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Glucose-dependent Insulinotropic Peptide: A Link between Nutrition and Metabolism

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ABBREVIATIONS

ALT	Alanine transaminase
AMP	Adenosine monophosphate
AUC	Area under the curve
Cd36	Cluster of differentiation 36
GH	Growth hormone
GIP	Glucose-dependent insulinotropic peptide
GLP-1	Glucagon-like peptide-1
HFD	High fat diet
HOMA	Homeostatic model assessment
MRI	Magnetic resonance imaging
NAD	Nicotinamide adenine dicucleotide
NAFLD	Non-alcoholic fatty liver disease
Ppara	Peroxisome proliferator-activated receptor a
Socs2	Suppressor of cytokine signaling 2
TG	Triacylglycerol
WISP1	WNT-inducible signaling pathway protein-1
WNT	Wingless-type
WT	Wild type

SUMMARY OF PUBLICATION-BASED THESIS

1. TITLE

Glucose-dependent Insulinotropic Peptide: A Link between Nutrition and Metabolism

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3. ABSTRACT

Non-alcoholic fatty liver disease, insulin resistance and chronic low-grade inflammation are major risk factors for the metabolic syndrome, which is a consequence of multiple factors, particularly hormonal and nutritional imbalances. The gut hormone glucose-dependent insulinotropic peptide (GIP) is secreted in response to nutrient ingestion and regulates glucose and energy homeostasis through enhancing postprandial insulin secretion and anabolic effects in adipose and other tissues. Genetic ablation of GIP receptors and even chronic reduction of GIP secretion alleviates obesity and insulin resistance under high fat diet (HFD) conditions. Multiple evidence suggests that adipokines, secreted bioactive substances from adipose tissue, trigger chronic low-grade inflammation and are implicated in the regulation of insulin resistance.

We examined the importance of endogenous GIP secretion and the function of GIP receptors for the control of fatty liver and energy metabolism through analysis of GIP receptor knockout (*Gipr*$^{-/-}$) mice and by examining carbohydrates with different potential to stimulate GIP secretion. In one study, animals were fed diets containing sucrose (resorbed proximally) and its isomer Palatinose (resorbed distally) to investigate glucose metabolism and fatty liver. In a separate experiment, C57Bl/6J mice were exposed to a HFD to evaluate the expression of a novel adipokine belonging to WNT signaling which regulates adiposity and low-grade inflammation.

Compared with sucrose, Palatinose intake resulted in slower glucose absorption and reduced postprandial insulin and GIP levels. Following 22 weeks of Palatinose containing diets, mice exhibited reduced hepatic triacylglycerol content (48.5%) and preserved glucose tolerance, without differences in body composition and food intake. Ablation of GIP signaling in *Gipr*$^{-/-}$ mice completely prevented the deleterious metabolic effects of sucrose feeding.

Moreover, we observed that mice fed a HFD showed increased body weight and fat mass. *Wisp1* gene expression was significantly up-regulated in the epididymal adipose tissue of HFD-fed mice. Stimulation of human macrophages with WISP1 resulted in pro-inflammatory responses.

WISP1 expression in adipose tissue was associated with markers of insulin resistance and inflammation.

Taken together, our data indicate that the site of glucose absorption and the GIP response determine liver fat accumulation and insulin resistance. Palatinose, as a food ingredient, attenuates postprandial GIP release. Additionally, our findings point to WISP1 as a novel adipokine linking obesity to inflammation and insulin resistance which could be considered as a novel target for obesity treatment.

4. ZUSAMMENFASSUNG

Nicht-alkoholische Fettleber, Insulinresistenz und chronische *low-grade inflammation* sind hauptsächliche Risikofaktoren für das Metabolische Syndrom, einem komplexen Krankheitsbild, welches vornehmlich durch hormonelle und nutritive Imbalancen gekennzeichnet ist. Das Darmhormon *glucose-dependent insulinotropic peptide* (GIP) wird nach Nahrungsaufnahme von enteroendokrinen K-Zellen ausgeschüttet. GIP verstärkt die postprandiale, pankreatische Insulinsekretion, sowie die anabolen Effekte im Fettgewebe und reguliert somit die Glukose- und Energiehomöostase. Die genetische Ablation des GIP-Rezeptors und die chronische Reduktion der GIP-Sekretion verringert das Auftreten von Adipositas und Insulinresistenz unter einer Hochfettdiät (HFD). Adipokine, sezernierte bioaktive Substanzen von Fettzellen, begünstigen nachweislich eine chronische *low-grade inflammation* und sind darüber hinaus an der Regulation der Insulinresistenz beteiligt.

Wir haben die Bedeutung der endogenen GIP-Sekretion und die Funktion des GIP-Rezeptors bezüglich der Entstehung der Fettleber und die Regulation des Energiestoffwechsels erforscht. Hierzu haben wir GIP-Rezeptor *knockout* (*Gipr*$^{-/-}$) Mäuse und Kohlenhydrate mit unterschiedlichen Potentialen zur Stimualtion der GIP-Sekretion untersucht. In der ersten Studie erhielten die Mäuse ein saccharosereiches (proximal resorbiert) bzw. ein palatinosereiches (distal resorbiert) Futter, um den Effekt der Diäten auf den Glukosestoffwechsel und die Fettleber zu untersuchen. In einer zweiten Studie erhielten die C57Bl/6J Mäuse eine HFD, um die Expression eines neu identifizierten Adipokins zu evaluieren, welches dem WNT Signalweg angehört und sowohl Adipositas als auch chronische *low-grade inflammation* reguliert.

Im Vergleich zu Saccharose, führte die Aufnahme von Palatinose zu einer langsameren Glukoseabsorption und zu niedrigeren postprandialen Insulin- und GIP-Spiegeln. Nach 22 Wochen des palatinosereichen Futters, zeigten die Mäuse eine konservierte Glukosetoleranz und reduzierte Triazylglyzerin-Spiegel (48,5%) bei unveränderter Körperzusammensetzung und

Nahrungsaufnahme. Die Ablation des GIP-Signals in *Gipr*$^{-/-}$ Mäusen konnte die schädlichen metabolischen Effekte einer saccharosebetonten Fütterung gänzlich vermeiden.

Wir konnten ferner beobachten, dass Mäuse, die eine HFD erhielten, ein erhöhtes Körpergewicht und eine vermehrte Körperfettmasse aufwiesen. Bei HFD-gefütterten Mäusen konnte eine signifikante Hochregulation der Genexpression von *Wisp1* im epididymalen Fettgwebe beobachtet werden. Eine Stimulation von humanen Makrophagen mit WISP1 resultierte in einer pro-inflammatorischen Antwort. Die *WISP1*-Expression im Fettgewebe war mit Insulinresistenz und Inflammation assoziiert.

Unsere Daten zeigen, dass der Ort der Glukoseabsorption (distal/proximal) und die GIP-Antwort maßgeblich an der Entstehung von Insulinresistenz sowie einer Fettakkumulation beteiligt sind. Der Lebensmittelinhaltsstoff Palatinose verringert die postprandiale GIP-Sezernierung. Zusätzlich konnten wir zeigen, dass WISP1, welches Adipositas mit Inflammation und Insulinresistenz assoziiert, ein neu entdecktes Adipokin ist. WISP1 könnte zukünftig als ‘‘Novel target“ für die Etablierung neuer Therapien gegen Adipositas eingesetzt werden.

5. INTRODUCTION

Metabolic syndrome is a grave public health problem and associated with obesity and a variety of metabolic disorders such as type 2 diabetes, non-alcoholic fatty liver disease (NAFLD) and insulin resistance. For instance, the prevalence of overweight and obesity rose worldwide by 27.5% for adults and 47.1% for children between 1980 and 2013, which means that around 2.1 billion individuals are overweight or obese and at risk for metabolic disorders [1; 2]. Both genetic and environmental factors including nutritional and hormonal aspects contribute to the development of metabolic syndrome. In order to develop successful therapies, we need to consider the interaction between these factors. Accumulating evidence suggests that ectopic deposition of triacylglycerol (TG), influenced by nutrition, is as an important contributor to NAFLD which encompasses a broad spectrum of liver diseases from simple, benign fatty liver to steatohepatitis (NASH) [3].

Gut hormones secreted in response to nutrient ingestion play essential roles in the regulation of energy homeostasis [4; 5]. Gastrointestinal peptides like glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide-1 (GLP-1) function as incretin hormones to convey the insulinotropic signals from the gut to the pancreatic islets in the postprandial state, with GIP having a predominant role [5; 6]. GIP is a 42-amino acid peptide [7] that is synthesized in and secreted from enteroendocrine K-cells in the proximal intestine in response to glucose and fat,

whereas GLP-1 is secreted from L-cells in the distal small intestine and colon [8; 9]. Following secretion, GIP exerts its effects through specific G protein coupled receptors and its activation leads to cyclic AMP (cAMP) generation and insulin secretion from pancreatic β-cells. GIP receptors are also widely expressed in non-islet cells, including the gastrointestinal tract, heart, adipose tissue, adrenal cortex and brain [9; 10]. In addition to its major action in glucose-dependent stimulation of insulin secretion, GIP elicits extra-pancreatic effects and is implicated in metabolic control [11; 12]. GIP links overnutrition to obesity [13]. A growing body of evidence supports a role for GIP in the regulation of fat metabolism and its anabolic characteristics [14]. In adipocytes, GIP stimulates adipogenesis and enhanced activity of lipoprotein lipase (LPL) activity [15-17].

Recently, genetic reduction of GIP secretion has been shown to alleviate obesity and insulin resistance under high fat diet (HFD) conditions [18]. Miyawaki, et al. demonstrated the inhibition of GIP signaling in the regulation of insulin resistance and fatty liver in mice. [19]. Consistent with these results, using a specific GIP receptor antagonist has been associated with enhanced insulin sensitivity [20]. Thus, GIP appears to play a different role in fat and glucose metabolism promoting effective assimilation and storage of food. Despite evidence for GIP effects in adipose tissue, the role of GIP has not been investigated in diet induced fatty liver. This challenge has fostered a research effort using animal models, especially GIP receptor knockout (*Gipr*$^{-/-}$) mice to better understand the etiology and pathology of metabolic disorders like fatty liver and glucose intolerance.

There is considerable interest in identifying extra-pancreatic and indirect actions of GIP. The role of GIP in the liver, presumably via an indirect mechanism, needs to be investigated. High intake of carbohydrates, particularly sucrose, in Western societies is associated with the development of NAFLD and type 2 diabetes mellitus. It is unclear whether this is related primarily to the carbohydrate quantity or to the hormonal responses, particularly GIP, which is released in the proximal intestine. To delineate the physiological importance of GIP action, we used different sugars, two glucose–fructose dimers, sucrose and Palatinose (isomaltulose), with dissimilar ability to release GIP and then studied the metabolic effects in experiments with *Gipr*$^{-/-}$ mice.

The second focus of the current thesis is regarding insulin resistance. Obesity, as a major risk factor of both NAFLD and type 2 diabetes provides the common link through insulin resistance [21]. Interestingly, despite known alterations of insulin sensitivity by aging, genetics and environment, it may change circannually. Therefore, finding the putative times of insulin sensitivity is valuable for interpretation of cross-sectional and prospective studies.

Insulin resistance is likely mediated by adipose tissue inflammation and dysregulated adipokine production in obesity [21]. GIP induces the secretion of adipokines such as resistin and adiponectin [22]. Interestingly, *gip* gene expression and incretin production were also been shown to be stimulated by the Wingless type (WNT) signaling cascade. The canonical WNT signaling pathway is closely related to the production of incretin hormones, since the transcription factor TCF7L2 affects glucagon and GIP gene expression in L and K enteroendocrine cells [23]. WISP1 (Wnt1 inducible signaling pathway protein 1) is the target gene of WNT signaling pathway. The protein WISP1 is present in multiple organs throughout the body and is expressed in the epithelium, heart, kidney, lung, pancreas, placenta, ovaries, small intestine, spleen, and brain. WISP1 has multiple cellular functions that include skeletal system development, vascular repair, cellular survival and extracellular matrix growth [24]. Evidence shows that WNT signaling regulates adipogenesis and low-grade inflammation in obesity. Here, the aim was to validate WISP1 as a novel adipokine.

In general, our aim was to investigate the effect of nutrition on stimulating GIP release and define divergent effects of GIP, beyond pancreatic roles, on liver and muscle and how endogenous GIP modulates fatty liver and glucose metabolism. In addition, we aimed to define WISP1 as a marker of HFD-induced obesity and to study its association with insulin resistance.

6. Material and Methods

6.1. Animals

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

Experiments were performed in male C57Bl/6J mice (Janvier Labs, Saint Berthevin, France), unless otherwise stated. Mice were housed in individual cages with free access to water and standard rodent chow, with a 12:12 h light–dark cycle and a temperature of 23±2°C. Mice were allowed a 1-week acclimatisation period before starting the experiments. *Gipr*$^{-/-}$ mice and wild-type (WT) littermates on a C57Bl/6J strain background were generated as described [25] .In order to explant organs, overnight fasted mice were sedated using isoflurane (Baxter, Unterschleissheim, Germany) and killed by cervical dislocation. Organs were isolated rapidly, snap frozen in liquid nitrogen and kept at -80°C for RNA isolation.

6.2. Diets

In study [26], animals were fed either a control diet with 10% of fat and energy density of 3.8 kcal/g (catalog no. D12450B; Research Diets Inc., New Brunswick, NJ, USA) or a HFD with 60% of fat and energy density of 5.2 kcal/g (catalog no. D12492; Research Diets Inc.) following to the experimental designs for 6 weeks.

In experiment [27], mice were fed isoenergetic diets containing 40.5% (wt/wt) carbohydrate, 41.5% (wt/wt) fat and 18% (wt/wt) protein. The diets differed in terms of the type of carbohydrate used, which was either palatinose or sucrose (table 1[27]).

6.3. Feeding test

In a cohort of animals, mice were trained for 4 days to consume either a Palatinose- or a sucrose-containing diet as detailed previously [28]. Briefly, individually housed mice were given 500 mg of the experimental diets following an overnight fast. Blood samples from the tail vein were drawn at 0 (overnight fasted), 30, 60, 90 and 120 min of consuming the whole portion of test meals within 15 min.

6.4. Dietary intervention

Body weight matched mice were fed the above-mentioned diets of identical macro- and micro-nutrient composition for 22 weeks [27].

To elucidate the role of GIP in mediating the sucrose induced hepatic fat accumulation, another long-term experiment was performed in *Gipr*$^{-/-}$ and WT littermates. In order to receive comparable information, age and body weight matched *Gipr*$^{-/-}$ and WT mice were placed on the aforementioned diets. All the experimental measurements are indicated below.

6.5. Body composition and food intake

Body weight and cumulative food intake were measured weekly for individually housed mice. Body fat and lean mass were determined at indicated times using nuclear magnetic resonance spectroscopy (Mini Spect MQ10 NMR Analyser Bruker, Karlsruhe, Germany) in conscious mice.

6.6. Glucose tolerance test

A glucose tolerance test (GTT) was performed by i.p. glucose (2 g/kg BW) injection after overnight fasting. Blood samples were taken from the submandibular vein plexus for glucose and insulin measurements before and at 10, 30, 60 and 120 min after the glucose challenge.

6.7. Incretin measurement

Plasma GIP levels were quantified using a rat/mouse total GIP ELISA kit (EMD Millipore). Blood samples were collected in tubes containing heparin lithium (Sigma-Aldrich Co, St Louis, MO, USA). Rat/Mouse GIP standard with the concentration of 2000 pg/ml was used for the assay. Levels of plasma GLP-1 were determined by the GLP-1 (active) ELISA kit (Shibayagi Co, Gunma, Japan). Recombinant GLP-1 (7-36) was used as the standard. Blood samples were collected in tubes containing EDTA-Na^{2+} (Sigma-Aldrich) and aprotinin (Carl Roth Co, Karlsruhe, Germany) with the final concentration of 1mg/ml and 500 KIU/ml, respectively. To avoid the degradation of GLP-1, a DPP-IV inhibitor (EMD Millipore) was added to the plasma samples. All samples were stored at -80°C until performing the assay.

6.8. Quantification of liver TG

Frozen liver samples were powdered in liquid nitrogen. 2.5 ml of 10mM sodium phosphate buffer containing 1mM EDTA and 1% polyoxyethylene 10 tridecylethan was added to 50 mg of the samples. They were homogenized and centrifuged (10 min, 20,000 x g). Then the supernatant was incubated at 70°C for 5 min. TG (triacylglycerol reagent, Sigma-Aldrich) and protein (DC protein assay, Bio-Rad, Hercules, CA, USA) levels were quantified in duplicate.

6.9. RNA extraction and quantitative RT-PCR

Total RNA was purified and quantified from liver, gastrocnemius muscle, hypothalamus and epididymal white adipose tissue samples as described [28]. Quantitative RT-PCR was performed using ABI Prism 7900 HT Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The quantity of target and the housekeeping gene (*Hprt*) were calculated according to a standard curve. Primer sequences are listed in the supplementary data [26; 27].

6.10. Microarray analysis

Total RNA (300 ng), quantified and qualified by Agilent 2100 Bioanalyser, was amplified using the Illumina TotalPrep RNA Amplification kit (Ambion, Carlsbad, CA, USA). Amplified cRNA was hybridised to Mouse Ref-8 v2.0 Expression BeadChips (Illumina, San Diego, CA, USA).

Staining and scanning were done according to the Illumina expression protocol. Transcriptome analyses were performed by the statistical programming environment R implemented in CARMAweb [29]. Genewise testing for differential expression was done using the Limma *t* test and Benjamini–Hochberg multiple testing corrections (FDR < 10%) and significant terms ($p<0.01$) were determined. Pathway enrichment analyses were done with the Ingenuity pathway software (Qiagen, Hilden, Germany).

6.11. Data analysis

Data were analysed using IBM SPSS statistics 20 (SPSS, Chicago, IL, USA). Comparisons between two groups were performed using unpaired Student's *t* test. Multiple comparisons were tested by one-way ANOVA, followed by post hoc Tukey or Games–Howell tests according to the homogeneity of variances (Levene's test). To test longitudinal changes over time, ANOVA with repeated measurement was used. Statistically significant effects of genotype and diet were determined using two-way ANOVA. The area under the curve (AUC) was calculated by the trapezoid rule. Statistical significance was defined as $p<0.05$. Results are presented as mean ± SEM.

7. RESULTS AND DISCUSSION

7.1. Nutritional strategy to prevent fatty liver and insulin resistance independent of obesity by reducing GIP responses in mice

The most common cause of NAFLD can likely be attributed to an exaggerated intake of dietary energy, especially carbohydrates, inducing a strong insulin response. Therefore, dietary components capable of decreasing postprandial glucose and insulin levels are promising approaches to reduce the development of NAFLD. Studies indicate that the intake of rapidly digestible sugars such as sucrose, known as high glycaemic index sugars, as compared with Palatinose, a slowly and completely resorbed sucrose analogue composed of α-1,6-linked glucose and fructose, has deleterious effects on postprandial glucose, insulin and TG levels, which are associated with the risk of obesity, insulin resistance and fatty liver [30; 31]. An inhibition of carbohydrate absorption by acarbose, an inhibitor of α glycosidase, was shown to reduce GIP release in humans [32]. Since genetic reduction of GIP secretion was shown to prevent HFD-induced obesity and insulin resistance [18], we decided to explore a dietary strategy to reduce GIP secretion to study the possible benefits of dietary lessened GIP release.

For this approach, we used sucrose and its isomer Palatinose. We speculated that Palatinose, composed of glucose and fructose, should also reduce GIP release and other consequences of high sucrose intake. To determine the role of GIP in diet-induced fatty liver and impaired glucose homeostasis, we performed a long-term diet intervention in *Gipr*$^{-/-}$ mice and WT littermates.

We first investigated the acute oral response of Palatinose and sucrose in glycaemic homeostasis and in the release of intestinal incretins. Sucrose caused an expected rapid increase in glucose accompanied by GIP and insulin release, while GLP-1 levels did not differ between groups. By contrast, Palatinose caused a more delayed increase in glucose, which resulted in little GIP secretion and, accordingly, much lower insulin secretion (supplement Fig. 1 [27]). Our next question was whether or not these differences might be maintained by diet. Therefore, we established isoenergetic diets based on Palatinose and sucrose.

Indeed, the diet containing sucrose induced a rapid and strong increase in GIP and insulin, as shown by the AUC, (2.5- and 1.5-fold, respectively), whereas Palatinose was not associated with a major increase in GIP and, accordingly, resulted in a smaller increase in insulin (Fig. 1). We observed differences in oral and meal tests on plasma glucose levels, which are well-known [33], and relate to the content of fat and protein in the whole diet, which slows gastric emptying and thereby delays glucose absorption in the small intestine. The most likely explanation for the differences in GIP release refers to the more distal absorption of Palatinose, which bypasses the proximally located GIP-producing K-cells in the small intestine.

In our study, plasma GLP-1 levels remained unchanged in mice administered sucrose or Palatinose (supplement Fig. 1 [27]), as reported earlier in models of GIP receptor antagonism and genetically reduced GIP secretion [18; 34], indicating that the reduction of GIP secretion does not affect GLP-1 secretion.

We further analysed the long-term metabolic response of sugars in a hypercaloric diet. Ad libitum access to diets containing Palatinose and sucrose resulted in similar body weight, body fat and cumulative food intake between the two groups (Fig. 2). Energy content of the diets was quantified by calorimeter and was comparable between groups (Palatinose, 4.81 kcal/g; Sucrose, 4.83 kcal/g). Digestibility of the diets was 89% with sucrose and 88.5% with palatinose and the digested energy between groups, as estimated over one week, was not significantly different (Table 2 [27]). However, the Palatinose-fed mice exhibited reduced hepatic TG and were protected from diet-induced impaired glucose tolerance (Fig. 3 [27]). A study in rats has reported that Palatinose-fed animals had higher hepatic insulin sensitivity [35], reduced hepatic TG, and lower postprandial insulin and glucose levels, which is consistent with our results, although that

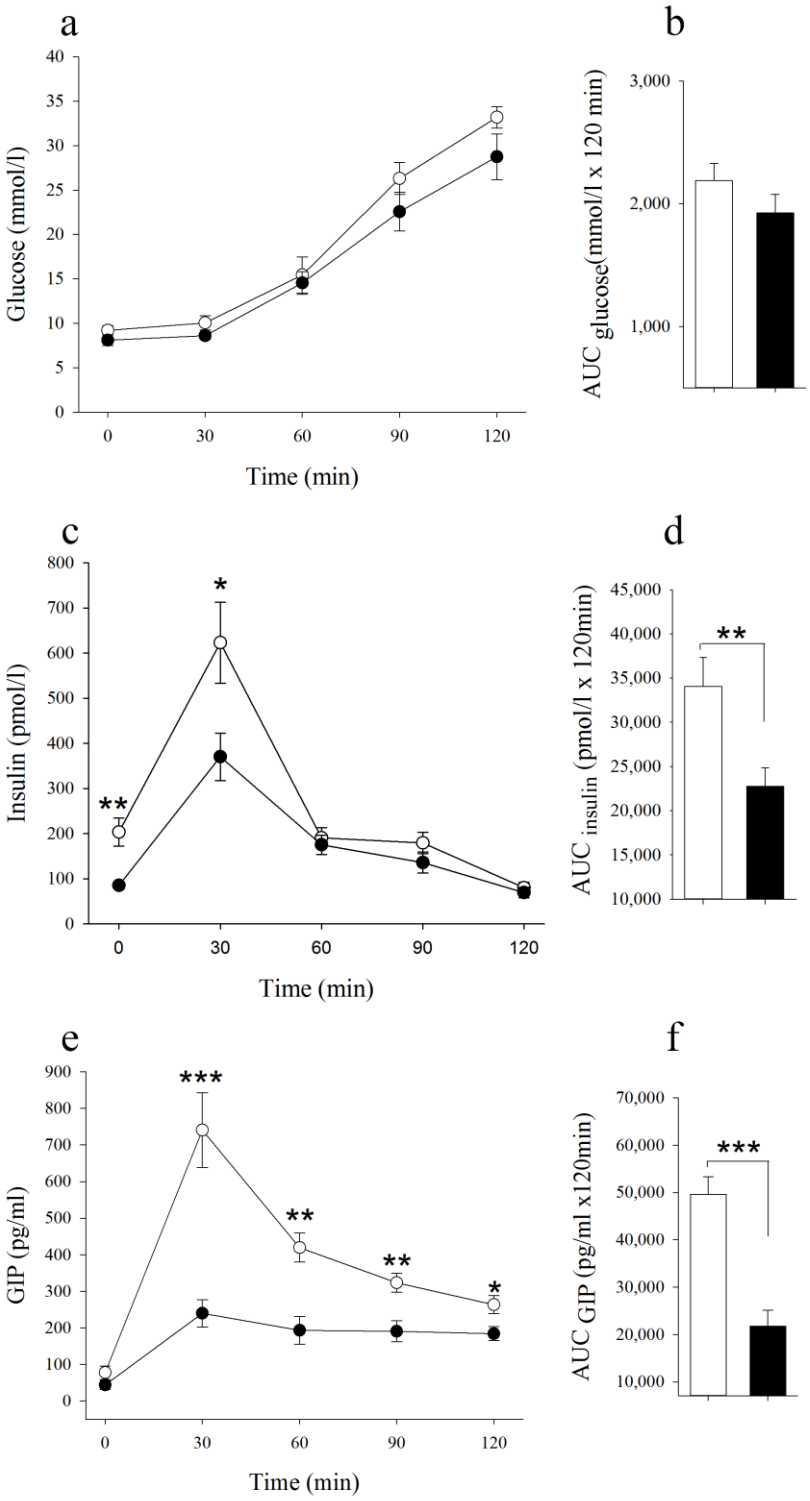

Fig. 1 Glycemic, insulinemic and GIP responses to sucrose (white circles and bars) and Palatinose (black circles and bars) intake.
To characterize the impact of the sugars as an ingredient of dietary pattern, a meal test was performed. Plasma levels of (**a**) glucose, (**c**) insulin, (**e**) GIP over 120 min were evaluated after ingestion of 500 mg of experimental diets. AUC for (**b**) glucose, (**d**) insulin and (**f**) GIP shows that the diets containing sucrose induced rapid and large increases of GIP and insulin compared to the Palatinose diet, while the glucose levels were not overall different between two diets.
Values are mean ± S.E.M of 8 male mice in each group.
* $p<0.05$, ** $p<0.01$ and
*** $p<0.001$

study was confounded by differences in body weight. Indeed, liver fat is associated with and is likely to be a cause of hepatic insulin resistance [36]. The 2-fold increase in liver TG levels with increased levels of ALT, a key indicator of hepatotoxicity, supports the presence of liver damage in our sucrose-fed mice. Palatinose intake resulted in a modest reduction in postprandial glucose levels and a 40% reduction in glucose-stimulated insulin response.

The most compelling evidence in support of the contribution of GIP to fatty liver was the *in vivo* experiment in *Gipr*$^{-/-}$ and WT mice. *Gipr*$^{-/-}$ mice behaved similarly to WT mice fed Palatinose, and were protected from fatty liver. Remarkably, liver TG levels in *Gipr*$^{-/-}$ mice fed sucrose and Palatinose were 2-fold and 3-fold less than those of the WT sucrose-fed mice, respectively. The levels of liver TG in WT mice fed sucrose were approximately twice those of Palatinose-fed mice (Fig. 2). In addition, during GTT, lower glucose levels in WT mice fed Palatinose and

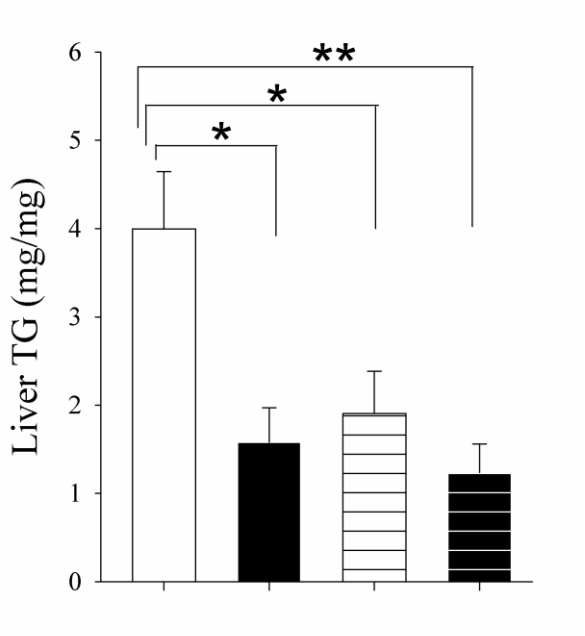

Fig. 2 Liver TG content in wild type (WT) and *Gipr*$^{-/-}$ mice exposed to diets containing Palatinose or sucrose for 22 weeks. Diets containing sucrose in WT mice induced significant higher levels of hepatic TG compared to WT Palatinose fed and *Gipr*$^{-/-}$ mice. White circles and bars: WT, sucrose; black circles and bars: WT, Palatinose. White triangles and hatched bars: *Gipr*$^{-/-}$, sucrose; black triangles and hatched bars: *Gipr*$^{-/-}$, Palatinose. Values are mean ± S.E.M of 9 WT and 11 *Gipr*$^{-/-}$ mice in each group. . *p<0.05, **p<0.01

Gipr$^{-/-}$ mice than WTs fed sucrose suggests that glucoregulation was similarly achieved in conditions of suppression of GIP signaling and reduced GIP production by Palatinose intake. At the same time, the circulating insulin levels were lower in *Gipr*$^{-/-}$ mice and WTs fed Palatinose that those of WT mice on sucrose diet (Fig. 4 [27]).

In our study, comparable body weight and food intake confirm similar energy intake and digestibility of diets; therefore, the observed metabolic and hormonal differences are related to absorption differences in the gut. Palatinose and sucrose were fully hydrolysed and absorbed in the small intestine, confirming finding from previous studies [37].

Although not significant, Palatinose feeding enhanced leptin release, which did occur independently of changes in fat mass and food intake most likely as a consequence of central regulation.

To evaluate the mechanisms for the observed effects in liver TG without differences in body weight and digested energy, we assessed the mRNA expression of transcription factors and genes involved in metabolic pathways leading to the development of NAFLD, including de novo lipogenesis (*Acca*, *Fas*, *Srebp1c* and *Chrebp*), lipid beta-oxidation (*Ppara* and *Cpt1a*) and secretion from liver (*ApoB100* and *Mtp*). However, the expression of these genes did not differ

between Palatinose and sucrose-fed mice. This finding is in contrast to other reports that increased *Ppara* and hepatic fat oxidation were observed for attenuated liver fat in Palatinose feeding [38]. However, in this study body weight differed between Palatinose and sucrose-fed mice.

Indeed, Palatinose improved glucose homeostasis and liver fat metabolism in the context of HFD in part by enhancing muscular fatty acid uptake and causing a shift towards fat oxidation instead of fat deposition in the liver, as indicated by increased expression of *Cd36* and *Ppara* (Fig 5. [27]).

For additional mechanisms we performed microarray analyses in liver and observed that Palatinose reduced 2.3-fold the mRNA expression of *Socs2*, suppressor of cytokine signaling 2. Studies have revealed changes in *SOCS2* mRNA levels in human steatotic livers [39; 40]. Recently, it was shown that *Socs2*$^{-/-}$ mice are protected from HFD-induced hepatic steatosis [41]. The reduced expression of *Socs2* mRNA observed in the Palatinose-fed mice could be due to reduced plasma growth hormone (GH) levels. It is reported that the GIP receptor mediates an increase in GH after glucose challenge [42; 43]. Our results suggest that Palatinose might indirectly contribute to GH modulation by inhibiting postprandial GIP release and *Socs2* expression in the liver. However, pathway analysis of microarray data indicated upregulation of glycogen and nicotinamide adenine dinucleotide (NAD) biosynthesis pathways in the sucrose group (supplement Table 3). Liver NAD biosynthesis is controlled by a salvage pathway using nicotinamide as a precursor and a *de novo* pathway using tryptophan. Increased NAD in the liver may enhance gluconeogenesis [44].

In summary**,** the main finding of our study was that Palatinose feeding prevented the development of fatty liver and improved glucose metabolism in the setting of a HFD, without differences in energy intake and body weight between groups. The highly significant prevention of hepatic fat accumulation was mediated by a reduced GIP response, avoiding postprandial hyperinsulinaemia. The results in *Gipr*$^{-/-}$ mice suggest that GIP may mediate the deleterious metabolic effects of sucrose induced insulin resistance and fatty liver. Therefore, Palatinose as a food ingredient reduces postprandial GIP secretion by evading upper intestinal absorption. By this mechanism, Palatinose feeding results in reduced postprandial glucose and insulin levels and represents a promising approach for the prevention and/or treatment of fatty liver and insulin resistance in humans.

7.2. WISP1 is a novel adipokine linked to inflammation in obesity

Over the past decade, obesity as the major risk factor for metabolic syndrome has been associated with chronic low-grade inflammatory responses. Adipose tissue has a major endocrine function secreting multiple adipokines which are involved in energy homeostasis and inflammation. In obesity, the adipocyte is integral to the development of obesity-induced inflammation by increasing secretion of various pro-inflammatory cytokines which have been reported to promote insulin resistance [45]. Furthermore, evidence from animal studies links the Wingless-type (WNT) signaling pathway to the regulation of adipogenesis [46] and inflammation [47] in obesity. Members of the WNT-signaling family are secreted glycoproteins that are acting in both autocrine and paracrine fashions to regulate cell proliferation, cell fate, differentiation and organism development. The WNT signaling network is composed of several canonical and non-canonical pathways which are strongly controlling cell remodelling [24; 48].

WNT-inducible signaling pathway protein-1 (WISP1 also known as FCCN4) belongs to the CCN family extracellular matrix proteins and is a downstream target gene of the canonical WNT signaling pathway [49]. WISP1 is expressed in various organs and tissues including heart, pancreas, lung, small intestine, spleen and brain. In some of these tissues it acts anti-apoptotic through PI3K and Akt pathways. WISP1 has a regulatory function in skeletal growth and bone repair [49]. Recent reports show that other members of CCN family are tightly related to adipogenesis. No data are currently available regarding the role of WISP1 on adiposity and its effects in insulin target tissues, including liver and fat. Therefore, we combined *in vitro* experiments with human studies accompanied by animal experiment to show that WISP1 is a novel marker of obesity regulated by HFD and validate its association with parameters of metabolic syndrome.

We could show that *WISP1* gene expression and WISP1 protein production is up-regulated during human adipocyte differentiation. *WISP1* was highly expressed in human visceral adipose tissue and moderately expressed in subcutaneous adipose tissue. In addition, *WISP1* expression correlated negatively with insulin sensitivity, circulating adiponectin levels, and with visceral fat content as measured by MRI, suggesting that WISP1 may be a useful marker of visceral fat accumulation and insulin resistance. Interestingly, WISP1 increased pro-inflammatory cytokine production in cultured macrophages and had a positive correlation with macrophage infiltration in subcutaneous and visceral adipose tissue. Moreover, it induced macrophage polarization towards the inflammatory M1 phenotype. Since WISP1 modulates macrophage infiltration and polarization and is also released by differentiated adipocytes, it may be characterized as an adipokine that participates in the control of macrophage function.

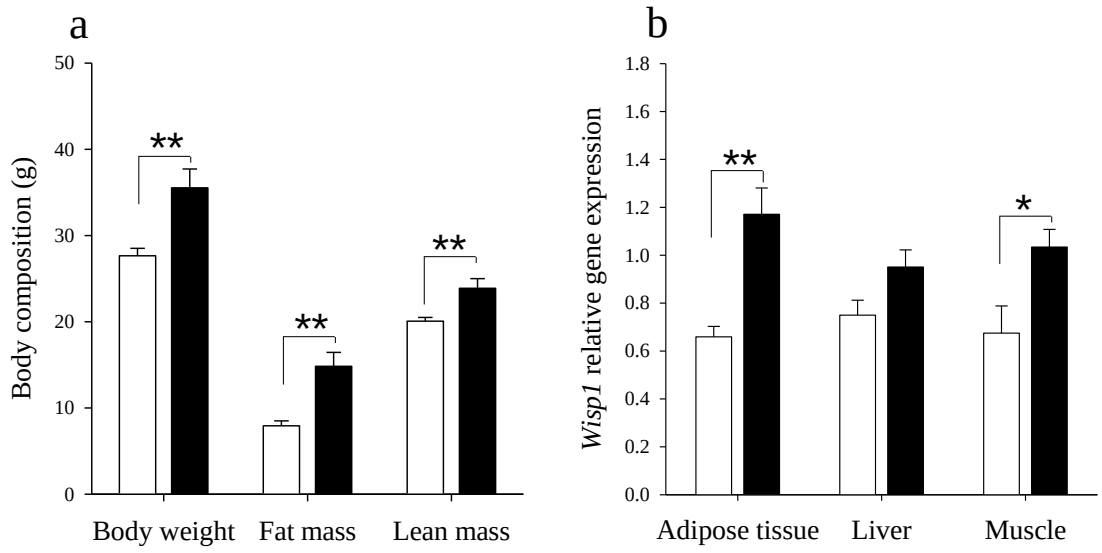

Fig. 2 (**a**) Changes of body weight, body composition and (**b**) *Wisp1* mRNA expression in epididymal fat tissue, liver and muscle in mice after 6 weeks of control diet (white bar) or high fat diet (black bar). Values are mean ± S.E.M of 7 wild type mice in each group. **p*<0.05, ***p*<0.01

Furthermore, to test the hypothesis whether WISP1 is regulated in diet-induced obesity, we studied male mice, which were randomized into either a control diet or HFD. After 6 weeks of diet intervention, the HFD-fed mice showed increased body weight, fat mass and lean mass compared with their control littermates (Fig. 4. [26]). *Wisp1* gene expression was significantly up-regulated in the epididymal fat tissue and muscle of HFD-fed mice. However, in the HFD fed mice *Wisp1* gene expression was not significantly increased in liver compared to mice on a control diet. Additionally, *WISP1* expression in liver was moderate and was not up-regulated in the human subjects with NAFLD. Furthermore, we observed no association between different biochemical and anthropometrical markers of obesity and hepatic gene expression of *WISP1*, suggesting that WISP1 is not involved in hepatic fat accumulation. Thus, body weight gain resulted in changes in circulating WISP1 originated most likely from adipose tissue rather than from the liver or other organs.

In conclusion, WISP1 is released by completely differentiated human adipocytes and stimulated macrophages. It is substantially expressed in adipose tissue in obesity and HFD feeding and reflects adipose tissue inflammation and insulin resistance. WISP1 as an adipokine plays a role in linking obesity to inflammation and insulin resistance.

7.3. Annual changes in insulin sensitivity

The elevated incidence of type 2 diabetes mortality during winter [50] may indicate an annual periodic change in insulin sensitivity. We therefore analysed putative annual periodic changes of insulin sensitivity in a large cross-sectional human cohort. To address this, we applied HOMA-%S, as an estimation of fasting glucose and insulin, and also Matsuda Sensitivity Index, as an index of glucose metabolism during glucose challenge. Calculations were performed within the Metabolic-Syndrome-Berlin-Potsdam (MeSyBePo) study group consisting of volunteers from the Berlin/Potsdam region in Germany enrolled during 2002-2009.

The study cohort consisted of 2385 participants (mean age 51.9 ± 0.3 years, mean BMI 29.8 ± 0.1 kg/m^2, mean HOMA-%S 104.9 ± 1.9%, 1992 participants without diabetes). We found a significant periodic oscillation (β=0.08, *P*=0.02) for HOMA-%S with a period length of 9.1 months. This corresponds throughout the year to a 1.08-fold increase of ln HOMA-%S, or a change of 8%. Restriction to the participants without diabetes (n=1992) confirmed the result and strengthened the model fit (β=0.07, *P*=0.009), again with a similar period length of 9.2 months (Fig 1 [51]).

To further demonstrate the impact of this annual change we compared insulin sensitivity between subjects enrolled during the first half of the year and those enrolled during the second half (January to June vs. July to December). Insulin sensitivity was significantly improved in subjects enrolled during the second half of the year (HOMA-%S 112.0±3.0 % vs. 97.4±2.4 %, *P*=0.00003). Using Matsuda Index instead of HOMA-%S revealed comparable results.

We here report annual changes of insulin sensitivity in a large cohort of more than 2000 participants. Our data are in agreement with the observed annual changes in blood glucose [52]. However, although statistically significant, the here observed 8% annual oscillation of insulin sensitivity was only moderate and is unlikely to relevantly affect general interpretation of studies in this field although it may become relevant in case of small changes in insulin sensitivity.

In principle, potential explanations for the periodic change in insulin sensitivity may be changes in vitamin-D metabolism known to be affected by annual changes and the exposure to sunlight [53; 54], and being discussed in the context of insulin sensitivity. Serum levels of 25-hydroxyvitamin D were described highest in autumn and lowest after winter [55], which fits well with the annual change in insulin sensitivity observed here.

In summary, we found a rather small annual periodicity in insulin sensitivity which is unlikely important for the general interpretation of studies but which may become relevant in case of small differences in insulin sensitivity.

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SELECTED PUBLICATIONS

1- Keyhani-Nejad F, Irmler M, Isken F, Wirth EK, Beckers J, Birkenfeld AL, Pfeiffer AF: Nutritional strategy to prevent fatty liver and insulin resistance independent of obesity by reducing glucose-dependent insulinotropic polypeptide responses in mice. Diabetologia 2015;58(2):374-383
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2- Murahovschi V, Pivovarova O, Ilkavets I, Dmitrieva RM, Docke S, Keyhani-Nejad F, Gogebakan O, Osterhoff M, Kemper M, Hornemann S, Markova M, Kloting N, Stockmann M, Weickert MO, Lamounier-Zepter V, Neuhaus P, Konradi A, Dooley S, von Loeffelholz C, Bluher M, Pfeiffer AF, Rudovich N: WISP1 is a novel adipokine linked to inflammation in obesity. Diabetes. 2015;64(3):856-66
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3- Isken F, Abraham U, Weickert MO, Keyhani-Nejad F, Arafat AM, Spranger J, Pfeiffer AF, Mohlig M: Annual change in insulin sensitivity. Hormone and metabolic research 2011;43:720-722
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Publication 1

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Murahovschi V, Pivovarova O, Ilkavets I, Dmitrieva RM, Docke S, Keyhani-Nejad F, Gogebakan O, Osterhoff M, Kemper M, Hornemann S, Markova M, Kloting N, Stockmann M, Weickert MO, Lamounier-Zepter V, Neuhaus P, Konradi A, Dooley S, von Loeffelholz C, Bluher M, Pfeiffer AF, Rudovich N: WISP1 is a novel adipokine linked to inflammation in obesity. Diabetes. 2015;64(3):856-66
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Publication 3

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CURRICULUM VITAE

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

COMPLETE LIST OF PUBLICATIONS

Original papers

1- Keyhani-Nejad F, Irmler M, Isken F, Wirth EK, Beckers J, Birkenfeld AL, Pfeiffer AF: Nutritional strategy to prevent fatty liver and insulin resistance independent of obesity by reducing glucose-dependent insulinotropic polypeptide responses in mice. Diabetologia 2015 Feb;58(2):374-383

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3- Murahovschi V, Pivovarova O, Ilkavets I, Dmitrieva RM, Docke S, Keyhani-Nejad F, Gogebakan O, Osterhoff M, Kemper M, Hornemann S, Markova M, Kloting N, Stockmann M, Weickert MO, Lamounier-Zepter V, Neuhaus P, Konradi A, Dooley S, von Loeffelholz C, Bluher M, Pfeiffer AF, Rudovich N: WISP1 is a novel adipokine linked to inflammation in obesity. Diabetes 2015 Mar;64(3):856-66

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Conference contributions and presentations

1- Keyhani Nejad F, Isken F, Osterhoff MA, Nitz B, Ludwig T, Grallert H, Pfeiffer AFH, Kruse M "A high fat diet during pregnancy and lactation affects the metabolic fate of Gipr-/- mice via hypothalamic insulin signaling and DNA-methylation of lipid metabolism genes" [Oral presentation] Diabetologia (2014) 57:[Suppl1]S41

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AFFIDAVIT (STATEMENT OF CONTRIBUTIONS)

I, Farnaz Keyhani-Nejad, certify under penalty of perjury by my own signature that I have submitted the thesis on the topic "Glucose-dependent Insulinotropic Peptide: A Link between Nutrition and Metabolism" I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My contributions in the selected publications for this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author of correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Date _____ Signature

Declaration of any eventual publications

Farnaz Keyhani-Nejad has contributed to the following publications as follows:

Publication 1: Keyhani-Nejad F, Irmler M, Isken F, Wirth EK, Beckers J, Birkenfeld AL, Pfeiffer AF: Nutritional strategy to prevent fatty liver and insulin resistance independent of obesity by reducing glucose-dependent insulinotropic polypeptide responses in mice. Diabetologia 2015 Feb;58(2):374-383

85% contribution

Detailed involvement: Designing the experiment, acquisition of results, statistical analysis and interpretation of the data, drafting and writing the manuscript in addition to review procedure.

Publication 2: Murahovschi V, Pivovarova O, Ilkavets I, Dmitrieva RM, Docke S, Keyhani-Nejad F, Gogebakan O, Osterhoff M, Kemper M, Hornemann S, Markova M, Kloting N, Stockmann M, Weickert MO, Lamounier-Zepter V, Neuhaus P, Konradi A, Dooley S, von Loeffelholz C, Bluher M, Pfeiffer AF, Rudovich N: WISP1 is a novel adipokine linked to inflammation in obesity. Diabetes 2014; [Epub ahead of print]

10% contribution

Detailed involvement: Design and performance of animal experiment, analysis the results, drafting the manuscript and reviews

Publication 3: Isken F, Abraham U, Weickert MO, Keyhani-Nejad F, Arafat AM, Spranger J, Pfeiffer AF, Mohlig M: Annual change in insulin sensitivity. Hormone and metabolic research 2011;43:720-722

25% contribution

Detailed involvement: Manuscript conception, compilation and analysis of the data, drafting and writing the manuscript

Signature, date and stamp of the supervising University teacher

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Signature of the doctoral candidate

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