TIMELINE OF INTESTINAL ADAPTATION AFTER MALABSORTIVE SURGERY. EFFECT OF LUMINAL NUTRIENTS, BILIOPANCREATIC SECRETION AND GLUTAMINE SUPLEMENTATION

Original Contribution

José Manuel Martínez Moreno *(Martínez-Moreno JM[1]) PhD. e-mail: jmmartinezm@uma.es*

Alexander Reyes-Ortiz (Reyes-Ortiz A[2]) PhD. e-mail: alexxanderhola@yahoo.com.mx>

José María Lage Sánchez (Lage-Sánchez JM[3]) MD. e-mail: jmlagesanchez@gmail.com

Pilar Sánchez-Gallegos (Sánchez-Gallegos P[1]) Prof. e-mail: pilars@uma.es

**Manuel Garcia-Caballero (Garcia-Caballero M[1]) Prof. (*Deceased)*

1. DEPARTMENT OF SURGICAL SPECIALITIES, BIOCHEMISTRY AND IMMUNOLOGY. FACULTY OF MEDICINE UNIVERSITY OF MÁLAGA, (SPAIN).
2. AUTONOMOUS UNIVERSITY OF THE STATE OF MÉXICO (UAEMex).
3. "VIRGEN DE LA VICTORIA" UNIVERSITY HOSPITAL.

This work was performed in Department of Surgical Specialities, Biochemistry and Immunology. Faculty of Medicine University of Málaga, (Spain)

Correspondence: Dr. José Manuel Martínez Moreno. Department of Surgical Specialities, Biochemistry and Immunology. Faculty of Medicine, University of Málaga, 29071-Málaga. Fax: 952-132678 E-mail: *jmmartinezm@uma.es*

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In memory of Prof. Manuel Garcia-Caballero

ABSTRACT

Background & Aims: The objective of the present work is to study the intestinal adaptation in in three portions of the small intestine (biliary limb, alimentary limb and common channel) that are submit to different stimuli (physical forces and nutrients caused by the transit of the bolus, just bile acid effects and mix of both) after malabsorptive bariatric procedure. And the effect of a glutamine supplementation diet in these changes.

Methods: We used tree different types of surgery, biliopancreatic by-pass like model of the study, small bowel resection (75%) like positive control of intestinal adaptation and transection like negative control. We measured height and width of intestinal villi, HDC activity and mRNA quantity in animals with standard diet and supplemented with

Results: We measured an increase in HDC activity and mRNA agrees with increasing in the length and width of the villi. According to our data, the increase in length of intestinal villi starts immediately after surgical invasiveness, their maximum values are obtained 2 weeks later and remains elevated above the transection measured values for the remaining 4 weeks.

Conclusions: The intensity of intestinal adaptation process in BPBP animals is lower than in the resection. Adaptation occurred maximally in intestinal segments stimulated by nutrients. Partial adaptation in the biliary limb may reflect the effects of systemic hormones. Glutamine supplementation does not improve any of the parameters studied, but accelerates the process of adaptation to the level of intestinal villi.

1. INTRODUCTION

Some of the surgical techniques used today for weight loss and control of diabetes mellitus are based on the alteration of gastrointestinal transit. The malabsorptive component of surgeries like biliopancreatic diversion (BPD) with or without duodenal switch (+SD/-SD), Roux-in-Y gastric bypass (RYBP) and one anastomosis gastric by-pass (OAGB), is obtained bypassing some of the absorptive capacity of the intestine. This intestinal rearrangement causes an adaptive response that should be different in the 3 new intestinal segments (biliary limb, food handle and common channel), as each one is exposed to different stimuli: jejunal alimentary loop is exposed to undigested food, biliary portion of jejunum to bile and to the pancreatic secretions, without nutrients, while the ileal common channel is exposed to both, nutrients and biliopancreatic secretion [1-3]. If these stimuli are studied separately and chronology of intestinal adaptation is established in each of the intestinal segments, new data can be obtained helping us to understand this process and improve the development of therapeutic agents or diets primarily aimed at reducing the recovery time of patients with short bowel problems [4].

The aim of this work is to study the intestinal adaptation, and their chronology, in the three portions of the small intestine after malabsorptive procedure. And the effect of a glutamine supplementation diet in these changes. We use biliopancreatic by-pass (BPBP) procedure as a model because it offered us all of these situations. Additionally, we also wanted to study whether it is possible to measure the cellular proliferation through the change of the levels of histidine decarboxylase enzyme (HDC) respect to the physiologic status of the gastrointestinal tract.

2. MATERIAL AND METHODS

Experimental design.

Domestic pigs were used from 5 to 8 weeks and between 15-20 Kg of weight. 5 animals were used by treatment group and measure point (n=30). The lodging conditions and the care of the animals were carried out following the effective legal guidelines (European Agreement on Protection of the vertebrate animals used with experimental ends and other scientific ends (DOCE L 222; 24-08-1999).

The animals were distributed according to the diet type (Table 1).

Operative technique.

***-Bilipancreatic by-pass*:** A jejunum segment was taken (distant 150 cm of the ileocecal valve and an ileum segment (distant 50 cm of the ileocecal valve). Next the bilio-digestive derivation was carried out. For it was divided it the intestine portion that is after the proximals 150 cm to the ileocecal valve in two parts of same longitude. One will derive the biliopancreatic secretion and the other one the nutritious bolus (luminal nutrients) until the handle common to level of the ileum.

The intestinal continuity was reestablished by means of anastomosis in a single layer with suture end to end resorbable at level of the ileum and end-lateral in the gastro-jejunal and jejunum-ileal unions.

We used a massive 75% resection of the small bowel and transection as positive and negative control of intestinal adaptation respectively

Intestinal cells isolation from ileum and jejunum handles.

Ileal intestinal cells were isolated from a piece of small bowel taken to 5m from the ileocecal valve. Jejunum intestinal cells were isolated from a piece of small bowel obtained to 5m of the first sample. Both fragments with 10 to 15cm length. Tissue removals were made in agreement with relevant local animal welfare laws, guidelines and policies. After 3 washes in 37°C divalent ion free PBS (PBS: 13.7 mM NaCl, 0.27 mM KCl, 0.43 mM Na2HPO4, 0.14 mM KH2PO4, pH 7.4), pieces were washed in warm PBS supplemented with 1% Antibiotic-Antimycotic solution (Gibco/BRL), 2.7 mg/ml DGlucose (Sigma), and 4 mM L-Glutamine (Gibco/BRL). Intestinal fragments were then immersed in the same warm supplemented PBS and transported within 20 minutes to the laboratory. All following steps were performed under a laminar flux hood. In this point, ileum and jejunum pieces were filled with PBS containing 1 mM 1.4-dithiothreitol and, after closing their extremities, incubated for 5 minutes in a shaking bath at 37°C, to get the epithelium surface rid of mucous before performing the cell isolation procedure. Then this incubation medium was replaced by the digesting solution consisting in supplemented PBS added of collagenase and dispase, for 15 minutes at 37°C in a shaking bath. Using again this medium, a second digestion step lasting 45 minutes was carried out in the same conditions. Then, each intestinal segment was longitudinally wide opened and the pre-digested epithelium was scraped from the digestive mucosa using a sterile scalpel blade. The resulting material was incubated in PBS containing 1 mg/ml dispase for 10 minutes whilst active pipetting movements were done to help the dissociation of epithelium fragments. Then the cells were centrifuged at 1500 rpm for 5 minutes. The cellular precipitate was washed three times with 50 ml of SPB. Finally, cells were pelleted by centrifugation at 1500rpm for 5 minutes, the SPB was retired and the pellet were frozen as a dry precipitate at -80ºC. For cell count was used trypan blue.

HDC activity.

Measure of HDC activity was carried out by adapting the technique of CO^2 trapping used to measure ODC activity [5]. To adapt the method we use HDC buffer measure (dithiothreitol 0.2 mM, pyridoxal phosphate 10 μM, polyethylene glycol-300 10mg/ml, potassium phosphate 100mM, pH 6.8) and L-[1-14C] histidine with an specific activity of 11.2 GB/mmol.

Additive RT-PCR

For the isolation of total RNA, it was employee the method described by Chomczynski and Sacchi, (1987) [6].

The primers used in the RT-PCR corresponds to human HDC.

-Human HDC-PR5: 5´-AAT CTT CAA gCA CAT gTC-3´. (1219-1236). -Human HDC-PR6: 5´-CTg gAT AgT ggC Cgg gAT gA-3´. (1406-1425).

* These primers were given by the Prof. ANDRAS FALUS from the Sommelweis University of Budapest (Hungary). They have been used in several studies published in: Invest Dermatol, 115, 345-352, 2000; Immunol Lett, 76, 175-182, 2001; Infl Head, 50,428-434, 2001.

We used the protocol described for the product Titan One Tube RT-PCR System from Roche.

The quantification of a determined mRNA by means of RT-PCR is based on the lineal form of the exponential growth obtained after the amplification of different concentrations of oneself sample. We have used a method described for Reyes-Engel *et al* [7].

Morphometry of the intestinal villi.

Jejunum and ileum samples of a one centimeter were fixed by direct immersion of the tissue in Bouin. The fixation with Bouin lasted from 24 to 48 hours to ambient temperature.

After the fixation, the pieces were included in paraffin, Histosec (Merk) was used.

The paraffin was removed with dimethyl benzene during 10 minutes, and the samples were dehydrated by means of successive passes in alcohol. Next, the samples were tinted with hematoxylin of Harris during 3 minutes.

When the samples were tinted, a new dehydration was carried out through a several alcohol bath. Before installing them with Eukitt, the samples were clarified through two baths in dimethylbenzene of 10 minutes each one.

50 measures of the height and width of the intestinal villi were carried out under light microscope by their half portion in each histological sample.

Statistical analysis.

We used descriptive statistics (mean and standard deviation). To test significant differences of parameters between the different study groups, we used Student's t test for independent samples. The differences less than 5 % were considered significant. Analyses were performed using SPSS (version 21 for Windows, SPSS, Chicago, IL, USA) and Excel 2013.

RESULTS

- Morphometry of the ileum intestinal villi after surgery (Fig. 1):

• *Standard Diet without glutamine (Fig.1 (A and C)):*

- ✓ BPBP vs Transection (C-): Significant differences were observed in villi height (430.05±85.27 µm (BPBP) and 342.06±7.03 µm (C-)).
- ✓ BPBP vs Resection (C+): Results showed significant differences in the evolution of the width (136.94±4.33 µm (BPBP) and 149.19±11.44 µm (C+)).

• *Diet supplemented with 250 mg/kg/day of glutamine (Fig1 (B y D)):*

- ✓ BPBP vs Transection (C-): When the diet was supplemented with glutamine, both the height (460.54±77.36 µm (BPBP) and 348.38±12.40 µm (C-)) and width (139.53±6.10 µm (BPBP) and 133,66±2.36 µm (C-)) showed significant differences.
- ✓ BPBP vs Resection (C+): In this case, differences in the villi width were statistically significant (139.53±6.10 µm (BPBP) and 149.36±11.39 µm (C+)).

• *Standard Diet without glutamine vs Diet supplemented with 250 mg/kg/day of glutamine (Fig1 (A vs. B) and (C vs. D)):*

No statistically significant differences were found in any of the studied parameters.

- Morphometry of the jejunum (LN) intestinal villi after surgery (Fig2):

• *Standard Diet without glutamine (Fig.2 (A and C)):*

- ✓ BPBP vs Transection (C-): Differences in the height of the villi among the animals of the study group (BPBP) and negative control animals (transection) were statistically significant (454.96±92.75 µm (BPBP) and 351.80±15.44 µm (C-)).
- ✓ BPBP vs Resection (C+): In this case, the differences found in both groups between the height and width of the villi were not statistically significant.

• *Diet supplemented with 250 mg/kg/day of glutamine (Fig2 (B y D)):*

- ✓ BPBP vs Transection (C-): In animals receiving supplementation of 250 mg / kg / day of glutamine in the diet, significant differences were observed in the height (471.75±87.78 µm (BPBP) and 351.18±16.41 µm (C-)) and in the width (142.18±11.10 µm (BPBP) and 133,34±5.80 µm (C-)).
- ✓ BPBP vs Resection (C+): When we compared the study group with the positive control, the differences found in both groups between the height and width of the villi were not statistically significant.

• *Standard Diet without glutamine vs Diet supplemented with 250 mg/kg/day of glutamine (Fig2 (A vs B) y (C vs D):*

No statistically significant differences were found in any of the studied parameters.

- Differences in histidine decarboxylase (HDC) activity and quantity of mRNA in intestinal cells from ileal villi after surgery (Fig.3):

• *Standard Diet without glutamine (Fig3 (A y C)):*

✓ BPBP vs Transection (C-): The observed differences in the values of HDC activity between animals of both groups were statistically significant (3.07±0,91 *10^{-4}nmol CO_2/h/10^6 cells* (BPBP) and 2.38±0.30 *10^{-4}nmol CO_2/h/10^6 cells* (C-)). Also the differences in the amount of mRNA were statistically significant (854.75±133.30 *Pixels/inch* (BPBP) and 712.75±41.04 *Pixels/inch* (C-)).

✓ BPBP vs Resection (C+): In this case, no statistically significant differences were observed.

• *Diet supplemented with 250 mg/kg/day of glutamine (Fig3 (B y D)):*

✓ BPBP vs Transection (C-): The results obtained when the animal diet was supplemented with glutamine were similar to those obtained in the group without supplementation. There were significant differences in both HDC activity (3.27±0.91 *10^{-4}nmol CO_2/h/10^6 cells* (BPBP) and 2.28±0.35 *10^{-4}nmol CO_2/h/10^6 cells* (C-)) and the amount of mRNA (806.50±134.10 *Pixels/inch* (BPBP) and 721.25±42.04 *Pixels/inch* (C-)).

✓ BPBP vs Resection (C+): As in the unsupplemented group, no significant differences were found.

• *Standard Diet without glutamine vs Diet supplemented with 250 mg/kg/day of glutamine (Fig3 (A vs B) y (C vs D):*

No statistically significant differences were found in any of the studied parameters**.**

- Differences in histidine decarboxylase (HDC) activity and quantity of mRNA in intestinal cells from jejunum (LN) after surgery (Fig.4):

• *Standard Diet without glutamine (Fig4 (A y C)):*

✓ BPBP vs Transection (C-): As in the ileal limb, statistically significant differences were observed in HDC activity (3.53±1.27 *10^{-4}nmol CO_2/h/10^6 cells* (BPBP) and 2.47±0.34 *10^{-4}nmol CO_2/h/10^6 cells* (C-)). Statistically significant differences were found when comparing the amount of mRNA in both groups (846.83±142.88 *Pixels/inch* (BPBP) and 712.58±46.92 *Pixels/inch* (C-)).

✓ BPBP vs Resection (C+): In this case, no significant differences were observed.

• *Diet supplemented with 250 mg/kg/day of glutamine (Fig4 (B y D)):*

✓ BPBP vs Transection (C-): The results obtained when the animal diet was supplemented with glutamine were similar to those obtained in the group without supplementation. There were significant differences in both HDC activity (3.35±1.06 *10^{-4}nmol CO_2/h/10^6 cells* (BPBP) and 2.42±0.35 *10^{-4}nmol CO_2/h/10^6 cells* (C-)) and the amount of mRNA (826.50±128.54 *Pixels/inch* (BPBP) and 729.92±63.61 *Pixels/inch* (C-)).

✓ BPBP vs Resection (C+): As in the without supplementation group, no significant differences were found.

• *Standard Diet without glutamine vs Diet supplemented with 250 mg/kg/day of glutamine (Fig4 (A vs B) y (C vs D):*

No statistically significant differences were found in any of the studied parameters**.**

- Effect of BPS, luminal nutrients and glutamine on the amount of mRNA, HDC activity, height and width of the villi after BPBP (Fig 5)

• *Standard diet without glutamine (Fig 5):*

The intestinal portion stimulated only by the BPS, obtained the lowest values in all the parameters studied (amount of mRNA (698.75±37.89 *Pixels/inch*) activity HDC (2.33±0.24 10^{-4}*nmol* $CO_2/h/10^6$ *cells*), height (342.65±12.17µm) and width (132.77±5.40µm)) of the villi. The differences between this intestinal portion (jejunum BPS) and those that receive the trophic stimulus of nutrients (ileum and jejunum LN) were statistically significant for the amount of mRNA (ileum (854.75±133.3 *Pixels/inch*) and jejunum LN (846.83±142.88 *Pixels/inch*)), HDC activity (ileum (3.07±0.91 *10-4nmol CO2/h/106 cells*) and jejunum LN (3.53±1.27 10^{-4}*nmol* $CO_2/h/10^6$ *cells*)) and height (ileum (430.50±4.33µm) and jejunum LN (454.96±92.75µm)) of intestinal villi. However, villi width values were very similar in all intestinal limbs (jejunum BPS (132.77±5.40µm), ileum (136.94±85,27µm) and jejunum LN (140.94±10.74µm)) and no significant differences were found in this case.

• *Diet supplemented with 250 mg / kg / day glutamine (Fig 5):*

As in the diet without glutamine, intestinal portion without trophic stimulus (jejunum SBP) showed the lowest values in amount of mRNA (688.92±24.47 *Pixels/inch*) activity HDC (2.10±0.12 10^{-4}*nmol* $CO_2/h/10^6$ *cells*), height (335.31±11.12µm) and width (132.63±5.33µm) of the villi. The differences between these values and those obtained in the two intestinal portions that were stimulated by the transit of nutrients, ileum (amount of mRNA (806.50±134.10 *Pixels/inch*) activity HDC (3.27±0.91$10^{-4}$*nmol* $CO_2/h/10^6$ *cells*) and height (460.54±77.36µm)) and jejunum LN (amount of mRNA (826.50±128.54 *Pixels/inch*) activity HDC (3.35±1.06 10^{-4}*nmol* $CO_2/h/10^6$ *cells*) and height (471.75±87.78µm) were statistically significant.

Furthermore, in this case the differences in the width of the villi were not statistically as in the standard diet group.

• *Standard Diet without glutamine vs Diet supplemented with 250 mg/kg/day of glutamine (Fig5):*

No statistically significant differences were found in any of the studied parameters

DISCUSSION

The first step of this work was to verify that really a surgical procedure such as biliopancreatic bypass provoked a process of intestinal adaptation in the three portions of intestine studied (biliary limb, alimentary limb and common channel) [8-10]**.** For this purpose, the values of height and width of the villi were measured and were compared with animals that had been subject only to sample collection process (transection/negative control). In all studied cases, significant differences in villi height and width were measured, except the portion that is stimulated only by biliopancreatic secretion (fig. 1 y 2). In this case the villus height does not increase, however, the width, remained at the same level as it had in the other two intestinal portions (fig 5). These data correspond to those described by Li et al in Zucker rats [11] To know the intensity of this process in surgeries that cause malabsorption, we compare the results with those obtained in pigs subjected to massive intestinal resection of 75%, used in many studies like example of short bowel syndrome [12, 13]. The villi height and, width values measured in animals subject to BPBP were not as elevated as those measured in resection, in any of the cases studied (Fig. 1 and 2). It seems that the malabsorption provoked by biliopancreatic bypass causes an intestinal

adaptation process with a lower intensity to that caused by a massive intestinal resection of 75%.

In 1996 our group related cell proliferation in the intestinal villi crypts with HDC activity increases after massive intestinal resection in dogs [14]. We wanted to check again the role of this enzyme, directly related to cell proliferation [15, 16] in the process of intestinal adaptation. In addition, data have been implemented with the study of its mRNA. In all cases studied, we can measure an increase in HDC activity which was corresponded with an increase in the amount of its mRNA. According Kazuma Fujimoto work the HDC role not be only related to cell proliferation, also intervene in intestinal mucosa repair by its receptor H1 [17]. Although there are no significant differences between BPBP and resection, it seems that the intensity of the process (as happened with the length of the villi) is lower in the BPBP (fig. 4 y 5). This can be reflected especially in HDC activity values, because the amount of mRNA values is practically the same in the two surgeries.

Regarding the chronology of the events, based on the morphology of the villi we can say that the process of intestinal adaptation is very similar in the BPBP and resection. In both cases it begins rapidly after surgical aggression and held until 6 weeks later. Getting the maximum at 2 weeks after started (fig. 2 y 3). The process of cell proliferation, measured by HDC activity levels and expression of its mRNA, appears to have a different timing. Both the mRNA and the protein activity reached their peak during the first 10 days, however, after this time mRNA levels begin to drop sharply over the next 32 days, while the HDC activity remained high values until the end of the study (fig 4 y 5). This behaviour could be the result of post-translational regulation [18]. Although this was not the aim of this study, additional work that would attempt to identify and measure the amount of inactive proenzyme could clarify this point.

We know that glutamine is one of the main sources of energy for enterocytes, [19] a fact that stoked the interest in exploring the role of glutamine in the intestinal adaptation. However, when the animal diet was supplemented with 250 mg/ kg/day of glutamine were not observed significant changes in any of the parameters studied compared to animals receiving standard diet, these data are consistent with those obtained by Yang Hua in rats [20]. However, we note something interesting. The villi of animals which received glutamine in the diet reached their maximum length and width before the animals with a standard diet. The time required to reach the maximum width was reduced from 14 days to 10 days and in the case of the length moved from about 30 days to 11 days. We are aware that the clinical effect it could have on patients who undergo bariatric/metabolic surgery is very low. Most of these patients return home after a hospital stay of a few days with hardly any complications. However, if we clarify the role of glutamine as well as many other factors in the process of intestinal adaptation, perhaps in the future we are able to handle it by nutritional or pharmacological therapies.

CONFLICT OF INTEREST: *The authors declare that they have no conflict of interest***.**

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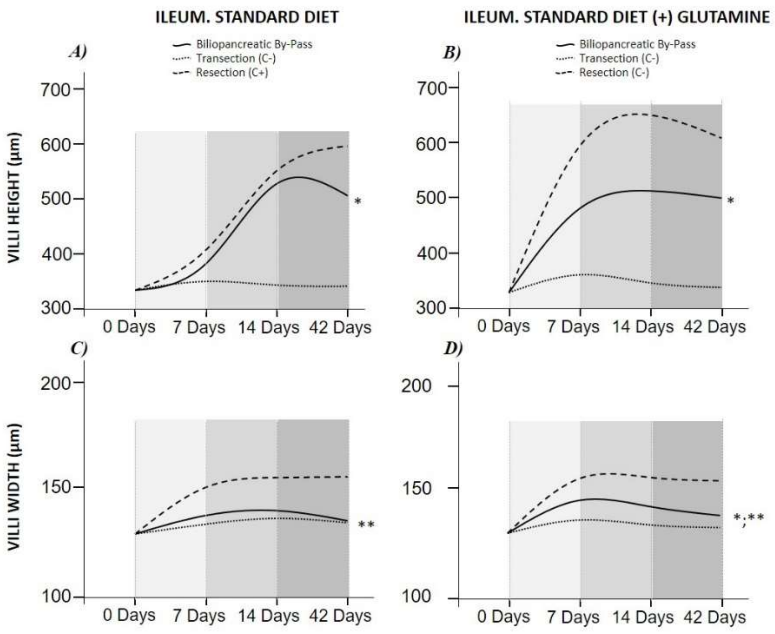
Table 1. Table of experimental design in which the number of used animals is represented in each group as well as the types of diets.

GROUP	BILIOPANCREATIC BY-PASS				TRANSECTION				RESECTION OF 75%			
DAYS / DIET	0	7	14	42	0	7	14	42	0	7	14	42
ORAL STANDARD DIET	5	5			5	5			5	5		
	5		5		5		5		5		5	
	5			5	5			5	5			5
ORAL STANDARD DIET WITH GLUTAMINE (250mg/kg/day)	5	5			5	5			5	5		
	5		5		5		5		5		5	
	5			5	5			5	5			5

Oral standard diet: 18% proteins, 69% hydrates of carbon, 4% fatty and 4% fiber by oral way.

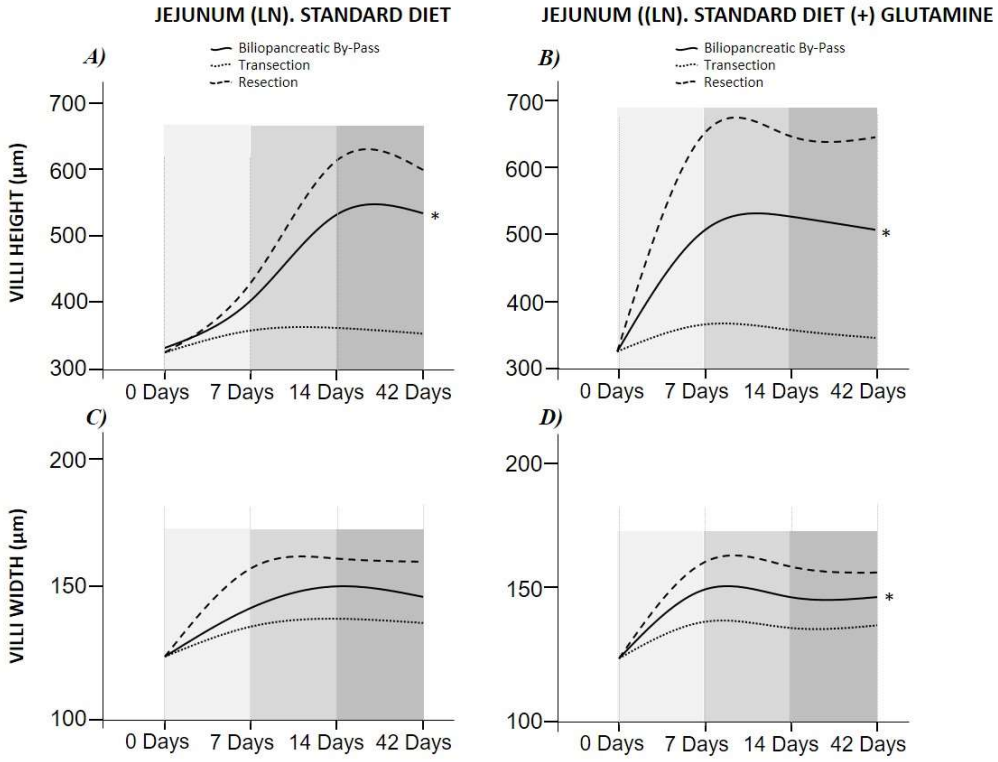
Oral standard diet with glutamine (250mg/kg/day): 18% proteins, 69% hydrates of carbon, 4% fatty, 4% fiber and 250 mg of glutamine by Kg and day by oral way. Both diets contribute the same number of calories.

Fig.1: Evolution of the ileum intestinal villi in pigs undergoing to biliopancreatic bypass, intestinal transection (C-) and intestinal resection of 75% (C +)



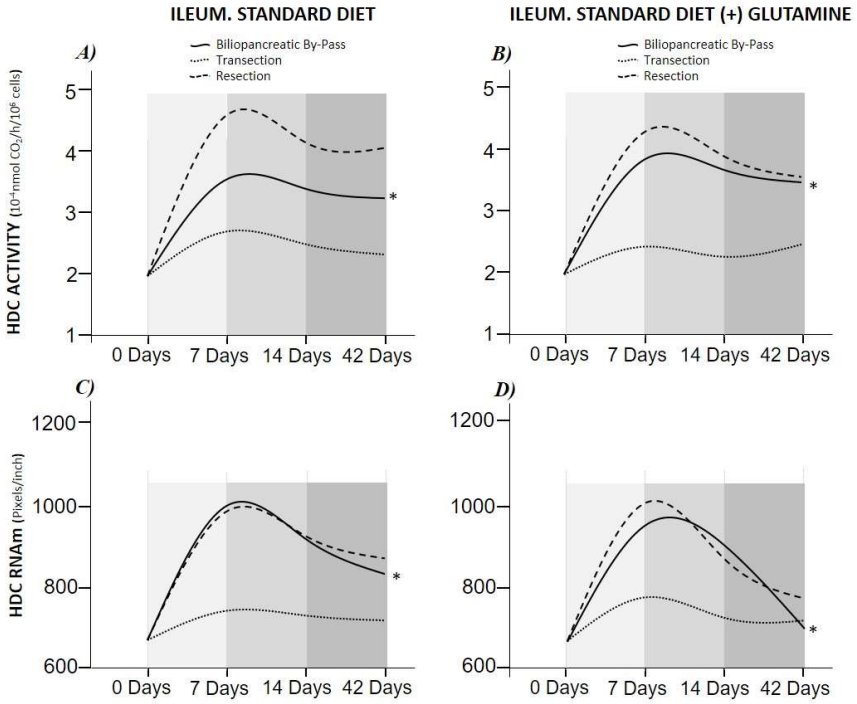
The graphics represent mean values of height and width villi. (C-)=negative control. (C+)=positive control. Student's T test for independent samples was realized to statistical analysis.
**Statistically significant (p<0.05) regarding negative control.*
***Statistically significant (p<0.05) regarding positive control*

Fig.2: Evolution of the jejunum (LN) intestinal villi in pigs undergoing to biliopancreatic bypass, intestinal transection (C-) and intestinal resection of 75% (C +)



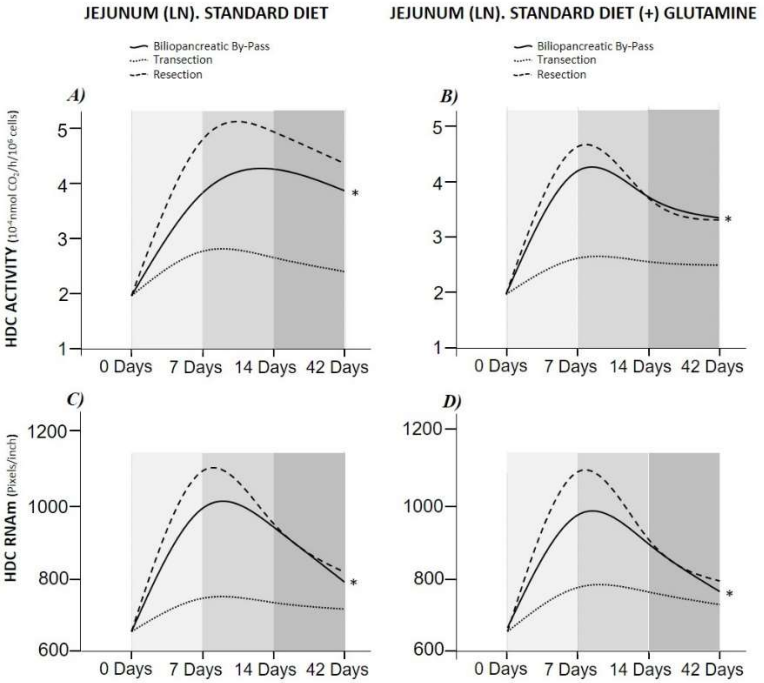
The graphics represent mean values of height and width villi. (C-)=negative control. (C+)=positive control. (LN)=luminal nutrients. Student's T test for independent samples was realized to statistical analysis.
**Statistically significant (p<0.05) regarding negative control.*
***Statistically significant (p<0.05) regarding positive control*

Fig.3: Evolution of the ileum RNAm quantity and HDC activity in pigs undergoing to biliopancreatic bypass, intestinal transection (C-) and intestinal resection of 75% (C +)



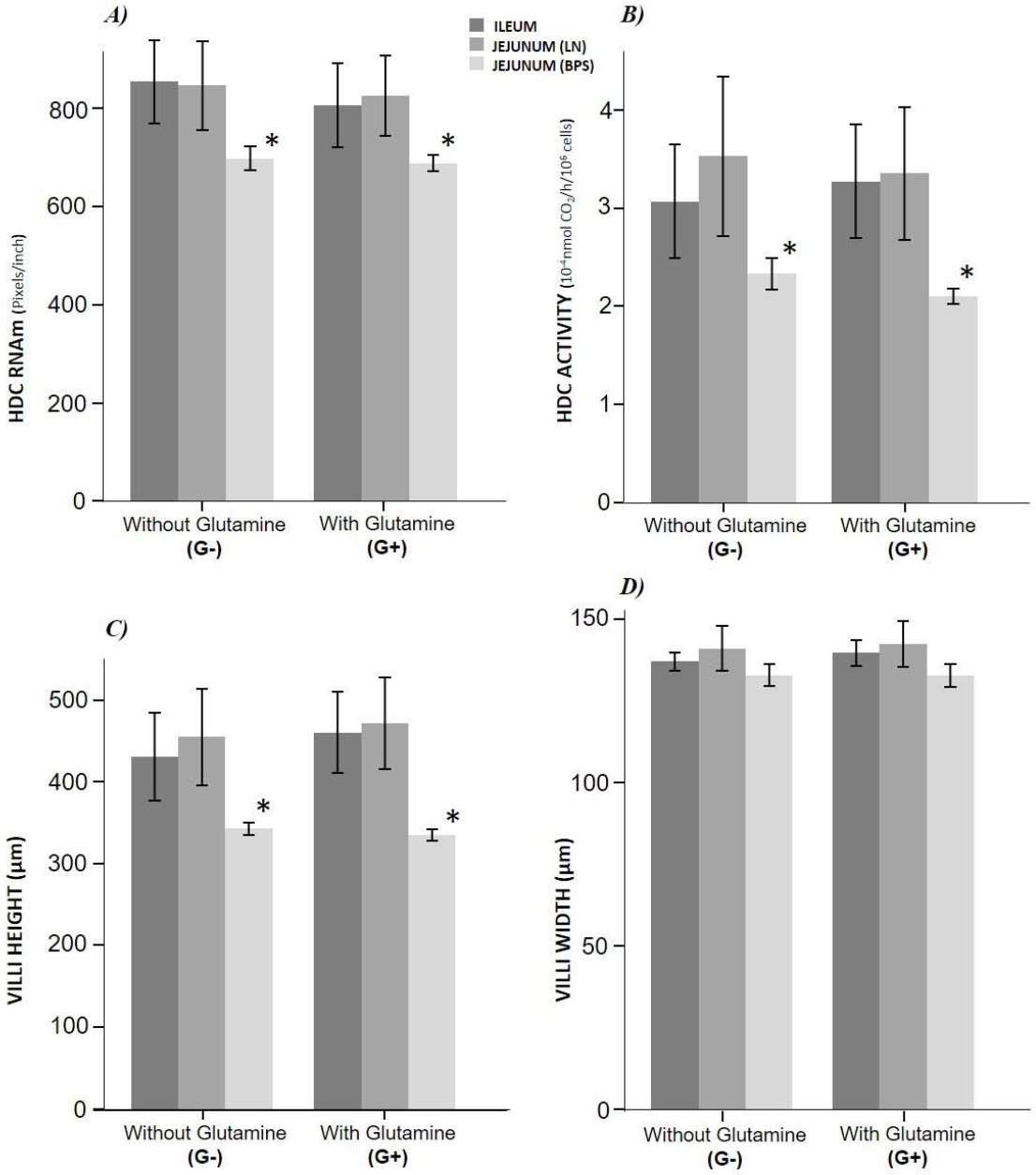
The graphics represent mean values of amount of messenger RNA and histidine decarboxylase activity. (C-)=negative control. (C+)=positive control. Student's T test for independent samples was realized to statistical analysis.
**Statistically significant (p<0.05) regarding negative control.*
***Statistically significant (p<0.05) regarding positive control*

Fig.4: Evolution of the jejunum RNAm quantity and HDC activity in pigs undergoing to biliopancreatic bypass, intestinal transection (C-) and intestinal resection of 75% (C +)



*The graphics represent mean values of amount of messenger RNA and histidine decarboxylase activity. (LN)=luminal nutrients. (C-)=negative control. (C+)=positive control. Student's T test for independent samples was realized to statistical analysis. *Statistically significant (p<0.05) regarding negative control. **Statistically significant (p<0.05) regarding positive control*

Fig.5: Evolution of RNAm quantity, HDC activity, height and width of the villi in pigs undergoing to biliopancreatic bypass, according to intestinal portion. With or without glutamine



*The graphics represent mean values of amount of messenger RNA, histidine decarboxylase activity, height and width of the villi. (BPS)=Biliopancreatic Secretion, (LN)=luminal nutrients, (G+)=With Glutamine, (G-)=Without Glutamine. Student's T test for independent samples was realized to statistical analysis. *Statistically significant (p<0.05)*