the meal replacement powder of LF diet is rich in isolated milk protein and contains defined fats. Whey protein and casein are high quality proteins based on bioavailability, digestibility, amino acid requirements and also bioactive peptides. Currently, there is substantial interest in milk proteins to improve vascular health which has been extensively reviewed [26]. Different research groups showed this positive effect of whey protein [27,28] and casein [29] supplementation on FMD. Although the molecular mechanism behind is still unclear, the present study corroborates that protein quality and quantity do play a part in FMD response. However, it is very challenging to separate the effect of single macro-(micro) nutrients without reflecting the modifications of other macronutrients, since they are connected (discussed below).

4.2. Fat Intake

Our analysis suggests that the intake of energy restricted LF diet leads to an enhanced endothelial function attributed perhaps in part to reduced fat composition of the LF diet. However, it seems that subjects with the lowest fat consumption had the lowest FMD, while subjects with the highest intake of fat had the highest FMD. On the contrary, in the VLC group, although the correlation was not significant, subjects with the lowest fat consumption tended to have the highest FMD and subjects with the highest intake of fat had the lowest FMD. This suggests a potential interaction of protein and fat intake. Indeed, we previously reported a key role of protein intake in modulating fat metabolism particularly with regard to lipolytic activity and modulation of free fatty acids (FFA) [23]. The n-3 FFA alpha-linolenic acid (ALA) and n-6

FFA linoleic acid compete for the delta5 and delta6 desaturases and elongase pathways resulting in the generation of either eicosapentaenoic acid or arachidonic acid. The latter is a precursor for numerous bioactive lipids of the prostanoid family with powerful endothelial activity [30]. On the other hand, the long chain n-3 fatty acids display anti-inflammatory properties, although the supplementation of n3 polyunsaturated fatty acids (PUFA) did not result in improved FMD in a recent study [31].

4.3. Carbohydrate Intake

A hand full of comparable short term studies investigating the dietary influence on FMD have been performed. Phillips et al. [17] and Varady et al. [16] observed this endothelial "LF benefit" after 6 weeks of diet. However, both research groups also described an endothelial "VLC impairment". Our study cannot confirm this observation, since we found no significant difference within (V1 vs V2) this diet group. Furthermore, correlation analysis between carbohydrate intake and FMD was not significant in either diet group.

In contrast to the VLC diet of the present study the high fat (VLC) diet was characterized by a carbohydrate intake of ca. 20g/d and by a higher intake of SFA, which probably has adverse effects on endothelial function [15,18].

Outcomes with VLC diets may improve by decreasing SFA while increasing PUFA and MUFA and perhaps by introducing whey protein and casein as main protein sources.

The main limitation of our study is that the self-reported diet records to assess food intake are a subjective tool, which may be an important confounder. Further limitations are that this study is limited to subjects of Caucasian ethnicity and the small sample size.

5. Conclusions

In conclusion, we demonstrate that both, VLC and LF, diets are effective tools for rapid reduction of weight, TAT, VAT, IHL and blood lipids in T2DM patients. Nevertheless, the LF diet elicited advantageous effects on flow mediated arterial dilation despite the greater HbA1c decrease of the VLC diet. The potential driver of this acute endothelial response may be the interaction of protein and fat metabolism saturated fats. Identification of molecular mechanisms underlying FMD may provide a greater understanding for diet induced effects on endothelial function.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: Nutritional table of MODIFAST® drink vanilla flavor.

Author Contributions: The authors' responsibilities were as follows—A.F.H.P., U.D. and S.K. designed the research and A.F.H.P. acquired funding; U.D., S.K. and J.M. conducted the research; U.D. and L.L. collected the referring data. R.L.B.-Y., performed the statistical analysis, analyzed data and wrote the manuscript; R.L.B.-Y. has primary responsibility for the final content. All authors read and approved the final manuscript.

Funding: General funding for this study was provided by the German Center for Diabetes Research, which itself is funded by the German Federal Ministry for Education and Research. The funding party had no involvement in study design, data collection, data analysis, interpretation, and writing of this publication.

Acknowledgments: We acknowledge all study participants for their cooperation. We gratefully thank our technical assistants and study nurses Anja Henkel and Carolin Lehn, for their help in acquiring the study data and their crucial work with the participants. We would like to acknowledge and thank the German Diabetes Society (DDG) to make the presentation of part of this work at the EASD (European Association for the Study of Diabetes) conference 2017 possible

Conflicts of Interest: The authors declare no conflict of interest.

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VIII Curriculum Vitae

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

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IX List of publications

Original papers

R. L. Barbosa-Yañez, M. Markova, U. Dambeck, C. Honsek., J. Machann, R. Schüler, S. Kabisch, A.F.H. Pfeiffer; Predictive effect of GIPR SNP rs10423928 on glucose metabolism liver fat and adiposity in prediabetic and diabetic subjects, [Peptides, Vol. 125, p.]

F. Keyhani-Nejad, R. L. Barbosa-Yañez , M. Kemper, R. Schueler, O. Pivovarova, N. Rudovich, A.F.H. Pfeiffer; Endogenously released GIP reduces and GLP-1 increases hepatic insulin extraction, [Peptides, Under revision]

R. L. Barbosa-Yañez, U. Dambeck, L. Li, J. Machann S Kabisch, A.F.H. Pfeiffer; Acute Endothelial Benefits of Fat Restriction over Carbohydrate Restriction in Type 2 Diabetes Mellitus: Beyond Carbs and Fats, Nutrients 10(12): 1859 (Open Access) (2018). https://doi.org/10.3390/nu10121859

Conference contributions and presentations

R. L. Barbosa-Yañez, U. Dambeck, C. Honsek, S Kabisch, A. F. H. Pfeiffer; The rs10423928 GIP Receptor "A" Allele Contributes to an Improved ß-Cell Response in Prediabetes Patients, Diabetes 2019 Jun. https://doi.org/10.2337/db19-1720-P

R.L. Barbosa-Yañez, M. Markova, R. Schüler, S. Kabisch, A.F.H. Pfeiffer; High protein diet induced reduction of fatty liver is dependent on GIPR SNP s10423928 genotype in type 2 diabetes patients, Diabetologie und Stoffwechsel 2019; 14(S 01): S32 DOI: 10.1055/s-0039-1688198

R.L. Barbosa-Yañez, M. Markova, R. Schüler, S. Kabisch, A.F.H. Pfeiffer "A Nutrigenomic Approach to Fatty Liver Response in Type 2 Diabetes Patients: Potential nexus between GIPR SNP s10423928 and liver fat". 37th annual meeting DNSG, Kerkrade, Holland, 2019. Poster

R.L. Barbosa-Yañez, M. Markova, D. Sonnenburg, M. Kemper, S. Rohn, A.F.H. Pfeiffer "Endogenous glucagon secretion in response to carbohydrate- or protein-rich meals is not regulated by endogenous GIP or GLP-1 in healthy or type 2 diabetic humans". Third EASD Incretin Study Group Meeting, Bochum, 2019. Oral presentation

R. L. Barbosa-Yañez: "Targeting diet induced differential MicroRNA expression in adipose tissue of type 2 diabetes mellitus patients", 36th annual meeting DNSG, Opatija, 2018. Oral presentation

Poster Session: "Typ-2-Diabetes – Pathophysiologie" German Diabetes Congress (DDG), Berlin, 2018. Chair

R. L. Barbosa-Yañez: “Dietary regulation of Facilitative Glucose Transporters GLUT3 and GLUT5 in abdominal subcutaneous adipose tissue of type 2 diabetes patients”, 61. German Congress for Endocrinology, DGE, Bonn, 2018. Poster

R. L. Barbosa-Yañez: "Effects of 3-week low-carbohydrate diet on endothelial function, visceral fat and liver fat in T2DM patients compared to a low-fat diet ", 53rd annual meeting EASD, Lisbon, 2017. Oral presentation

R. L. Barbosa-Yañez: "Effect of low-carbohydrate / low-fat dietary intervention on gene expression of GLUT transporters in abdominal subcutaneous adipose tissue of type 2 diabetes patients", NuGOweek 2017, Varna, 2017. Oral Presentation

R. L. Barbosa-Yañez: "Effects of short-term low-carbohydrate diet on flow mediated-dilation in T2DM patients compared to a low-fat diet", 35th annual meeting DNSG, Skagen, 2017. Oral presentation

X Acknowledgements

First of all, I would like this opportunity to thank **my family** for supporting me unconditionally and for giving me the opportunity to live this wonderful experience, especially my Uncle Raúl.

I am deeply grateful to the department of clinical nutrition (KLE-DZD) from the German Institute of Human Nutrition (DIfE), principally to Prof. Dr. Andreas F.H. Pfeiffer as the first supervisor and Dr. Stefan Kabisch as second supervisor, for believing in my capacity to accomplish this PhD project. I am very grateful for the permanent support, scientific guidance, constant optimism and encouragement throughout this long walk with all its "ups and downs". I consider myself extremely lucky to have had the opportunity to work and learn from Prof. Dr. Andreas F.H. Pfeiffer and Dr. Stefan Kabisch.
I would like to express a special appreciation to Olga Ramich for her scientific guidance, the good advices, cheerful mood and support whenever needed, and Vanessa Scheling for her amazing work and helpfulness.

I deeply appreciate and thank **all** my colleagues from the KLE (**former and present**) for their constant support during these years.

Many thanks to the competence MRL team in German Institute of human Nutrition (DIfE) for their support and friendly assistance.

Special thanks to my friends, for the constant motivation and moral support. I also would like to express my gratitude to everyone who helped and supported me during the writing of this thesis. A special thanks to Johanna Hansmann and Camila Barbosa Yañez for the diligent proofreading of this thesis.