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(FEDERAL UNIVERSITY OF SÃO PAULO)  
ESCOLA PAULISTA DE MEDICINA  
(SCHOOL OF MEDICINE)  
UNIFESP – EPM



PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA TRANSLACIONAL  
(POST-GRADUATION PROGRAM IN TRANSLATIONAL MEDICINE)

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Ph.D. Research Project

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*Isolated ileal interposition  
in enteroendocrine L cells differentiation.*

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SÃO PAULO, BRAZIL - 2011

## ABSTRACT

**INTRODUCTION:** Due to the progressive nature of type 2 diabetes, its complexity and drug treatment perpetuity, there is currently a search for surgical procedures that can promote euglycemia also in non-obese patients. Diabetic patients glycemic control can be achieved by increasing the blood concentration of GLP-1, a hormone produced by L cells that are more densely concentrated in the terminal ileum. Early and extended improvement of diabetes in patients submitted to bariatric surgeries awakened the necessity of investigating the isolated ileal interposition as surgical alternative for the treatment of diabetes. The interposition of this ileal segment to a more anterior region (proximal jejunum) can promote a greater stimulation of the L cells by poorly digested food, increasing the production of GLP-1 and reflecting on glycemic control. However, in order to consolidate the ileal interposition as a surgical treatment of diabetes it is necessary that the interposed ileum keep the same differentiation rate into L cells for a long period to justify the intervention.

**AIMS:** To investigate the isolated ileal interposition influence on the differentiation of intestinal precursor cells into enteroendocrine L cells over time.

**METHODS:** Twelve 12-week-old male Wistar rats (*Rattus norvegicus albinus*) of the WAB strain (heterogeneous) will be used. All animals will receive a high-calorie, high-fat diet for 16 weeks or more until they develop glucose dysmetabolism confirmed by glycemic test. They will be divided into two groups of 10 animals each: the isolated ileal interposition group (GI) and the control group (GC), comprising animals that will not be submitted to any surgical intervention. Blood samples will be collected under anesthesia at the weeks 12, 26, 36 and 44 for the determination of serum levels of glucose, insulin, GLP-1, glucagon, C-peptide and glycosilated hemoglobin. The insulin tolerance test will be performed and insulin resistance will be calculated. For the comparative analysis of the ileal precursor cells differentiation into enteroendocrine cells among the two groups, the following intestinal fragments will be collected after euthanasia: interposed ileum and remaining ileum from GI, jejunum and ileum from GC. These fragments will be analyzed by imunofluorescence and also by Real Time PCR using PCR Arrays for target genes including the main ones related to stem cell, stem cell signalling, diabetes, Wnt and Notch signaling pathways and other genes and pathways involved in the differentiation of intestinal precursor cells into enteroendocrine cells, especially GLP-1-producing L cells that play important role in euglycemia.

## 1. INTRODUCTION

Diabetes is one of the most common chronic diseases and a growing global epidemic (Danaei *et al.*, 2011).<sup>1</sup> Type 2 diabetes (T2D) is a serious metabolic disease characterised by high glucose levels. Complications from T2D usually result in poor quality of life, disability, and early death.<sup>2</sup> Despite efforts to control glycaemia in diabetes patients, no therapeutic approach has significantly impacted the progression of this disease (Tahrani *et al.*, 2010).<sup>3</sup> There is, however, a simple and reversible surgical procedure that might become a valid therapeutic alternative in non-obese patients. This procedure, isolated ileal interposition (III), results in a dramatic increase in the incretin glucagon-like peptide 1 (GLP-1).

### Epidemiology and aetiopathogenesis of diabetes

In 2000, diabetes caused the death of about three million people.<sup>4</sup> In 2010, the number of affected individuals was approximately 285 million, and this number is projected to increase 54% by 2030. A much greater increase in the number of adults with diabetes is projected in underdeveloped countries than in developed countries: the number of affected individuals is projected to increase approximately 70% in underdeveloped countries and 20% in developed countries.<sup>1</sup> T2D, also known as non-insulin-dependent diabetes, is responsible for 90% to 95% of diabetes cases.<sup>5</sup>

T2D is a progressive heterogeneous disease that mainly involves peripheral insulin resistance and gradual dysfunction of pancreatic beta cells.<sup>2,6</sup> It develops when pancreatic islets can no longer maintain insulinaemia at levels sufficient to overcome peripheral tissue resistance. Throughout the course of the disease, diabetes passes through intermediate stages. Initially, diabetes is treated only with modifications in lifestyle and specific diet. Later in the disease, patients are treated with drugs that promote insulin secretion. As insulin deficiency unavoidably increases, however, the combination of therapeutic oral agents often fails to control hyperglycaemia efficiently, and the use of insulin is eventually required.<sup>6-9</sup>

In the long run, patients with T2D have an increased risk of complications that are a substantial cause of morbidity and mortality, including macrovascular disease, nephropathy, retinopathy, and neuropathy.<sup>10</sup> Further complications of diabetes include ischemia of the limbs resulting in amputation, dental problems, and pregnancy disorders.<sup>5</sup> The risk of developing diabetes-associated complications is related to the duration of diabetes and the level of glycaemic control.<sup>11</sup>

Although 90% of T2D cases can be attributed to excessive weight,<sup>12</sup> its incidence is growing among individuals with body mass index (BMI) in the normal and overweight ranges.<sup>13,14</sup>

### **Treatment of diabetes**

T2D is a multifaceted condition requiring an integrated and individualised approach to each patient's care that can prove quite challenging (Freeman, 2010).<sup>15</sup> Treatment must initially be based on substantial lifestyle changes, including diet adjustment and regular physical activity. In most patients, these behavioural measures eventually do not suffice to keep glycaemia within appropriate levels, and oral antidiabetic agents are added.<sup>17</sup> Currently, there are several drug classes with different mechanisms of action that might be used singly or in various therapeutic combinations.<sup>16</sup> Many patients eventually require combinations of two or more oral drugs.<sup>17-19</sup> Despite these treatments, glycaemia increases over time as the disease progresses, requiring the use of insulin in combination with oral agents and eventual full insulinisation. Although these interventions might decrease peripheral blood glycaemia, none of these actions is effective in stopping disease progression.<sup>9</sup> Intensification of insulin therapy is the most appropriate intervention used to attempt to achieve normoglycaemia and reduce complications through early, strict, persistent, and effective control of glycaemia.<sup>16,20,21</sup> Thus, insulin is currently the only effective conservative therapeutic option for achieving metabolic control.<sup>6,7,22</sup>

However, the use of external insulin is not easy to manage. Attaining optimal glycaemic levels, i.e., glycaemia levels as close as possible to those of a non-diabetic individual<sup>23</sup> in order to prevent complications, still poses a major challenge in clinical practice.<sup>2,21</sup> Due to the limitations of most of the available therapeutic measures, only a small fraction of the diabetic population meets therapeutic goals.<sup>23</sup> These limitations include poor compliance with diets, resistance to physical exercise programmes, limited efficacy and significant side effects of current therapeutic agents, delays in insulin therapy onset, and patient aversion to the multiple parenteral injection regimes required for the administration of insulin.<sup>21-24</sup> Successful insulin therapy requires accurate information, motivation, high socioeconomic and cultural levels, high adherence and learning ability, availability of resources, and participation of and support by a multiprofessional team.

Some antidiabetic drugs accelerate beta cell apoptosis,<sup>2</sup> whereas others reduce bone mineral density and promote weight gain due to volume expansion and oedema, potentially causing or exacerbating heart failure and triggering ischemic cardiac events.<sup>17,18</sup> Some insulin

analogues are associated with a more physiological pattern of recovery; these drugs are more flexible to use and convenient to prescribe, and they allow greater freedom in diet while still providing improved quality of life.<sup>6,7,25</sup> Formulations eliminating the need for subcutaneous injections might correct some limitations of typical insulin therapy, thus improving glycaemic control and increasing patient quality of life.<sup>21</sup> Some formulations that allow non-invasive administration of insulin are in the testing phase, including the inhalable insulin powders Exubera (Pfizer), Technosphere (MannKind), Aerdose (Aerogen), BAI (Kos), Alveair (Coremed), and Bio-Air (BioSante). Therapy selection must take into account tolerability, non-glycaemic effects of antidiabetic agents, effects on associated comorbidities, and cost (Stolar *et al.*, 2008).<sup>17</sup>

The limitations of conventional treatments that fail to preserve pancreatic beta cell function over time have resulted in a critical need to find new means to attain appropriate glycaemic control and avoid or delay the need for additional measures (Hansen *et al.*, 2010).<sup>2</sup> Thus, antidiabetic treatments seeking to preserve beta cell function and integrity and halt T2D progression are clearly needed.<sup>8</sup> More effective measures based on the disease aetiopathogenesis are necessary; these measures must control both fasting and postprandial glycaemia.<sup>22</sup>

A new approach to T2D treatment involves the use of therapies based on incretins such as dipeptidyl peptidase-4 (DPP-4) inhibitors and GLP-1 receptor agonists, which are GLP-1 analogues and bind to GLP-1 receptors on pancreatic beta cells to inactivate them.<sup>2,3</sup> Both groups of drugs have proven safe and effective in reducing glycaemia and have yielded favourable effects regarding weight, lipid profile, and blood pressure.<sup>9,17,26-28</sup> Both are associated with insulin release and glucose-dependant glucagon suppression with consequent low hypoglycaemia risk. Experimental studies showed that these therapies prolong the survival, delay the dysfunction, and promote the regeneration of pancreatic beta cells and thus theoretically hold the potential to halt T2D progression.<sup>3,9,28</sup>

Although these studies have demonstrated the therapeutic promise of DPP-4 and GLP-1 analogues, the high cost of these new agents and the lack of studies on their long-term safety must be considered. Nausea, headache, acute pancreatitis, upper airway infection, depression, severe hypoglycaemia, and skin allergic reactions have been reported with the use of these drugs. Some individuals also exhibit moderate reduction of glycated haemoglobin levels when compared to patients treated with insulin and older agents. Moreover, the

development of carcinomas has been associated with the use of these agents in guinea pigs; however, this finding has not been confirmed in humans.<sup>18,19,28</sup>

In addition to the wide variety of pharmacological options for multidisciplinary treatment aimed at weight loss and glycaemic control, bariatric and metabolic surgical techniques can be included among the therapeutic approaches to T2D and insulin resistance. These surgical interventions have been shown to provide long-term control of T2D.<sup>29,30</sup>

### **Surgical interventions**

Bariatric surgery can improve and eventually completely reverse obesity-associated comorbidities in 70% to 100% of patients,<sup>31</sup> thus increasing their life expectancy,<sup>32</sup> partially reversing hypothalamic dysfunction, and increasing the anti-inflammatory activity of the cerebrospinal fluid.<sup>33</sup>

Improved glycaemic control is observed months after adjustable gastric band surgery, and improvement is faster and more complete with ROUX-en-Y bypass. Both strategies can improve or even cure T2D, potentially through different mechanisms (Meijer *et al.*, 2011).<sup>34</sup> Vertical gastrectomy with or without contention ring and Roux-en-Y gastrojejunal bypass – thought to be the gold standard surgical intervention in the treatment of morbid obesity – are known to achieve the goals of weight loss and control of comorbidities and to maintain these goals over time.<sup>35</sup> This control of comorbidities is usually attributed to body mass reduction; however, a potentially glycaemia-controlling endocrine effect has been observed even before any significant weight loss.<sup>36</sup> After gastrojejunal bypass, levels of substances directly secreted by the bowel such as GLP-1 were found to be elevated in the peripheral blood; these substances can stimulate insulin production by pancreatic beta cells, facilitate insulin-mediated glucose transport into cells, and induce a feeling of satiety.<sup>37</sup>

Roux-en-Y gastrojejunal bypass favours the stimulation of GLP-1-producing cells by foods arriving at the distal portions of the small intestine incompletely digested, as food transit is diverted to the proximal jejunum.<sup>38</sup> Jejunal bypass and other highly effective bariatric and metabolic surgical interventions deliver nutrient-rich chyme to the distal bowel earlier than normal. Its arrival directly to the ileum activates a negative feedback mechanism known as the “ileal brake”,<sup>39</sup> which involves neuronal and endocrine mechanisms that influence stomach voiding, intestinal motility, and satiety.<sup>40</sup>

As early as 1998, it was already thought that T2D might be an anterior bowel disease.<sup>41</sup> Currently, several clinical, physiological, biological, anthropological, epidemiological, anatomical, and evolutionary lines of evidences together with surgical results have confirmed that the proximal small intestine – whose size was appropriate for our ancestral environment – might have become too large due to the modern industrialised diet. Consumers of a rich and modified modern diet developed a much larger anterior intestine (jejunum) than desired. A shorter jejunum prevent ingested food from being fully absorbed in the proximal portion of the intestine, allowing it to arrive at the distal intestine (ileum) almost *in natura* in order to stimulate L-type endocrine cells to produce substances such as GLP-1 that promote insulin production by the endocrine pancreas, facilitate glucidic metabolism in peripheral tissues, and induce satiety through selective hypothalamic inhibition.<sup>42</sup>

The genesis of disruptions in glucose metabolism involves a multifaceted range of intimately intertwined factors. Of these factors, hormones are the most significant; of particular interest is GLP-1, which is produced by enteroendocrine L-cells of the small intestine, which are more densely concentrated at the terminal ileum.<sup>32</sup> GLP-1 is produced by tissue-specific post-translational processing of its precursors, namely, the peptide proglucagon, by pro-hormone convertase enzymes.<sup>43</sup> Post-translational modifications of the glucagon gene give rise to five different products in the bowel: glycentin, oxyntomodulin (OXM), intervening peptide-1 (IP-1), GLP-1, and GLP-2.<sup>44</sup> Proglucagon is processed in bowel L-cells by PC1/3 pro-hormone convertase.<sup>43</sup> GLP-1 secretion occurs in response to stimuli generated by nutrients with an incretin effect.<sup>45</sup> Incretins are hormones secreted into the blood circulation by the gastrointestinal tract in response to intake of certain nutrients. This results in increased insulin production and consequent glucose uptake. GLP-1 has well-defined functions, such as stimulation of glucose-dependent insulin secretion, upregulation of insulin gene transcription, induction of Langerhans islet beta cell neogenesis and proliferation, inhibition of beta cell apoptosis, increased phenotypic differentiation of beta cells, stimulation of somatostatin production, and reduction of glucagon production.<sup>46,47</sup>

Advances in neurogastroenterology have provided a better understanding of gastrointestinal physiology. Therefore, bariatric surgery intervention modalities evolved into the current mixed procedures that take into account neurohormonal and metabolic factors in addition to restriction and dysabsorption features. Thus, the term *baroendocrine surgery* is increasingly used, especially in the treatment of T2D.<sup>38,40</sup> Currently, mixed (restrictive and dysabsorptive) bariatric surgery is the most efficacious treatment in patients with morbid

obesity, resulting in significant improvement of associated comorbidities (Melissas, 2008).<sup>49</sup> In studies of these interventions, enhanced GLP-1 release and improved glycaemic control have been observed even before significant weight loss, demonstrating that the control of diabetes might be related to hormonal effects secondary to the surgical technique performed.<sup>47,50</sup>

### Isolated ileal interposition

In the early 1980s, enhanced GLP-1 release had already been shown to suffice for body weight control in obese rats treated with the interposition of a 5- or 10-cm terminal ileum fragment.<sup>51</sup> The effects of this surgery are illustrated in Figure 1.

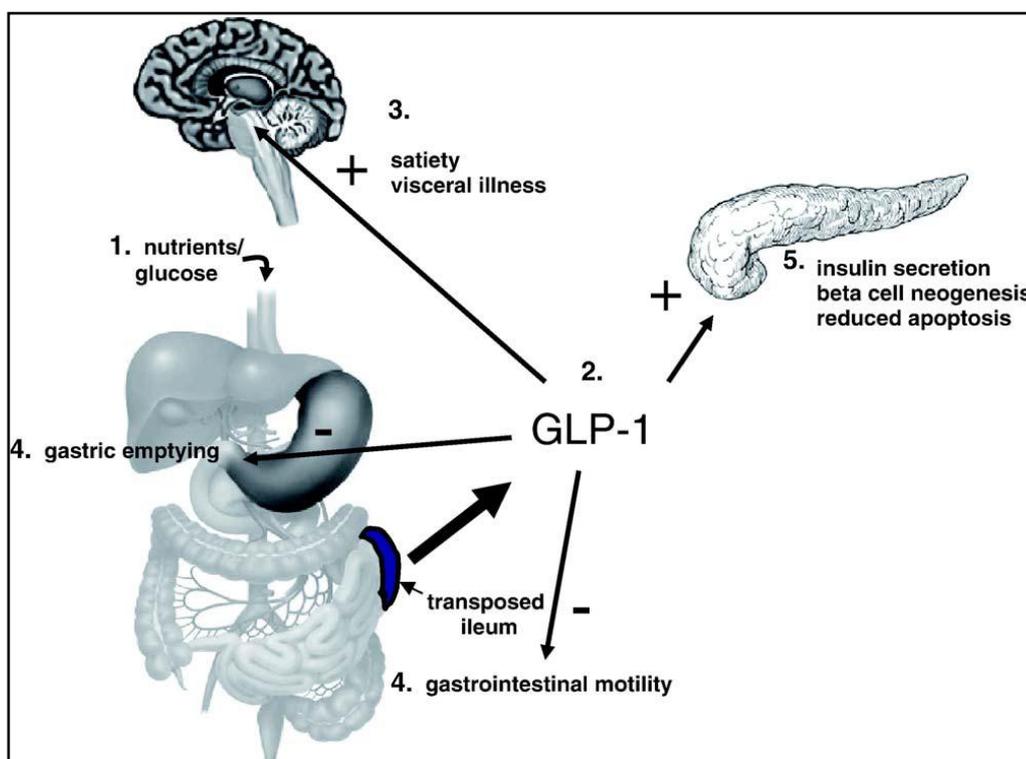


Figure 1. Effects of ileal interposition on glucose control. (1) Dietary nutrients enter the bowel lumen and stimulate neuroendocrine cells causing (2) early and long-lasting GLP-1 release, (4) which will affect gastrointestinal motility and stomach voiding. (5) GLP-1 is an incretin and will also mediate insulin secretion and endocrine pancreas protection.<sup>52</sup>

Another study in rats showed hypertrophy of transposed ileum together with increased serum GLP-1 levels.<sup>53</sup> In humans, GLP-1 increased after jejunoileal and biliopancreatic bypass in morbidly obese individuals.<sup>54</sup> Another study published in 1998 showed that patients exhibited high levels of GLP-1 even 20 years after jejunoileal bypass.<sup>55</sup>

Together with a study published in 1999, these findings suggested that ileal interposition might be used as a treatment for T2D.<sup>56</sup> Fast and permanent improvements in glucose control were observed in patients immediately after bariatric surgery, and surgery eliminated the need for glycaemia-controlling drugs in most cases.<sup>57,58</sup> The beneficial effects of bariatric surgery on glycaemic control also depend on the duration of disease<sup>59</sup> and the type of surgical intervention; interventions based solely on stomach restriction, such as adjustable gastric bands, proved to be less effective in improving T2D than procedures involving substantial amounts of intestinal bypass<sup>55,60,61</sup> or significant increases in digestive transit speed, such as vertical gastrectomy.<sup>62,63</sup>

Vertical gastrectomy is aimed mainly at achieving weight loss. It is a restrictive procedure due to the significant reduction in stomach reservoir capacity; moreover, it is considered a metabolic procedure, as it results in reduced circulating levels of the orexigenic hormone ghrelin, which is produced at the fundus and greater curvature of the stomach.<sup>62,63</sup> The good long-term postoperative<sup>64</sup> glycaemic control achieved by vertical gastrectomy<sup>63</sup> might be due to the arrival of incompletely digested food at the distal ileum as a result of increased voiding speed after surgery, which in turn results in increased GLP-1 secretion by L-cells.<sup>66</sup>

Ileal interposition has frequently been performed in obese and non-obese humans; however, it is never performed in isolation. Promising results were recently obtained in humans using techniques combining vertical gastrectomy with the interposition of a segment of distal ileum in the trajectory of the proximal jejunum.<sup>67-72</sup> This procedure can induce early satiety along with benefits to glucidic metabolism and cause short- and long-term weight loss. In non-obese diabetic patients, vertical gastrectomy combined with ileal interposition effectively controlled T2D.<sup>67-71</sup> Analysis of the effects of vertical gastrectomy combined with ileal interposition on humans 6 and 18 months after the procedure demonstrated T2D remission in 80% of patients, who were freed from treatment with hypoglycaemic agents or diet. The remaining 20% of patients showed significant improvement despite the need to continue oral treatment (Tinoco, 2011).<sup>72</sup>

Despite these findings, however, there is a conceptual problem in proposing to perform vertical gastrectomy in non-obese diabetic patients: the metabolic benefits reported in the literature<sup>67-72</sup> were probably due almost exclusively to ileal interposition rather than to the partial gastric restriction procedure (vertical gastrectomy).

The early postoperative improvement in glucose metabolism observed in obese and diabetic patients subjected to vertical gastrectomy alone<sup>63</sup> is most likely due to the increase in intestinal transit speed induced by intervention,<sup>66</sup> which favours quicker arrival of undigested food to the terminal ileum, where it stimulates L-cells to produce endogenous GLP-1. We conclude that in these non-obese patients with glucidic dysmetabolism, vertical gastrectomy improves glucose metabolism, but this effect is only due to the acceleration of gastrointestinal transit, which causes incompletely digested food to arrive at the terminal ileum, where it triggers GLP-1 production by L-cells. Other consequences of vertical gastrectomy, such as the restriction caused by making the gastric reservoir a small-calibre tube, and the anorexigenic effect caused by reducing levels of the orexigenic hormone ghrelin,<sup>63</sup> arise from the removal of the fundus and greater curvature of the stomach, where X/A-type neuroendocrine cells are more concentrated. However, vertical gastrectomy is a major surgical procedure that is not without significant complications, such as torpid evolution of fistulas and reflux esophagitis. There is no reason to apply this procedure to non-obese diabetic patients, in whom isolated ileal interposition may be highly effective. Ileal interposition in rats involves placing a 10- to 20-cm segment of distal ileum with its nerves and vessels intact into the proximal jejunum,<sup>73</sup> resulting in significant hyperplasia, hypertrophy, and even full “jejunisation” of the transposed ileum.<sup>74-78</sup>

Isolated ileal transposition proved efficacious in correcting dysmetabolism in several studies of experimental animals;<sup>51,79,80</sup> however, in the context of bariatric and metabolic surgery, this surgical modality involving only ileal interposition has not been assessed in humans.

Increased synthesis and release of GLP-1 can be attributed to increased stimulation of L-cells located in the interposed ileum segment in response to the presence of a greater amount of partially digested food, resulting in direct effects on glucidic metabolism (Patrity *et al.*, 2007).<sup>81</sup> Serum levels of GLP-1 increase in response to alimentary stimulation, resulting in a satiating effect on the central nervous system,<sup>82</sup> reduced fat absorption by the gastrointestinal tract,<sup>83</sup> and reduced gastric<sup>84</sup> and intestinal motility.<sup>85</sup> The most remarkable effects of GLP-1 are reduced peripheral insulin resistance, decreased apoptosis of pancreatic beta cells, increased differentiation of primitive pancreatic canaliculus cells into adult beta cells, and increased proliferation of beta cells.<sup>86,87</sup>

GLP-1 is the incretin hormone most associated with the antidiabetic effects of bariatric surgery. Stimulation of ileal L-cells to cleave proglucagon and release GLP-1 seems to be the

most effective means of inducing the incretin effect in diabetic patients subjected to bariatric surgery. Several techniques might be applied to achieve this effect. All of these techniques are derived from the hindgut theory, which states that contact of partially digested food with the ileum corrects the deleterious effects of “empty ileum syndrome” caused by the lack of L-cell stimulation.<sup>61,81,88</sup> The results of the simple interposition of an ileum segment into the proximal segments of the small intestine are the strongest arguments supporting this hypothesis. A study of interposition in experimental animals subjected to a model of diet-induced obesity showed a significant increase in GLP-1 levels (Strader, 2006).<sup>52</sup> Similarly, interposition of a 50-cm ileum segment distal to Treitz’s angle in combination with vertical gastrectomy resulted in improved diabetes symptoms in a clinical trial.<sup>89</sup>

In addition to the “ileal brake” (a reaction that decreases proximal gastrointestinal transit motility and enterohormone<sup>90,91</sup> production following ileal interposition surgery), ileal interposition also improved glucose tolerance in experimental, euglycaemic rats.<sup>52,92</sup>

These findings suggest that isolated ileal interposition might be a valid alternative for the treatment of diabetes. Due to the intestines’ great capacity for adaptation, further research is necessary to justify ileal interposition as a surgical treatment for diabetes. Specifically, studies addressing the ability of interposed ileum L-cells to continue to differentiate with a density similar to intact ileum; to fulfil their functions, including GLP-1 production, over time; and to contribute to the metabolic control of glucose levels will be necessary.

### **Cell differentiation and intestinal adaptation**

Cells of the small intestine constantly proliferate and differentiate, and they are able to adapt after injury, inflammation, or resection.<sup>93</sup> The intestine is known to be able to adapt morphologically and functionally in response to internal and external stimuli. Intestinal adaptation, also known as enteroplasticity, is a complex and multifaceted process that serves as a paradigm for gene-environment interactions. Adaptation can occur after the loss of a small portion of intestine, in diabetes, with age, or due to malnutrition.<sup>94-97</sup> Increased nutrient absorption after intestinal resection compensates for absorption surface loss and minimises malabsorption<sup>98</sup> by increasing crypt depth, villus length, enterocyte proliferation, and absorption of electrolytes, glucose, and amino acids.<sup>99,100</sup>

Several studies aiming to elucidate the basis of the adaptation response showed that digestive and absorptive properties are increased coordinately with the expression of

enteroendocrine genes.<sup>101-103</sup> Peptides derived from proglucagon, an adaptation response marker, act as humoral mediators; ileal levels of proglucagon increase after small intestine resection. Proglucagon messenger RNA (mRNA) levels increase specifically in the ileum. This increase is immediate and persists for up to three weeks after resection.<sup>104</sup> Multiple complex factors, including humoral factors such as the growth factors insulin-like growth factor 1 (IGF-1), peptide YY (PYY), epidermal growth factor (EGF), and GLP-2, biliopancreatic secretions, and nutritional factors (glutamine, arginine, fatty acids, and triglycerides), also influence the mechanism of intestinal adaptation.<sup>93</sup>

Dietary components supply continual signals inducing the expression of genes that influence intestinal adaptation.<sup>105</sup> The amount and type of dietary fat consumed can influence intestinal function.<sup>106</sup> Carbohydrates can induce the intestinal adaptation response by increasing hexose transporter levels in order to increase sugar absorption.<sup>107</sup> Alterations in the amount of ingested protein induce adaptations in the transport of non-essential amino acids.<sup>108</sup> Biliary acids solubilise fat and participate in complex hormone metabolism by activating nuclear receptors, which control the transcription of genes also involved in glucose metabolism, and the cell surface receptor TGR5, which modulates energy expenditure in muscle and adipose cells. TGR5 has been shown to be expressed by enteroendocrine GLP-1-secreting L-cells. TGR5 activation by biliary acids results in intestinal secretion of GLP-1, thus improving post-prandial tolerance to insulin in mice, which might have implications for T2D treatment (Knop, 2010).<sup>109</sup> TGR5, GPR119, and GPR120 are G protein-coupled receptors known to mediate the release of GLP-1 by L-cells.<sup>110-113</sup>

The proglucagon gene encodes two hormones with important functions and opposite effects on glucose homeostasis: glucagon, which is expressed in pancreatic islets, and GLP-1, which is expressed in the bowels. The tissue-specific regulation of proglucagon expression remains poorly understood. In endocrine cell lines, the glucagon promoter is stimulated by beta-catenin, which is the main effector of the Wnt signalling pathway. GLP-1 synthesis and mRNA expression are activated by the inhibition of glycogen synthase kinase 3 $\beta$ , which is the main negative modulator of the Wnt pathway. In addition to demonstrating a specific mechanism for the regulation of proglucagon expression in intestinal endocrine cells, this study suggests that tissue-specific expression of the factors TF and TF-4 plays a role in the diverse Wnt signalling pathways.<sup>114</sup>

Several hormones, such as glucocorticoids and the growth hormones IGF-1, EGF, keratinocyte growth factor (KGF), leptin, ghrelin, and GLP-2, can modify intestinal shape and

function. It is clear that genes regulating cell cycle, proliferation, differentiation, and apoptosis are important components of the adaptation process (Drozdowski *et al.*, 2009).<sup>97</sup> Several studies in humans and laboratory animals showed that after massive resection of the proximal small intestine, the remaining ileum exhibits morphological and functional adaptation in an attempt to preserve nutritional health by increasing ileal absorption of dietary nutrients. The authors of these studies concluded that the ileal adaptation mechanisms for peptide absorption are mediated by cell proliferation, i.e., villus hyperplasia and intestinal dilatation, which serves to increase the absorption surface.<sup>115</sup>

Gastrointestinal epithelial cells are under constant regenerative pressure. To maintain homeostasis, there must be a balance among cell apoptosis, senescence, proliferation, and differentiation. The maintenance of this balance is attributed to gastrointestinal stem cells. These cells are able to replicate and give rise to cells identical to themselves and to cells that will differentiate into each of the different cell types present in this tissue.<sup>116</sup> Small numbers of intestinal stem cells (between one and three) are found at the bottom of each of the Lieberkühn crypts, which are the invaginations that make up the intestine's proliferative component. These cells give rise to a transient population of progenitor cells, which divide quickly while migrating along villi towards the bowel lumen. During migration, these cells commit to one of three different cell lineages: secretory (goblet cells), absorptive (enterocytes), or enteroendocrine. This is a continual process in which the most differentiated cells are replaced at the top of the villi every four or five days. Another type of secretory cell, called Paneth cells, differentiates at the bottom of the crypts (Figure 2).<sup>117</sup>

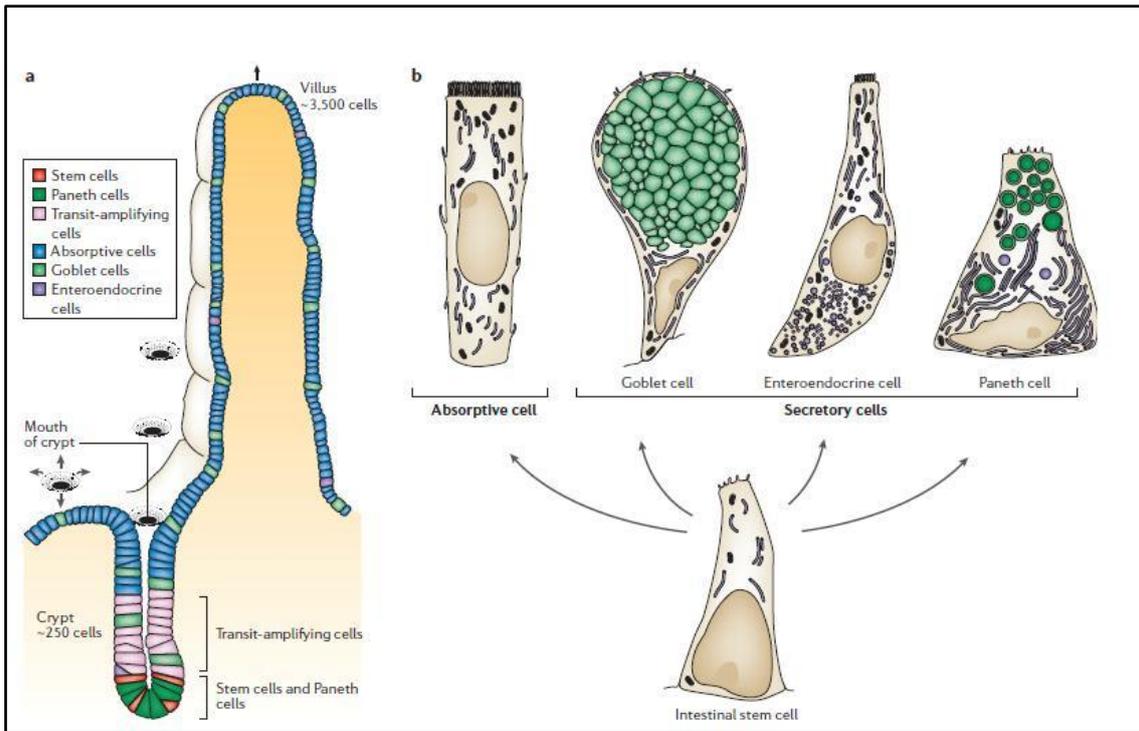


Figure 2. Intestinal epithelial cell types. A) One villus with one of the crypts contributing to its formation. Stem cells and Paneth cells are located at the crypt bottom. Above these cells are the multiplying progenitor cells, and higher up are the differentiated cells (absorptive, goblet, and enteroendocrine). B) The four types of differentiated cells.<sup>118</sup>

Enteroendocrine cells (EECs) comprise approximately 1% of all gastrointestinal tract cells, and although they are sparse, they are essential regulators of digestion, intestinal motility, appetite, and metabolism. How the relative proportions of individual subtypes and the endocrine compartment itself are maintained in this epithelium under rapid and constant renewal remains unclear (May and Kaestner, 2010).<sup>119</sup> There are at least 14 different types of EECs; each type of EEC secretes one or more hormones, such as GLP-1 and GLP-2, or hormone-like substances that are released directly in the lamina propria and diffuse through the capillary vessels.<sup>119,120</sup> EECs are polarised, with projections extending towards the intestinal lumen containing chemoreceptors sensitive to several classes of nutrients and other compounds present in the bowel contents. The base of these cells is close to capillary vessels and nerve endings, and they contain secretory vesicles with peptides secreted by cells in response to stimuli received by chemoreceptors. Secreted peptides act as classic hormones, travelling through the blood stream to act on receptors in distant organs, and as neuromodulators, acting on receptors expressed by autonomic nerve endings close to the peptide secretion site.<sup>121</sup> Mechanisms regulating enteroendocrine cell differentiation are important during embryonic development and for the constant renewal

of the intestinal epithelium in the adult. The identification of transcription factors and DNA regulating elements that contribute to the specific genetic profile of each cell type is increasing the understanding of the different networks controlling the spatial and temporal activation of enteroendocrine differentiation programmes.<sup>122</sup> EECs secrete several regulatory molecules that control physiological and homeostatic functions, mainly post-prandial secretion and motility, and act as sensors of lumen contents.<sup>123</sup> Although EECs secrete transcriptional regulators, they differentiate from pluripotent stem cells located in crypts. Their origin is therefore endodermal as with all epithelial mucosa cell types.<sup>124-126</sup>

The Wnt signalling pathway plays an important role in the proliferative activity of the normal intestinal crypt.<sup>127</sup> The *Lgr5/GPR49* gene is only expressed in the stem cell compartment located at the crypt bottom, demonstrating that all epithelial lines derive from *Lgr5*-expressing intestinal stem cells.<sup>125</sup> EEC spatial orientation along the crypt-villus axis is known to be closely associated with differentiation. Although most EECs differentiate and migrate towards the villus top, a recent study showed that a small EEC subpopulation that migrates towards or remains at the crypt bottom expresses stem cell markers and post-mitotic endocrine markers.<sup>128</sup> A fourth type of secretory cell, referred to as tuft cells, was recently described. The differentiation of tuft cells depends on the transcription factors *Atoh1/Math1* but not on other factors, distinguishing them from EECs, Paneth cells, and goblet cells. These cells are the main source of endogenous intestinal opioids, and they are the only type of epithelial cell expressing cyclooxygenase enzymes, suggesting an important role in intestinal epithelial physiopathology.<sup>129</sup>

The Notch signalling pathway plays a crucial role in enteroendocrine differentiation. Notch is a transmembrane receptor protein that mediates cell-to-cell communication and coordinates a signalling cascade. Notch receives signals at the cell surface and functions in the nucleus to regulate gene expression.<sup>130</sup> In mammals, the main components of this pathway are ligands (*Delta* and *Jagged*), receptors (*Notch 1, 2, 3, and 4*), and a transcription factor (*RBP-Jk*) that activates the target genes. Ligand interaction promotes proteolytic cleavage of Notch, thus releasing the Notch intracellular domain from the cell surface.<sup>131</sup> Intracellular Notch is transported to the nucleus, where it interacts with *RBP-Jk* to form a complex that binds and activates the promoters of hairy/enhancer of split (*HES*) gene family members. *Hes1* or another *HES* family member then represses bHLH pro-endocrine transcription factors such as neurogenin 3 (*Ngn3*) by binding their promoters or enhancers (Figure 3).<sup>119,132,133</sup> *Hes1* is crucial for maintaining a reservoir of undifferentiated endocrine

precursors. Hes1-deficient mice have a greater number of serotonin-, cholecystokinin (CCK)-, proglucagon-, somatostatin-, and gastric inhibitory peptide (GIP)-producing cells in their bowels.<sup>132</sup>

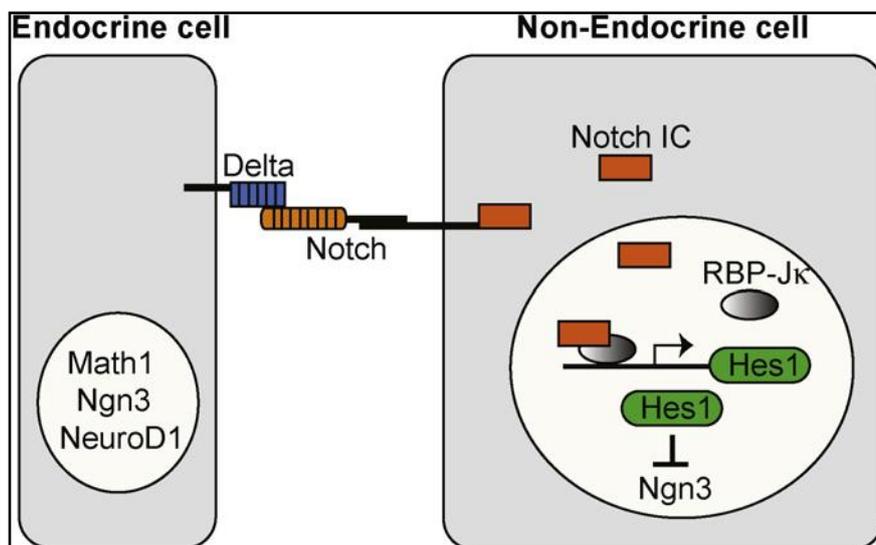


Figure 3. Participation of the Notch signalling pathway in endocrine cell differentiation. The Delta ligand is upregulated in differentiating endocrine cells. It binds to Notch in neighbouring non-endocrine cells, resulting in release of IC Notch, which is transported to the nucleus and interacts with RBP-Jk to activate target genes, such as Hes1, that inhibit bHLH pro-endocrine transcription factors such as Ngn3.<sup>119</sup>

A major function of Notch is to mediate lateral inhibition between adjacent cells, preventing neighbouring cells from adopting the same fate.<sup>130-134</sup> This function is evident in the findings that normal gastrointestinal epithelium never contains two adjacent EECs and that the loss of Notch function results in excess EECs. Notch signalling controls the differentiation programme directed by bHLH transcription factors to select individual EECs from the multiplying cell precursors.<sup>132-135</sup> In addition to its role in the initial decisions that determine epithelial lineage, Notch signalling is also important in the final stages of differentiation, during which it plays a role in fine tuning the number of cells of each different enteroendocrine cell type. After the initial choice between the secretory and absorptive lineages, EEC fate is decided differently from that of other secretory cells (goblet and Paneth cells) through different components of Notch signalling, including Ngn3 and BETA2/NeuroD.<sup>132,136-139</sup>

Loss-of-function studies in rats have shown that three bHLH pro-endocrine transcription factors, Math1 (also known as ATOH1), neurogenin 3 (Ngn3), and NeuroD, contribute to enteroendocrine cell differentiation.<sup>136,137,139,140</sup> These transcription factors function sequentially, with one factor activating another to control the initial specification and

final differentiation of EECs.<sup>119</sup> Math1 is necessary for the initial specification of all three intestinal secretory lines (goblet, Paneth, and EEC).<sup>138</sup> Later on, expression of lineage-specific transcription factors, such as Sox9 for Paneth cells, Klf4 for Goblet cells, and Ngn3/NeuroD for EECs, is necessary.<sup>136,141,142</sup>

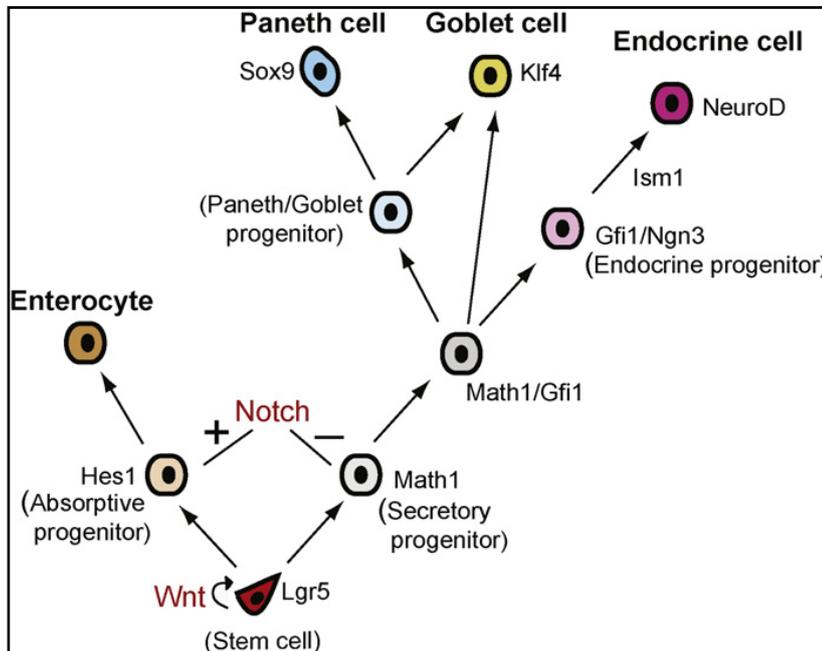


Figure 4. Overview of enteroendocrine differentiation in the intestinal epithelium. Lgr5-expressing stem cells in the crypts give rise to the four intestinal epithelial cell types. Expression of Math1 is restricted to the secretory lineage, and Hes1 is restricted to the absorptive lineage. After this initial specification is established, lineage-specific transcription factors, such as Sox9 for Paneth cells, Klf4 for goblet cells, and Gfi1/Ngn3/NeuroD for EECs, are necessary for differentiation into each specific secretory lineage.<sup>119</sup>

Some studies show that intestinal precursors preferentially differentiate into enterocytes in the intestine of adult Math1 knockout rats, thus confirming the importance of this gene in maintaining the balance between enterocytes and EECs.<sup>143</sup> In mice and humans, all intestinal EECs require Ngn3,<sup>136</sup> which acts downstream of the Math 1 transcription factor. Ngn3 is a bHLH transcription factor that is activated on embryonic day 11.5.<sup>144</sup> Its expression persists in the adult bowels and the glandular portion of the stomach.<sup>136</sup>

Genetic analyses of NeuroD provided the first link between Notch signalling and EEC differentiation.<sup>139</sup> In contrast to Ngn3, NeuroD expression is restricted to a subgroup of secretin-producing EECs.<sup>139,145</sup> NeuroD acts downstream of Ngn3, as demonstrated by the lack of NeuroD expression in Ngn3-deficient mice.<sup>146</sup> Notch signalling also controls the transcription

of Hes1, which encodes a bHLH transcriptional repressor that inhibits the activity of the bHLH transcriptional activators mentioned above.<sup>147</sup> Hes1 is crucial for maintaining pro-endocrine reservoirs in an undifferentiated state. Several bHLH factors are up-regulated in rats in the absence of Hes1, resulting in early differentiation of enteroendocrine subtypes and high numbers of serotonin-, CCK-, proglucagon-, somatostatin-, and GIP-positive cells in the intestine.<sup>132</sup>

Ngn3 and NeuroD specify the differentiation of EECs such as L-cells, in which Pdx4 and Pax6 play important roles in final differentiation.<sup>148</sup> A diet rich in oligofructose, a non-digestible carbohydrate, was shown to double the number of GLP-1-producing L-cells in the proximal colon of rats through a mechanism involving up-regulation of Ngn3 and NeuroD. This study suggests that the final products of oligofructose fermentation, such as acetate, propionate, and butyrate, might be involved in the induction of L-cell differentiation. Butyrate is thought to regulate intestinal cell differentiation, as it was shown *in vitro* to increase the expression of the glucagon gene in immortalised L-cells. Moreover, butyrate infusion in the colon *in vivo* increases proglucagon and GLP-1 levels.<sup>149,150</sup>

The transcription factors Foxa1 and Foxa2 are expressed throughout the gastrointestinal epithelium from embryo to adult. Mice lacking these factors also lack GLP-1- and GLP-2-expressing cells (L-cells), and they have reduced numbers of somatostatin-expressing D-cells and PYY-expressing L-cells. The mRNA levels of glucagon, somatostatin, PYY, and the transcription factors Isl-1 and Pax6 were reduced in the small intestine of these animals, demonstrating that Foxa1 and Foxa2 are involved in the network of transcription factors regulating enteroendocrine lineage differentiation.<sup>151</sup>

Transcription factors other than bHLH factors also seem to play important roles in EEC differentiation. In addition to the early EEC differentiation observed in Hes1-deficient animals, Pax4, Pax6, Nkx2.2, and Isl-1 genes were activated in the intestine.<sup>132</sup> Instead of controlling EEC global differentiation like bHLH genes, these factors seem to play important roles in the fine control of specification decisions among enteroendocrine populations.<sup>119</sup> LIM homeodomain genes encode a family of transcription regulating proteins involved in the control of several aspects of embryonic development and are responsible for some human diseases. Islet-1 (Isl-1) is expressed in gastrointestinal epithelium on approximately embryonic day 10, and in adults, its expression is restricted to EEC subgroups. Expression analysis suggests that Isl-1 might play an important role in the final differentiation and/or maintenance of mature enteroendocrine subtypes in the gastrointestinal epithelium.<sup>152</sup>

In the adult intestine, the zinc-finger transcription factor Gfi-1 is mainly found in crypts. Its expression is restricted to neuroendocrine lineages, and it acts downstream of Math1 in the differentiation process. Gfi-1 is a transcriptional repressor that acts in secretory lineage differentiation, specifically in the selection between enteroendocrine progenitors and other lineages (goblet and Paneth). Expression of Gfi-1 was detected in adult small intestine and colon crypts and villi. Gfi-1 co-localises with Math1, Ngn2, serotonin, and chromogranin A, but not with goblet and Paneth cells markers. These data suggest a model for intestinal epithelial differentiation in which Gfi-1 expression must be abolished for normal differentiation of goblet and Paneth cell progenitors (Figure 5). Alternatively, Gfi-1 might only be expressed in Math1/Ngn3-positive enteroendocrine precursors, whose production increases in the absence of Gfi-1, and serve to inhibit the production of goblet and Paneth cells from adjacent progenitors.<sup>140</sup>

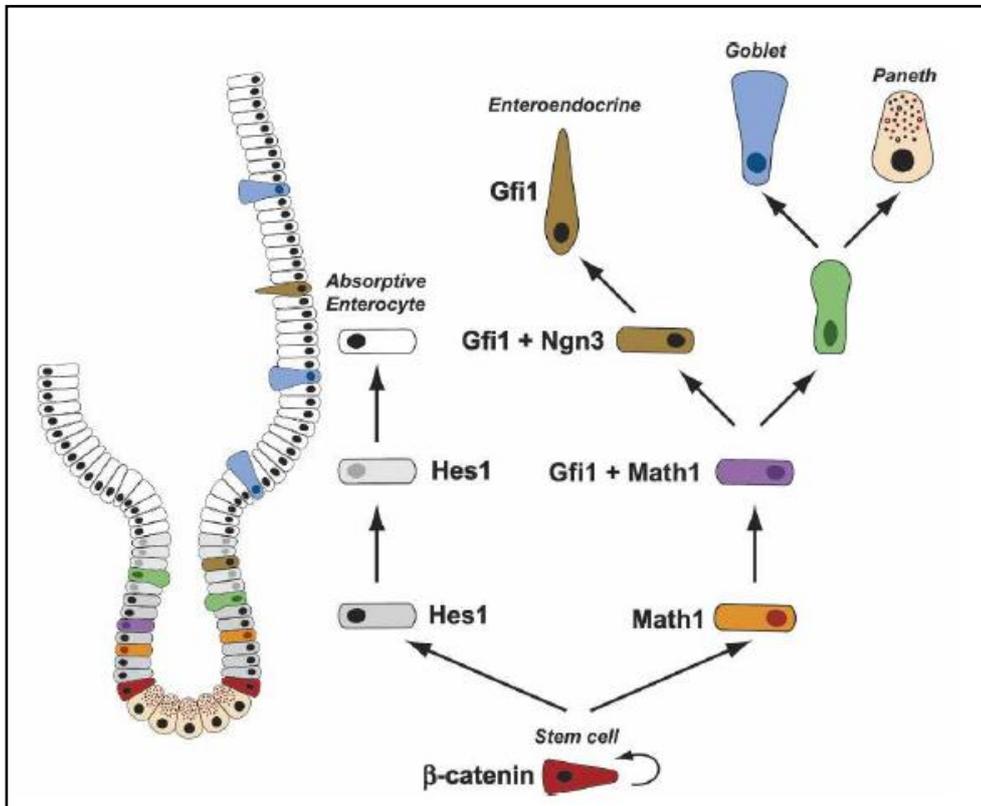


Figure 5. Model of intestinal epithelial cell differentiation. Stem cells give rise to highly proliferative multipotent progenitors that use Notch signalling to select Hes1- or Math1-expressing cells. Hes1-expressing cells differentiate into absorptive enterocytes, and Math1-expressing cells are the secretory lineage progenitors. Next, these progenitors co-express Gfi-1, which selects between goblet and Paneth cell progenitors (green) and enteroendocrine precursors (brown). Enteroendocrine precursors express Ngn3 and Gfi-1, which continues to be expressed in a subgroup of mature EECs.<sup>140</sup>

Another zinc-finger protein, Insm1 or IA-1, is found in proliferating cells, many of which co-express NeuroD1 and chromogranin A.<sup>153</sup> Insm1 has been shown to control the differentiation of specific intestinal enteroendocrine lineages and acts downstream of Notch and Ngn3 after the initial specification of enteroendocrine precursors.<sup>119</sup> Several homeobox genes, including Isl-1, Pdx1, Nkx6.1, and Nkx2.2, have also been shown to participate in EEC differentiation.<sup>154</sup>

Two paired box (Pax) genes, Pax 4 and Pax 6, are linked to pancreatic and intestinal endocrine cell differentiation.<sup>155,156</sup> Deletion of Pax4 in the duodenum disrupts the differentiation of serotonin-, secretin-, CCK-, GIP-, and PYY-expressing cells; however, there is no significant reduction in the number of EECs in the ileum and colon of Pax4-deficient mice. There is a severe reduction of somatostatin- and gastrin-expressing cells in the distal stomach of Pax6-deficient mice; however, serotonin-expressing cells are not affected. The number of GIP-expressing cells in the duodenum of Pax6-deficient mice is reduced.<sup>157</sup> Pax6 is crucial for proglucagon gene expression in intestinal epithelium. It is expressed in intestinal EECs and binds to G1 and G3 elements in the proglucagon promoter to activate its transcription.<sup>158,159</sup> Pax6 might also bind to the promoter and induce production of PC 1/3 pro-hormone convertase, an enzyme essential for the conversion of proinsulin into insulin<sup>160</sup> and of proglucagon into GLP-1.<sup>43</sup> Proglucagon processing is accomplished by PC1 in L-cells and by PC2 in pancreatic alpha cells to produce glucagon. Published data suggest that these tissue-specific processing mechanisms are due to differential expression of PC1 and PC2.<sup>113,161,162</sup> GIP, another important incretin, might be expressed alone or together with GLP-1. Cells expressing GIP and GLP-1 are Pax6- and Pdx1-positive, and cells expressing only GLP-1 are Pax6-positive and Pdx1-negative. This suggests that the presence of Pdx1 determines whether gastrointestinal L-cells will co-express GIP.<sup>163</sup>

Most Nkx6.1-expressing cells also express serotonin, and some express gastrin. Nkx6.1 is not expressed in Pdx1-deficient mice, indicating that Pdx1 acts upstream of Nkx6.1 in enteroendocrine differentiation.<sup>164</sup> Some enteroendocrine hormone-producing intestinal populations are reduced in Nkx2.2-deficient mice, including serotonin-, CCK-, GIP-, and gastrin-producing cells; however, the number of ghrelin-expressing cells is greater, and the numbers of Paneth cells, goblet cells, and enterocytes are unaltered. It seems that Nkx2.2 functions upstream of Pax6 in regulating the fate of intestinal cells, as Pax6 mRNA and protein levels are reduced in Nkx2.2-deficient cells. Specification of ghrelin-expressing cells is thought to occur at the expense of other intestinal enteroendocrine cells; however, the total number of EECs

remains the same.<sup>165</sup> Nkx6.3 knockout mice show a severe and selective reduction in gastrin-secreting G-cells and an increase in somatostatin-secreting D-cells; however, these animals express normal levels of the Pdx1, Pax6, and Ngn3 transcription factors, which are needed for G-cell development.<sup>166</sup> These results suggest that Nkx6.3 acts independently from other transcription factors in G-cell differentiation.<sup>119</sup>

GATA transcription factors regulate proliferation, differentiation, and gene expression in several organs. In the small intestine, they are needed for crypt cell proliferation, secretory lineage differentiation, and gene expression in absorptive enterocytes. GATA4 is expressed in the proximal region in 85% of the small intestine and regulates the jejuno-ileal gradient of gene expression in absorptive enterocytes. GATA6 is co-expressed with GATA4, but it is also expressed in the ileum. Deletion of GATA6 in the ileum causes decreased crypt cell proliferation, reduces the number of Paneth cells and EECs, increases the number of crypt goblet cells, and alters the expression of specific genes in absorptive enterocytes. Deletion of GATA4 and GATA6 in rats results in a jejunum and ileum phenotype almost identical to that of the GATA6-deficient ileum, suggesting that GATA4 and GATA6 share some functions.<sup>167</sup>

GATA4 is a zinc-finger transcription factor involved in jejunum gene expression. GATA4-deficient mice exhibit a dramatically decreased ability to absorb dietary cholesterol and fat. A study comparing global gene expression profiles in wild-type jejunum and ileum and GATA4-deficient jejunum demonstrated a 53% decrease in the expression of jejunum-specific genes and a 47% increase in the expression of ileum-specific genes in GATA4-deficient jejunum samples. Changes included decreased expression of mRNA encoding lipid and cholesterol transporters and increased expression of mRNA encoding proteins involved in bile absorption. This study showed that GATA4 is crucial for jejunum function and plays an essential role in the determination of jejunal versus ileal identity. It has been shown that GATA4 or GATA6 is necessary for secretory progenitors to commit to a neuroendocrine lineage.<sup>167-169</sup> Immunohistochemical analysis of mouse small intestine revealed that GATA4 is only expressed in villus enterocytes. GATA5 was not detected in enterocytes, but it was detected in other lineages. High levels of GATA6 were only detected in the neuroendocrine lineage. Together, these results suggest that GATA transcription factors may play different roles in the differentiation and/or allocation and maintenance of small intestine lineages.<sup>179</sup>

Some transcription factors and signalling molecules regulate the expression of the glucagon gene. The homeoprotein Cdx-2 activates the glucagon gene promoter in pancreatic and intestinal proglucagon-producing cell lineages.<sup>171</sup> The Pax-6 protein<sup>158</sup> and the kinase A

signalling pathway<sup>172</sup> are involved in the regulation of glucagon gene expression in pancreatic and intestinal glucagon-producing cell lineages, primary pancreatic islets, and intestinal cell cultures.<sup>114</sup>

Studies of intestinal adaptation have examined genes possibly involved in this process using differential display polymerase chain reaction (DD-PCR) and cDNA microarray. Examination of the patterns of gene expression in animals during the adaptation process has revealed that some metabolism-related genes are increased at least two-fold after resection. Changes in the expression of genes encoding ion channels, transport proteins, transcription factors, DNA-binding proteins, receptors, and cytoskeleton proteins were also observed.<sup>173,174</sup> One study using DD-PCR showed that adaptation after resection results from the up-regulation of genes not previously related to the adaptation response. Genes that exhibited differential regulation after resection were divided into three categories: amino acid transport, protein transport, and signal transduction molecules. This same study suggested that analyses using reverse transcriptase polymerase chain reaction (RT-PCR) can more precisely define the magnitude of the adaptation response.<sup>93</sup>

To study adaptation after 50% resection of the mouse intestine, cDNA microarrays were used to characterise the expression of individual genes and global gene expression patterns in the remaining ileum. The analysis of these microarrays revealed changes in the expression of several genes, including those involved in cell cycle regulation, apoptosis, DNA synthesis, and transcriptional regulation. Expression patterns were consistent with the increase in cell proliferation and apoptosis observed during intestinal adaptation; however, verification by RT-PCR and Northern blot are still necessary.<sup>173</sup> Another study examined gene regulation after resection in mice using cDNA microarrays and showed that 27 genes were altered after resection; these genes included *spr2*, which increased almost five-fold and had not previously been linked to intestinal adaptation.<sup>174</sup>

Very little is known about what guides region-specific expression of hormones in the bowels. Despite the discovery of the function of transcription factors important for neuroendocrine cell differentiation, it is still not known how these factors direct and control gene expression in the different enteroendocrine cell types. Nor is it known to what degree these transcription factors control hormone expression in adults.<sup>119</sup>

The differentiation mechanism of L-cells and the genetic and/or environmental factors that might influence the proliferation rate of this intestinal cell subtype, which is crucial for

maintaining normoglycaemia, remain unknown. Thus, comparison of intact and interposed ileum in terms of the differential expression of genes related to the differentiation of intestinal stem cells into each of the intestinal epithelial lineages, and specifically to the differentiation of neuroendocrine GLP-1-producing L-cells, is essential for the study of ileal interposition as a possible surgical treatment of T2D.

## **2. AIMS**

### **Overall**

To assess the effects of isolated ileal interposition on intestinal stem cells of rats with diet-induced glucidic dysmetabolism.

### **Specific**

To investigate changes in the relative expression of genes involved in intestinal stem cell differentiation into enteroendocrine cells after isolated ileal interposition.

To analyse changes in the number and function of L-cells after isolated ileal interposition.

## **3. METHODS**

### **3.1. Research Ethics Committee**

This research project will be presented to the Experimental Research Ethics Committee of the Federal University of São Paulo – São Paulo School of Medicine (UNIFESP/EPM).

### **3.2. Experimental procedures**

Experimental procedures will be performed according to guidelines in the manual “Care and Handling of Laboratory Animals”, Lapchick, Mattaria, Ko; Atheneu, 2009, CDD 636.0885.

All animals will be provided the same diet and will be subjected to surgery when they become dysmetabolic. Blood samples will be collected from all animals on the days of surgery and euthanasia for biochemical analysis. After euthanasia, samples will be collected from the interposed and remaining ileum of the interposition group and from the jejunum and ileum of the control group. These samples will be analysed using molecular biology and

immunofluorescence techniques to compare changes in the small intestine segment under study relative to the characteristic endocrine pattern of the ileum.

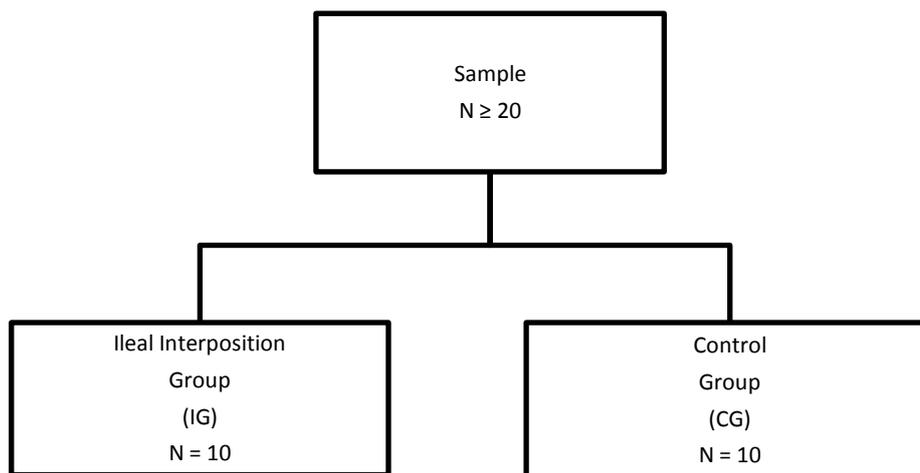
### 3.3. Sample

Twenty male Wistar – 2BAW heterogeneous male rats (*Rattus norvegicus albinus*) aged 12 weeks and weighing between 250 and 280 g will be used. The animals will be supplied by the Laboratory of Animal Experimentation at the Institute of Pharmacology and Molecular Biology of the Federal University of São Paulo – São Paulo School of Medicine (UNIFESP/EPM), where they will be housed throughout the study.

Animals will be housed in individual cages and kept for 32 weeks at a controlled ambient temperature of  $23 \pm 2^{\circ}\text{C}$ , relative humidity of  $55 \pm 15\%$ , and automatic 12-hour light–dark cycle (06:00 / 18:00) (Timer-Kienzle).

For surgical procedures, animals will be randomly distributed into two groups according to the flowchart defined below:

- Interposition Group (IG) – 10 animals subjected to ileal interposition
- Control Group (CG) – 10 animals not subjected to any surgical intervention



Surgical interventions will be performed at post-natal week 28 (W28) or as soon as glucidic dysmetabolism is established. All animals will be monitored until euthanised in the 16<sup>th</sup> post-operative week, which will be equivalent to post-natal week 44 (W44).

### 3.4. Diet

Throughout the experiment, all animals will be provided a lipid-rich pelletised hypercaloric diet (HD) *ad libitum* in alternating cycles of four feed types (1, 2, 3, and 4) to stimulate intake. The experimental feed will be alternated every 24 hours, and non-ingested amounts will be measured. Consumption of these diets will promote obesity in animals, which will exhibit characteristics commonly associated with human obesity, such as insulin resistance, hyperglycaemia, hyperinsulinaemia, dyslipidaemia, and liver steatosis. All groups will have free access to water.

Experimental feeds 1, 2, 3, and 4 will be industrially prepared by Rhoster®, Araçoiaba da Serra – SP according to guidelines in *Requirements of the Laboratory Rat* recommended by the *National Academy of Sciences* and will consist of standard rat feed with protein, vitamin, and mineral supplements. Additional hyperenergetic ingredients used in hypercaloric experimental diets will be (in grams per kilogram):

- HD1 – standard ration, 355; toasted peanuts, 176; casein, 123; corn oil, 82; chocolate, 88; corn biscuits, 176; and vitamins and minerals.

- HD2 – standard ration, 439; toasted peanuts, 218; casein 129; corn oil, 61; chips, 153; and vitamins and minerals.

- HD3 - standard ration, 371; toasted peanuts, 185; casein, 99; corn oil, 68; pasta, 185; grated cheese, 92; and vitamins and minerals.

- HD4 - standard ration, 359; toasted peanuts, 179; casein 105; corn oil, 80; condensed milk, 161; wafer biscuits, 116; and vitamins and minerals.

The macronutrient composition of the standard and hypercaloric-hyperlipidic experimental feeds prepared and analysed in the laboratory by Rhoster®, Araçoiaba da Serra – SP is described in Table 1.

Table 1 – Composition of standard and experimental diets

Compounds	Feed				
	Standard	HD1	HD2	HD3	HD4
Protein (%)	26	27	28	28	26
Carbohydrate (%)	54	43	36	33	43
Fat (%)	3	20	23	24	20
Other (%) <sup>*</sup>	17	10	13	15	11
Calories (Kcal/g)	3.5	4.6	4.6	4.6	4.6

<sup>\*</sup> - vitamins, minerals, humidity, ashes.

### 3.5. Weight and ration intake curve

As soon as the clear period begins, the animals will be weighed twice per week on pre-established days using appropriate high-precision scales (Filizola BP-6), and each animal's weight in grams will be recorded. Feed intake will be recorded three times per week on pre-established days; in each cage, the difference between offered and consumed feed will be calculated, and estimated daily feed intake will be expressed in grams. Weight and feed intake curves will be calculated for both groups throughout the study.

### 3.6. Blood collection and laboratory tests

Six hours before blood collection and surgical procedures, animals in all groups will be subjected to fasting with access to water *ad libitum*. Blood will be collected for laboratory tests on the week 12 (W12), two weeks before surgery (W26), eight weeks before surgery (W36) and on the day of euthanasia during the 16<sup>th</sup> post-operative week (W44), after weighing and under anaesthesia.

In total, 0.5 mL of blood will be collected from each animal in tubes containing 5 µl of DPP-IV inhibitor (an enzyme that degrades GLP-1) by caudal vein puncture. The blood samples will then be centrifuged, and serum will be stored at -20°C until analysis of serum levels of active glucagon-like peptide-1 (GLP-1), glucagon, insulin, and other diabetes-related analytes. For this purpose, we will use the Milliplex<sup>®</sup> Magnetic-Metabolism (Rat Metabolic Disease, Genese, SP) simultaneous quantification method.

Ten minutes after blood collection, serum glucose will be measured with reagent strips on a glucometer (Kit Accu-Chek Advantage<sup>®</sup>). Next, the animals will be subjected to an insulin tolerance test (ITT). Regular insulin will be injected in the penian vein at a concentration of 1 U/kg body weight. One drop of blood will be collected from the tail using a lancet 4, 8, 12, and

16 minutes after insulin injection for the detection of glycaemia. The results will be recorded for later analysis.

Serum glucose will also be measured weekly on pre-established days throughout the study (W14 to W44) using reagent strips and a glucometer (Kit Accu-Chek Advantage®).

### **3.7. Anaesthesia, weighing, and antibiotic prophylaxis**

Before the onset of anaesthesia, all animals will be weighed on high-precision scales (Filizola BP-6), and their weights will be recorded in grams. In both groups (IG and CG), general anaesthesia will be induced with halothane to collect tail blood. Dissociative anaesthesia will then be induced by simultaneously injecting the animals intramuscularly with Zoletil 50® (tiletamine+zolazepam) at a dose of 20 mg/kg and Fentanil® at a dose of 0.025 mg/kg to perform the insulin tolerance test and surgical intervention.<sup>170</sup>

Each animal will be maintained on spontaneous ventilation during the procedure, and the anaesthetic plane will be checked periodically every 45 minutes by assessing the auricular and interdigital reflexes, which are abolished during anaesthesia. When these reflexes reappear, one-third of the initial anaesthetic dose will be administered. The total amount of anaesthetics used will be recorded at the end of the surgical intervention.

Antibiotic prophylaxis will be performed by intramuscular injection of cefoxitin at a dose of 50 mg/kg body weight soon after anaesthesia for surgery.

### **3.8. Surgical procedures**

Rats in the IG group will be subjected to surgery upon developing diet-induced glucidic dysmetabolism, which should occur around 28 weeks of life (W28). All Halsted asepsis and antisepsis principles will be applied, and sterile microsurgical tools will be used.

The anaesthetised rats will be placed in horizontal dorsal decubitus on the surgical table with the legs and tail duly immobilised and secured with tape. Antisepsis of the abdominal area will be performed with chlorhexidine in an aqueous vehicle.

A sterilised fenestrated surgical drape will be placed on each animal's abdominal area, and a 5-cm long median longitudinal incision will be made in the wall with a disposable scalpel containing a number 15 blade.

The caecum will be identified in each IG animal and exposed together with the terminal ileum. Next, the small intestine will be sectioned perpendicularly in two areas 5 cm and 15 cm from the ileo-caecal transition, thereby producing a 10-cm segment of ileum. The ileum segment will be removed and wrapped in gauze moistened with 0.9% saline solution (Figure 6A). Next, the jejunum will be sectioned in an area 5 cm from the duodenum-jejunum transition (Figure 6A). The previously separated distal ileum segment will be interposed between the segments of sectioned jejunum in isoperistaltic position by means of enteroenteric anastomosis (Figure 6B). Next, anastomosis will be performed between the interposed ileum and the remaining ileum to re-establish the continuity of the digestive tube (Figure 6C).

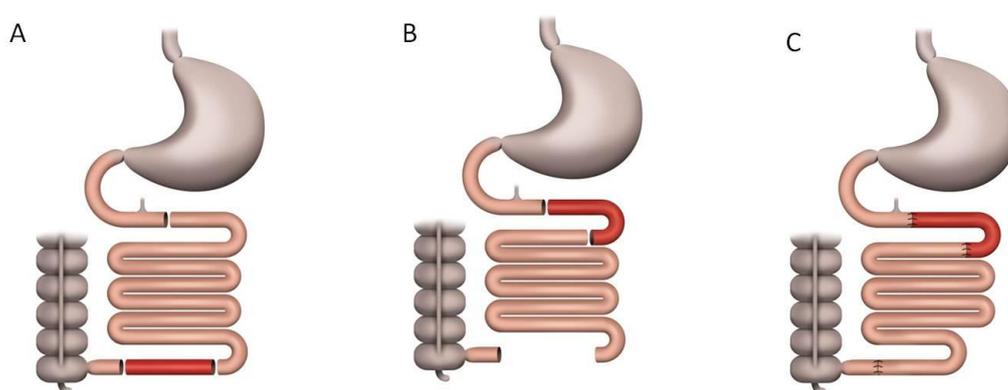


Figure 6 – Illustrative scheme of section and anastomosis areas in the Interposition Group. (A) Section of jejunum and the isolated ileal segment to be interposed (red). (B) Ileum interposed to jejunum. (C) Anastomoses.

No surgical intervention will be performed on the CG animals.

All intestinal anastomoses will be termino-terminal and will be performed with a total of 6 separated points using 7-0 polypropylene sutures pre-mounted on cylindrical needles.

After completion of the final revision surgery, the animals will be hydrated by intraperitoneal injection of 1.0 mL crystalloid solution (0.9% physiological saline) per 300 g body weight at 36°C.

Abdominal wall synthesis will be performed by monoblock continual suture of parietal peritoneum, muscle layer, and aponeurosis using 4-0 polyglactin sutures pre-mounted on

cylindrical needles. In the skin, continual suture with inverted stitches will be performed using 4-0 polyglactin sutures pre-mounted on cylindrical needles.

### **3.9. Postoperative assessment**

Animals will be kept warm and observed until full recovery from anaesthesia, when they will be placed in individual cages and transported to the housing room in the same laboratory and under the same environmental conditions as before surgery.

Before recovery from anaesthesia, animals will be given analgesic by gavage (0.5 mL of 3 g/mL dipyron in water). Water will be reintroduced *ad libitum* as soon as animals recover from anaesthesia. Dipyron (1 g/100 mL in water) will be given during the first 72 post-operative hours. Access to food will be allowed 12 hours after surgery.

The animals will be assessed and monitored until the 16<sup>th</sup> post-operative week (W44).

### **3.10. Euthanasia**

On the 44<sup>th</sup> post-natal week, the fasting, anaesthesia, weighing, and blood collection for laboratory tests will be repeated. Euthanasia will then be performed by decapitation according to the guidelines of the Brazilian Society of Laboratory Animals Science/Brazilian College of Animal Experimentation – SCAL-COBEA.

### **3.11. Study parameters**

- Body Weight: as soon as the clear cycle starts, each animal in each group (IG and CG) will be weighed on high-precision scales, and the weight will be recorded in grams (W1, W2, W3..., W32).
- Feed Intake: all animals will be given feed diurnally at a known amount sufficient for their daily needs; three times per week, non-ingested feed will be weighed on adequate scales, and daily feed intake will be calculated from the difference between supplied and remaining feed.
- Biochemical Tests: serum levels of active GLP-1 (GLP-1), glucose (GLI), insulin (INS), and glucagon (GLU) will be measured in all animals on week 12 (W12), two weeks before surgery (W26), eight weeks after surgery (W36) and euthanasia (W44).
- Insulin Resistance: on the days of surgery and euthanasia, insulin resistance (IR) will be calculated using the HOMA-IR indirect test (Homeostasis Model Assessment Insulin Resistance)

from the product of serum insulin (mU/mL), glycaemia (mg/mL), and the constant 0.05551 divided by 22.5.

- Insulin Tolerance Test (ITT): will be performed in all animals on the day of study onset (W12), two weeks before surgery (S26), eight weeks after surgery (W36), and euthanasia (W44). The rate of glucose removal will be calculated using the formula  $\ln 2/t_{1/2}$ ;  $t_{1/2}$  will be calculated for glucose from the minimum slope of the regression curve during the phase of linear decay of serum glucose concentration using PRISMA software.

### 3.12. Tissue collection

After euthanasia, the following fragments of intestinal tissue will be collected for histological analysis by immunofluorescence and gene expression analysis by molecular biology:

- Interposition Group: median segment of transposed ileum and segment adjacent to ileal anastomosis (ileum remaining in normal position) (Figure 7A).

- Control Group: ileum segment and jejunum segment (Figure 7B).

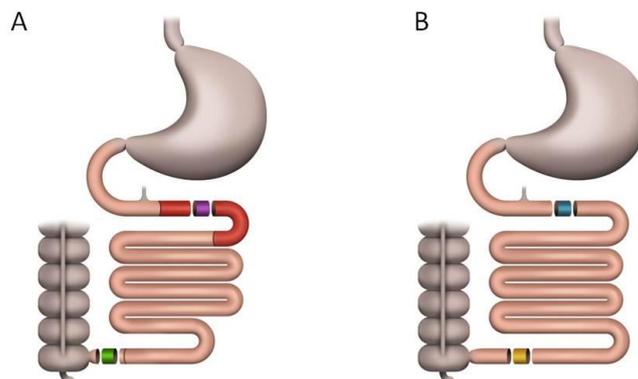


Figure 7 – Illustrative scheme of tissue collection for analysis. (A) Interposition Group: interposed ileum segment (purple) and remaining ileum adjacent to ileal anastomosis (green). (B) Control Group: segment of jejunum (blue) and ileum (yellow).

### **3.13. Histological analysis by immunofluorescence**

One-centimetre tissue samples for histological analysis by immunofluorescence will be cryopreserved in increasing sucrose concentrations (10% and 20% for 4 hours at 4°C and 30% for 12 hours at 4°C), frozen in liquid nitrogen, and kept at -80°C until analysis. Three- to five-micron-thick sections will be cut from the tissues using a cryostat (Leica) and mounted on silanated slides (BMF).

To block nonspecific staining, the slides will be incubated in phosphate-buffered saline (PBS) pH 7.4 + 1% bovine serum albumin (BSA) for 30 minutes at room temperature. For binding with primary antibody, the slides will be incubated with specific antibody (anti-GLP-1 and others, Abcam) diluted in 1% PBS/albumin buffer (Sigma A9647) for 1 hour at 37°C or for 16-18 hours at 4°C in a humid chamber. Next, they will be washed three times with 1% PBS/albumin for 5-10 minutes and incubated with a fluorophore-conjugated secondary antibody (FITC, Rhodamine, or Alexa Fluor 488, Invitrogen) for 90 minutes at 4°C in a humid chamber following the manufacturer's instructions. After incubation, the slides will be washed three times with 1% PBS/albumin for 5-10 minutes and incubated with the second primary antibody, and the incubation and washing steps described above will be repeated. Finally, the slides will be incubated with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen) for 30 minutes at 37°C, mounted on 1% glycerol-coated microscopy slides, and stored refrigerated and protected from light until analysis.

Immunoexpression of GLP-1, an L-cell marker, and other relevant genes will be assessed under a fluorescence microscope using appropriate filters for each fluorochrome.

### **3.14. Molecular biology: studying relative gene expression**

#### **3.14.1. Preparation of total RNA**

Collected segments (interposed and remaining ileum in the IG, jejunum and ileum in the CG) will be immediately immersed in RNA stabilisation reagent (RNAlater RNA Stabilization Reagent, Qiagen) and stored in tubes (RNAlater Tissue Protect Tubes, Qiagen) at -20°C according to the manufacturer's instructions until analysis.

To isolate total RNA, we will homogenise each sample using QIAzol reagent (QIAzol Lysis Reagent, Qiagen) on a Polytron tissue processor. After precipitation with ethanol, total RNA will be isolated using a commercial kit (RNeasy Mini Kit, Qiagen), and genomic DNA will be

eliminated (RNase Free-DNase Set, Qiagen). Total RNA concentration and purity will be assessed by spectrophotometry and agarose gel electrophoresis.

### 3.14.2. Synthesis of cDNA and fluorescence

To convert total RNA into DNA, cDNA will be synthesised using a cDNA synthesis kit (RT<sup>2</sup> Easy First Strand Kit, Qiagen). Next, we will add reagents from the RT<sup>2</sup> SYBR Green Master Mix kit (Qiagen) to the synthesised cDNA for fluorescent intercalator emission to allow detection and analysis of the relative expression of genes of interest.

### 3.14.3. Real-time PCR

Samples will be subjected to real-time polymerase chain reaction (real-time PCR) using an ABI 7500 thermocycler (Applied Biosystems, USA) with 5 specific kits, one per analysed pathway (Stem Cells, Notch, Diabetes, Stem Cell Signalling, and Wnt) and a kit specially customised for this study (Enteroendocrine Differentiation). The complete list of genes to be analysed with each kit is described in the tables below.

KIT RT <sup>2</sup> Rat Stem Cell PCR Array (PARN-405)	
Família de Genes	Genes
Stem Cell Specific Markers	Cell Cycle Regulators: Apc, Axin1, Ccna2, Ccnd1, Ccnd2, Ccne1, Cdc2a, Cdc42, Ep300, Fgf1, Fgf2, Fgf3, Fgf4, Myc, Notch2, Pard6a, Rb1. Chromosome and Chromatin Modulators: Gcn5l2, Hdac1, Hdac2, Myst1, Myst2, Rb1, Tert. Genes Regulating Symmetric/Asymmetric Cell Division: Dhh, Ihh, Notch1, Notch2, Numb, Pard6a. Self-Renewal Markers: Hspa9a, Myst1, Myst2, Neurog2, RGD1565646 (Sox2). Cytokines and Growth Factors: Bmp1, Bmp2, Bmp3, Cxcl12, Fgf1, Fgf2, Fgf3, Fgf4, Igf1, Jag1, LOC306312 (Gdf2), RGD1564178 (Gdf3). Genes Regulating Cell-Cell Communication: Dhh, Dll1, Gja1, Gjb1, Jag1. Cell Adhesion Molecules: Agc1, Apc, Bglap2, Cd4, Cd44, Cdh1, Cdh2, Catna1 (Ctnna1), Cxcl12, Ncam1. Metabolic Markers: Abcg2, Aldh1a1, Aldh2, Fgfr1.
Stem Cell Differentiation Markers	Embryonic Cell Lineage Markers: Actc1, Ascl2, Foxa2, Isl1, Ka15 (Krt15), Msx1, Myod1, Pdx1 (Ipf1), T. Hematopoietic Cell Lineage Markers: Cd19, Cd3d, Cd3e, Cd4, Cd8a, Cd8b, Mme. Mesenchymal Cell Lineage Markers: Agc1, Alpi, Bglap2, Col1a1, Col2a1, Col9a1, Pparg. Neural Cell Lineage Markers: Cd44, Ncam1, Oprs1, S100b, Tubb3.
Signaling Pathways Important for Stem Cell Maintenance	Notch Pathway: Dll1, Dll3, Dtx2, Dvl1, Ep300, Gcn5l2, Hdac1, Hdac2, Jag1, Notch1, Notch2, Numb. Wnt Pathway: Adar, Apc, Axin1, Btrc, Ccnd1, Fzd1, Myc, Ppard, Wnt1.

<b>KIT RT<sup>2</sup> Rat Notch Signaling Pathway PCR Array (PARN-059)</b>	
<b>Família de Genes</b>	<b>Genes</b>
Notch Signaling Pathway	Notch Binding: Dll1, Jag1, Jag2. Notch Receptor Processing: Adam10, Ncstn, Psen1, Psen2, Psenen.
Notch Signaling Pathway Target Genes	Apoptosis Genes: Cdkn1a (p21Cip1/Waf1), Cflar (Cash), Ifng, Nfkb1. Cell Cycle Regulators: Ccnd1, Cdkn1a (p21Cip1/Waf1). Cell Proliferation: Cdkn1a (p21Cip1/Waf1), Il2ra, Stat6. Genes Regulating Cell Differentiation: Ccnd1, Nr4a2 (Nur77), Pparg. Neurogenesis: Fos, Hes5, Nr4a2 (Nur77). Regulation of Transcription: Fos, Fos1 (Fra1), Hes1 (Hry), Hes5, Hey1, Ifng, Nfkb1, Nfkb2, Nr4a2 (Nur77), Pparg, Stat6. Others with Unspecified Functions: Cd44, Chuk (IKKa), Erbb2 (Her2), Il17b, Krt1, Map2k7 (Jnk2), Pdpk1 (Pdk1), Ptcr.
Other Genes Involved in the Notch Signaling Pathway	Apoptosis Genes: Gsk3b, Notch1, Notch2, Psen1. Cell Cycle Regulators: Ccne1, Cdc16, Figf (Vegfd), Gsk3b, Jag2, Notch2, Pcaf (Kat2b). Cell Growth and Migration: Jag2, Lrp5, Notch2, Psen1, Shh, Wisp1. Cell Proliferation: Ctnnb1, Figf, Gsk3b, Jag1, Jag2, Notch2, Numb, Pcaf (Kat2b), Shh. Genes Regulating Cell Differentiation: Ctnnb1, Dll1, Gsk3b, Jag1, Jag2, Notch1, Notch2, Notch3, Notch4, Runx1 (Aml1), Shh. Neurogenesis: Dll1, Heyl, Jag1, Notch2, Numb, Pofut1, Rfng, Zic2 (Hpe5). Regulation of Transcription: Aes (Grg5), Ctnnb1, Ep300, Gli1, Hdac1, Heyl, Hoxb4, Hr, Mycl1 (Lmyc1), Ncor2, Notch1, Notch2, Notch3, Notch4, Pcaf (Kat2b), Rbpjl, Runx1 (Aml1), Tead1, Tle1. Other Genes with Unspecified Functions: Adam17 (Cd156b), Cbl, Gbp2, Il6st (Gp130), Lfng, Lmo2, Map1b, Mfng, Mmp7 (Matrilysin), Neurl (Neu1), Sel1l, Supt6h.
Genes from Signaling Pathways that Crosstalk with Notch Signaling	Sonic Hedgehog (Shh) Pathway: Gli1, Gsk3b, Shh, Smo, Sufu. Wnt Receptor Signaling Pathway: Aes (Grg5), Axin1, Ctnnb1, Fzd2, Fzd3, Fzd4, Fzd5, Fzd6, Fzd7, Gsk3b, Lrp5, Tle1, Wisp1, Wnt11.

<b>KIT RT<sup>2</sup> Rat Diabetes PCR Array (PARN-023)</b>	
<b>Família de Genes</b>	<b>Genes</b>
Receptors, Transporters & Channels:	Adra1a, Adrb3, Aqp2, Ccr2, Cd28, Ceacam1, Ctla4, Gcgr, Glp1r, Icam1, Il4ra, Nsf, Rab4a, Sell (LECAM-1), Slc2a4 (GLUT4), Slc14a2, Snap23, Snap25, Stx4a, Stxbp1, Stxbp2, Stxbp4, Tnfrsf1a, Tnfrsf1b, Vamp2, Vamp3, Vapa.
Nuclear Receptors:	Ppara, Pparg
Metabolic Enzymes:	Ace, Acly, Parp1 (Adprt), Dpp4, Enpp1, Fbp1, G6pc, Gck, Gpd1, Gsk3b, Hmox1, Ide, Nos3, Pygl, Sod2
Secreted Factors:	Agt, Ccl5 (Rantes), Gcg, Ifng, Il6, Il10, Il12b, Ins1, Retn, Tgfb1, Tnf, Vegfa.
Signal Transduction:	Akt2, Dusp4, Igfbp5, Ikbkb (IKKbeta), Inpp1 (SHIP2), Irs1, Irs2, Mapk8 (JNK1), Mapk14 (p38 MAPK), Pik3cd, Pik3r1, Ptpn1 (PTP-1B), Trib3 (Skip3).
Transcription Factors:	Cebpa, Foxc2, Foxg1, Hnf4a, Foxp3 (LOC317382), Neurod1, Nfkb1, Nrf1, Pdx1 (Ipf1), Ppargc1a, Srebf1, Tcf2 (HNF1b), Ttf1
Others	Serpine1 (PAI-1), Ucp2.

KIT RT <sup>2</sup> Rat Stem Cell Signalling PCR Array (PARN-047)	
Família de Genes	Genes
Pluripotency Maintenance Pathway	Receptors: Il6st (Gp130), Lifr. Transcription Factor: Stat3.
Fibroblast Growth Factor (FGF) Signaling Pathway:	Receptors: Fgfr1, Fgfr2, Fgfr3, Fgfr4. Transcription Factor: Cdx2.
Hedgehog Signaling Pathway:	Receptors & Coreceptors: Ptch1, Ptchd2, Smo. Transcription Factors & Cofactors: Gli1, Gli2, Gli3, Sufu.
Notch Signaling Pathway:	Receptors & Coreceptors: Ncstn, Notch1, Notch2, Notch3, Notch4, Psen1, Psen2, Psenen. Transcription Factor: Rbpjl.
TGF $\beta$ Superfamily Signaling Pathway:	Receptors & Coreceptors: Acvr1 (Alk2), Acvr1b, Acvr1c, Acvr2a, Acvr2b, Acvr1l (Alk1), Amhr2, Bmpr1a (Alk3), Bmpr1b (Alk6), Bmpr2, Eng (Evi-1), Ltbp1, Ltbp2, Ltbp3, Ltbp4, Rgma, Tgfbr1 (Alk5), Tgfbr2, Tgfbr3, Tgfbrap1. Transcription Factors & Cofactors: Crebbp, E2f5, Ep300, Rbl1, Rbl2, Smad1, Smad2, Smad3, Smad4, Smad5, Smad6, Smad7, Smad9, Sp1, Zeb2.
Wnt Signaling Pathway:	Receptors: Fzd1, Fzd2, Fzd3, Fzd4, Fzd5, Fzd6, Fzd7, Fzd8, Fzd9, Lrp5, Lrp6, Vangl2. Transcription Factors & Cofactors: Bcl9, Bcl9l, Ctnnb1, Lef1, LOC679869 (Tcf7l2), Nfat5, Nfatc2, Nfatc3, Nfatc4, Pygo2, RGD1560225 (Nfatc1), Tcf3, Tcf7

KIT RT <sup>2</sup> Rat Wnt Signalling PCR Array (PARN-043)	
Família de Genes	Genes
WNT Signaling Pathways	Canonical: Apc, Apc2_predicted, Axin1, Axin2, Bcl9_predicted, Csnk1a1, Csnk1d, Csnk2a1, Csnk2b, Ctnnb1, Dixdc1_predicted, Dkk1_predicted, Dkk3, Dkk4_predicted, Dvl1, Dvl2, Dvl3_predicted, Ep300, Fzd1, Fzd2, Fzd3, Fzd4, Fzd5, Fzd6, Fzd7_predicted, Fzd9, Gsk3a, Gsk3b, Lef1, Lrp5_predicted, Lrp6_predicted, Nkd1_predicted, Nkd2_predicted, Ppp2ca, Ppp2r1a, RGD1308535_predicted (Pygo2), RGD1564947_predicted (Porcnd), Senp2, Sfrp1, Sfrp2, Sfrp4, Sfrp5_predicted, Tcfe2a, Tcf3_predicted, Tcf4, Tcf7_predicted, Wif1, Wnt1, Wnt10a_predicted, Wnt10b_predicted, Wnt2, Wnt2b, Wnt3, Wnt3a_predicted, Wnt4, Wnt6_predicted, Wnt7a, Wnt7b, Wnt8a_predicted, Wnt8b_predicted. Planar Cell Polarity (PCP): Daam1_predicted, Dvl1, Dvl2, Dvl3_predicted, Nkd1_predicted, Nkd2_predicted, Rhoa, Wnt9a_predicted, Wnt9b_predicted. Wnt/Ca+2: Fzd2, Wnt1, Wnt10a_predicted, Wnt10b_predicted, Wnt11, Wnt2, Wnt2b, Wnt3, Wnt3a_predicted, Wnt4, Wnt5a, Wnt5b, Wnt6_predicted, Wnt7a, Wnt7b, Wnt8a_predicted, Wnt9a_predicted, Wnt9b_predicted.
WNT Signaling Negative Regulation:	Apc, Apc2_predicted, Axin1, Axin2, Btrc (bTrCP), Ccnd1, Dkk1_predicted, Dkk3, Dkk4_predicted, Fbxw11_predicted, Fbxw2_predicted, Frzb (FRP-3), Kremen1, Lrp6_predicted, Nkd1_predicted, Nkd2_predicted, RGD1561440_predicted (Nik), Senp2, Sfrp1, Sfrp2, Sfrp4, Sfrp5_predicted, Tle1_predicted, Tle2, Wif1.
WNT Signaling Target Genes:	Btrc (bTrCP), Ccnd1, Ccnd2, Ccnd3, Jun, Mitf, Myc, Pitx2
Developmental Processes:	Cell Fate: Ctnnb1, Dkk1_predicted, Dkk3, Dkk4_predicted, Wnt1, Wnt3, Wnt3a_predicted. Tissue Polarity: Fzd2, Fzd3, Fzd5, Fzd6. Cell Growth & Proliferation: Apc, Apc2_predicted, Ccnd1, Ccnd2, Ccnd3, Ctnnb1, Ep300, Fgf4, Fzd3, Jun, Lrp5_predicted, Myc, Ppp2ca, Ppp2r1a, Wisp1, Wnt3a_predicted.
Cell Migration:	Apc, Apc2_predicted, Dkk1_predicted, Dkk3, Dkk4_predicted, Lrp5_predicted, Lrp6_predicted, Wnt1.
Cell Cycle:	Apc, Apc2_predicted, Btrc (bTrCP), Ccnd1, Ccnd2, Ccnd3, Ctnnb1, Ep300, Jun, Myc, Rhoa, Tcfe2a, Tcf3_predicted
Cellular Homeostasis:	Apc, Apc2_predicted, Fzd2, Jun, Myc

KIT RT <sup>2</sup> Rat Customized Enteroendocrine Differentiation PCR Array
<b>Genes</b>
ATOH1 (ou Math1)
Ngn3, BETA2/NeuroD, Pax4, Pax6, Nkx2.2, Nkx6.1, Isl-1, Insm1 (ou IA-1), Pdx1, CCK
Foxa1, Foxa2
Gfi-1
GATA4
GATA5
GATA6
Proglucagon, Glucagon, GLP-1, GLP1-r, GLP-2, PYY, TGR5, GPR-119, GPR-120, PC-1, GIP
TCF-4, Glicentin, Oxyntomodulin
Other genes related to enteroendocrine differentiation

#### 3.14.4. Gene expression

The relative expression of genes of interest will be analysed in real-time by using the PCR equipment software Abi Prism 7000 Sequence Detection System version 1.6 to compare gene expression levels among tissue fragments from the different groups of animals.

During real-time PCR, fluorescence increases with every PCR cycle until reaching a threshold where all samples can be compared. The threshold corresponds to the time-point used for fluorescence analysis. This time-point is defined by the researcher and must be within the range in which fluorescence generated by sample amplification becomes significantly higher than background fluorescence.

The threshold is defined during the PCR exponential phase, when the amount of product formed can be used to calculate the initial concentration of template strands (cDNA) amplified by the reaction. The exact cycle when fluorescence crosses the threshold is called the threshold cycle (Ct). More concentrated samples (with greater numbers of initial template strands) reach the threshold faster and have lower Ct values. When PCR efficiency is close to 100%, the number of copies generated increases exponentially and doubles with each reaction cycle.

The number of initial template strands can be calculated from the Ct. The equation  $N_s = N_0(1+E)^n$  describes exponential PCR amplification, where “N<sub>s</sub>” is the number of template strands at a given reaction cycle, “N<sub>0</sub>” is the number of initial template strands, “E” is efficiency of the amplification reaction, and “n” is the number of cycles. Assuming that reaction efficiency is 100% (log E=1) and “n” is a selected amplification point identical for all samples, we can calculate the number of initial template strands using the following equation:  $N_0 = N_s \times 2^{-Ct}$ .

The method of analysing gene expression by real-time PCR proposed here represents a relative quantification of genes of interest because it requires an endogenous control (a gene whose expression does not exhibit statistically significant variation among the analysed samples). Relative quantification of a given sample by real-time PCR results from the relationship between the Ct of the gene of interest (i) and the Ct of the endogenous control (e):

$$\begin{aligned} N_{si} &= N_{oi} \times 2^{-C_{ti}} \text{ (gene of interest)} \\ N_{se} &= N_{oe} \times 2^{-C_{te}} \text{ (endogenous gene)} \end{aligned}$$

Because  $N_{si}/N_{se}$  is equal to the normalised number of molecules of the gene of interest relative to the number of endogenous control molecules that we will call X, and  $N_{se}$  and  $N_{si}$  is a constant that we will name K, the equation becomes:

$$X = K \cdot 2^{-\Delta Ct}$$

Relative quantification of gene expression is used to describe changes in the expression of a gene of interest in a group of samples relative to a control group. In this study, we will compare ileum and jejunum samples in their normal localisations with interposed ileum samples and with ileum samples from animals in the sham-treated group. Thus, the value of X will be measured under two different conditions, and by dividing the value of X under condition A by the value of X under condition B, we obtain  $2^{-\Delta\Delta Ct}$ , as shown by the following equation:

$$\frac{X_a}{X_b} = \frac{K \cdot 2^{-\Delta Ct_a}}{K \cdot 2^{-\Delta Ct_b}} = 2^{-\Delta\Delta Ct}$$

This method will be used in our study to calculate the relative expression levels of genes of interest. The relative expression levels of genes of interest will be compared among the different segments from different groups of animals (IG ileum, SG ileum, CG jejunum, and ileum).

### 3.14.5. Statistical analysis

The variables will be summarised for each study group using the pertinent descriptive statistics: absolute (n) and relative (%) frequency, or mean, standard deviation (s.d.), median, maximum, and minimum values. The data will be analysed using parametric or non-parametric tests according to the observed distribution.

For normally distributed data, analysis of variance will be applied with two fixed factors: groups (4 levels: IG, SG, CG1, and CG2) and assessment time-point (3 levels: Week 12 (W12), Week 20 (W20), and Week 28 (W28)). For the variables assessed weekly, the test will have 16 levels corresponding to each week. For non-normally distributed data, the Mann-Whitney test will be applied to compare techniques within each assessment, and the Friedman test will be applied to related samples to compare variations within each technique. The presence of an association between qualitative variables will be assessed by the chi-squared test or Fisher's exact test. The level of significance will be set to 0.05 ( $\alpha = 5\%$ ), and p values lower than this value will be considered significant.

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