

## Communication

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# Ileal transposition in rats influenced glucose metabolism and HSP70 levels

**Abstract:** Objective: Ileal transposition procedure (IT), in combination with sleeve gastrectomy, is widely used to induce diabetes remission and to control related metabolic abnormalities. A transposition of a long segment of distal ileum in obese Zucker rats improved glucose tolerance 6 months after IT. The premise of our study was to examine the long-term effects of ileum transposition on the liver glycolytic enzymes content in a euglycemic group of operated Zucker rats. Methods: Twenty male Zucker rats underwent either the transposition of 50% distal ileum or a sham surgery. Six months after surgery, liver tissue concentrations of glycogen synthase kinase alpha (GSK-3 $\alpha$ ), glucose 6-phosphatase (G6PC), glycogen phosphorylase (PYGM) and phosphofructokinase (PFK) and HSP70 were assessed by immunoenzymatic methods. Results: HSP70 values were significantly higher in the IT group compared to SHAM. G6PC liver concentrations in the IT group were almost 1.45-fold lower than in the SHAM operated rats. Statistical analyses (F-test) showed HSP70 levels were significantly related to caveolin-1 and SHAM group. Conclusions: Lowered glycolytic enzyme concentrations assessed in the liver suggest positive effects on glucose metabolism in long-term observations.

**Keywords:** HSP70, ileal transposition, type 2 diabetes mellitus, obesity surgery, metabolic surgery

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## 1 Introduction

One of the effects of oxidative stress disorders in obesity and diabetes mellitus (type 2) is liver dysfunction manifested with deterioration in carbohydrate and lipid metabolism. These metabolic abnormalities promote steatosis, fibrosis, cirrhosis, hepatitis and biliary disease. The increased hepatic glucose production, hepatic glucose uptake and glycogen storage is one of the effects of insulin resistance and hyperglycemia observed in Zucker diabetic fatty (CrI:ZUC-Lepr<sup>fa</sup>) rats, a widely used obese metabolic syndrome and pre-diabetes model [1]. Ileal transposition procedure (IT) is widely used to induce diabetes remission and to control related metabolic abnormalities. A transposition of a long segment of the distal ileum in obese Zucker rats improved glucose tolerance for 6 months after IT [2,3]. This was the premise to examine the long-term effects of ileum transposition on liver glycolytic enzymes and HSP 70 content in a euglycemic group of operated Zucker rats.

Heat shock proteins (Hsps) are up-regulated in response to cellular stresses such as inflammation and oxidative stress and are considered to be molecular chaperones that assist in the folding, assembly and degradation of other cell proteins [4,5]. In general, Hsps are known to regulate signal transduction, protein trafficking, cellular proliferation, and cell differentiation [6]. HSP70 is a big family of ATP-dependent chaperones, which takes part in protein folding and protect against cellular stress and toxicity [7]. In the conditions of insulin resistance, HSP levels are low in the insulin dependent tissues like liver, skeletal muscle and adipose tissue [8].

The improvement in glucose metabolism after bariatric surgery may be achieved through physiological and biochemical mechanisms contributing to weight loss, improved hepatic and peripheral insulin sensitivity

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[9]. Enzymes assayed in this project were selected due to their central role in the regulation of carbohydrate and energy homeostasis. It has been recognized that glucose-6-phosphatase (G6PC) is one of the crucial regulatory enzymes of gluconeogenesis and is expressed in the liver, skeletal muscle and small intestine [10]. Glycogen phosphorylase (PYGM) is the major enzyme in glycogenolysis responsible for the glucose-1-phosphate release from glycogen [9]. Glycogen synthase kinase-3 (GSK-3 EC 2.7.11.26) is involved in glycogen synthase (GS) regulation, phosphorylation and its deactivation. GSK-3 shows enhanced activity in insulin-resistant states and has been proposed as a potential therapeutic target for the treatment of diabetes [11]. Phosphofructokinase (PFK) is a sensitive indicator of the glycolytic pathways in diabetic states and a key rate-limiting enzyme in glycolysis [12].

This paper is a continuation of a research project examining the long term effect of ileal transposition in term of physiologic background and long-term adaption in Crl:ZUC-Lepr<sup>fa</sup> Zucker rats [3,13]. The principal goal of this study was to investigate the impact of transposition of 50% of distal ileum on separated, selected parameters of glucose administration and HSP70 concentration levels during *in vivo* experiments on the Crl:ZUC-Lepr<sup>fa</sup> rat's liver six months after IT surgery.

## 2 Materials and Methods

This paper is the third part of a wider experiment which concerned ileal transposition treatment of obesity and insulin resistance, in the obese rat model: Crl:ZUC-Lepr<sup>fa</sup> Zucker rat [3,13]. All surgical and experimental procedures were precisely described earlier by our research group [3,13], thus in this paper we briefly present only the main points. The animal experimental protocols were approved by the Ethics Committee of the University of Freiburg [3,9,13]. In short, 11 to 12-week-old, 200–220 g, obese male Zucker rats (Crl:ZUC-Lepr<sup>fa</sup>) were purchased from Charles River Breeding Laboratories (Wilmington, Mass). The rats were housed individually and maintained on a 12/12-hour light/dark cycle with free access to water and rat chow diet (ProvimiKliba AG, Kaiseraugst, Switzerland). The chow contained 24% protein, 4.9% fat, 7% crude ashes, 4.7% crude fiber, lysine (13.6 g/kg), calcium (12 g/kg), methionine (4.5 g/kg), and phosphorus (8.3 g/kg).

### 2.1 Surgery

After a 7–8 day acclimation period, 10 rats underwent an ileal transposition surgery on 50% of distal ileum [3]. The

animals were fasted for 12 hours before surgery. Anesthesia was induced and maintained using Isoflurane 2% (Abbott GmbH & Co. KG, Wiesbaden, Germany) and oxygen flow at 2 L/min under spontaneous breathing. After a midline incision of 4–5 cm to gain abdominal access, Bauhin's valve was identified. Previously, we had determined the total small bowel length of Zucker rats to be approximately 85 cm. Transections of 50% distal segment (25 cm) was conducted in relation to Bauhin's valve.

#### 2.1.1 IT

For IT, the Treitz's ligament was identified and the jejunum divided 5 cm aborally. The ileal loop was then interpositioned in an isoperistaltic fashion forming two end-to-end anastomoses. Mesenterial openings were closed with Prolene 6/0 (Ethicon). Fascia and skin closure were performed as a continuous suture using Monocryl 4/0 and Vicryl 4/0. Postoperative analgesia was ensured via subcutaneous Carprofen (Rimadyl, Pfizer, Switzerland) injection (4 mg/kg) at the beginning of the operation. Animals were fasted on day 1 after the operation with free access to tap water, and oral food was continuously built up to free access until day 6 [3,13].

#### 2.1.2 SHAM

Ten male Zucker rats were selected for SHAM surgery. A division of small intestine with subsequent anastomoses was performed at the 3 corresponding positions without IT. Pre and postoperative treatment was identical for the IT group [3,13].

### 2.2 Blood and tissue collection

Six months after surgery, anesthesia was induced and maintained using isoflurane 2% and oxygen flow at 2 L/min under spontaneous breathing. A cannula (26-gauge) was placed in the tail vein for blood collection. We drew 400 µL of whole blood *via* the cannula at 0 and 20 minutes after oral glucose (1.5 mg/kg) was given via tubes containing 10 mL EDTA (Sigma-Aldrich, St. Louis, Mo). After centrifugation at 4.000 rpm for 10 minutes at 4°C, plasma samples were collected and snap frozen in liquid nitrogen and stored at -80°C until analysis. After blood sampling, the animals were sacrificed and tissues were harvested. Liver tissue was explanted and snap frozen in liquid nitrogen and stored at -80°C until further analysis.

## 2.3 Tissue HSP70 assessment

HSP70 liver tissue concentrations were assessed in duplicates by immunoenzymatic method with the commercially available ELISA kits (USCN Life Science Inc., USA). To assess the validity and predict tissue concentration of chosen parameters in 100 mg of tissue, the optimal sample dilutions were preliminarily determined. Tissue samples were prepared by homogenization and sonification (15 s) on ice in tissue cell lysis buffer containing protease inhibitors (Gold Biotechnology, USA). After that, the homogenates were centrifuged at  $5000 \times g$  for 15 minutes at  $4^{\circ}\text{C}$ , then the supernatant was removed and diluted according to previously assessed protocols and used in the ELISA assay. The microtitre plate was pre-coated with a monoclonal antibody specific to rat HSP70. Standards and samples were added to microtitre plate coated with biotin-conjugated polyclonal antibody preparation specific for HSP70. Subsequently, a mixture of a biotin specific antibody and horseradish peroxidase (HRP) was added to each well and incubated. For detection, each well was incubated with a substrate solution and measured in a microplate reader at a wavelength of  $450 \text{ nm} \pm 10 \text{ nm}$ . For those wells that contained HSP70, biotin-conjugated antibody and enzyme-conjugated Avidin exhibited a change in color. The concentration of HSP70 in the samples was determined by comparing the optical density O.D. of the samples to the standard curve. The data were expressed in ng/mL. The sensitivity of the kit was  $\leq 0.045 \text{ ng/mL}$ .

## 2.4 Tissue glycolytic enzymes assessment

Tissue concentration of glycogen synthase kinase alfa (GSK-3 $\alpha$ ), glucose 6-phosphatase (G6PC), glycogen phosphorylase (PYGM) and phosphofructokinase (PFK-1) in the liver was assessed in duplicate using immunoenzymatic methods with the commercially available ELISA kit (USCN Life Science Inc., USA) after optimization procedure. To assess the validity of our methods and to be able to predict the tissue concentration of chosen parameters in 100 mg of tissue, optimal sample dilutions for particular elements of the ELISA bioassay were preliminarily determined. Tissue samples were prepared by homogenization and sonification (15 s) on ice in a tissue cell lysis buffer containing protease inhibitors (Gold Biotechnology, USA). After that, the homogenates were centrifuged at  $5000 \times g$  for 15 minutes at  $4^{\circ}\text{C}$ , then the supernatant was removed and diluted according to the previously assessed dilution and used in the ELISA assay.

## 2.5 Statistics

Statistical analysis was conducted using the Statistica package (STATISTICA 10.0, StatSoft Inc., 2010). For HSP70 group comparison Mann-Whitney U post-hoc tests were used and  $p < 0.05$  was considered significant. Spearman's rank correlation coefficient was calculated for  $p < 0.05$ . For estimation of the relationships among the analyzed variables F-test was applied,  $p < 0.005$ . For enzymes, we used ANOVA and Tukey's post-hoc tests and the values are presented as mean  $\pm$  standard deviations (SD).

## 3 Results

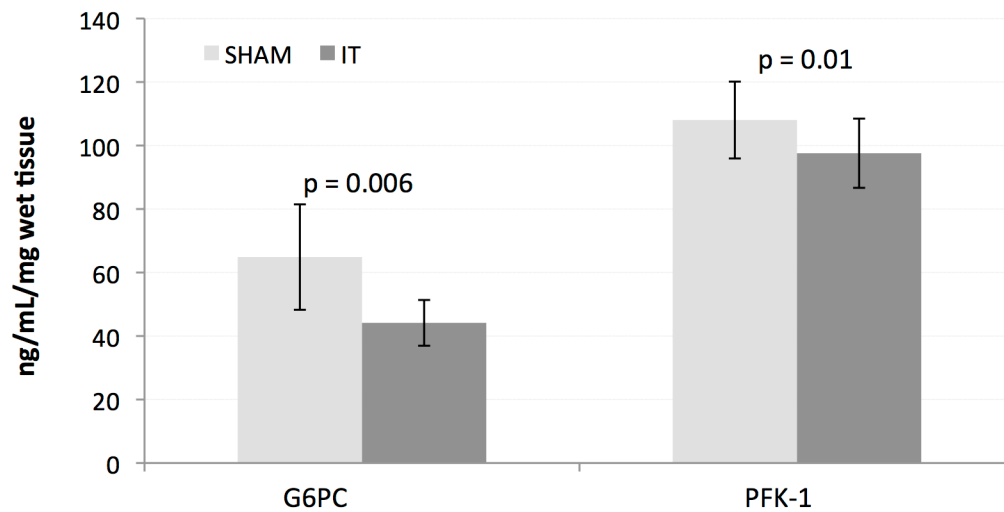
This study is the third part a of previously presented experiment [3,13]. The general characteristics of IT operated rats and the SHAM group with regards to body mass, the oral glucose tolerance test (OGTT), incretin hormones and insulin serum levels were presented by Grüneberger [13].

### 3.1 Glycolytic enzymes tissue concentration (GSK-3 $\alpha$ , PYGM, G6PC, PFK-1) and liver HSP70 concentrations

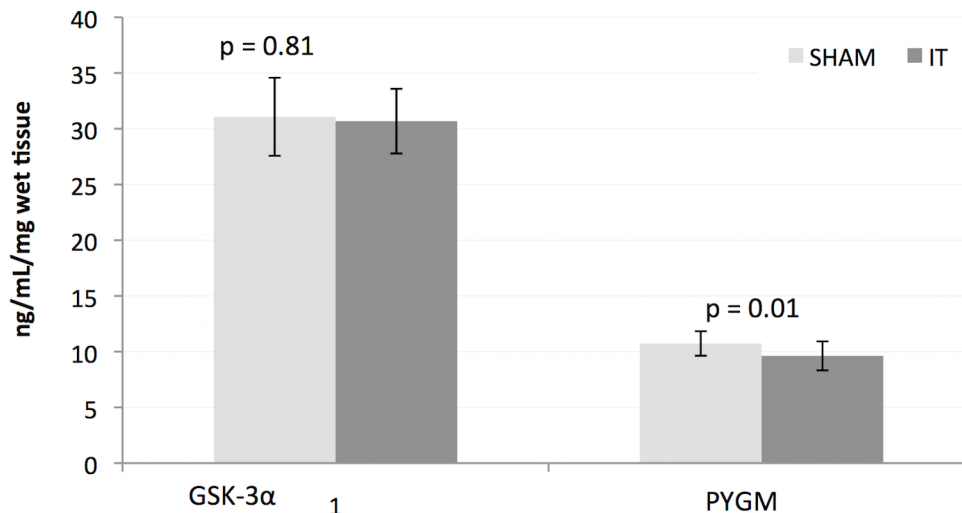
To assess the glycolytic enzyme concentrations, all values were calculated and presented as ng enzyme/mL/mg of wet tissue. Concentrations of PYGM, G6PC, PFK-1 were significantly different between the analyzed IT and SHAM groups. G6PC liver concentrations in the IT group were almost 1.45-fold lower than in the SHAM operated rats ( $64.868 \pm 26.6$  vs.  $44.150 \pm 7.2 \text{ ng/mL/mg w.t.}$ ,  $p < 0.006$ ; fig.1). The effects of IT on PYGM and PFK-1 liver concentrations were more discreet (respectively;  $10.734 \pm 1.1$  vs.  $9.623 \pm 1.3$ ,  $p < 0.01$  and  $108.029 \pm 12.1$  vs.  $97.572 \pm 10.9 \text{ ng/mL/mg w.t.}$ ,  $p < 0.01$ ; Fig.1,2.). No significant differences in liver concentration of GSK-3 $\alpha$  were found between IT and SHAM groups ( $31.072 \pm 3.5$  vs.  $30.678 \pm 2.9 \text{ ng/mL/mg w.t.}$ ,  $p < 0.81$ ; Fig.2.). There were significant correlations between selected enzymes (Tab.1). For HSP70 tissue assessment all values, calculated and presented as ng/mL/mg of wet tissue, were significantly higher in IT group compared to SHAM (Fig. 3.).

#### 3.1.1 F-test results

HSP70 levels were significantly related to SHAM group ( $\beta = -0.239$ ; +95% CI =  $-0.334$ ; -95% CI =  $-0.144$ ;  $p = 0.000001$ ).



**Fig.1:** The tissue concentrations of glucose 6-phosphatase (G6PC) and phosphofructokinase (PFK) in the liver. Optimized enzyme-linked immunosorbent assay assessment for tissue concentration [ng/mL/mg wet tissue], SHAM versus 50% distal IT, six months after surgery, Tukey test,  $p < 0.05$ .



**Fig.2:** The tissue concentrations of glycogen synthase kinase alpha (GSK-3α), glycogen phosphorylase (PYGM) in the liver. Optimized enzyme-linked immunosorbent assay assessment for tissue concentration [ng/mL/mg wet tissue], SHAM versus 50% distal IT, six months after surgery, Tukey test,  $p < 0.05$ .

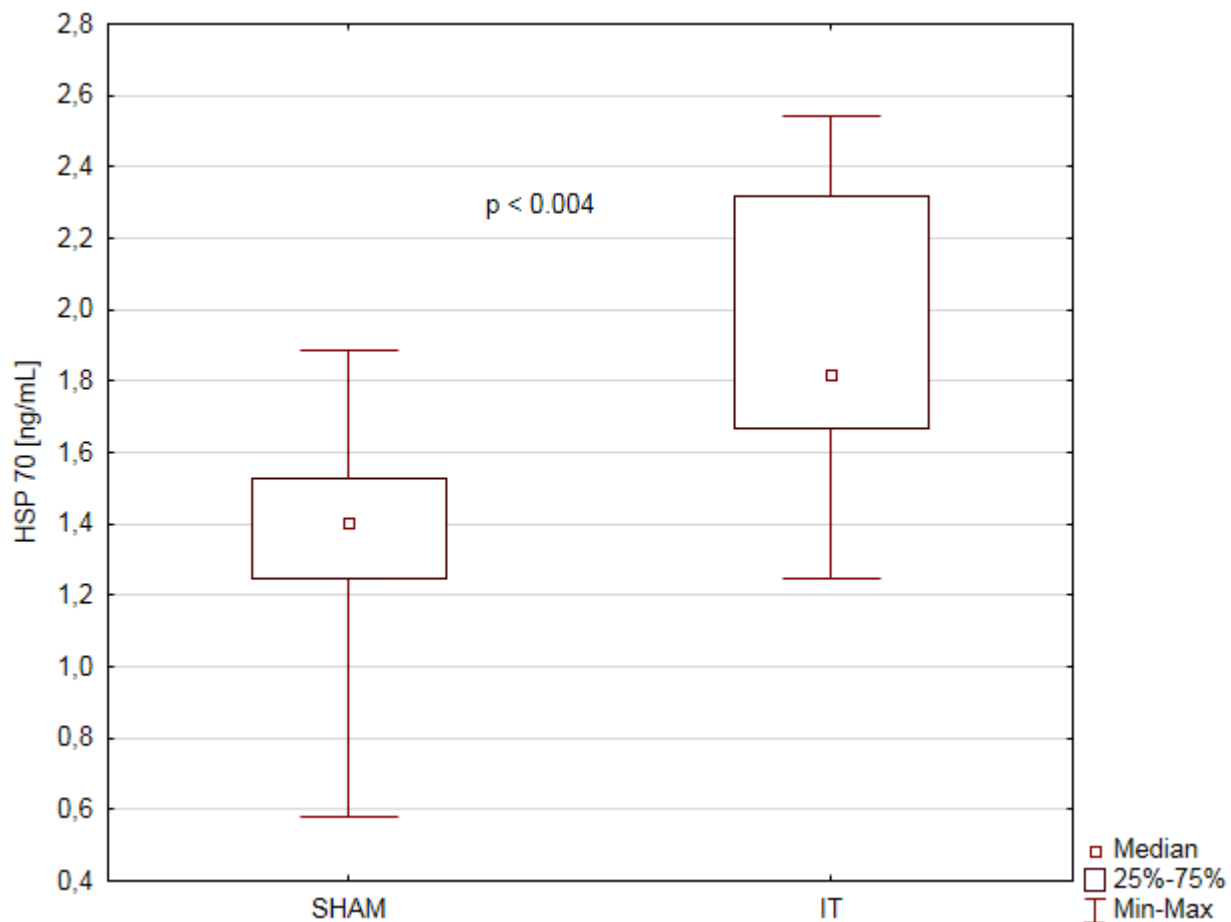
## 4 Discussion

As anti-inflammatory agents HSP70 plays role in response to oxidative cell stress, which is characteristic for obesity and insulin resistance. The levels of HSP70 were found lowered in all insulin-dependent tissues in conditions of obesity, diabetes 2 mellitus and insulin resistance which were also associated with low heat shock factor-1

(HSF-1), low HSP expression, and low HSP levels in insulin-sensitive tissue [8,11]. Decreased HSF1 levels correlate to lowered HSP concentrations in liver and skeletal muscles tissues of diabetic animals in the streptozotocin-induced animal model. In this study, serum HSP70 levels were significantly higher in IT group compared to SHAM, which may be associated with improved insulin resistance. García et al. (2012) presented very interesting

**Table 1:** Significant correlations between analyzed parameters in IT and SHAM groups, spearman correlations,  $p < 0.05$ .

ANALYSED PARAMETERS	R Spearman	t (N-2)	p
G6PC : GSK-3 $\alpha$	0.720	3.448	0.005
G6PC : PYGM	0.722	3.468	0.005
PYGM : PFK-1	0.647	2.817	0.016
PFK-1 : GSK-3 $\alpha$	0.604	2.516	0.028
PFK-1 : G6PC	0.773	4.041	0.001

**Fig.3:** Comparison of HSP70 concentration [ng/mL] in SHAM and IT groups. Mann-Whitney U test,  $p < 0.05$ . Results are presented as median with first and third quartile.

study where downregulation of caveolin-1 related to upregulation of eNOS/HSP70 in tubulointerstitial fibrosis injury in neonatal early kidney obstruction treated with rosuvastatin [14]. HSP70 liver concentration can be higher in IT group due to increased expression, which was not assessed in this experiment [15]. Influencing the induction of heat shock proteins was widely proven as highly beneficial, particularly in patients suffering from diabetes or being insulin-resistant, protecting them from complications. HSP family reduces ischemia-reperfusion injury in the heart [16 powers] and provides favorable

effects on neuropathy and nephropathy in diabetic rodents [10,17]. Induced expression of HSP70 can provide broader health advantages in patients after IT. To increase the picture of changes in glucose metabolism after ileal transposition, we assessed the content of key rate-limiting glycolytic enzymes. The concentrations of PYGM, G6PC, PFK-1 in liver tissue of rats after IT were significantly lowered compared to SHAM groups (Figs 1, 2). Glucose-6-phosphatase level was the most decreased in comparison with other analyzed enzymes. Glucose homeostasis in the liver consists of a number of mechanisms, but low release

of glucose into the blood from the liver could be the dominant cause of lower glucose fasting concentrations after IT [3,13]. Glucose-6-phosphatase is widely expressed in skeletal muscle, the liver and the small intestine. Gluconeogenesis also occurs in the kidney, thus the future investigation of expression of G6PC can be assessed also in that tissue. Perhaps, glucose management can also be altered in the kidney. In this project, G6PC was chosen as one of the key enzymes involved in glucose metabolism to examine the reasons of benefits linked with an improvement in glucose tolerance after IT procedure. Both gluconeogenesis and glycogenolysis result in the formation of glucose 6-phosphate, which has to be hydrolyzed by glucose-6-phosphatase (G6PC) before entering the bloodstream as glucose. The inhibition of glucose-6-phosphatase results in hepatic entrapment of glucose and *de novo* lipogenesis, leading to massive steatosis within several hours. Also, acute inhibition of glucose-6-phosphate translocator activity leads to increased *de novo* lipogenesis and development of hepatic steatosis without affecting VLDL production in rats [18]. It is worth mentioning that 20 weeks after duodenal - jejunal bypass in Goto-Kakizaki rats, G6PC levels within the liver were lower than in the SHAM operated group [9]. DJB operation consists of elements other than IT, but the effect could be similar, meaning low levels of G6PC in the liver. The effects of IT and duodenal-jejunal bypass on G6PC levels could be compared. It may suggest lower utilization of glucose and glycogen with a concomitant increase in the level of plasma triglycerides. In our study, glycogen phosphorylase (PYGM) and phosphofructokinase (PFK1) levels were also lowered 24 weeks after IT surgery. PYGM is responsible for the release of glucose-1-phosphate from glycogen. Glycogenolysis is catalyzed in liver, muscle and brain by tissue specific isoforms of glycogen phosphorylase and the regulation of the hepatic glucose output through glycogenolysis is an important target for type 2 diabetes therapy [19]. Low hepatic PYGM levels in IT animals may suggest that during periods of fasting, the liver releases less glucose than in SHAM animals. This is the probable reason for lower glucose fasting levels in IT animals, therefore this mechanism is not marked like in G6PC changes. Due to the central role of this enzyme in glycogen metabolism, PYGM has been exploited as a model for structure-assisted design of potent inhibitors, which may be relevant to the control of blood glucose concentrations in type 2 diabetes [20]. PFK-1 is the other key regulatory enzyme in glycolysis. In IT animals we observed lower PFK-1 levels in the liver, which could suggest that glucose is more likely to be stored in the liver than released into the bloodstream. A recent study reported PFK-1 upregulation

changes after IT in Sprague-Dawley rats, in the adipose tissue and skeletal muscle [21]. According to literature data and to our study, the IT operations could reproduce the correct storage of glucose in the liver to allow? utilization in peripheral tissues, such as adipose tissue and skeletal muscle. GSK3- $\alpha$  shows a modulating effect on heat shock protein expressions through heat shock factor-1 (HSF-1) [11]. Hooper suggested that the condition of reduced level of HSP is an effect of failed inactivation of GSK-3, and results in phosphorylation and deactivation of HSF-1 [11]. In this study the liver tissue concentration of GSK3- $\alpha$  remained unchanged between IT and SHAM groups. All those findings suggest a suppressed hepatic glucose production, uptake and also reduced metabolism and storage of glycogen. The present study for the first time presents the long-term effect of ileal transposition on HSP70, and glycolytic enzyme concentrations in the liver. In our experiment, six months after transposition of 50% of the ileum, levels of glycolytic enzymes assessed in the liver were lowered.

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**Conflict of interest:** The authors have no conflicts of interest or financial ties to disclose.

**Statement of Animal Rights:** Procedures followed were in accordance with the ethical standards of the Ethics Committee of the University of Freiburg, Germany.

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