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ORIGINAL CONTRIBUTIONS

Putting the Hindgut Hypothesis to the Test in a Diabetic Zucker Rat Model

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Abstract

Background The hindgut theory hypothesizes a key role of differential hindgut stimulation following metabolic procedures in ameliorating diabetes mellitus. We used two strategies to remove the hindgut from intestinal continuity in order to analyze its impact on diabetes mellitus.

Methods Loop duodeno-jejunostomy (DJOS) with exclusion of one-third of total intestinal length was performed in 3 groups of 9-week-old Zucker diabetic fatty rats. In group 1, no further alteration of the intestinal tract was made. Group 2 received additional ileal exclusion (IE). Group 3 underwent additional resection of 50% of the ileum with side-to-side ileocecal anastomosis (IR). One, 2, and 4 months after surgery, fasting blood glucose levels, oral glucose tolerance tests (OGTT), and glucose-stimulated hormone analyses were conducted, and bile acid blood levels were compared. Body weight was documented weekly.

Results In relation to DJOS, glucose control was not impaired in IR or IE. On the contrary, only IR could maintain preOP glucose values until 4 months. There were no significant weight differences between the groups. Confirming effective ileal diversion, bile acid blood levels were significantly higher in the DJOS group compared with both IR and IE ($p = 0.0025$ and $p = 0.0047$). Operative interventions had no impact on GLP-1 levels at any time point (ANOVA $p > 0.05$ for all). Insulin secretion was preserved in all groups.

Conclusion This data supports the hypothesis that the mechanisms driving amelioration of diabetes mellitus are complex and cannot be reduced to the ileum.

Keywords Metabolic surgery · Duodeno-jejunal bypass · Diabetes · Zucker rat

Introduction

Metabolic surgery is a highly effective treatment for a defined group of obese patients with type 2 diabetes (T2DM) [1–3]. Despite this fact, there has long been a gap between evidence and clinical practice. Only recently, metabolic surgery has been accepted as an adequate diabetes treatment by most leading diabetic societies worldwide [4]. This was only possible

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on the grounds of ample evidence for surgical therapy that not only consists of evident clinical data but also on well-defined pathophysiological models.

One of the most renowned models, the "hindgut hypothesis," relies on anatomical changes after Roux-en-Y gastric bypass (RYGB) and biliopancreatic diversion (BPD), as these operations create the largest antidiabetic effect [5, 6]. This hypothesis is mainly carried by glucagon-like peptide 1 (GLP-1) released from ileal L-cells that have been stimulated through rapid food delivery to the ileum [7]. Primarily, this theory seems conclusive, as indeed RYGB or BPD stimulates GLP-1 secretion and this incretin in turn exerts antidiabetic effects [8, 9]. However, there is an increasing doubt that the most prominent hypothesis plays the most prominent pathophysiological role [10].

Feeding this doubt is the fact that antidiabetic medication stimulating the GLP-1 axis does not carry the effect of a metabolic operation [10]. Blocking agents of GLP-1 again only mildly impair a RYGB effect [11, 12]. Potential ileal effects on glucose control are described to originate from luminal but also endocrine or neurohumoral stimulation [13–15].

We hence put the hindgut hypothesis to the test by either bypassing, resecting, or stimulating the ileum in a Duodeno-jejunal bypass model in diabetic Zucker (ZDF) rats.

Material and Methods

Diets and Animals

Seven-week-old male obese Zucker diabetic fatty rats (ZDF-laprfa/CRL) were acquired from Charles River Breeding Laboratories (Wilmington, MA). Animal care and description of feed were undertaken as described previously [16]. Rats were fasted 4 h before surgery and 6 h before oral glucose tolerance tests (OGTT) and hormone measurement. All animal experimental protocols were approved by the local animal welfare committee under the auspices of the responsible regional commission. All applicable institutional and national guidelines for the care and use of animals were followed.

Experimental Protocols

Rats were acquired and left to acclimatize with free access to food and water for 14 days. Rats were randomly assigned to the 3 operative groups: duodeno-jejunostomy (DJOS, $n = 13$), duodeno-jejunostomy with ileal resection (IR, $n = 13$), and duodeno-jejunostomy with ileal exclusion (IE, $n = 13$) using sealed envelopes. For evaluation of glucose metabolism, oral glucose tolerance tests (OGTT) were performed in all groups 1, 2, and 4 months after surgery. In 12 rats, we performed an OGTT 2 days prior to surgery. Hormone measurements were conducted 2 days after each OGTT and 20 min after glucose stimulation. Following the last procedure, the rats were euthanized with a lethal intracardial injection of potassium chloride (2 mmol/kg body weight) under general anesthesia. The terminal ileum, duodenum, biliopancreatic limb, and ascending colon were removed for immunohistochemical staining.

Body weight was recorded twice daily in the first week, once a week for the remaining period. Food and water consumption were recorded in the first week after surgery only. Figure 1 illustrates the experimental protocol as well as numbers of animals operated and lost to follow-up.

Surgery

DJOS operations were performed as described previously [16, 17]. In brief, after a midline incision of 3–4 cm, the total length of the small intestine was determined. The duodenum was then divided in pars 1. The remaining duodenal stump was closed using PDS 6/0 (Ethicon). The earlier defined jejunum was anastomosed via an end-to-side antecolic duodeno-jejunostomy, excluding the duodenum and 1/3rd of total intestinal length. Mesenteric defects (Petersen space) were closed with PDS 6/0 (Ethicon). For the IR procedure, the distal 50% of the ileum (25% of total intestinal length) was removed and the continuity was reconstructed with a side-to-side ileocecal anastomosis with a running PDS 6/0 (Ethicon) suture. For IE, 50% of terminal ileum was measured. The ileum was then divided at this position, followed by a closure of the distal lumen with a Vicryl 4/0 (Ethicon) ligature and interrupted PDS 6/0 (Ethicon) sutures. Continuity was restored by a side-to-side ileocecal anastomosis using the proximal part of the divided ileum with a running PDS 6/0 (Ethicon) suture analog to the IR procedure (Fig. 2).

Anesthesia was induced and maintained using isoflurane 2% (AbbVie Deutschland GmbH&Co.KG, Ludwigshafen, Germany) and oxygen flow at 2 l/min under spontaneous breathing [18]. Perioperative analgesia was conducted via subcutaneous carprofen (Rimadyl, Pfizer, Switzerland) injection (4 mg/kg body weight) at the beginning of the operation, and the next 3 days postoperatively. Additionally, Buprenorphine (MSD SHARP & DOHME GmbH, Haar, Germany) was injected (0.05 mg/kg body weight) every 8 h for the first 24 h. Animals were kept with free access to water and Fresubin energy drink (Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany) on day 1. Oral food was continuously increased to free access until day 5 after operation.

Perioperative mortality was 10%. Five further animals deceased during OGTTs due to aspiration. Overall mortality added up to 23%.

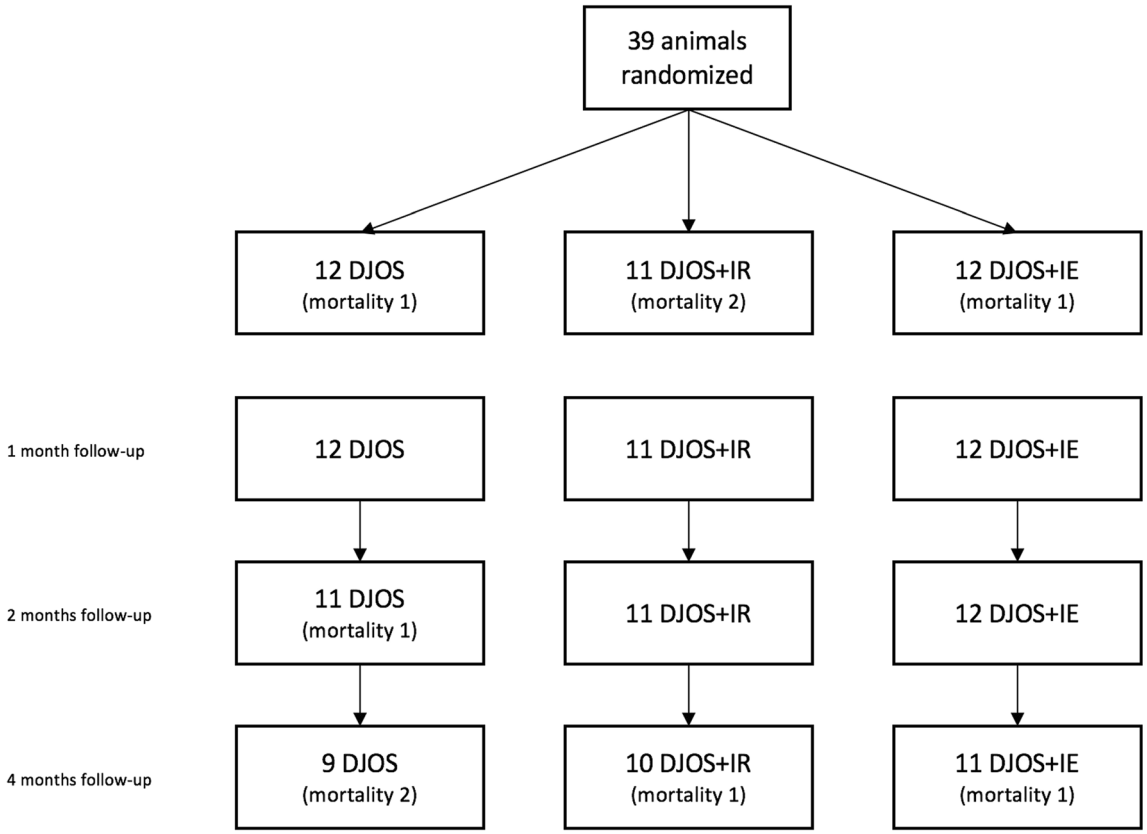

Fig. 1 Flow chart of experimental protocol and mortality

Oral Glucose Tolerance Test

OGTTs were performed under general anesthesia and were initiated after placement of an orogastric tube (central venous catheter, Arrow Deutschland GmbH, Kernen, Germany) via infusion of a 70% glucose solution at the dosage of 1 mg/kg body weight. Glucose was determined via tail tap at 0, 30, 60, 90, 120 min using a glucose meter (Accu-Check Aviva, Roche Diagnostics Deutschland GmbH, Mannheim, Germany).

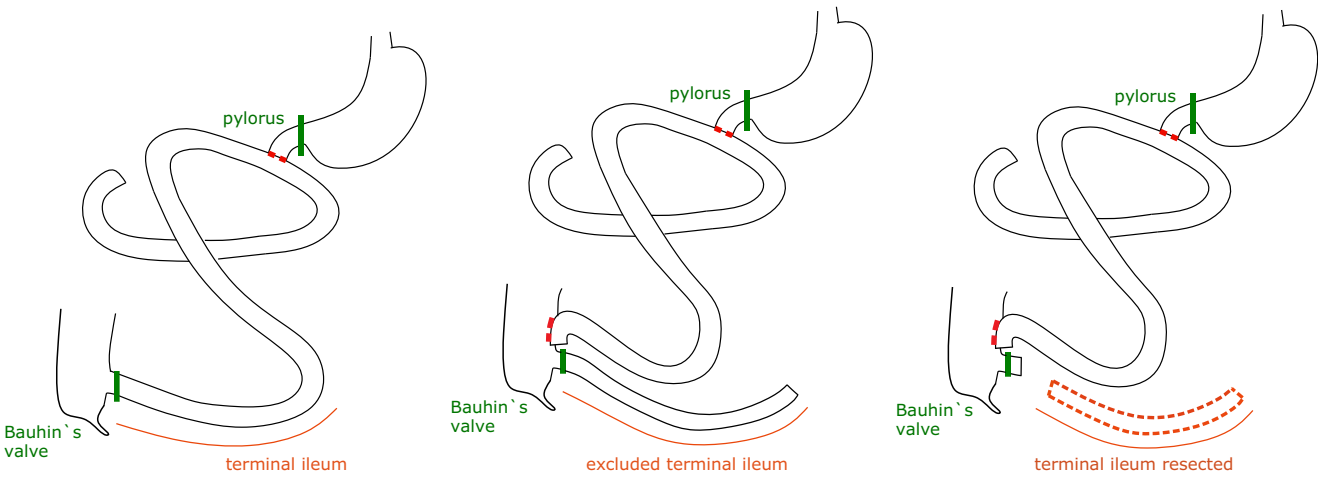

Fig. 2 Mouse models of DJOS, IR, and IE

Hormone Measurement

The experimental setting was identical to the OGTT and as described previously. Twenty minutes after gastric glucose infusion, 400 μl blood was drawn via cannulization of the tail vein, using tubes containing 0.69 mg K3EDTA (Sarstedt AG & Co, Nümbrecht, Germany).

High-range rat insulin ELISA was a solid phase two-site enzyme immunoassay using HRP reaction for detection (DRG Instruments GmbH, Marburg, Germany). GLP-1 (7–36) samples were added directly to a straptavidin-coated microtitre plate (EMD Millipore Corporation, Darmstadt, Germany). Bile acids were measured with the total bile acids assay kit (Diazyme Laboratories, MDSS, Hannover, Germany).

Immunostaining

Specimens of duodenum, biliopancreatic limb, terminal ileum, and ascending colon were fixed in paraformaldehyde solution and embedded in paraffin. Sections of 3 μm were cut. To analyze intestine structures, tissue samples were stained for GLP-1. In brief, sections were deparaffinized in Rotihistol (Roth, Karlsruhe, Germany) and subjected to antigen retrieval in 10 mM Tris/1 mM EDTA pH 9. Sections were then blocked for endogenous peroxidase activity and non-specific binding with 5% goat serum followed by incubation with primary rat anti-GLP-1 antibody (1 : 5000, GLP1-#22625, Abcam, Cambridge, UK). Sections were visualized using Dako Envision (K4003-HRP, Dako North America, Carpinteria, California) and developed using 3-Amino-9-ethylcarbazol as a chromagen before they were dehydrated with Rotihistol (Roth, Karlsruhe, Germany) and preserved with Roti-Histokitt (Roth, Karlsruhe, Germany) (Fig. 5).

To quantify the total number of L-cells, slides were scanned and digitalized using Zeiss Axio Scan.Z1 slide scanner. For histological evaluation, the number of GLP-1 positive cells in randomly selected cross sections adding up to 3000 μm luminal length in each defined segment of the intestine was determined by two independent examiners (one surgeon and one pathologist).

Statistical Analysis

Statistical analyses were conducted using Prism 8 for Mac OS X (GraphPad Software Inc.). ANOVA and two-way ANOVA were used for group comparison. A student's *t* test was applied for changes from 1- to 4-month follow-up within each group. Data were tested for normality using a D'Agostino and Pearson normality test. Weight gain of animals was analyzed using linear regression. Applicable values of $p < 0.05$ were considered significant.

Results

Body Weight

Animals in all groups continuously gained weight. Weight gain occurred to a similar amount and at a similar rate (linear regression $p = 0.7319$; Fig. 3).

Glucose Control

Animals were diabetic preoperatively (mean fasting glucose 224.2 ± 61.14 mg/dl, range 119–325). IR and IE but not DJOS presented with improved fasting glucose values at first follow-up (Tukey's test preOP vs. IR $p = 0.0248$, IE $p = 0.0153$). In relation to preoperatively, OGTT revealed no further improvement in any group at that time (Tukey's test DJOS $p = 0.1822$; vs. IR $p = 0.5578$; vs. IE 0.9767, Fig. 4). Glucose control deteriorated in DJOS and IE starting 2 months after surgery (Tukey's test preOP vs. DJOS 2 months $p = 0.0002$, 4 months $p = 0.0021$; vs. IE 2 months $p = 0.0035$, 4 months < 0.0001, Fig. 4). Ileal resection led to a maintenance of glucose control at all examined time points (Tukey's test preOP vs. IR 2 months $p = 0.2406$, 4 months $p = 0.0812$).

Comparing IR with DJOS or IE at each single time point revealed only small differences that largely reflected the trend of a better performance in the IR group (Tukey's test IR vs. DJOS 1 month $p = 0.005$; IR vs. IE 4 months $p = 0.021$).

Insulin

Insulin levels were similar in between groups at all examined time points (ANOVA 1 month $p = 0.2445$; 2 months $p = 0.0926$; 4 months 0.1817). Overall insulin production was preserved in all 3 groups (*t* test 1 vs. 4 months: DJOS $p = 0.4297$; IR $p = 0.2766$; IE $p = 0.0204$; Fig. 5a).

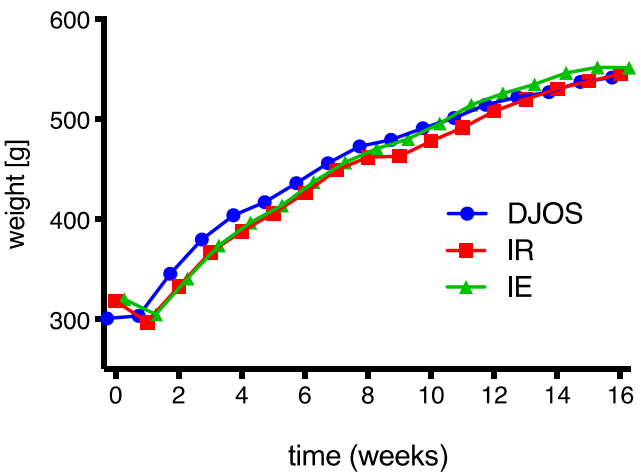

Fig. 3 Plot of total body weight (linear regression $p = 0.7319$)

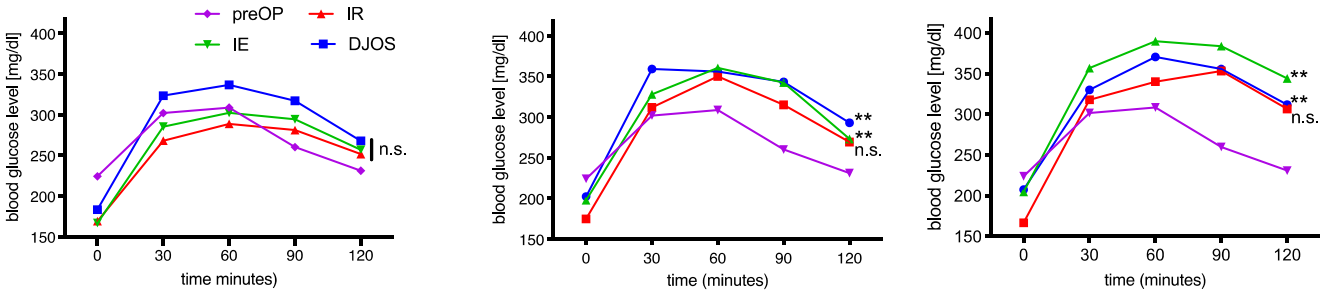

Fig. 4 Plot of blood glucose levels during OGTT 1 (**a**), 2 (**b**), and 4 (**c**) months after surgery. As reference, preoperative glucose levels are displayed. **a** Glucose levels equal to preOP in all groups. **b** Impaired glucose control in DJOS and IE animals (**Tukey's test DJOS vs. preOP $p = 0.0002$; IE vs. preOP $p = 0.0035$). **c** Impaired glucose control in DJOS and IE animals (**Tukey's test DJOS vs. preOP $p = 0.0021$; IE vs. preOP $p < 0.0001$)

Bile Acids

Resection or exclusion of the ileum led to lower bile acid levels in relation to DJOS at 2 and 4 months postoperatively (Tukey's test DJOS vs. IR $p = 0.0057$ and $p = 0.0025$; vs. IE $p = 0.0056$ and $p = 0.0047$ after 2 and 4 months, resp.). Exclusion of the ileum led to a similar observation already 1 month after surgery (Tukey's test DJOS vs. IE $p = 0.0146$, Fig. 5b). Of note, bile acid levels increased from 1 to 4 months in DJOS animals only (t test 1 vs. 4 months $p = 0.0099$).

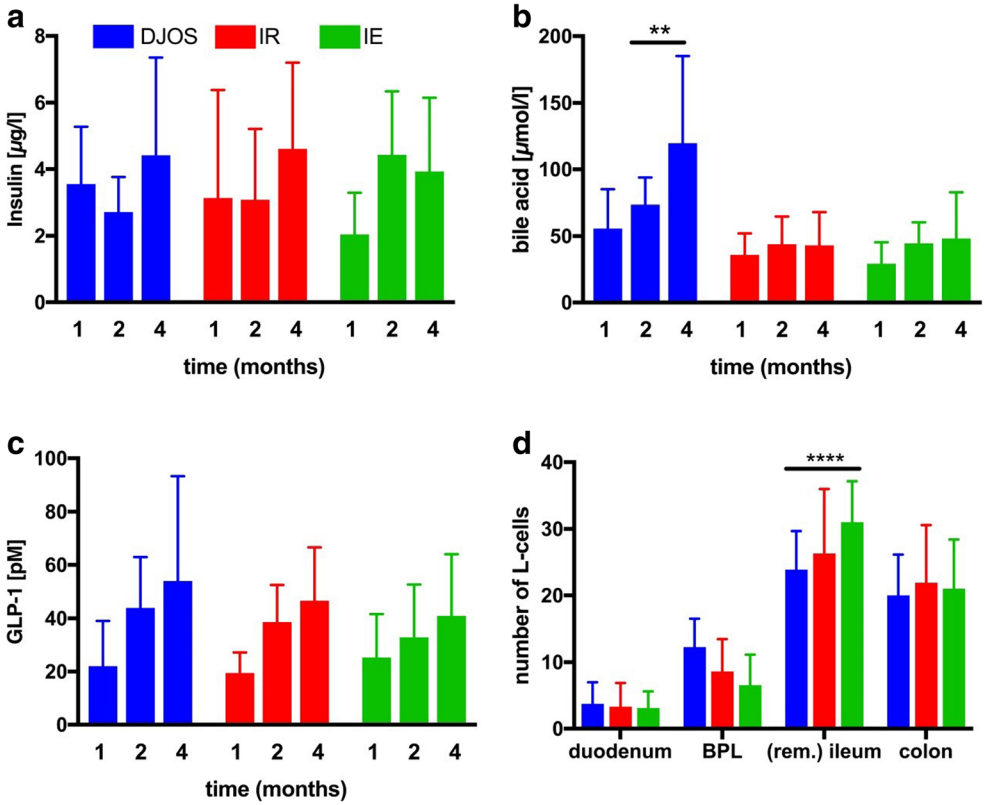

Fig. 5 Box plot of insulin (**a**), bile acid (**b**), GLP-1 levels (**c**) ± SD 20 min after oral glucose infusion at 1, 2, and 4 months, and number of GLP-1 positive L-cells (**d**). **a** Similar insulin levels in between groups at all examined time points (ANOVA $p > 0.05$ for all). Sustained insulin secretion (1 vs. 4 month) in all groups (t test 1 vs. 4 months: DJOS $p = 0.4297$; IR $p = 0.2766$; IE $p = 0.0204$). **b** Lower bile acid levels in IR and IE in relation to DJOS (Tukey's test DJOS vs. IE 1 month $p = 0.0146$; **DJOS vs. IR and IE 2 and 4 months $p < 0.01$ for all). Significant increase from 1- to 4-month follow-up in DJOS only (t test $p = 0.0099$). **c** Similar GLP-1 levels in between groups (ANOVA $p > 0.05$ for all). Significant increase of GLP-1 in DJOS and IR from 1 to 4 months (t test DJOS $p = 0.0275$; IR $p = 0.0006$). **d** L-cell expression not different in between groups (ANOVA $p > 0.05$ for all). L-cell expression in the (remaining) ileum significantly higher in relation to duodenum (*** Tukey's test $p < 0.0001$ for all groups) and biliopancreatic limb (BPL, ***Tukey's test $p < 0.0001$ IR and IE, $p = 0.0007$ for DJOS). Similar number of L-cells in colon and (remaining) ileum in DJOS and IR (Tukey's test ileum vs. colon DJOS $p = 0.4286$; IR $p = 0.5260$)

Glucagon-Like-Peptide 1

GLP-1 levels were similar in between groups at each examined time point (ANOVA 1 month $p = 0.6217$; 2 months $p = 0.3915$; 4 months $p = 0.6217$). In the DJOS and IR groups, GLP-1 levels increased significantly from 1 to 4 months postoperatively (t test 1 vs. 4 months DJOS $p = 0.0275$; IR $p = 0.0006$; Fig. 5c). By trend, the same observation could be noted for IE (t test 1 vs. 4 months IE $p = 0.0773$).

Regional Number of L-Cells

L-cell numbers gradually increased in the small intestine with lowest counts in the duodenum and highest counts in the ileum in all groups (Tukey's test duodenum vs. ileum $p < 0.0001$ for all). Colonic L-cell expression was similar to the ileum in DJOS and IR (Tukey's test DJOS $p = 0.4286$, IR $p = 0.526$). Overall, all parts of examined intestine expressed similar L-cell numbers in all 3 groups (ANOVA duodenum $p = 0.9141$; biliopancreatic limb $p = 0.0667$; ileum $p = 0.1428$; colon $p = 0.8703$, Fig. 5d).

Discussion

The complex mechanisms of post-bariatric diabetes improvement remain elusive. One of the most prominent concepts states that stimulation of the ileum leads to secretion of entero-endocrine hormones and improvement of glycemic control [7, 9]. Excluding or resecting large parts of the ileum in a loop duodeno-jejunostomy rat-model, we put this hypothesis to the test.

In the ZDF animal model, the ileum was not responsible for glucose control. Left untreated, ZDF rats develop severe, progredient diabetes [16, 19]. A DJOS operation transiently preserved preoperative glucose levels, yet did not lead to improved glucose control. Additionally, resecting the terminal ileum, and hence excluding that this part of the intestine is responsible for diabetes improvement, did not impair the DJOS effect. On the contrary, IR sustained preoperative glucose levels at all examined time points and furthermore transiently improved fasting glucose values. Bypassing the ileum did not have the same effect, yet did not lead to impaired glucose control, either.

This stands in clear contrast to earlier observations. Imoto et al. performed a partial or complete duodeno-jejunal bypass in Long-Evans rats. Only a complete bypass led to improved glucose control in this model. His group therefore concluded that the ileum is responsible for the observed effect [11]. Leaving the foregut untouched, our own group demonstrated an amelioration of glucose control after different lengths of ileal transposition. Transposing a long, distal ileal segment and hence the potentially largest amount of L-Cells was favorable in this experiment. Our data furthermore suggested that glucose control was GLP-1 associated [20].

We cannot fully explain these contrasting observations. Overall, our current findings corroborate some of our earlier data. Bypassing different lengths of jejunum, a similar improvement of glucose control was observed compared with SHAM. At that time, we had speculated that not the ileum, but duodenal exclusion, was the driving force behind enhanced glucose control [16]. A very recently published article examined glucose control after ileal resection in a RYGB diabetic rat model. Resecting the ileum had no negative effect in this study [21]. Coming from a different perspective, Buchwald et al. resected or excluded the ileum in Goto-Kakizaki rats, assuming that this would impair glucose control. However, neither of these interventions had the anticipated effect [22]. Assuming there are two main mechanisms underlying improved glucose control after metabolic surgery, i.e., ileal stimulation and duodenal exclusion, these data suggest that the latter may be the more potent one.

Resection of the ileum did not lead to diminished GLP-1 levels. It is assumed that GLP-1 originates from the ileum, as GLP-1 levels typically rise after ileal transposition and GLP-1 staining shows large numbers of GLP-1 positive L-cells in the transposed segment [23]. The current study also shows that the highest expression of GLP-1 positive cells within the small intestine is located in the ileum (Fig. 6). However, similar GLP-1 levels in all groups despite resection or exclusion of the ileum raises the question of alternate sources of GLP-1. Analyzing the effect of ileal resection in Goto-Kakizaki rats, Buchwald et al. demonstrated an unforeseen rise in GLP-1 levels. His group hypothesized that this increase was due to stronger simulation of colonic L-cells [22]. In our study, histological staining revealed a similar L-cell number in the colon and the (remaining) ileum of DJOS and IR animals. GLP-1 serum levels increased from 1- to 4-month follow-up in these

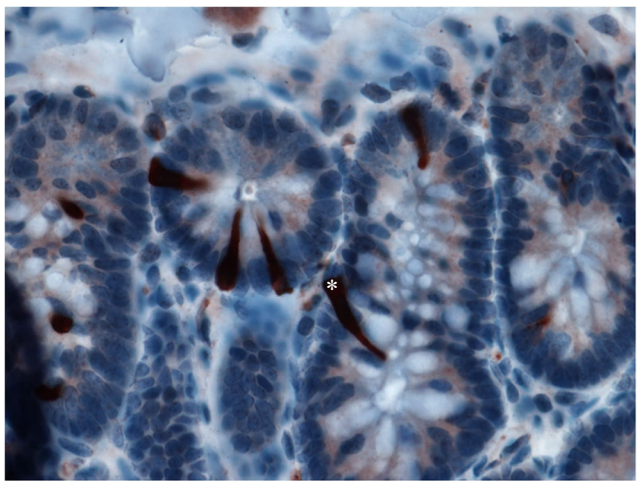

Fig. 6 Representative graph of GLP-1 immunostaining in the intestine. The asterisk shows a L-cell

two groups. This suggests that the colon was the likeliest alternate source of GLP-1 in ZDF rats [24].

GLP-1 had no relevant effect on glucose control in ZDF rats. Both DJOS and IR presented with significantly rising GLP-1 levels during follow-up. At the same time, glucose control deteriorated in DJOS and remained unchanged in IR animals. There is ample evidence that GLP-1 has a relevant effect on diabetic control in many animal models, yet our data is not isolated [25, 26]. Rising GLP-1 levels after ileal resection despite unchanged glucose control led Buchwald et al. to question the role of GLP-1 in Goto-Kakizaki rats [22].

Insulin secretion did not depend on a fully functioning ileum in the current model. In the normal course of ZDF rats, insulin secretion deteriorates after approximately 8 weeks and animals develop severe diabetes [16, 19]. This typically leads to a weight stagnation [16, 19].

In the current examination, insulin secretion remained intact in all groups, regardless of ileal resection or exclusion. This observation stands in line with steadily increasing bodyweight in all groups.

There was no evidence for a significant influence of bile acids on glucose control in ZDF rats. On the contrary, associations of elevated bile acid levels and improved glucose tolerance after metabolic surgery are commonly found [27, 28]. As one example, Cummings et al. demonstrate improved glucose control in line with significantly elevated bile salts following ileal transposition in UCD-T2DM rats. His group furthermore identified a bile acid associated reduction of endoplasmatic reticulum stress in peripheral insulin-sensitive tissue as a potential mechanism [29]. In a proof of principle experiment, diverting the bile directly to the ileum via a gallbladder-ileostomy and ligation of the common bile duct lead to similar improvement of glucose tolerance compared with RYGB in C57BL/6 mice [27].

In line with previous literature, our own published data showed elevated bile acid levels in DJOS compared with sham-operated control animals [16]. Current data furthermore demonstrate a significant increase in bile acid levels over time after DJOS. In theory, a rise in bile acids after metabolic surgery is explained by decreased hepatic uptake or increased intestinal absorption [30, 31]. Evidence for these mechanisms is not conclusive at present [32]. Extensively analyzing bile acid transport-related gene and mRNA expression in Zucker rats after RYGB, Bhutta et al. found upregulation of bile acid uptake in the biliopancreatic limb. However, this does not explain the observed steady increase in bile acids during this experiment [30]. The current study was not designed to examine the mechanism of bile acid absorption, yet it underlines the role of the ileum in this matter. Without the intact ileum, serum bile acid levels were significantly lower reflecting a reduced capacity of bile acid reabsorption. A steady increase was not observed. However, this impairment had no effect on glucose control.

This study had several limitations. The described effects of all hormone measurements are based on statistical associations and do not rely on independently tested functions. To limit malabsorption, the ileum was not resected completely. We resected the distal and potentially functionally more important 25% of total intestinal length in the IR group. We hence cannot exclude a rest-function of the remaining ileum. For reasons of animal protection, we carried no additional sham surgery group in this experiment.

The current study tested the effect of the terminal ileum on glucose tolerance in ZDF rats. In this animal model, the ileum was not responsible for glucose control suggesting a more potent role of antidiabetic foregut mechanisms. These data have no immediate implications for humans.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Informed Consent Statement Does not apply.

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