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Direktor: Professor Dr. med. Andreas F. H. Pfeiffer

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Role of gastrointestinal hormones in the metabolic dysfunction in obesity and associated diseases

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Dr. med. Natalia Rudovich
geboren in Leningrad/Russland

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Dekanin: Prof. Dr. med. Annette Grüters-Kieslich

1. Gutachter: Prof. Dr. med. Jochen Seufert/Freiburg

2. Gutachter: Prof. Dr. med. Horst Harald Klein/Bochum

“But love is dark, like truth, and has the bitter bite
Of wormwood, while the salt of sweat grows still more salty.
Time for a change, you cannot live with all doors bolted,
A diehard beast, ad finem, to the final rites.

The mill of learned books has barely reached its youth.
Clutching a textbook in a wasteland isn't canny.
Blessed is he, whose will is strong, and who knows Truth,
But truths are many, many...”

M. Scherbakov

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Abbreviations

BMI	Body Mass Index
CI	Confidence Interval
CCK	Cholecystokynine
EC	Euglycemic hyperinsulinemic clamp
EPIC	European Prospective Investigation into Cancer and Nutrition study
FDR	First degree relative of T2DM
GIP	Glucose-dependent insulinotropic peptide
GIPR	Glucose-dependent insulinotropic peptide receptor
GLP-1	Glucagon-like-peptide-1
HBA1C	Haemoglobin A1c
HDL-C	High-Density Lipoprotein-Cholesterol
HHEX	Hematopoietically expressed homeobox protein gene
HPRT1	Hypoxanthine phosphoribosyltransferase 1
$HOMA_{IR}$	Homeostasis Model Assessment of Insulin Resistance
HC	Hyperglycemic hyperinsulinemic clamp
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IDE	Insulin degrading enzyme
LDL-C	Low Density Lipoprotein-Cholesterol
LMCT	Liquid meal challenge test
MESYBEPO	Metabolic Syndrome Berlin Potsdam study
NAFLD	Non-alcoholic fatty liver disease
NGT	Normal glucose tolerance
OGTT	Oral glucose tolerance test
OR	Odds ratio
RPLP0	Ribosomal protein large protein 0
qRT-PCR	Quantitative real-time PCR
SDHA	Succinate Dehydrogenase Complex subunit A
SNP	Single Nucleotide Polymorphism
T2DM	Type 2 diabetes mellitus
TG	Triglycerides

1. Introduction

Obesity is one of the five major health risks in modern societies and considered a wordwide epidemic [1]. Beside the genetic predisposition, low physical activity and hyperalimentation are the environmental factors which lead to the development of obesity. Obesity significantly increases the risk of developing type 2 diabetes, non-alcoholic fatty liver disease, cardiovascular complications and several types of cancer [2]. Early detection and appropriate lifestyle modification coupled with early therapeutical intervention are essential for preventing the progression of obesity in the population.

Despite there being more than a million adipose subjects worldwide and an enormous medical and socio-economic burden due to the progression of the associated diseases such as type 2 diabetes, hypertension, stroke and cancer, the aetiology of obesity is still not yet completely understood. The gastrointestinal tract is the largest endocrine organ in the body [3]. The gastro-intestinal hormones have an important sensing and signaling role in the regulation of appetite and energy expenditure **(Figure1)**. In humans this is best revealed by the striking success of bariatric surgery for the treatment of morbid obesity [4]. There is also increasing evidence that bariatric surgery reduces hunger and improves glucose homeostasis partly by altering circulating gut hormones such as Glucagon-like Peptide-1 ($GLP\text{-}1_{7\text{-}39}$) and also Glucose-dependent insulinotropic peptide receptor (GIP) and Peptide YY (PYY) [5, 6]. GLP-1 and GIP are so-called “incretins”, intestinal hormones released by food components which cause insulin release [7].

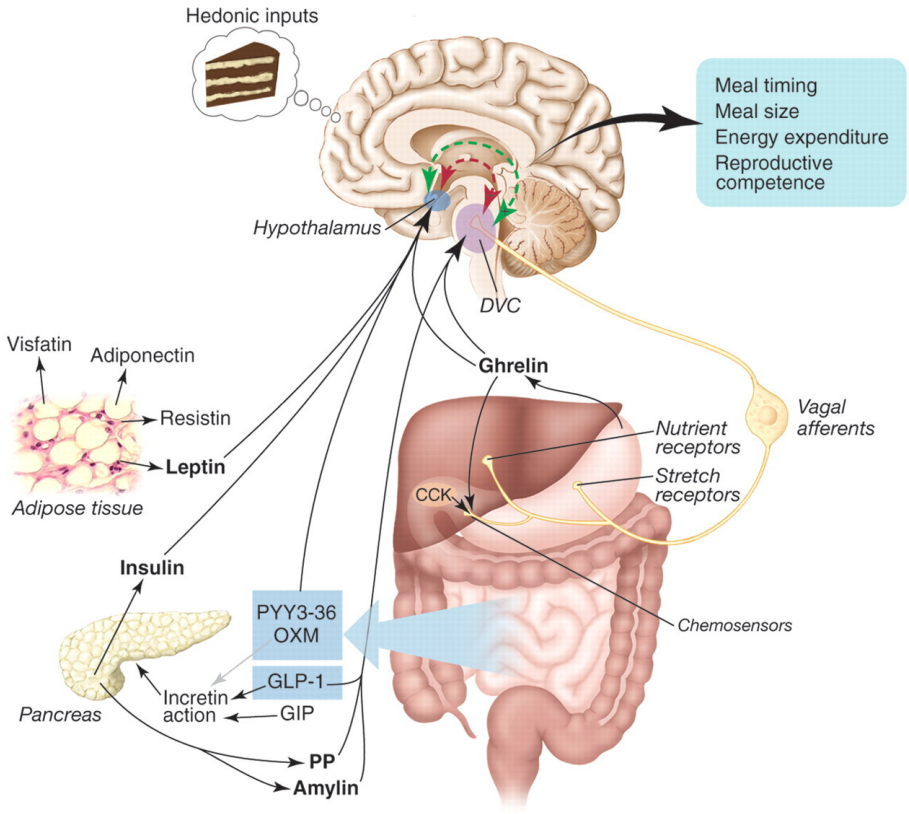

Figure. 1. The brain integrates long-term energy balance **(*Badman & Flier Science 2005;307:1909-1914; Ref. 19)***

Incretins normally account for approximately 60 – 70% of the insulin response to orally administered glucose but also elicit additional effects on metabolic control [7]. Today, two classes of antidiabetic drugs have been developed that work through the stimulation of GLP-1 receptors [8] or through inactivation of dipeptidyl peptidase-4, an enzyme which cleaves incretin [9].

Glucose-dependent insulinotropic peptide (GIP) is an insulinotropic hormone, secreted from enteroendocrine K-cells of the duodenum in response to food ingestion containing glucose or fat [10-12], which modulates glucose-dependent insulin secretion [7, 13]. However, its other physiologically relevant

role appears to be as an anabolic agent with a stimulatory effect on fat synthesis in adipocytes and glucose utilisation in muscle [10, 11, 14]. GIP increases the glucose transport into tissue, stimulates the lipoprotein lipase activity and the conversion of free fatty acids to triglycerides in presents of insulin in fat cells [10, 11, 15, 16].

The secretion of GIP is not decreased in type 2 diabetes mellitus, while the insulinotropic effect gets lost to a great extent [17]. Obese adults and overweight patients with type 2 diabetes mellitus show high postprandial levels of circulating GIP [18]. It was suggested that GIP is important in stimulating insulin secretion and weight gain [11, 19, 20]. Furthermore, postprandial GIP levels are exaggerated by high-fat diet suggested, that increased GIP signalling promotes fat accumulation into adipocytes [12, 21, 22].

The GIPR is a glycoprotein belonging to the class II G protein-coupled receptor superfamily that includes receptors for glucagon, GLP-1, secretin, vasoactive intestinal polypeptide, and pituitary adenylyl cyclase-activating protein [23]. Ligand binding to the GIPR activates a heterotrimeric Gs protein that in turn activates adenylate cyclase, elevates intracellular cAMP and ionized Ca levels, and activates PKA, as well as a host of other signaling pathways, including PI-3K, PKB, MAPK, and phospholipase A2 [10, 23]. GIP receptors (GIPR) are widely distributed in animals and are found not only on beta cells of the pancreatic islets of Langerhans but also on fat cells, in gastric mucosa, the lungs and other organs [15, 16, 24, 25].

Remarkably, a genetic deletion of GIP receptors ($GIPR^{-/-}$) was shown to protect mice from developing obesity [21]. In normal mice, high-fat diet leads to

increased adipocyte mass and insulin resistance, whereas GIPR$^{-/-}$ mice fed high-fat diets do not become obese or develop insulin resistance. The mice seem to have a higher energy expenditure rather than lower energy intake compared with wild-type mice [21]. Consistent with these results, the chemical ablation of GIP receptor action in obesity-related diabetic mice using a specific GIP receptor antagonist was associated with enhanced insulin sensitivity by lowering the meal induced excursions of blood glucose and insulin concentrations [26]. Thus, GIP appears to play a different role in fat and glucose metabolism promoting effective assimilation and storage of food.

Recently published data have suggested that GIP may play an etiologic role in the pathogenesis of obesity and type 2 diabetes mellitus [19, 20, 27] and the inhibition of GIP signalling as a new target for anti-obesity drugs was discussed [20, 26, 28, 29]. Despite evidence for GIP effects on fat metabolism in humans, the role of GIPR in the pathogenesis of human obesity and insulin resistance has not been completely understood. Recent work of the applicant extended this field with respect to the metabolic syndrome and obesity.

The second focus of the applicant was the role of impaired insulin metabolism in the pathogenesis of obesity and type 2 diabetes mellitus (T2DM). T2DM is characterized by insulin resistance, pancreatic beta-cell dysfunction, and probably alterations of insulin metabolism [30-32]. Decreased hepatic insulin degradation is an early phenotypical marker of disturbances in the insulin metabolism and it was observed in first degree relatives of type 2 diabetes patients [32], in obese insulin resistant persons and children with metabolic

syndrome [33-36]. Decreased insulin degradation may intensify the insulin resistance via chronically elevated circulating fasting and postprandial insulin levels (**Figure 11**). However, mechanisms leading to the alteration in the insulin degradation remain unclear.

Insulin-degrading enzyme (IDE) is thought to be a major enzyme responsible for insulin degradation [37]. IDE is a 110 kDa Zn requiring metalloproteinase localized in the cytoplasm, cell membranes, in some cell organelles (endosomes, peroxisomes, mitochondria) and secreted into the extracellular space [37]. Insulin is the preferred substrate for IDE, but a large body of other substrates including glucagon, atrial natriuretic peptide and beta-amyloid peptide were reported. IDE has also regulatory functions for proteasome activity, steroid receptors, peroxisomal fatty acid oxidation, growth and development [37]. IDE is ubiquitously expressed, both in insulin-sensitive and in non-insulin-sensitive cells, supporting a multifunctional role for this protein [37]. All insulin-sensitive cells contain IDE and remove and degrade insulin. However, the liver is the main site of insulin clearance removing approximately 50 % during the first portal passage [38]. Amazingly, extremely few reports to date concerning the functional regulation of IDE in liver or liver cells have been published [39-41].

The linkage with IDE chromosome region 10q23-q25 was identified for T2DM and related quantitative traits [42, 43]. *IDE* knockout mice are characterized by classic features of T2DM: decreased insulin degradation, hyperinsulinemia and glucose intolerance [44]. Loss-of-function mutations or pharmacological inhibition of *IDE* increases amyloid accumulation in pancreatic beta-cells and in the central nervous system [44-46]. On the other side, *IDE*

overexpression increases insulin degradation and decreases the efficiency of insulin stimulation in the insulin signalling pathway [47]. These data demonstrate that the regulation of the *IDE* expression level and/or its activity may contribute to the T2DM pathogenesis.

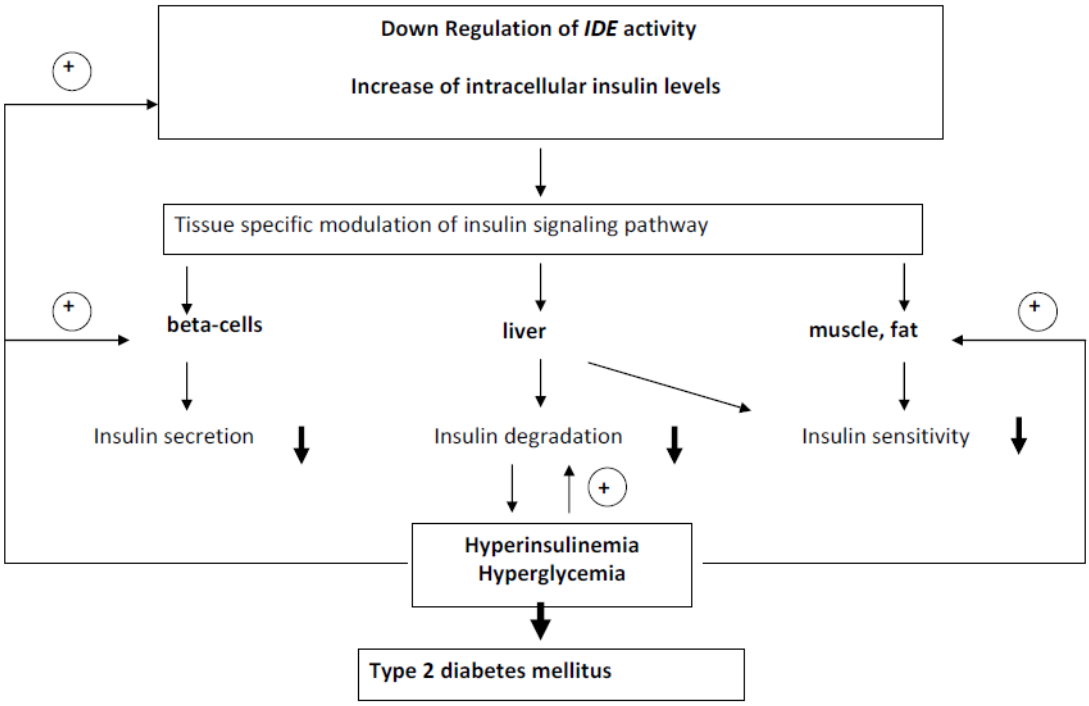

Figure 11: Schematic view on IDE-mechanisms in the development of T2DM. ***(Rudovich N, unpublished observation).***

However, no clear data were available in human, especially the effect of *IDE* gene polymorphisms on T2DM risk had not been investigated in large prospective population studies. We analysed the diabetes risk of two *IDE* polymorphisms in the prospective nested case control study and made detailed phenotypisation of IDE-dependent effects on insulin metabolism in additionally cross-sectional cohort of non diabetic subjects [48]. Moreover we performed a

detailed analysis of the regulation of IDE function by different concentrations of insulin and glucose in *in vitro* and *in vivo* models [49].

Although the manuscript will primarily focus on the applicant's previously published work, some yet unpublished data will also be shown, if appropriate. All data will be demonstrated within the actual scientific context.

2. Gastrointestinal hormones and metabolic dysfunction in obesity

2.1 GIP receptor mRNA expression in different fat tissue depot and effect of the moderate weight loss

Human *GIPR* is a member of the secretin-vasoactive intestinal polypeptide family of G-protein-coupled receptors [50, 51]. The expression of the *GIPR* in the human tissues except pancreas and an aberrant *GIPR* gene expression in causes of GIP-dependent Cushing disease are purely investigated [51, 52]. We studied in details *GIPR* in human subcutaneous and visceral adipose tissue [53]. We also aimed to investigate whether such a metabolic syndrome characteristics are primarily influenced *GIPR* expression in the different fat tissue depots and whether the weight loss may modulate *GIPR* expression.

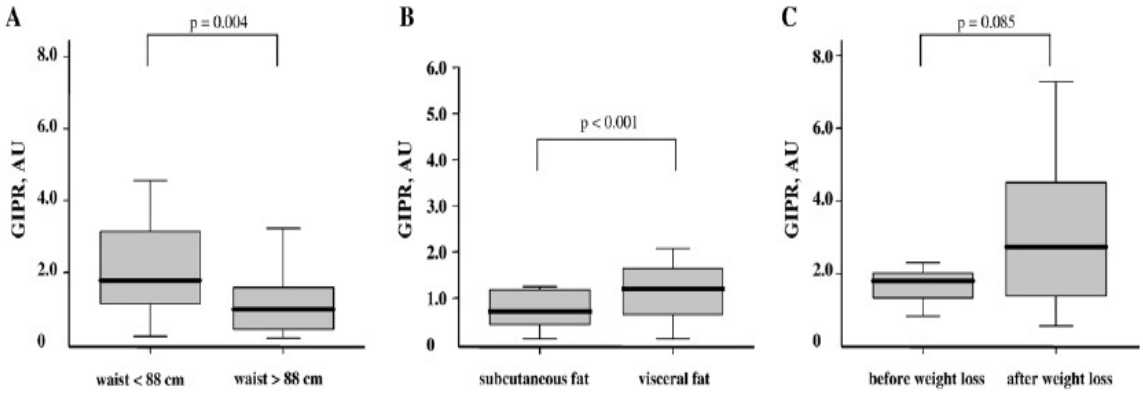

Figure 2: *GIPR* gene expression in subcutaneous and visceral fat tissues. **1-A:** Participants from the first cross-sectional study (*n*=70) were divided into two groups of waist circumference (below and above 88 cm). **1-B:** *GIPR* gene expression in paired samples of visceral and subcutaneous adipose tissues (*n*=25), who underwent open abdominal surgery. **1-C:** Effect of weight reduction (5% weight loss) on subcutaneous adipose *GIPR* in a subset of women from the cross-sectional study (*n*=14). ***(Rudovich N et al., Reg Peptides, 2007)***

In our cohort of 95 postmenopausal women subcutaneous adipose-tissue *GIPR* gene expression was significantly reduced in woman with central obesity compared with normal weight individuals **(Figure 1-A)**. The visceral fat *GIPR* mRNA expression was higher as the subcutaneous fat *GIPR* mRNA expression **(Figure 1-B)**.

The subcutaneous fat *GIPR* mRNA expression was negatively correlated with fasting insulin, waist circumference, fasting blood glucose, LDL-cholesterol and $HOMA_{IR,}$ and positively correlated with HDL-cholesterol levels but not with age **(Table 1)**. The stepwise multiple linear regression analysis revealed waist circumference as the most significant predictor of *GIP*R gene expression whereas fasting blood glucose and fasting triglyceride concentrations had no influence (data not show).

***GIPR* gene expression in subcutaneous adipose tissue**		
Parameter	***r***	***p***
Age	-0.124	NS
Waist circumference	-0.399	0.0001
HOMA-IR	-0.424	0.0001
Fasting glucose	-0.400	0.0001
Fasting insulin	-0.427	0.0001
TG	-0.381	0.0001
HDL-cholesterol	0.393	0.0001
LDL-cholesterol	-0.365	0.001

Table 1: Correlation of *GIPR* gene expression in subcutaneous adipose tissue samples with metabolic parameters in non diabetic postmenopausal women from first and second study population (*n=95*). Data are given as Pearson´s correlation coefficient r. NS = not significant. TG - tryglycerides0, HDL - high density lipoprotein, LDL - low density lipoprotein. All variables were adjusted for BMI. ***(Rudovich N et al., Reg Peptides, 2007)***

***GIPR* gene expression in visceral adipose tissue**		
Parameter	***r***	***p***
Age	0.103	NS
Waist circumference	-0.581	0.002
HOMA-IR	0.539	0.005
Fasting glucose	0.637	0.001
Fasting insulin	0.556	0.004
TG	0.192	NS
HDL-cholesterol	0.243	NS
LDL-cholesterol	-0.464	0.019
GIPR gene expression in SAT	0.826	0.0001

Table 2: Correlation of *GIPR* gene expression in visceral adipose tissue samples with metabolic parameters in non diabetic postmenopausal women from second study population (n=25). Data are given as Pearson´s correlation coefficient r. NS - not significant. TG - tryglycerides, HDL - high density lipoprotein, LDL - low density lipoprotein. All variables were adjusted for BMI. SAT – subcutaneous adipose tissue. ***(Rudovich N et al., Reg Peptides, 2007)***

The visceral fat *GIPR* mRNA expression was negatively correlated with waist circumference and LDL-cholesterol levels, and positively correlated with fasting insulin, fasting glucose and $HOMA_{IR}$ but not with age, triglycerides and HDL-cholesterol **(Table 2)** and fasting insulin levels were the best predictors of the *GIPR* gene expression [53]. We found a higher level of the *GIPR* gene expression in visceral fat tissue compared with subcutaneous fat tissue. In contrast to data from subcutaneous fat tissue, we found a positive correlation between insulin levels and visceral fat *GIPR* gene expression. This phenomenon could be the result of a different regulatory mechanism of subcutaneous and visceral fat gene expression by insulin [54]. Moreover, there are regional differences in the intrinsic characteristics of the preadipocytes in the different fat depots, with those of subcutaneous adipose tissue presenting greater

differentiation and fat cell gene expression but less apoptosis than that of visceral fat tissue [55]. The regulation of *GIPR* expression in human fat tissue has until present not been investigated in detail. Thus we also aimed to investigate the effect of moderate weight reduction (>5% of initial body weight) on *GIPR* expression in obese women (*n*=14). Weight loss was accompanied by a median reduction of BMI (median and range; 31.7 (27.1-40.1) kg/m^2 vs. 30.4 (26-37.8); p<0.05). However, expression levels of the *GIPR* gene in adipose tissue did not change significantly with weight loss **(Figure 1-C)**. Similarly, no significant changes were found for basal serum insulin concentrations and $HOMA_{IR}$. Given the possible determinant effect of insulin on *GIPR* gene expression, the lack of change in insulin concentrations after moderate weight loss may explain the absence of change in gene expression levels.

Additionally, we detected the *GIPR* mRNA in different human tissues including brain, small intestine, kidney, salivary gland, heart, lung, thyroid, placenta, lymph node, stomach, testis, colon, thymus, spleen, cervix, uterus, pituitary gland, trachea, prostate, pancreas, subcutaneous and visceral fat and liver but not in the skeletal muscle **(Figure 2)**. This finding is partially consistent with previous reports demonstrating that *GIPR* is widely distributed in human tissues [56] and suggested the possibly function of GIP as a pleiotropic modulator in the pathogenesis of obesity and associated diseases [23].

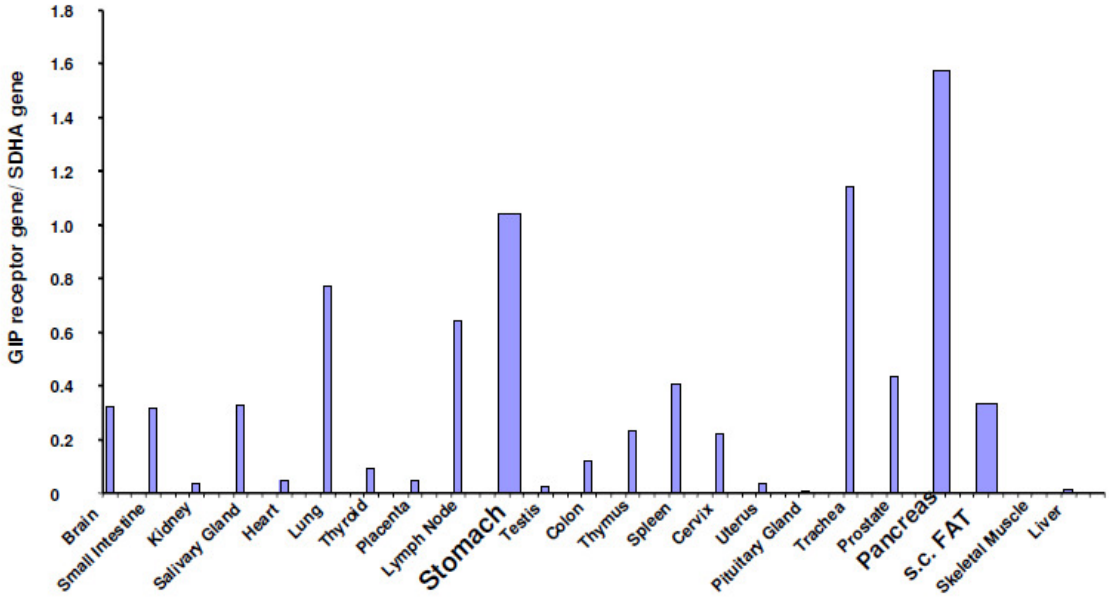

Figure 3. *GIPR* gene expression in human multiple tissue set. ***(Rudovich N et al., Reg Peptides, 2007)***

In summary, decreased expression of the *GIPR* gene in subcutaneous fat tissue is associated with signs of insulin resistance in non diabetic women with central obesity and demonstrates that insulin is a possible regulator of *GIPR* gene expression in adipocytes. Opposite correlation between of *GIPR* gene expression and insulin concentration in visceral and subcutaneous fat tissue reflects regional differences in adipose tissue biology. Weight reduction in a moderate range, did not change gene expression levels of *GIPR* in subcutaneous adipose tissue ***(Rudovich N et al., Reg Peptides, 2007)***.

2.2 GIP and insulin metabolism in obesity

There is increasing evidence that genetic defects in insulin secretion represent a major factor predisposing to the development of T2DM [30, 31]. In addition, several studies of diabetes in humans have shown decreased insulin clearance and degradation associated with insulin resistance [34, 37, 57].

The fine mechanisms of regulation of insulin clearance in humans remain unclear. In non-diabetic insulin-resistant individuals, the hyperinsulinemia resulted from an increase in glucose-stimulated insulin secretion as well as from a decrease in insulin clearance [36]. Moreover, increased insulin sensitivity results in low insulin secretion and increased insulin clearance [37]. On the other hand, a chronically impaired ability to clear insulin from the circulation, as is seen in certain liver diseases, causes hyperinsulinemia with attendant peripheral insulin resistance as a result of impaired insulin action [58]. Thus, altered insulin clearance may not only represent an adaptive mechanism but also one of the primary defects in the pathogenesis of type 2 diabetes mellitus. The regulation of insulin clearance has profound pathophysiological consequences for fuel metabolism. In this context it is of importance to understand not only how insulin secretion, but also how insulin clearance is altered in insulin resistance. One of the potential mechanisms appears to be the effect of incretin hormones on insulin secretion and insulin clearance [59].

We thus measured the ability of the ß-cell to maintain the insulin secretion and mechanisms to regulate the peripheral insulin concentration in normal-weight glucose tolerant young first degree relatives of subjects with T2DM and healthy

subjects **(Table 3)** [32]. We designed a hyperglycemic clamp that evaluates multiple parameters during a single session: first- and second-phases of glucose-induced insulin secretion; insulin secretion during an incretin stimulus (hyperglycemia plus 1 h infusion of gastric inhibitory polypeptide); and first and second phases of an arginine induced insulin secretion (hyperglycemia plus GIP-infusion plus 30 min arginine bolus and infusion).

Parameters	Control	First-degree relatives of T2DM	p
Gender (female/male)	6w/8m	8w/5m	0.35
Age (years)	34 ± 2.5	29 ± 2.8	0.37
Body mass index (kg/m^2)	23.5 ± 1.1	23.9 ± 1.2	0.68
Fasting blood glucose (mmo/l)	4.5 ± 0.1	4.9 ± 0.1	0.30
Fasting insulin, (pmol/l)	22 ± 2	40 ± 4	0.001
Fasting C-peptide, (pmol/l)	346 ± 47	493 ± 62	0.038
ISR, basal (pmol/min)	99 ± 23	132 ± 17	0.004
HOMA-IR [fold normal]	0.99 ± 0.1	2.03 ± 0.5	0.003
HOMA-IS [% of normal]	94 ± 13	169 ± 45	0.067

Table 3: Subjects characteristics. Data are means ± S.E.M. ***(Rudovich N et al., Diabetes 2004)***

In contrast to control subjects, FDR´s had higher fasting peripheral insulin concentrations and were more insulin resistant **(Table 3)**. During the infusion of GIP and arginine, similar blood glucose levels were achieved in control subjects and FDR´s. The glucose infusion rates necessary to maintain hyperglycemia did

not differ between the control group and FDR´s (data not shown). During the hyperglycemic clamp, insulin concentrations were significantly higher in the group of FDR´s at the time 25-70 min **(Figure 4-A)**. In contrast, C-peptide concentrations were not significant different between both groups during the hyperglycemic clamp **(Figure 4-B)**. Insulin secretion rate was significant different between both groups only at time-point 7.5 min after glucose-bolus (p=0.017) **(Figure 4-C)**. The increase in insulin concentration closely followed the increase in insulin secretion rate in the first hour of the hyperglycemic clamp. Then, at 70 min in the FDR´s and at 85 min in control subjects, the increase in the insulin concentrations began to exceed the increase in insulin secretion rate. Subsequently, with continuing stimulation of insulin secretion with three secretagogues, this difference became more pronounced. Thus, whereas the insulin secretion rate increased to 2272.4±228.4 % of basal in the control group and to 1634.4±116.2 % of basal in FDR´s (p=0.04) at 115 min of the hyperglycemic clamp, the peripheral insulin concentration increased to a significantly greater extent, reaching 7333.3±1562.5 % of basal in control group and to 7345.8±1455.7 % of basal in FDR´s (p=0.09) **(Figure 5)**. The observation that the increase in the serum insulin concentration was relatively greater than the increase in the insulin secretion rate can explain only by a decrease in the clearance of endogenously secreted insulin during the clamp.

We measured insulin clearance indirectly in three ways based on previously reported techniques of calculation of insulin clearance: (a) as relationship between changes in ISR and changes in peripheral insulin concentrations during the hyperglycemic clamp, both in respect to basal levels

[60, 61]; (b) as ratio of AUC_{ISR} to $AUC_{insulin}$ for basal and stimulated state of the hyperglycemic clamp [60, 61]; (c) as the molar ratio of $AUC_{C\text{-}peptide}$ to $AUC_{insulin}$ for basal and stimulated state of the hyperglycemic clamp [62, 63].

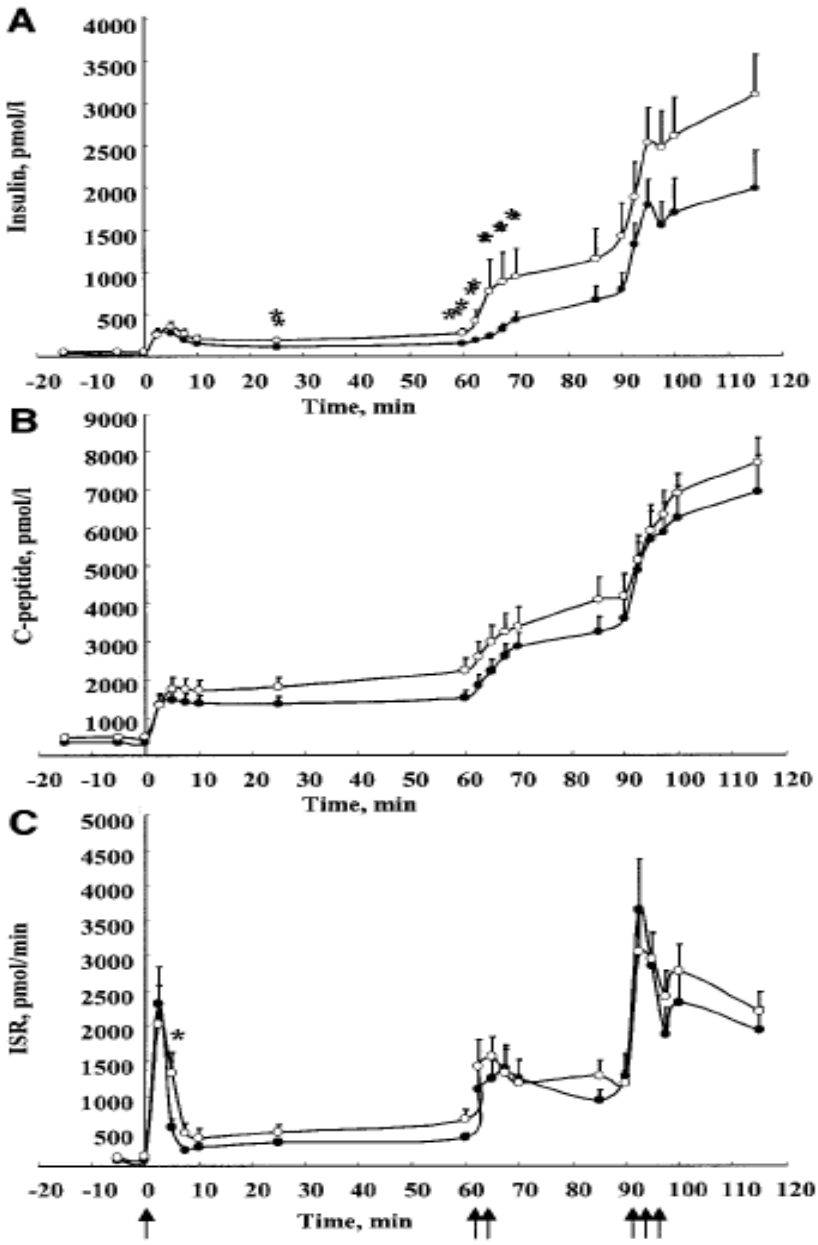

Figure 4 A-C: Mean insulin (A), C-peptide (B) and ISR (C) values during hyperglycemic clamps. White circles = first-degree relatives of patients with type 2 diabetes mellitus, black circles = control subjects. Arrows indicate initiation of glucose- , GIP- and arginine infusion.* $p < 0.05$. ***(Rudovich N et al., Diabetes 2004)***

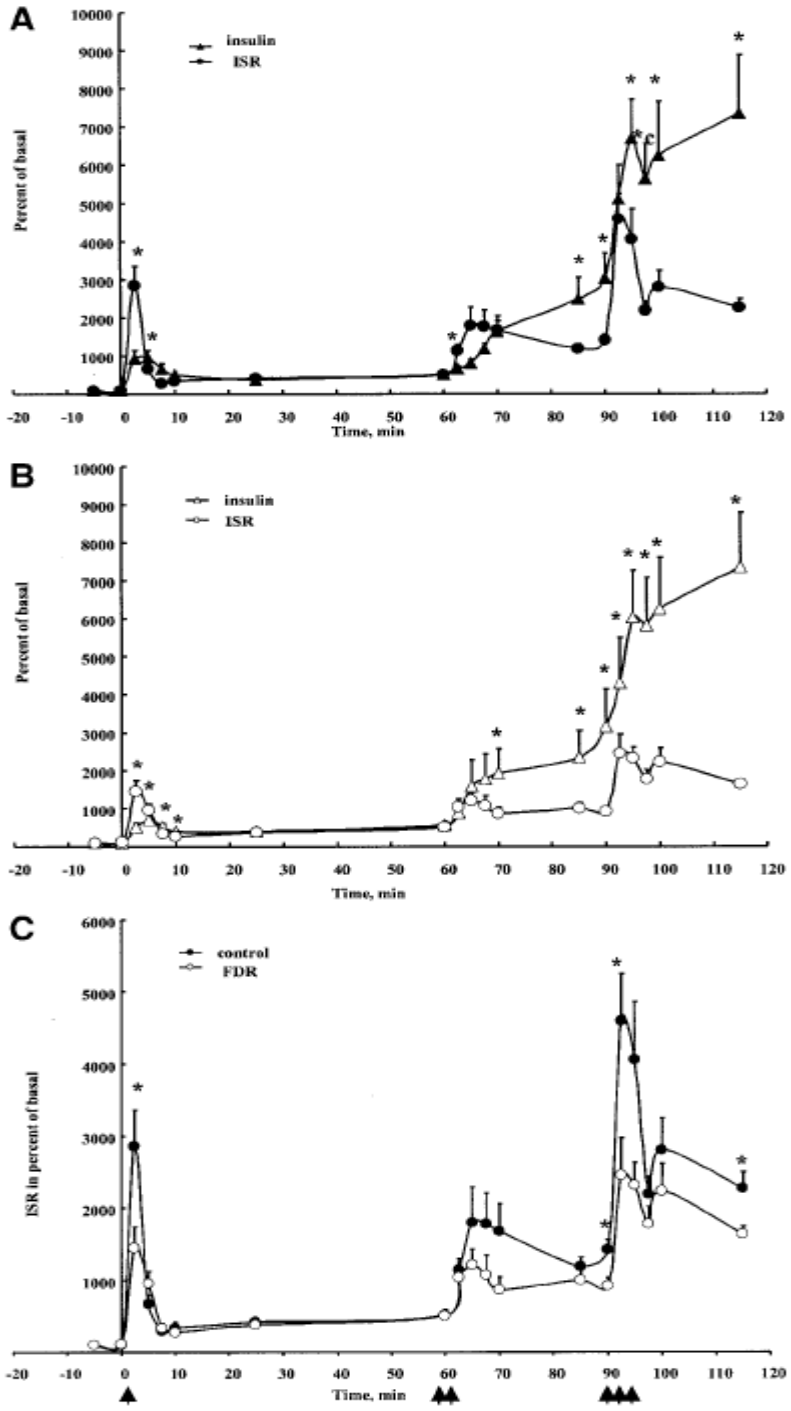

Figure 5 A-C: Peripheral insulin concentration and insulin secretion rate (ISR) as percentages of their respective mean basal values, in response to three different secretagogues in the hyperglycemic clamps (mean ± s.e.m). A, control group; B, first-degree relatives of patients with type 2 diabetes mellitus. Rhombus = insulin, circles = ISR. C, ISR in percent of basal: control subjects vs. first-degree relatives of patients with type 2 diabetes mellitus. White circles = first-degree relatives, black circles = control subjects. Arrows indicate initiation of glucose-, GIP- and arginine infusion. *p < 0.05. ***(Rudovich N et al., Diabetes 2004)***

We observed the reduction of insulin clearance in the basal state and under GIP-infusion in FDR by calculation of insulin clearance using method (a) and (b) (**Figure 5-B, 6-A**). In addition, we observed the reduction of insulin clearance under GIP-infusion using method (c) and saw a borderline significant change in the basal insulin clearance in FDR´s (**Figure 6-B**). $HOMA_{IR}$ correlated negatively with insulin clearance under basal conditions (MCR basal) ($r = -0.96$; $p<0.01$) and under GIP infusion ($r = -0.56$; $p = 0.003$).

An important and novel finding of our study was the reduction of insulin clearance under GIP-infusion in the group of FDR´s (**Figure 6 A-B**). This decrease in insulin clearance allowed maintaining normal even increased peripheral insulin concentrations despite a reduced insulin secretion capacity. This specific effect in GIP action applies to conditions of increased ß-cell demand as occurs in postprandial situations. However, FDR´s did not show a general impairment in insulin extraction since the clearance of insulin was not different compared to controls in the presence of glucose, GIP and arginine.

In summary, we demonstrated the *in vivo* abnormality in the insulin handling in marginally insulin-resistant FDR´s in the hyperglycemic clamp with three different secretagogues. The FDR´s presented relative and global impairment of insulin secretion in response to glucose, GIP and arginine, as a sign of limitation of ß-cell secretion capacity. The reduction of insulin clearance under GIP–infusion in FDR`s was significantly greater than in control subjects

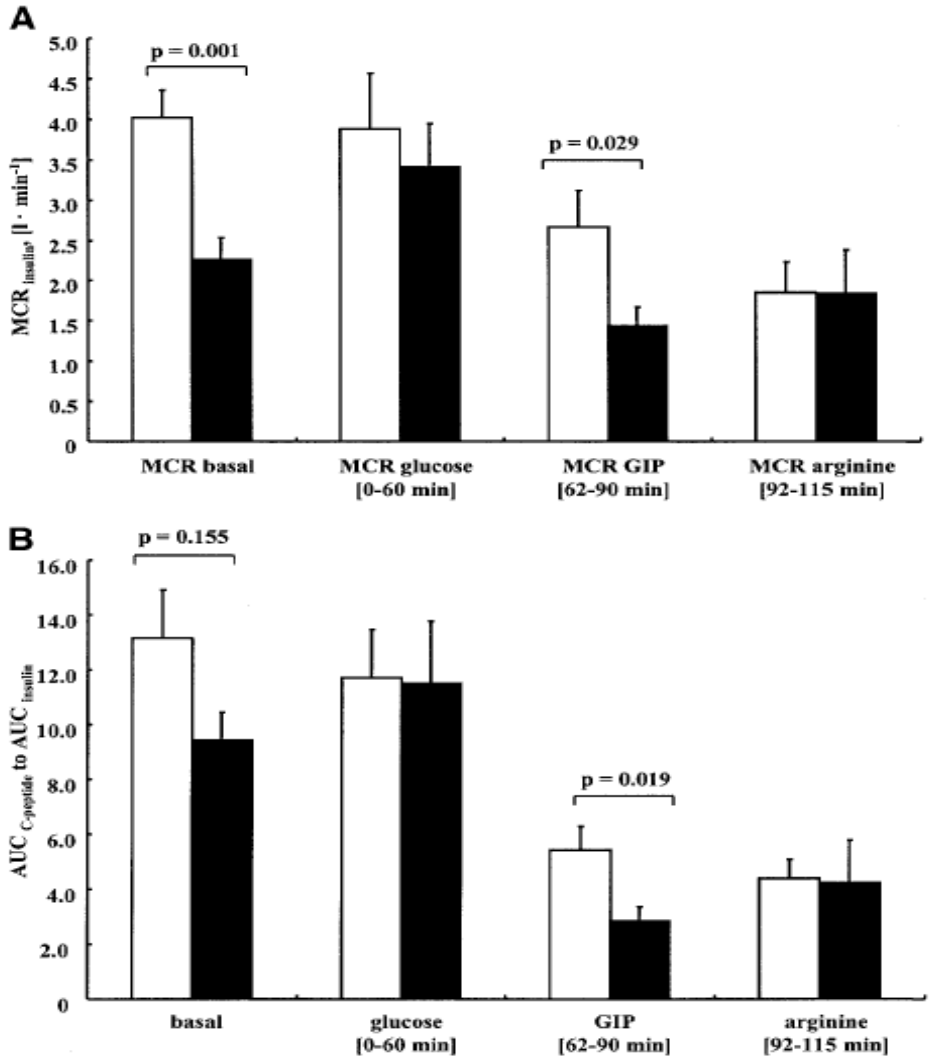

Figure 6: Metabolic clearance rate of endogenous insulin ($MCR_{insulin}$) (A) and ratio of $AUC_{C\text{-}peptide}$ to $AUC_{insulin}$ as indirect index of the change in insulin extraction (B) at each period of the hyperglycemic clamp. Dark columns = present first-degree relatives of type 2 diabetes mellitus patients, white columns = control subjects. ***(Rudovich N et al., Diabetes 2004)***

and negatively correlated with the degree of insulin resistance. This suggests that decreased insulin clearance in response to GIP may represent a further pathophysiological mechanism involved in the development of type 2 diabetes mellitus ***(Rudovich N et al., Diabetes 2004)***.

2.3 GIP and hormonal appetite regulation: GIP to ghrelin interactions

Energy homeostasis and food intake behaviour are regulated by a complex system of endocrine, neuronal and nutrient signals including an increasingly well understood communication channel called the brain-gut-axis [64]. Ghrelin, an acylated gut peptide, which is primarily produced by endocrine cells of the gastric mucosa [65], stimulates food intake and promotes adiposity [66]. Feeding suppresses ghrelin production and fasting stimulates ghrelin release [66-68]. The exact molecular underpinnings controlling these processes are still not entirely understood. Parenteral glucose and/or insulin infusions clearly suppress ghrelin levels when administered for prolonged periods or at supraphysiological doses in animals and humans [69-74]. Physiological doses of glucose and/or insulin that mimic postprandial fluctuations however do not seem to affect circulating ghrelin in humans [75-77]. In contrast, enteral nutrients consistently suppress ghrelin levels, even at low doses [69, 71, 72, 77]. Moreover, selective gastric distension, chemosensation or nutrient exposure are insufficient to induce a ghrelin response in animals [78], while small intestinal nutrient exposure, is sufficient for food-induced plasma ghrelin suppression [79, 80]. Thus, it is likely that nutrient-related ghrelin suppression is based on nutrient sensing or uptake distal of the stomach [81].

Recent data indicate that the orexigenic effect of ghrelin might also be influenced by other gastro-intestinal peptides such as cholecystokinine (CCK), bombesin, PYY(3-36), glucagon-like peptide-1 (GLP-1), suggesting relevant cross-talk among peripheral orexigenic and anorexigenic signals in the control of

appetite and body weight [82]. The incretin GIP, another nutrient dependent factor secreted by intestinal enteroendocrine cells has recently received considerable attention as a potential drug target and endogenous regulator of energy metabolism. A potentially important interaction between GIP and ghrelin was suggested from initial experiments with isolated rat stomach, which suggested a potentially direct influence of GIP on ghrelin secretion [83]. In addition, descriptive association studies indicate that postprandial ghrelin concentrations are inversely correlated with postprandial GIP concentrations in healthy subjects [84], as well as in obese subjects or patients with T2DM [18]. These and other data suggest GIP as an intriguing candidate for the molecular interface controlling postprandial ghrelin secretion: 1) GIP is secreted distal to the stomach from intestinal K-cells in response to nutrient ingestion and acts to augment insulin secretion in pancreas [7]; 2) the stimulation of insulin secretion by GIP occurs only in the presence of elevated glucose levels [11]; 3) GIP receptors are presented in gastric mucosa [24, 53] and are down-regulated in central obesity [53].

2.4 Ghrelin is not suppressed in hyperglycemic clamps by gastric inhibitory polypeptide and arginine

We hypothesized that GIP may be involved in regulation of ghrelin release in vivo [85]. Firstly, we thus measured the effect of a dose-dependent stimulation of insulin secretion by GIP and arginine, an other insulin secretagogues under clamped hyperglycemia on ghrelin release in healthy non-obese subjects. In the first hour of the hyperglycaemic clamp, circulating insulin concentration increased to 166±98 pmol/l ($p<0.01$), but did not affect ghrelin concentrations ($p= 0.208$). An additional exogenous GIP-infusion increased circulating insulin concentration to 1,109±942 pmol/l ($p<0.02$) and suppressed ghrelin to 86.2% of baseline, $p=0.050$ vs. baseline). During administration of arginine and GIP together, insulin concentration further increased progressively to 3,005±1,604 pmol/l ($p<0.01$). Ghrelin concentration remains unchanged at 98.9 % of baseline; (**Figure 7**).

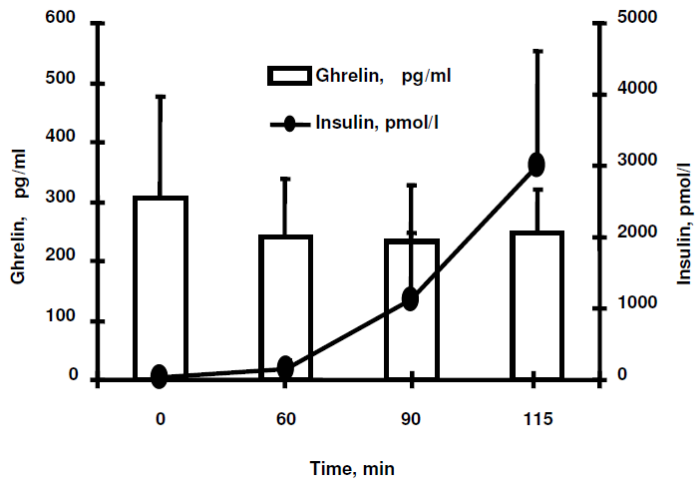

Figure 7: Changes in serum ghrelin (columns) and insulin (points) concentrations during hyperglycemic clamp combined with GIP- and arginine infusion. Data are means ± SE. ***(Rudovich N et al., Regul Pept. 2005)***

The lack of ghrelin suppression under supraphysiological concentrations of insulin and glucose confirms results of a recent study in which intravenous glucose loading failed to suppress systemic ghrelin concentrations[75, 76]. In contrast, another study has reported a rapid and transient decrease in plasma ghrelin after a high-dose glucose bolus [86, 87]. Although it is possible that changes in ghrelin concentrations within periods shorter than 30 min could have been missed in our experiment, it is unlikely that hyperglycemia combined with hyperinsulinemia in the high supraphysiological range results in ghrelin suppression in healthy individuals.

In summary, hyperglycemic hyperinsulinemia and further increases of hyperinsulinemia to supraphysiological and high supraphysiological concentrations under GIP- and arginine- infusion do not significantly decrease ghrelin concentrations in healthy subjects. Moreover, there is no dose-dependent suppression of ghrelin by insulin in the hyperglycemic condition ***(Rudovich N et al., Regul Pept. 2005)***.

2.5 Metabolomic linkage unveils functional interaction between Glucose-dependent Insulinotropic Peptide (GIP) and ghrelin in humans

There is growing evidence that GIP is involved in the control of fuel metabolism beyond its role as an incretin [9, 10, 19, 20]. Given the fact that GIP, like insulin, is involved in the regulation of carbohydrate and fat metabolism [10, 20], indirectly synergistic and/or combinatorial action of GIP and/or insulin metabolites on ghrelin secretion don't seem unlikely. Moreover, controversy results from different techniques of glucose administration leading to differential influences on metabolically important hormones, such as insulin, somatostatin and growth hormone and ultimately their impact on ghrelin secretion [70-75] may reflect the under-estimated multiplicity of their effects. It is therefore not known to which extent the changes observed in these studies are directly related to hormonal alterations or secondary to metabolic changes such as, for example, the modulation of free fatty acids [88, 89]. Complex functionality of hormone-to-hormone interactions are frequently forming complex patterns of combinatorial or/and synergistic effects in a dense network of molecular information exchange. We therefore applied metabolomic systems analysis to search for novel endocrine and metabolite signal patterns regulating ghrelin regulation [90].

The objective of this study was to investigate whether GIP regulates ghrelin secretion, either dependent or independent of circulating blood glucose and insulin concentrations. To address this issue, we employed a GIP-infusion test and euglycemic- and hyperglycemic glucose clamp experiments combined with infusion of GIP or placebo in a cross-over manner in moderate overweight

male subjects. The hyperglycemic hyperinsulinemic condition was chosen to mimic postprandial conditions, while the euglycemic hyperinsulinemic condition would allow detecting interactions of GIP with insulin independent of elevated glucose concentrations, more similar to metabolic situations typically encountered in obesity. Moreover, we applied network correlation analysis of metabolic profiles to study the generated data for molecular links reflecting GIP - ghrelin interactions.

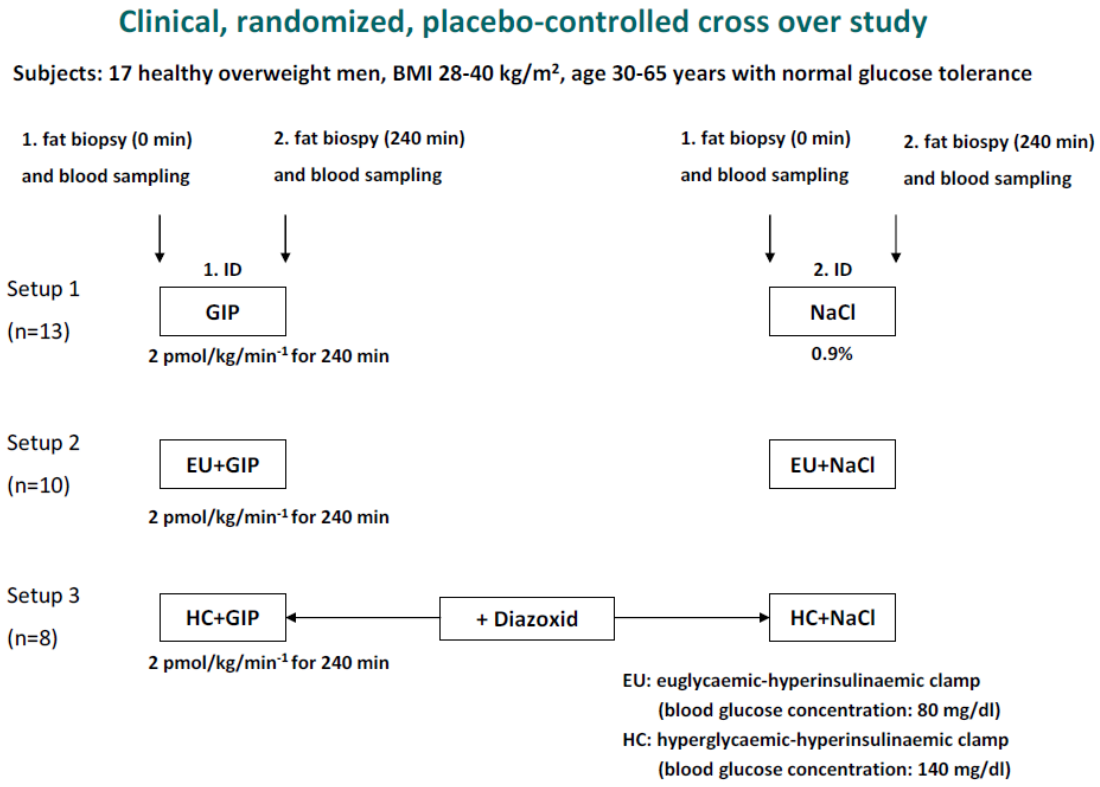

Figure 8. Design of the study. ***(Rudovich NN et al., Am J Physiol Endocrinol Metab. 2011)***

Obese subjects (*n=14*) were studied on four occasions and exposed every time to two of four different conditions (**Figure 8**): They were infused with GIP (2.0 pmol kg ^{1} min ^{1}) or placebo, either in the fasting state, during euglycemic-

hyperinsulinemic clamps (EC) or during hyperglycemic- hyperinsulinemic clamps (HC). Apart from analysis of plasma ghrelin and insulin levels, GC-TOF/MS analysis was applied to decipher relevant metabolomic patterns from the same plasma samples. We created a hormone-metabolite network for each experiment and analysed the GIP and insulin effects on circulating ghrelin levels within the framework of those networks.

In the HC total ghrelin levels decreased significantly in the absence (18.8 % vs. baseline; p=0.028) as well as in the presence of GIP (34.3%; p=0.018). Total ghrelin levels were significantly lower during HC with GIP than with placebo, in spite of insulin levels not differing significantly **(Figure 9)**. In the GIP network combining data on GIP- infusion, EC+GIP and HC+GIP experiments, ghrelin was integrated into hormone-metabolite networks through a connection to octadecanoic (directly) and a group of other long-chain fatty acids (indirectly) **(Figure 10)**. In contrast, ghrelin stayed completely disintegrated from a metabolomics network built from data generated by placebo-infusion with and without EC and HC tests.

In summary GIP decreases circulating levels of the only hormone promoting food intake and body adiposity and may affect the ghrelin system via modification of circulating long-chain fatty acids pool. These observations were independent from insulin and offer potential mechanistic underpinnings for the involvement of GIP in systemic control of energy metabolism.

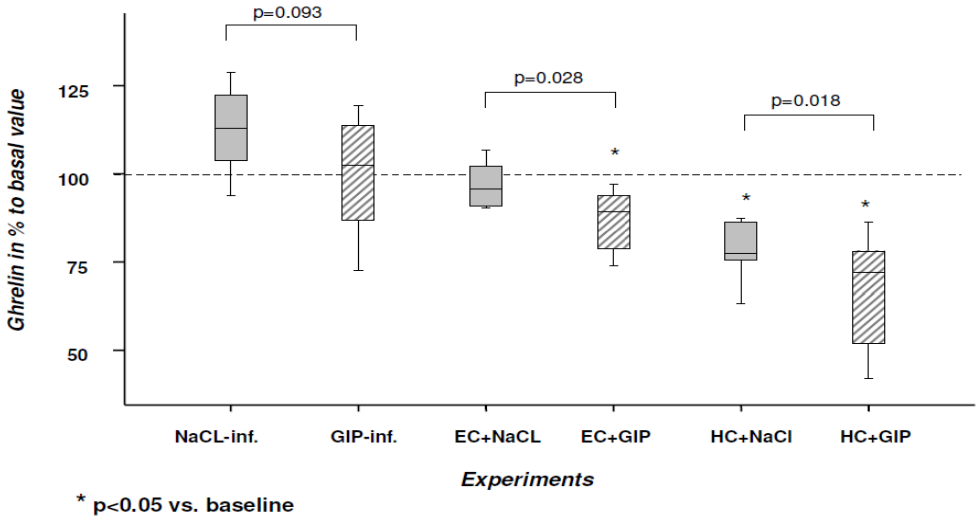

Figure 9. Changes of serum total ghrelin concentrations (mean of values 210-240min) in % to basal level of the each experiments: GIP-infusion (GIP-inf), NaCl-infusion (NaCl-infusion), hyperinsulinemic, euglycemic clamps with NaCl (EC+NaCl) and GIP-infusion (EC+GIP); hyperinsulinemic, hyperglycemic clamps with NaCl (HC+NaCl) and GIP-infusion (HC+GIP). (□) - experiments with NaCl-infusion; (▨) - experiments with GIP-infusion at infusion rate of [2.0 pmol kg $^{-1}$ min^{-1}*p < 0.05 vs. basal level in the Wilcoxon test. ***(Rudovich NN et al., Am J Physiol Endocrinol Metab. 2011)***

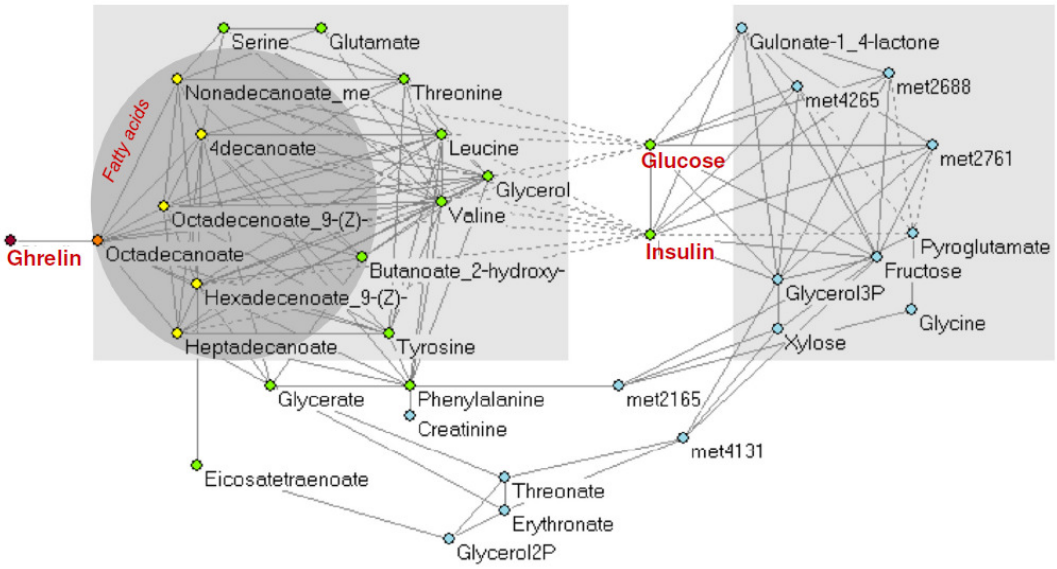

Figure 10. Involvement of ghrelin in the GIP-dependent hormone-metabolite network combining the data of three experiments: GIP-infusion test, hyperinsulinemic, euglycemic clamps with GIP and hyperinsulinemic, hyperglycemic clamps with GIP. Color code for hierarchical distance from ghrelin: ***(Rudovich NN et al., Am J Physiol Endocrinol Metab. 2011)***

3. Insulin degradation, obesity and type 2 diabetes mellitus

3.1 Modulation of IDE activity by glucose and insulin *in vivo* and *in vitro* models

We performed a detailed analysis of the regulation of IDE function by different concentrations of insulin and glucose in HepG2 cells [49]. In this liver cell model, IDE regulation was analysed at three levels: IDE transcription, IDE translation and IDE protein activity. Moreover, in humans we assessed the regulation of IDE by different clamped glucose and insulin concentrations *in vivo* in subcutaneous adipose tissue.

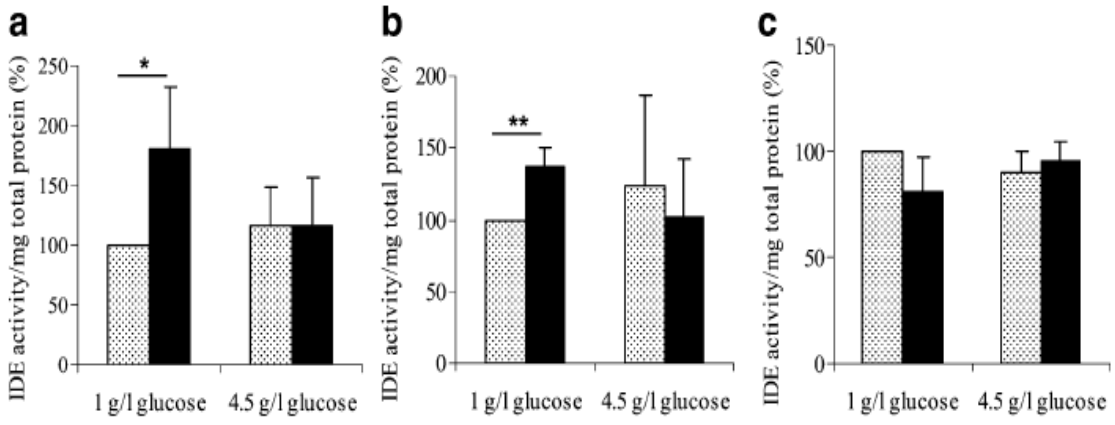

Figure 12: Effects of insulin and glucose on the catalytic activity of IDE in the HepG2 cells. Enzyme activity is assessed as relative fluorescent units per mg of total protein. The data are expressed as percentage of control activity. A, total cell lysate; B, cytosolic fraction; C, membrane fraction. * p<0,05; ** p<0.01. ***(Pivovarova, O., Gögebakan, O., Pfeiffer, A. F., Rudovich, N. Diabetologia, 2009)***

Insulin increases IDE activity in HepG2 cells in normal but not in high glucose conditions **(Figure 12)**.

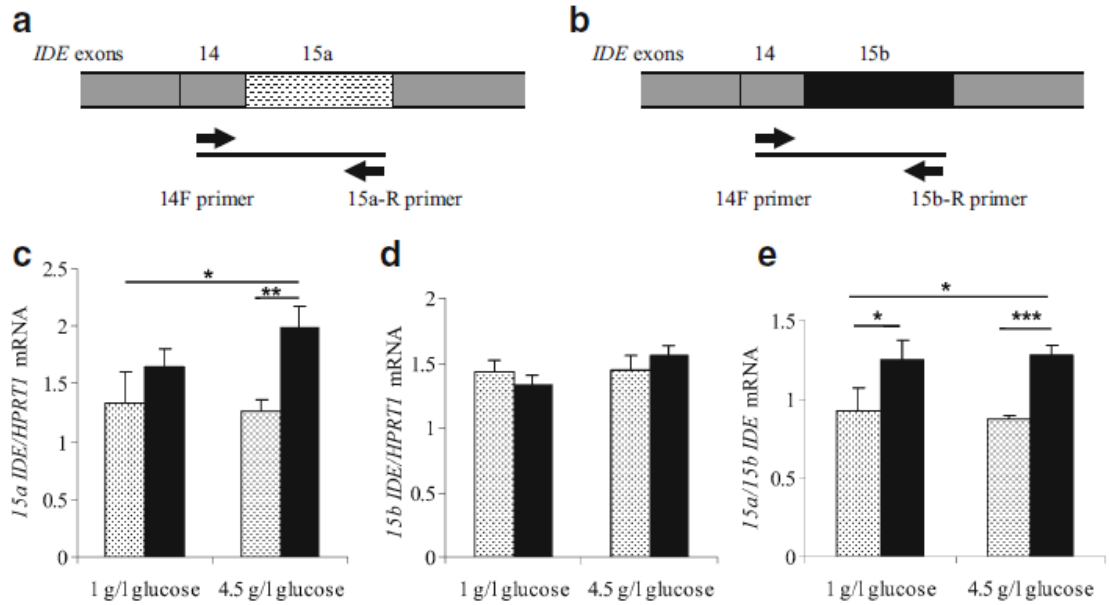

Figure 13: The influence of insulin and glucose on the levels of 15a- and 15b-IDE isoforms in HepG2 cells. *A*, Positions of forward (14F) and reverse (15a-R/15b-R) primers used for detection of 15a/15b-mRNA levels of IDE isoforms by exon-specific qRT-PCR; B, levels of 15a-IDE mRNA; C, levels of 15b-IDE mRNA; D, 15a/15b ratios in control vs. insulin and/or glucose treated cell samples. * $p<0.05$; ** $p<0.01$; *** $p<0.001$. ***(Pivovarova, O., Gögebakan, O., Pfeiffer, A. F., Rudovich, N. Diabetologia, 2009)***

This disturbance cannot be explained by corresponding alterations in IDE protein levels or IDE splicing. The IDE isoform produced by the transcript in which exon 15b replaces canonical exon 15a was described to have less catalytic efficiency for insulin and amyloid beta-protein in comparison to the wild-type isoform [91]. Therefore, our next hypothesis was that insulin and/or glucose may alter the 15a/15b-isoform ratio in HepG2 cells. We assessed the levels of the 15a- and 15b-IDE mRNA by qRT-PCR using a common forward primer residing in exon 14 and exon 15-specific reverse primers for exons 15a and 15b (**Figure 13, A**). After the 10 nM insulin treatment, the level of 15a-isoform of IDE demonstrated a trend to an increase in normal glucose medium and was significantly increased in high glucose conditions in comparison to untreated cell samples (**Figure 13, *B***), while

insulin and/or high glucose induced no alteration of 15b-isoform transcription (**Figure 13, C**). The ratio of 15b-*IDE* to 15a-*IDE* mRNA was also significantly increased after the insulin treatment independently of glucose concentration in the cell medium (**Figure 13, *D***).

To study the regulation of *IDE* expression by insulin and glucose *in vivo* in humans we performed qRT-PCR in cDNA from subcutaneous adipose tissue taken before (-40 min) and after (240 min) of the clamp procedures. Healthy obese non diabetic men (N=17; age 47.4±8.4 years, BMI 32.5±2.2 $kg \bullet m^{-2}$, waist circumference 110.5±7.1 cm) participated in two different clamp experiments (EC, euglycemic, hyperinsulinemic clamp and HC, hyperglycemic, hyperinsulinemic clamp) and in the control NaCl-infusion, each of 240 minutes duration. *IDE* mRNA levels were highly variable across the individuals studied (22.5±13.5%). No correlations between *IDE* mRNA levels in subcutaneous adipose tissue and anthropometric data (age, BMI, waist circumference, percent of body fat) and basal and steady state insulin and glucose concentration were detected (data not shown). In the NaCl-infusion test, no alterations of *IDE* expression were observed. However, in EC a trend towards an increase of *IDE* mRNA levels at the end of the clamps was observed (by 17.1%, p=0.097) and was more pronounced in HC (increase by 45.6%, p=0.091) (**Figure 14**).

The observed increase of IDE activity after insulin treatment in human hepatoma cells under normal glucose concentration may be expected. This effect may be a part of a physiological negative feedback mechanism of the regulation of insulin action: insulin induces an increase of IDE activity which leads to the increased insulin degradation and decreased insulin signaling.

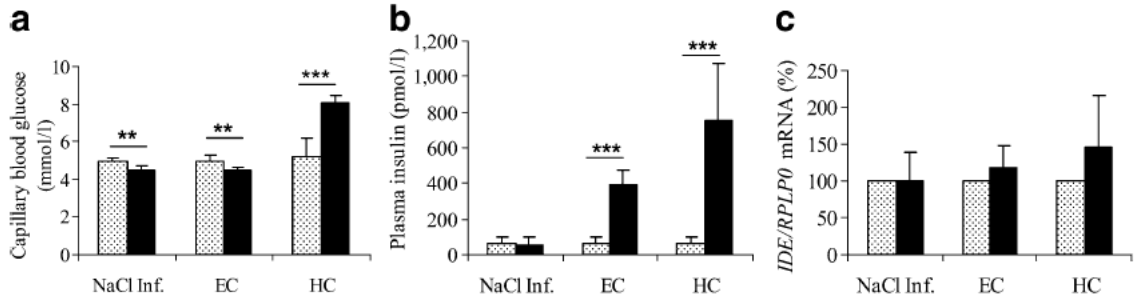

Figure 14. The influence of insulin and glucose on IDE mRNA levels in human subcutaneous adipose tissue in the clamp study. a Capillary blood glucose. b Plasma insulin (dotted columns, basal values; black columns, steady-state values). c IDE mRNA levels. Data are percentages of basal IDE mRNA levels. Dotted columns, biopsy samples taken before the start of clamp tests; black columns, biopsy samples taken at 240 min of clamp tests. EC, euglycaemic–hyperinsulinaemic clamps; NaCl Inf., NaCl infusion; HC, hyperglycaemic–hyperinsulinaemic clamps. *p<0.05; **p<0.01; ***p<0.001. ***(Pivovarova, O., Gögebakan, O., Pfeiffer, A. F., Rudovich, N. Diabetologia, 2009)***

However, we detected no regulation of IDE expression by insulin under normal glucose concentration as described in other tissues. Particularly, IDE in brain was shown to be a downstream target of the insulin signaling cascade regulated through the phosphatidylinositol-3 kinase pathway [98]. Moreover, we observed a loss of insulin-induced changes of IDE activity in high glucose conditions accompanying with increasing of IDE gene expression. Thus, the combination of hyperglycemia and hyperinsulinemia leads to increase of intracellular insulin concentration, which in turn increases expression of its own degradations enzyme, i.e. feed-forward regulation of enzyme expression by its substrate. Our finding suggests that hyperglycemia may provoke the known disturbance of IDE activity in T2DM. Results of analyses of IDE activity in biological fluids (blood cells, plasma, wound fluid, cerebrospinal fluid) of diabetic patients reported in the literature are controversial and apparently depend on a range of factors including design of study, the type of diabetes mellitus and type of antidiabetic treatment

[40, 92-94]. The underlying mechanism of hyperglycemia induced down regulation of IDE-activity remains unclear and is possibly of metabolic nature.

In summary, insulin enhances IDE activity under normal glucose concentration without changes in IDE expression or translation in a liver cell model. This positive regulation of IDE-activity by insulin is loss under high glucose conditions accompanied with increase of expression of most active 15a-IDE isoform. Our data suggested a presence of feed-forward regulation of IDE gene expression by insulin. This observation is supported by data from hyperglycemic hyperinsulinemic clamp study with similar changes in IDE expression in subcutaneous fat tissue in vivo. The observed effects of the insulin and high glucose on the IDE activity may contribute to the regulation of insulin degradation in liver and to reduced hepatic extraction of insulin in T2DM ***(Pivovarova, O., Gögebakan, O., Pfeiffer, A. F., Rudovich, N, Diabetologia, 2009)***

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3.3 *IDE* gene polymorphisms affect type 2 diabetes risk and insulin metabolism

The human insulin-degrading enzyme (*IDE)* gene is located on chromosome 10q23-25, within 4–30 Mb from markers that have shown suggestive linkage for type 2 diabetes (T2DM) and related phenotypes in a number of whole-genome linkage studies [42, 43, 95]. About five years ago, *IDE* genetic polymorphisms were found to be associated with T2DM [96, 97]. This association of *IDE/HHEX* genomic region has been well replicated in genome wide application studies [98-101] and candidate gene approaches in different ethnic populations [102-105]. In 2006 in a cohort including 4,206 Caucasian individuals, no significant evidence of an association between *IDE* and T2DM was observed [106]. A recently published meta-analysis which included seven study samples amounting to a total of 5,799 participants found only marginal significant association of *IDE* polymorphisms with T2DM [102]. This evidence, therefore, opened the question about the influence of genetic variation of *IDE* on the T2DM pathogenesis.

We investigated the effect of two *IDE* polymorphisms on diabetes risk in two independent German populations: we designed a nested case-cohort study within the prospective population-based EPIC-Potsdam cohort (*n=3,049*) and in the Metabolic Syndrome Berlin Potsdam (MESYBEPO) cohort (*n=1026*), from the same geographical region. The EPIC-Potsdam cohort is a part of the multi-centre, population-based cohort study “European Prospective Investigation into Cancer and Nutrition” (EPIC). In brief, 27,548 subjects mainly aged 35–65 years, were recruited from the general population [107, 108]. The baseline examination

included anthropometric measurements, blood sampling, a self-administered validated food-frequency questionnaire, and a personal interview on lifestyle habits and medical history. Follow-up questionnaires have been administered every 2 to 3 years to obtain information on current medication and newly developed diseases, including T2DM. Case subjects were defined as those individuals, who were disease-free at baseline of the study and subsequently developed T2DM during the follow-up (mean 7.1±1.8 years). Cases were identified by self-report questionares and verified by recalls to the respective clinican, who needed to confirm the diagnosis. Only those individuals with a verified diagnosis were included into the analysis. As expected, case subjects were more obese, exercised less **(Table 8)**.

The association between *IDE* polymorphisms and metabolic traits affecting glucose and insulin metabolism was additionally investigated in this second cohort. As a major finding we demonstrate the association of genetic variants in the *IDE* gene with the risk of T2DM in one German cohort, providing first evidence of the association in a prospective case-cohort study design. In the EPIC-Potsdam cohort, both polymorphisms rs1887922 and rs2149632 showed a strong association with increased T2DM risk **(Table 9)**. Both SNPs exhibited significant *p* values, with the highest increased risk of disease being found for the T allele of SNP rs2149632 in the EPIC-Potsdam cohort. We also found that in the EPIC-Potsdam cohort haplotypes included one or both risk alleles of studied *IDE* SNPs also increase the relative risk of T2DM according to the number of risk alleles. In the cross-sectional MESYBEPO study, only SNP rs1887922 demonstrated a trend to association with T2DM risk in the general genetic model

(OR 2.624, 95 % CI 0.873–7.890, p=0.086 for recessive vs. dominant homozygotes). This discrepancy between two studied populations may be explained due relatively small number of diabetic subjects (*n=227*) in the MESYBEPO cohort.

	Incident cases ***(n = 801)***	**Non-cases** ***(n = 2248)***	***p- value***
Males (%)	58.0	38.0	< 0.0001
Age (years)	54.7 ± 7.4	49.3 ± 8.9	< 0.0001
BMI (kg/m^2)	30.4 ± 4.6	25.8 ± 4.1	< 0.0001
WHR	0.939 ± 0.087	0.844 ± 0.095	< 0.0001
Sport activites (>2 h/w)	9.4	16.1	< 0.0001
General* activities (>30 min/day)	55.8	58.5	0.2
BMR (basic metabolic rate)	7.1 ± 1.1	6.4 ± 0.9	< 0.0001
Energy intake	9182 ± 3086	8860 ± 2868	0.01
CRP [mg/dl]	3.1 ± 4.3	1.6 ± 2.8	< 0.0001
Total cholesterol	181.1 ± 37.3	173.2 ± 38.1	< 0.0001
HDL-cholesterol	39.8 ± 10.3	48.0 ± 13.1	< 0.0001
	N = 109	N = 313	
Insulin [pmol/l] †	79.2 ± 47.9	43.9 ± 32.9	< 0.0001
Glucose [mmol/l] †	6.2 ± 1.3	4.8 ± 0.7	< 0.0001
	N = 239	N = 632	
Glucose [mg/dl] ‡	109.5 ± 28.5	85.2 ± 12.5	< 0.0001

Triglyceride [mg/dl] ‡	149.2 ± 105.9	94.1 ± 69.0	< 0.0001

Table 8: Baseline characteristics of subjects with incident type 2 diabetes case-cohort study from EPIC-Potsdam population. Data are presented as mean ± SD or %; †, values obtained from subjects after an overnight fast without beverage intake; ‡, values obtained from subjects after an overnight fast with beverage intake before blood collection; *sports, biking, gardening ***(Rudovich et al., J Mol Medicine, 2009)***.

SNP ID (RAF)		**Non-cases/Incident cases**	**RR (95% CI)**	***P***
rs1887922 (19.5%)	TT	1469 / 483	1.0	
	CT	658 / 272	1.26 (1.04-1.53)	**0.02**
	CC	**79** / **43**	1.57 (1.03-2.39)	**0.04**
	additive*		1.26 (1.08-1.47)	**0.003**
rs2149632 (67.4%)	CC	248 / 70	1.0	
	C**T**	1004 / 319	1.16 (0.85-1.60)	0.36
	TT	**954** / **409**	1.63 (1.18-2.23)	**0.003**
	additive*		1.33 (1.15-1.52)	**<0.0001**
Haplotypes	T^a- C^b and C^a-C^b	33.8 / 28.3% 2.1 / 0.4%	1.0	
	T^a-T^b	47.7 / 49.3%	1.63 (1.21-2.20)	**0.001**
	C^a-T^b	18.3 / 22.0%	2.07 (1.45-2.96)	**<0.0001**

Table 9: Association analysis of IDE polymorphisms in the type 2 diabetes case-cohort study from EPIC-Potsdam. Relative Risks (RR) adjusted for age and gender; *RRs determined for the risk alleles (shown in bold letters). [a] rs1887922 allele; [b] rs2149632 allele ***(Rudovich et al., J Mol Medicine, 2009).***

To investigate the pathophysiological mechanisms, by which polymorphisms in *IDE* gene influence diabetes risk, we tested both *IDE* SNPs for association with quantitative traits in non-diabetic subjects (NGT+IFG/IGT; **Table**

10). We observed clearly association between the T allele of SNP rs2149632 and decreased insulin secretion in this sub-cohort. Even thought our statistical data were not corrected for multiple comparisons, this SNP's association with measures of insulin secretion derived from insulin and C-peptide levels largely excludes the possibility of a coincidental finding. Unexpected, we detected no significant alterations of glycemic traits demonstrated by other authors [97]. Moreover, we detected a trend to decreased insulin sensitivity in homozygote carriers of the risk allele rs1887922.

Based on the multiple metabolic abnormalities observed in the subjects with IFG/IGT, we refined the statistical analyses and tested the effects of both *IDE* polymorphisms on insulin and glucose metabolic traits in the subcohort of NGT subjects (*n=440*). The risk allele of rs2149632 remained strongly associated with impairment of insulin secretion measured as C-peptide levels at 30 min of OGTT and reduced insulinogenic index (p=0.049 and p=0.021, respectively) (data not show). The risk alleles of polymorphisms rs1887922 was associated with decreased insulin clearance and had borderline association with increased fasting insulin (p=0.025 and p=0.047, respectively). However, this association with insulin clearance phenotype disappeared when 299 subjects with IFG/IGT were added to the analyses. Thus, we cannot exclude a type 1 error leads to overestimation of effect on insulin clearance in rested small subcohort of NGT subjects. Thus, the association of *IDE* polymorphisms regarding the decreased insulin clearance needs to be replicated in the large cohort of the subjects with NGT. Mechanisms mediating the influence of *IDE* variants on insulin metabolism remain a matter of speculation. Firstly, *IDE* polymorphisms might impair the

embryonic organogenesis of liver and pancreas and result in the disturbance of insulin secretion and insulin degradation [109]. Secondly, the beta-cell secretion may be influenced via different *IDE*-dependent mechanisms such as amyloid accumulation [46], activation of downstream insulin signaling pathways via

SNP genotype	rs1887922			*P*	p^a	rs2149632			*p*	p^a
	TT	CT	CC			TT	CT	CC		
n (%)	471 (69.0%)	198 (29.0%)	14 (2.0%)			319 (46.6%)	288 (42.1%)	77 (11.3%)		
Insulin $_{0\ min}$ [pmol· l^{-1}]	49 ± 33	54 ± 36	58 ± 29	0.18	0.06	49 ± 30	53 ± 39	48 ± 20	0.83	0.49
Insulin $_{30\ min}$ [pmol · l^{-1}]	378 ± 221	392 ± 217	359 ± 154	0.89	0.75	365 ± 212	396 ± 230	394 ± 196	0.17	0.09
C-peptide$_{0\ min}$ [ng · ml^{-1}]	2.0 ± 0.9	2.2 ± 0.9	2.2 ± 1.0	0.61	0.32	2.0 ± 0.9	2.1 ± 1.0	2.1 ± 0.6	0.69	0.56
C-peptide$_{30min}$ [ng · ml^{-1}]	6.5 ± 2.7	6.6 ± 2.5	6.3 ± 2.2	0.87	0.92	6.3 ± 2.6	6.7 ± 2.7	6.7 ± 2.6	0.43	0.27
Insulin clearance [l · min^{-1}]	6.4 ± 2.6	6.1 ± 2.4	5.7 ± 2.0	0.66	0.36	6.5 ± 2.6	6.2 ± 2.6	6.2 ± 2.4	0.57	0.74
ISI Matsuda [AU]	5.8 ± 3.6	5.3 ± 3.1	4.3 ± 1.9	0.16	0.054	5.7 ± 3.4	5.7 ± 3.7	5.2 ± 2.5	0.61	0.59
1st phase IS [pmol]	867 ± 510	913 ± 506	852 ± 398	0.74	0.88	840 ± 495	922 ± 534 †	884 ± 436	0.024	0.07
2nd phase IS [pmol]	246 ± 115	257 ± 115	244 ± 87	0.83	0.87	241 ± 111	259 ± 121 †	251 ± 97	0.052	0.09
II [pmol · $mmol^{-1}$]	81.0 ± 53.6	83.0 ± 50.5	74.3 ± 37.6	0.82	0.73	76.8 ± 48.9	85.2 ± 56.1	86.5 ± 51.1	0.050	0.038
DI [1/$mmol^2$]	8.7 ± 5.7	8.1 ± 4.8	6.4 ± 3.5	0.13	0.049	8.1 ± 5.0	8.8 ± 5.5	9.0 ± 6.3	0.17	0.16

Table 10: Table 3 Quantitative metabolic traits in non-diabetic subjects from MESYBEPO cohort.
Only subjects with BMI≤50, complete data from OGTT (0-120 min) and untreated with antidiabetic drugs were included in statistical analysis. Data are presented as mean ± SD or %. *p*- Value for general genetic model, p^a - dominant vs. recessive homozygotes. Genetic models were adjusted for BMI, age, gender and waist circumference; II- Insulinogenic index, IS- Insulin secretion and DI- Disposition index. † $p < 0.05$ CT vs. TT. ***(Rudovich et al., J Mol Medicine, 2009)***

increased intracellular insulin concentrations and alteration in the mitochondrial metabolism [110].

Since both diabetes-associated *IDE* SNPs analysed in the present study locate within the intron region of the *IDE* gene, they do not alter protein sequences directly. However, these polymorphisms may affect the level of gene expression by altering transcription, splicing or message stability. We cannot exclude the possibility that one of the other genes within the locus – *HHEX* or *KIF11* – is the causal one for the observed effects on insulin metabolism because of the high degree of LD within that region. In particular, genetic variations within the *HHEX* locus are associated with the reduced beta-cell secretion capacity and T2DM risk as shown in the range of recent studies including GWA studies [99-101, 111].

In summary, we validated the association of two *IDE* polymorphisms with T2DM risk and firstly confirmed this finding in the prospective case-cohort association study ***(Rudovich et al., J Mol Medicine, 2009)***. Moreover, we present a novel finding of influences of *IDE* genetic variants on all three components of insulin metabolism: insulin secretion, insulin sensitivity and insulin degradation.

3.4 *HHEX* gene polymorphisms, risk of type 2 diabetes and insulin metabolism

Recently published genome–wide association studies revealed a range of novel type 2 diabetes (T2DM) predisposing loci [98]. Particularly, the association of two single nucleotide polymorphisms (SNP), rs1111875 and rs7923837, in the 3'-flanking region of *HHEX* locus with T2DM risk was confirmed in some European and Japanese populations [111-113]. *HHEX* (hematopoietically expressed homeobox protein) gene encodes a member of the homeobox family of transcription factors involved in Wnt signaling and being required for early development of ventral pancreas and liver [114, 115]. However, for polymorphisms within this gene region, no phenotype except T2DM and possibly impaired β-cell function has been demonstrated [112, 113].

We aimed to investigate the association of SNPs rs1111875 and rs7923837 with insulin metabolism [116]. We hypothesized that given the prior knowledge of the biological significance of the *HHEX* gene; the diabetes-associated variants may be associated with each of three key mechanisms or combinations between them in the pathogenesis of T2DM: impaired insulin secretion combined with decreased hepatic insulin degradation and insulin resistance. Two *HHEX* polymorphisms (rs1111875 and rs7923837) were genotyped in 1026 subjects from the German MESYBEPO cohort. Complete OGTT data were available for a subset of 420 with normal glucose tolerance (NGT), 282 with impaired glucose tolerance/impaired fasting glucose (IGT/IFG) and 146 diabetic subjects.

In the NGT and IFG/IGT subjects, the risk alleles of rs7923837 and rs1111875 were significantly associated with decreased first- and second phases

of insulin secretion and lower insulinogenic index after oral glucose loading (**Table 11**). In healthy, normal glucose tolerant subjects, the same association of *HHEX* SNP rs1111875 with OGTT-derived phases of insulin secretion were detectable, however rs7923837 was only weakly associated with reduced insulinogenic index. For both polymorphisms, no significant correlations with insulin sensitivity were obtained. Reduced insulin clearance was also observed for the heterozygous carriers of rs1111875 (p=0.05).

In the present study, we found that genetic variations within the *HHEX* locus were associated with the reduced β-cell secretion capacity measured as first and second phase of insulin response in the OGTT. This finding is in accordance with previously published evidence of different alteration in insulin secretion by variants in the *HHEX* gene region including decreased acute insulin response after OGTT or tolbutamide challenge [117] and decreased insulin secretion after OGTT or intravenous glucose challenge [111, 113]. Mechanisms of the influence of HHEX variants on the insulin secretion remain a matter of speculation. Recently, Tanaka et al. [118] demonstrated that *HHEX* may regulate β-cell development and/or function through the activation of hepatocyte nuclear factor 1α. Functional studies on *HHEX* knockout mouse showed alterations in the embryonic organogenesis of the ventral pancreas [114]. Based on these data, a decreased ß-cell secretory capacity or decreased ß-cell mass could be suggested in carriers of risk alleles in humans. Recently presented evidence of association of the common risk alleles in *HHEX* gene with reduced birth weight through a predominant effect of fetal genotype [119] supports this hypothesis. On the other side, both variants in the HHEX gene were not susceptible for MODY diabetes [120]. These discrepancies

allow proposing same compensatory mechanisms for protection of ß-cell secretion. Moreover, we firstly demonstrated an association with decreased hepatic insulin clearance for heterozygous normal glucose tolerant carriers of risk allele rs1111875.

In summary, we validated the association of polymorphisms of the *HHEX* gene with type 2 diabetes in MESYBEPO cohort. Impaired insulin secretion accompanied with decreased hepatic insulin degradation is the phenotypical marker for carriers of the risk alleles. No association of diabetes-associated *HHEX* SNPs with alterations in insulin sensitivity was observed ***(Pivovarova O, Nikiforova VJ, Pfeiffer AFHP, Rudovich N, Diabetes Metab Res Rev, 2008)***.

SNP genotype	rs1111875			*p*	p^a	p^b	rs7923837			*p*	p^a	p^b
	CC	CT	TT				GG	GA	AA			
n (%)	258 (37.9%)	310 (45.5%)	113 (16.6%)				282 (41.5%)	307 (45.1%)	91 (13.4%)			
BMI ($kg \cdot m^{-2}$)	29.3 ± 5.4	29.5 ± 5.3	30.6 ± 6.2	0.18			29.1 ± 5.48	29.9 ± 5.62	29.9 ± 5.47	0.23		
Waist-to-hip ratio	0.91 ± 0.47	0.92 ± 0.48	0.96 ± 0.89	0.54	0.89		0.90 ± 0.45	0.92 ± 0.49	0.99 ± 1.00	0.26	0.72	
$Glucose_{0\ min}$ [$mmol \cdot l^{-1}$]	5.0 ± 0.5	5.0 ± 0.5	5.0 ± 0.5	0.97	0.91	0.93	5.0 ± 0.5	5.0 ± 0.5	5.0 ± 0.6	0.75	0.63	0.64
$AUC_{gluc\ 0\text{-}120\ min}$ [$mmol \cdot l^{-1} \cdot h^{-1}$]	6.3 ± 2.2	6.3 ± 2.4	6.3 ± 2.6	0.62	0.46	0.45	6.3 ± 2.2	6.4 ± 2.5	6.3 ± 2.3	0.54	0.28	0.29
$Insulin_{\ 0\ min}$ [$pmol \cdot l^{-1}$]	50.8 ± 32.4	52.3 ± 37.4	51.5 ± 27.8	0.78	0.99	0.96	48.7 ± 30.6	54.6 ± 39.0	50.0 ± 24.9	0.19	0.60	0.58
$Insulin_{\ 30\ min}$ [$pmol \cdot l^{-1}$]	367.7 ± 209.8	392.1 ± 230.0	393.0 ± 210.8	0.39	0.56	0.54	358.0 ± 213.1	405.1 ± 232.2	385.8 ± 188.1	0.02	0.12	0.11
$AUC_{ins\ 0\text{-}120\ min}$ [$pmol \cdot l^{-1} \cdot h^{-1}$]	628.7 ± 360.9	659.1 ± 400.6	655.9 ± 370.6	0.71	0.98	0.99	608.6 ± 357.3	690.0 ± 410.6	625.3 ± 332.4	0.06	0.13	0.14
$C\text{-peptide}_{\ 0\ min}$ [$ng \cdot ml^{-1}$]	2.1 ± 0.9	2.1 ± 1.0	2.1 ± 0.8	0.46	0.85	0.80	2.0 ± 0.9	2.1 ± 1.0	2.1 ± 0.8	0.32	0.84	0.83
$C\text{-peptide}_{\ 30\ min}$ [$ng \cdot ml^{-1}$]	6.5 ± 2.6	6.5 ± 2.7	6.6 ± 2.6	0.73	0.93	0.92	6.4 ± 2.6	6.6 ± 2.7	6.7 ± 2.4	0.20	0.76	0.76
$AUC_{C\text{-}pep\ 0\text{-}120\ min}$ [$ng \cdot l^{-1} \cdot h^{-1}$]	10.5 ± 3.9	10.4 ± 3.9	10.5 ± 4.0	0.91	0.74	0.71	10.3 ± 3.9	10.6 ± 4.0	10.6 ± 3.8	0.36	0.85	0.88
Insulin clearance [$l \cdot min^{-1}$]	**6.5 ± 2.6**	**6.2 ± 2.5**	**6.3 ± 2.7**	**0.44**	**0.54**	**0.23** [c]	**6.6 ± 2.7**	**6.1 ± 2.5**	**6.5 ± 2.6**	**0.07**	**0.26**	**0.26** [c]
HOMA-IR [$mmol \cdot mU \cdot l^{-2}$]	1.9 ± 1.3	2.0 ± 1.5	1.9 ± 1.1	0.78	0.99	0.98	1.8 ± 1.2	2.1 ± 1.5	1.9 ± 1.0	0.19	0.65	0.63
ISI Matsuda [AU]	5.6 ± 3.2	5.7 ± 3.8	5.4 ± 3.1	0.67	0.99	0.99	5.9 ± 3.5	5.4 ± 3.5	5.5 ± 3.0	0.22	0.43	0.46
1^{st} phase IS [pmol]	**845.7 ± 499.7**	**908.0 ± 521.1**	**897.6 ± 486.7**	**0.02**	**0.16**	**0.01** [c]	**822.3 ± 498.1**	**932.1 ± 536.1**	**898.5 ± 421.5**	**0.02**	**0.15**	**0.03** [c]
2^{nd} phase IS [pmol]	**242.3 ± 112.4**	**256.1 ± 118.9**	**254.1 ± 108.8**	**0.16**	**0.25**	**0.03** [c]	**236.8 ± 112.0**	**262.1 ± 121.9**	**253.4 ± 95.0**	**0.007**	**0.097**	**0.03** [c]
II [$pmol \cdot mmol^{-1}$]	**76.9 ± 48.7**	**84.2 ± 55.3**	**85.7 ± 52.8**	**0.09**	**0.22**	**0.04** [c]	**75.0 ± 49.5**	**86.7 ± 55.7**	**84.8 ± 49.1**	**0.01**	**0.06**	**0.02** [c]
DI [$1/mmol^2$]	8.2 ± 5.2	8.6 ± 5.3	8.9 ± 6.2	0.39	0.26	0.23	8.3 ± 5.5	8.5 ± 5.2	9.1 ± 6.0	0.22	0.36	0.33

Table 11. Quantitative metabolic traits in subcohort of MESYBEPO population including NGT and IFG/IGT subjects stratified according to genotype. This subcohort was restricted to subjects with bmi≤50. Completely OGTT data (0-120 min) were obtained for 420 subjects with NGT and 282 subjects with IFG/IGT. Data are presented as mean ± SD or %. *p*- value for general linear model; [a] - adjustment for age, gender and BMI; [b] - adjustment for age, gender, BMI and waist circumference; [c] – variables were additionally adjusted for ISI Matsuda; II- Insulinogenic index, IS- Insulin secretion and DI- Disposition index. ***(Pivovarova O, Nikiforova VJ, Pfeiffer AFHP, Rudovich N, Diabetes Metab Res Rev, 2008)***

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6. Relevant publications of N. Rudovich

1. Rudovich NN, Nikiforova VJ, Otto B, Pivovarova O, Gögebakan O, Erban A, Möhlig M, Weickert MO, Spranger J, Tschöp MH, Willmitzer L, Nauck MA, Pfeiffer AF. Metabolomic linkage unveils functional interaction between Glucose-dependent Insulinotropic Peptide (GIP) and Ghrelin in Humans. *Am J Physiol Endocrinol Metab.* **2011** May 17. [Epub ahead of print]

2. Rudovich NN, Weickert MO, Pivovarova O, Bernigau W, Pfeiffer AF. Effects of acarbose treatment on markers of insulin sensitivity and systemic inflammation. *Diabetes Technol Ther.* **2011**;13(6):615-23.

3. Rudovich NN, Weickert MO, Machann J, Pfeiffer AF. Combination of acarbose and ezetimibe prevents non-alcoholic fatty liver disease: a break of intestinal insulin resistance? *J Hepatol.* **2010**;52(6):952-3.

4. Rudovich N, Möhlig M, Otto B, Pivovarova O, Spranger J, Weickert MO, Pfeiffer AF. Effect of meglitinides on postprandial ghrelin secretion pattern in type 2 diabetes mellitus. *Diabetes Technol Ther.* **2010**;12(1):57-64.

5. Rudovich N, Pivovarova O, Fisher E, Fischer-Rosinsky A, Spranger J, Möhlig M, Schulze MB, Boeing H, Pfeiffer AF. Polymorphisms within insulin-degrading enzyme (IDE) gene determine insulin metabolism and risk of type 2 diabetes. *J Mol Med (Berl).* **2009**;87(11):1145-51

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11. Rudovich NN, Leyck Dieken MG, Rochlitz H, Pfeiffer AF. Enhancement of early- and late-phase insulin secretion and insulin sensitivity by the combination of repaglinide and metformin in type 2 diabetes mellitus. *Exp Clin Endocrinol Diabetes.* **2004**;112(7):395-400.

12. Roudovitch N, Nauck MA, Schatz H, Pfeiffer AF. Modified yperinsulinaemic, eu- and hypoglycaemic clamp technique using lispro-insulin for insulinoma diagnostic. *Exp Clin Endocrinol Diabetes.* **2001**;109(8):397-401.

7. Declaration

Hiermit erkläre ich, dass weder früher noch gleichzeitig ein Habilitationsverfahren durchgeführt oder angemeldet wird bzw. wurde. Die vorgelegte Habilitationsschrift wurde ohne fremde Hilfe verfasst. Die beschriebenen Ergebnisse wurden selbst gewonnen und die verwendeten Hilfsmittel, die Zusammenarbeit mit anderen Wissenschaftlerinnen oder Wissenschaftlern und technischen Hilfskräften und die Literatur wurden vollständig angegeben.

Mir ist die geltende Habilitationsordnung bekannt.

Potsdam, 31.01.2011